EVALUATION OF MICROBIAL COMMUNITIES AND THEIR RELATIONSHIPS WITH CHEMICAL PERFORMANCE IN PASSIVE ACID MINE DRAINAGE TREATMENT SYSTEMS AMENDED WITH CRAB SHELL

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by
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ABSTRACT

Acid mine drainage (AMD), a common environmental problem caused by mining activities, adversely impacts receiving watersheds, wildlife, and even public health due to its low pH and high concentration of toxic metals. The choice of organic substrates utilized in low-cost, low-maintenance, passive treatment systems for AMD is crucial to maintain a diverse microbial community for successful remediation. Recent studies by our research team have shown that crab shell (CS) amendments improve the longevity and performance of traditional AMD treatment systems containing spent mushroom compost (SMC). However, the microbial contribution to this has not been thoroughly explained. In this research, both chemical and biological techniques were used to comprehensively evaluate microbial communities and their relationship with chemical performance under varying environmental conditions in passive AMD treatment systems amended with CS. This might shed light on future design and operation strategies for AMD bioremediation systems.

Previous performance data of five sulfate-reducing columns treating AMD suggested that columns amended with higher fractions of CS supported more efficient and stable performance in terms of alkalinity generation, sulfate reduction, and metal removal, as compared to columns with traditional SMC and limestone substrates. Accordingly, microbial analyses conducted on packing materials of the five columns in this study revealed significant advantages of CS amendment in sustaining the growth of functionally more diverse microbial groups including cellulose degraders, chitin degraders, fermenters, and sulfur or sulfate reducers. Remarkably, the 100% CS column
supported the growth of sulfate-reducing bacteria (SRB) from eight different genera—key players in AMD treatment systems. PCoA and phylogenetic ARB trees showed that bacterial communities in 50% CS and 100% CS columns were more likely to cluster together. Archaea predominantly identified as methanogens and fungi capable of polysaccharide degradation were only detected in columns containing SMC. In the 100% CS column, copy numbers of the functional genes representing fermenters, sulfate reducers, and chitin degraders were approximately 2.4, 3.9, and 3.2 times higher than those in SMC column, respectively.

In a field study, four pilot-scale vertical flow pond (VFP) systems featuring different substrate combinations of CS and SMC and different underdrain materials were constructed and operated for 633 days at the Klondike-1 site in Cambria County, PA. Analyses of chemical performance data, 454 sequencing, and quantitative PCR (qPCR) data suggested that under changing environmental conditions at the Klondike-1 site, the VFPs containing CS sustained more efficient and reliable treatment of AMD coupled with functionally more diverse and stable microbial communities. Influent pH rapidly increased from < 3.0 to above 6.0 and was maintained circum-neutral by all VFPs. As compared to a control reactor containing SMC, the reactors containing CS sustained higher alkalinity (around 300 mg/L as CaCO₃), slower alkalinity exhaustion, and steadier acidity neutralization. Apart from the dissolution of CaCO₃ in CS and LS materials, fermentation and sulfate reduction contributed comparably to the generation of alkalinity in pilot VFPs. Correspondingly, copy numbers of genes representing fermenters and sulfate reducers were higher in the pilot reactors containing CS than in the SMC reactor. Similar to the column study, a higher diversity of SRB was observed in reactors
containing CS. Due to higher alkalinity levels and sulfate-reducing rates, more thorough metals removal was observed in reactors containing CS than SMC: >90% for the removal of iron (Fe) and aluminum (Al), >50% for manganese (Mn) and zinc (Zn), and no breakthrough above 50% of the influent concentration was observed for Fe, Al, and Zn throughout the course of the test. Geochemical modeling indicated that possible mineral phases of precipitation were goethite and mackinawite for Fe, gibbsite for Al, and rhodochrosite for Mn. Sorption onto the surface of Fe and/or Al minerals could be another mechanism for metal removal. Noticeably, the pilot VFP containing 70% CS was shown to maintain more stable performance while possessing comparable treatment efficiency to the 100% CS reactor over varying environmental and operational conditions during the pilot study. This agrees well with microbial observations under no flow condition, which is experienced by many field bioremediation systems due to seasonal changes or clogging: the relative abundance of core phyla shifted in all pilot reactors, but the smallest changes in functional gene copies were observed in the 70% CS reactor.

All of the observations summarized above make CS an apparently advantageous substrate for use in passive AMD treatment systems from the standpoints of both system performance and the support of robust microbial communities. Compared to the 100% CS reactor, the 70% CS pilot VFP sustained more diverse and stable microbial communities as well as comparable efficiency and more reliable system performance throughout the seasonal changes of environmental conditions at the field site. Therefore, the use of CS as a fractional amendment to substrate mixtures used in passive treatment systems for AMD or other environmental contaminants is attractive when considering both cost and system performance.
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CHAPTER 1
INTRODUCTION

1.1. Literature Review

1.1.1. Acid mine drainage: formation and environmental impact

Acid mine drainage (AMD), a common environmental problem caused by mining activities, poses increasing threats to receiving watersheds and even public health. Upon investigation, AMD impacts between 15,000 and 23,000 kilometers of streams in the United States (U.S.) (USEPA, 1994), and its remediation costs have been estimated in the billions of U.S. dollars (Feasby et al., 1991). In general, the formation of acidic mine drainage is caused by the exposure of sulfide minerals, especially pyrite (FeS$_2$), to air and water. The subsequent chemical and biological reactions release large amounts of sulfuric acid and ferric iron (Fe$^{3+}$), some of which precipitates as ferric hydroxide (Fe(OH)$_3$). The following reaction (Eq. 1.1) summarizes the weathering of pyrite initiated by pyrite oxidation at neutral pH (Benner et al., 1997):

$$4\text{FeS}_2(s) + 15\text{O}_2 + 14\text{H}_2\text{O} \rightarrow 4\text{Fe(OH)}_3(s) + 8\text{SO}_4^{2-} + 16\text{H}^+$$  \hspace{1cm} (1.1)

The generation of hydrogen ions (H$^+$) greatly increases the acidity of mine drainage, and correspondingly the pH can decrease to around 3.0 or less. Not only is the resulting low pH harmful to the biosphere, but the subsequent dissolution of toxic metals such as arsenic, cadmium, copper, lead, nickel, and/or zinc from the surrounding host
rock may also adversely impact wildlife and human beings (Benner et al., 1997; Gibert et al., 2003).

In reality, pyrite oxidation is a complex process involving oxygen-dependent reactions and an oxygen-independent reaction (Johnson and Hallberg, 2005). The oxygen-dependent reactions include the oxidation of ferrous iron (Fe\(^{2+}\)) to Fe\(^{3+}\) and the oxidation of sulfur compounds ultimately to SO\(_4^{2-}\), whereas the oxygen-independent reaction mainly involves Fe\(^{3+}\) attack on the pyrite mineral. Although the oxidation of sulfide minerals may be abiotic, the Fe\(^{2+}\) oxidation rate can be increased by several orders of magnitude by iron- or sulfur-oxidizing bacteria or archaea, and many of these microorganisms tend to be most active at pH 2.0 to 4.0 (Church et al., 2007). Some typical chemolithotrophic iron-/sulfur-oxidizing bacteria associated with the generation of acid mine drainage are *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, and *Gallionella ferruginea* (Baker and Banfield, 2003; Fowler et al., 1999; Johnson et al., 2005; Lear et al., 2009).

1.1.2. AMD treatment systems

The remediation of acid mine drainage involves three major processes: the neutralization of acidity, the reduction of sulfate, and the removal of dissolved metals. Current remediation techniques for AMD can be divided into two different approaches: ex-situ chemical treatment (active treatment) and in-situ biological treatment (passive treatment). Active AMD treatment involves the continuous addition of an alkaline substance such as limestone (CaCO\(_3\)) or a sulfide compound to stimulate metal precipitation as hydroxides or sulfides, respectively (Johnson and Hallberg, 2005). Major
problems associated with this kind of treatment systems are the increasing cost for the continuous supply of chemicals and the generation of large amounts of metal-containing sludge (Berghorn and Hunzeker, 2001; Johnson and Hallberg, 2005; Waybrant et al., 2002).

In contrast to active treatment, passive treatment systems allow the AMD to flow through a solid reactive mixture which requires only periodic maintenance and replenishment. Passive treatment can result from abiotic or biotic processes. The former achieves AMD treatment mainly through chemical means such as anoxic limestone drains and open limestone channels, while the latter remediates AMD using sulfate-reducing bacteria (SRB). Typical configurations of biological passive treatment systems include aerobic wetlands, vertical flow wetlands, compost bioreactors, and permeable reactive barriers (Lear et al., 2009).

Under favorable conditions, SRB can convert SO$_4^{2-}$ into hydrogen sulfide (H$_2$S) and increase alkalinity in the presence of carbon and nitrogen sources. For example, the complete oxidation of C-even fatty acids by SRB can be expressed in the following equation (Rabus et al., 2006; Eq. 1.2):

\[
4\text{H}(\text{CH}_2)_n\text{COO}^- + (3n+1)\text{SO}_4^{2-} + (2n+2)\text{H}^+ \longrightarrow (4n+4)\text{HCO}_3^- + (3n+1)\text{H}_2\text{S}
\]  

(1.2)

As a result, the precipitation of dissolved metals as sulfides can take place. Several studies have shown that passive treatment systems for AMD can be more cost-efficient than other treatment options due to their low operation and maintenance requirements as well as minimal production of metal-containing sludge (Berghorn and Hunzeker, 2001; Waybrant et al., 2002).
1.1.3. Background of Klondike sites in Central Pennsylvania

Little Laurel Run is located in northeastern Cambria County, Pennsylvania, and has a watershed of approximately 3 square miles. The stream is classified as “not attaining” its quality as a Cold Water Fishery because of the AMD it receives from the abandoned Klondike mine (Rose, 2005). In addition, the contaminated Little Laurel Run is a significant contributor to acidification of Clearfield Creek into which it flows. The Clearfield Creek eventually drains into the Susquehanna River and from there, into the Chesapeake Bay. The Klondike mine discharges two streams of AMD into Little Laurel Run from the southwest. The Klondike-1 discharge emerges from an abandoned strip mine on the Clarion, Lower Kittanning, and Middle Kittanning coal seams, with an average flow rate of 15 gallons per minute (gpm), acidity of 417 mg/L as CaCO$_3$, pH 3.4, 141 mg/L Fe, 4 mg/L Al, and 30 mg/L Mn (Rose, 2008). The Klondike-2 discharge flows from the abandoned underground Klondike Mine on the Lower Kittanning coal seam at a flow rate averaging 134 gpm with acidity 50 mg/L as CaCO$_3$, pH 3.5, 5.3 mg/L Fe, 2.0 mg/L Al, and 3.0 mg/L Mn (Rose, 2005).

Two passive treatment systems were constructed separately for acidic discharges from the Klondike-1 and Klondike-2 sites in order to remove acidity, metals, and generate a net alkalinity. Both treatment systems consist of a primary oxidation pond, a vertical flow pond (VFP), an aerobic settling pond, and a wetland. According to the monitoring data of the Clearfield Creek Watershed Association (CCWA), the passive treatment system at the Klondike-2 site functions well, reducing acidity and iron concentrations to an average of -21 mg/L as CaCO$_3$ and 3.3 mg/L, respectively, while
generating a net alkalinity of 50 mg/L. In contrast, within 9 months of operation, the VFP at the Klondike-1 site had clogged due to the formation of iron precipitates on top of the organic substrate layer. Even after the removal of the Fe precipitates and the construction of two new oxidation ponds prior to VFP, the system still does not meet effluent requirements for acidity and iron which were as high as 66 mg/L as CaCO$_3$ and 18 mg/L, respectively. Upon noticing the poor performance of the existing VFP and lack of free space to expand the current AMD treatment system at the Klondike-1 site, CCWA was interested in seeking potential alternative substrates to enhance treatment efficiency while keeping costs within the limited budget.

1.1.4. Organic carbon and nitrogen sources for AMD passive treatment

When it comes to the microbial processes involved in passive AMD treatment, substrate compositions affect the microbial diversity inside the system to a great extent. Since AMD is generally deficient in dissolved organic carbon and nitrogen (Kolmert and Johnson, 2001), the choice of substrates in passive treatment systems is crucial for successful remediation. In general, external carbon sources can be divided into two groups: simple organic carbon sources (methanol, ethanol, lactate, glucose, acetate, etc.) and complex organic carbon sources (cellulosic wastes and organic wastes) (Neculita et al., 2007). The selection of an appropriate substrate is important to reduce operational costs as well as to enhance SRB activity. As early as the 1980s, different substrates began to be evaluated for the purpose of improving the performance of AMD passive treatment systems, including sawdust, spent mushroom compost, whey, wood chips, methanol, lactate, and crab shell (Chang et al., 2000; Christensen et al., 1996; Daubert and Brennan,
Table 1.1 lists the source of organic carbon (i.e., substrate) of several passive treatment systems, as well as the $\text{Fe}^{2+}$ and $\text{SO}_4^{2-}$ concentrations being treated. As shown in Table 1.1, higher sulfate reduction rates were achieved with reactive substrate mixtures containing multiple organic carbon sources (Chang et al., 2000; Robinson-Lora and Brennan, 2009; Waybrant et al., 1998; Zagury et al., 2006).

Spent mushroom compost (SMC), typically consisting of manure, hay, straw, corn cobs, wood chips, and conditioned with gypsum and limestone (Dvorak et al., 1992), is one of the most commonly used organic substrates for AMD bioremediation. Nevertheless, the performance of AMD passive treatment systems supported by SMC have shown varying degrees of success: some function well for several years while others are subject to a short lifetime and/or cannot meet the requirements of the initial design (Benner et al., 1997; Blowes et al., 2000; Johnson and Hallberg, 2002). Besides SMC, a new alternative substrate, crab shell, has been investigated by our research team for its ability to remediate AMD-impacted watersheds (Daubert and Brennan, 2007; Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2009).

1.1.5. Crab shell chitin

Recent studies have shown that crab shell is a promising multifunctional substrate for enhancing the performance of AMD passive treatment systems. In the presence of SC-20 grade crab shell, pH increased from acidic to circum-neutral, alkalinity increased to approximately 200 mg/L as $\text{CaCO}_3$, dissolved iron and aluminum exhibited nearly complete removal, and dissolved manganese, the most recalcitrant metal present in AMD,
was reduced by at least 80% within 10 days of operation of AMD treatment units
(Daubert and Brennan, 2007; Newcombe and Brennan, 2010; Robinson-Lora and
Brennan, 2009).

Crab shells are composed of a mixture of chitin, protein, and calcium carbonate
(CaCO₃), and can be obtained from the waste products of the seafood industry. Chitin,
poly-N-acetyl-D-glucosamine linked by β-1,4 bonds (Fig. 1.1, Ko et al., 2011), is the
second most abundant biopolymer in nature, just after cellulose (Beaney et al., 2005).
Chitin and its deacetylated form, chitosan, are widely used in food industries,
pharmaceutical products, agriculture, membrane manufacture, and wastewater treatment
because of its biodegradability, biocompatibility, and non-toxicity. Generally, chitin can
be extracted from the shells of various crustaceans by chemical and/or biological
processes using strong acids and bases, and/or by organic acids and enzymes-produced
by microorganisms, respectively (Brine and Austin, 1981; Jung et al., 2007). The
nitrogen content of chitin is fairly high ((C₈H₁₃O₅N)ₙ), and its carbon-to-nitrogen ratio
(around 6.9 on mass basis) is nearly ideal to support the growth and activity of SRB, the
key players in AMD passive treatment systems. The fermentation of chitin mainly
generates volatile fatty acids (VFAs), some alcohols, and ammonium, creating a strong
reducing environment capable of sustaining anaerobic processes inside the systems
(Robinson-Lora and Brennan, 2009; Vera et al., 2001).

The effectiveness of crab shell to promote microbial activity in AMD remediation
systems is likely due to its ability to simultaneously serve as an electron donor source
(organic contents), neutralizing agent (CaCO₃), and nitrogen source (ammonium from
protein and chitin decomposition). In addition, crab shell has been proven to retain its
permeability during fermentation under continuous-flow conditions (Brennan, 2003), and its microporous structure contributes to the removal of metal contaminants from aqueous solutions through physical adsorption (Felse and Panda, 1999; Franco et al., 2004). In terms of economics, crab shell can be purchased at the price of $0.44/kg (dewatered) to $1.76/kg (dried) (JRW Bioremediation), which is relatively expensive in comparison to the cost of SMC at $0.055/kg. However, SMC is short of nitrogen and calcium carbonate, making it a limited source of nitrogen and vulnerable to fluctuations in pH. Moreover, the performance of AMD treatment systems containing SMC is sometimes compromised because of its limited longevity. Under the consideration of all these factors, it was hypothesized that both treatment efficiency and low cost can be achieved in AMD passive treatment systems by combining crab shell and SMC together in an optimal proportion. Previous continuous-flow column studies demonstrated that the substrate mixture of 70% crab shell and 30% SMC is the most cost effective to support the remediation of high-strength AMD (Grembi, 2011; Sick, 2010).

1.1.6. Microbial communities in AMD passive treatment systems

Since the 1990s, a variety of studies have been conducted to investigate the microbial assemblage in sulfate-reducing AMD treatment systems by using culture-based techniques (Benner et al., 2000; Hard et al., 1997; Johnson et al., 2005). More recently, DNA extraction, polymerase chain reaction (PCR), cloning, sequencing, and other molecular biological techniques have been applied to further examine the composition and activity of the microbial community in the system (Chandler et al., 2006; Clarke et al., 2004; Labrenz and Banfield, 2004; Pruden et al., 2007). Until now, most studies on AMD
treatment have focused on systems fed with a mixture of cellulosic wastes (sawdust, alfalfa, hay, woodchips, etc.) and organic wastes (manure, sludge, compost, etc.). In AMD treatment systems fed with SMC, only a very small fraction of the substrate is readily available to SRB (Benner et al., 1999) because SRB typically require short chain organic carbon compounds (alcohols, organic acids, or $\text{H}_2$) for energy and growth (Widdel, 1988). Therefore, the cooperation within and/or among different microbial communities is critical for the performance of the entire treatment system.

As pointed out by Logan et al. (2005), while sulfate reduction is located downstream of the total carbon and energy flow, the upstream anaerobic degradation of complex materials to simpler compounds by cellulolytic microbes and fermenters almost occupies the rest of the entire pathway and may limit the rate at which substrates become available to SRB. Specifically, it has been demonstrated that the four major metabolic groups present in AMD passive treatment systems are cellulose-degrading bacteria, other polysaccharide degraders, fermentative bacteria, and sulfur- and sulfate-reducing bacteria. Methanogens were also proven to exist in some treatment systems, although they did not form a significant part of the population (Hiibel et al., 2008; Logan et al., 2005; Pereyra et al., 2008; Pruden et al., 2007). Pereyra et al. (2008) compared the composition and activity of five microbial inocula with respect to their ability to remediate mine drainage, and pointed out that the cultures exhibiting the best performance contained microorganisms from all levels of carbon and energy flow in the system, including polysaccharide hydrolysis and degradation, fermentation of hydrolysis products, acetogenesis, and sulfate reduction.
Although microbial analysis of AMD treatment systems has been previously investigated, no microbial studies have addressed the effect of varying substrate compositions and operational conditions on the performance of AMD treatment systems amended with crab shell. Therefore, several molecular biological technologies (cloning, qPCR, metagenomics, etc.) have been utilized to investigate the diversity and abundance of microbial communities in sulfate-reducing AMD systems treated with crab shell under different environmental conditions. In recent years, because of the rapid development of culture-independent analyses and sequencing techniques in microbiology, the metagenomics era has come, wherein environmental DNA (metagenome) from the samples to be studied are directly isolated and analyzed. The application of the metagenomic approach relies on the high-throughput sequencing (HTS) technologies, such as 454 Titanium and Illumina sequencing platforms. In a 454 sequencing event, a basic workflow consists of DNA library preparation, emulsion PCR, and then sequencing. When compared to other traditional molecular approaches, metagenomics via direct sequencing has several advantages, such as minimal technical biases as introduced by PCR amplification of the 16S rRNA gene, high-throughput screening of multiple samples simultaneously, reliable identification of the majority of microbes in different environmental samples, and possible insights into taxonomy and diversity of microbial communities (Ercolini, 2013; Lewin et al., 2013). However, data analyses of metagenomic studies often require bioinformatics skills, and the cost of HTS is usually high. Therefore, determination of which molecular approaches should be used can be made by comprehensively considering the study objectives, sampling size, time and costs anticipated, and experimental and analytical skills owned.
1.2. Hypotheses and Objectives

The overall objective of this study was to comprehensively evaluate the relationship between AMD treatment performance and microbial community members in passive treatment systems featuring different substrate combinations of SMC and crab shell, thus serving as a reference to guide future remediation strategies. These objectives were met by applying molecular microbiology techniques to samples collected from experiments conducted at two different scales: continuous-flow laboratory columns and pilot-scale field reactors. For the laboratory study, it was hypothesized that columns with larger fractions of crab shell would tend to support functionally more diverse microbial communities, especially sulfate-reducing bacteria (SRB)-key players in the systems. The specific objectives of the column work were to:

a. Investigate the compositions of bacterial, archaeal, and fungal communities inside five columns with different substrate combinations;

b. Determine the phylogenetic relationships of microbial communities within/between different columns;

c. Assign putative functions to identified clone sequences, and classify and quantify key functional groups in the different columns to determine their relative abundance; and,

d. Evaluate the relationships between substrate materials, chemical performance, and microbial communities in these AMD treatment columns.

For the pilot-scale field study, which was performed at the Klondike-1 site, it was anticipated that reactors containing crab shell would outperform the SMC reactor in terms
of alkalinity generation, metal and sulfate removal, and diversity and stability of the microbial communities throughout system operation. The specific objectives of the field study were to:

a. Compare different combinations of substrates for treatment performance and efficiency in four pilot-scale reactors;

b. Determine the effect of scale-up and varying environmental conditions on treatment efficiency of the field systems;

c. Compare the microbial compositions of the pilot-scale field reactors, and quantify the key functional groups within each reactor;

d. Evaluate the changes in microbial composition within the pilot reactors over the course of the test based on water quality data (start up, pseudo-steady state, decline);

e. Compare microbial adaptability as well as chemical performance of different pilot reactors under environmental conditions of extremely low flow rate and low temperature.

1.3. Thesis Layout

To meet the objectives of this study, this thesis is composed of three manuscripts as specified below:

a. CHAPTER 2: “Comparative Analysis of the Impact of Crab Shell Amendments on Microbial Communities in Sulfate-Reducing Columns Treating Acid Mine Drainage”

Authors: Yishan Lin, Caroline E. Newcombe, and Rachel A. Brennan.
The content presented in this chapter will be submitted for publication in 

*Biotechnology and Bioengineering.*

b. CHAPTER 3: “Effect of Substrate Compositions and Varying Environmental Conditions on Treatment Efficiency of Pilot-scale Field Reactors Remediating High Strength Acid Mine Drainage”

Authors: Yishan Lin, Jessica A. Grembi, Sara S. Goots, Shanxing Lin, and Rachel A. Brennan.

Content presented in this chapter will be submitted for publication in *Bioresource Technology* or *Water Research.*

c. CHAPTER 4: “Development and Adaptability of Microbial Communities in Pilot-scale Field Reactors Treating Acid Mine Drainage Amended with Crab Shell”

Authors: Yishan Lin, Aswathy Sebastian, István Albert, and Rachel A. Brennan.

Content presented in this chapter will be submitted for publication in *The ISME Journal* or *Applied and Environmental Microbiology.*
1.4. References


Grembi, J.A. 2011. Remediation of high-strength mine impacted water with crab shell substrate mixtures: laboratory column and field pilot tests. in: *Civil and*

University park.


Jung, W.J., Jo, G.H., Kuk, J.H., Kim, Y.J., Oh, K.T., Park, R.D. 2007. Production of chitin from red crab shell waste by successive fermentation with Lactobacillus


### TABLE 1.1. Characteristics of several passive AMD treatment systems.

<table>
<thead>
<tr>
<th>Organic Carbon Source</th>
<th>System Mode</th>
<th>HRT(^a) (days)</th>
<th>Substrate Loading (kg/kg SO(_4^{2-}))</th>
<th>SO(_4^{2-}) (mg/L)</th>
<th>Total Fe (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>In</td>
<td>Out</td>
<td>In</td>
<td>Out</td>
</tr>
<tr>
<td>Spent mushroom compost</td>
<td>Continuous</td>
<td>5</td>
<td>2.27</td>
<td>1002</td>
<td>831</td>
<td>53</td>
</tr>
<tr>
<td>Whey</td>
<td>Batch</td>
<td>203</td>
<td>4.40</td>
<td>888</td>
<td>2548</td>
<td>29.0</td>
</tr>
<tr>
<td>Mixtures of sewage sludge, leaf mulch, wood chips, sheep manure, sawdust, and cellulose</td>
<td>Batch</td>
<td>65</td>
<td>21.90</td>
<td>1200-4800</td>
<td>&lt;35</td>
<td>1080</td>
</tr>
<tr>
<td>Spent mushroom compost, oak chips, spent oak, paper sludge, and organic-rich soil</td>
<td>Continuous</td>
<td>20</td>
<td>26.59</td>
<td>2580</td>
<td>200-650</td>
<td>500</td>
</tr>
<tr>
<td>Methanol</td>
<td>Continuous</td>
<td>0.3-2.7</td>
<td>1.00-5.00</td>
<td>1900-2100</td>
<td>832</td>
<td>92.5</td>
</tr>
<tr>
<td>Lactate</td>
<td>Continuous</td>
<td>0.7</td>
<td>2.00</td>
<td>2280-2315</td>
<td>&lt;400</td>
<td>5.1-50.8</td>
</tr>
<tr>
<td>Single source or mixture of maple wood chips, sphagnum peat moss, leaf compost, conifer compost, poultry manure, and sawdust</td>
<td>Batch</td>
<td>70</td>
<td>11.78</td>
<td>4244</td>
<td>163-5575</td>
<td>1683</td>
</tr>
<tr>
<td>Crab shell chitin (SC-20)</td>
<td>Batch</td>
<td>20</td>
<td>10.60-23.15</td>
<td>181-393</td>
<td>10-150</td>
<td>1.2-10</td>
</tr>
</tbody>
</table>

Note: \(^a\) HRT, hydraulic residence time
FIG. 1.1. Chemical structure of chitin.
CHAPTER 2

Comparative Analysis of the Impact of Crab Shell Amendments on Microbial Communities in Sulfate-Reducing Columns Treating Acid Mine Drainage

2.1. Abstract

It has been demonstrated that crab shell (CS) amendments improve the longevity and performance of traditional acid mine drainage (AMD) treatment systems containing spent mushroom compost (SMC), although the microbial contribution to this has not been thoroughly explained. In this work, clone libraries for 16S and 18S rRNA genes were constructed and real-time quantitative PCR (qPCR) was performed on packing materials collected from five continuous-flow columns containing different substrate compositions ranging from 0 to 100% CS after receiving a continuous flow of AMD for 148 days (428 pore volumes). The data revealed that the proportion of CS in the substrate was positively correlated with the diversity of both SRB and archaeal clones, but negatively correlated with fungal diversity. According to Unifrac Significance and PCoA tests, CS also had an impact on the cluster formation of microbial populations in different columns, indicating structural differences in the communities. Specifically, the column containing 100% CS supported microorganisms from all functional levels and 8 different genera of sulfate-reducing bacteria – the most ever observed in an AMD treatment system. Moreover, the copy numbers of the functional genes representing fermenters, sulfate reducers, and
chitin degraders increased with increasing proportions of CS. These observations agree well with the chemical performance data, further validating that by supporting functionally more diverse microbial communities, chitinous substrates may provide benefits for improving the treatment of not only AMD but also other environmental contaminants which are amenable to anaerobic bioremediation.

2.2. Introduction

The functions and roles of various microorganisms in the bioremediation of contaminated sites have gained more and more attention in recent years, especially after the introduction of culture-independent molecular techniques. Although the adaptability and activity of key microorganisms in bioremediation systems is crucial, the diversity and cooperation of the entire microbial community can still greatly improve system performance. This is particularly true in the remediation of certain recalcitrant, hazardous, and/or extreme sites, among which watersheds contaminated by acid mine drainage (AMD) represent a complex environment characterized by low pH, and high concentrations of sulfate (SO$_4^{2-}$) and dissolved metals. Generally, AMD is a common and recurring environmental problem caused by mining activities, impacting between 15,000 and 23,000 kilometers of streams in the United States (U.S.) (USEPA, 1994).

Passive treatment systems for AMD are widely used, have been shown to be more cost-efficient than other treatment options (Berghorn and Hunzeker, 2001; Waybrant et al., 2002), and can simultaneously support three major processes for AMD remediation: the neutralization of acidity, the reduction of sulfate, and the removal of dissolved metals.
Biological passive treatment remediates AMD by relying on a group of various microorganisms, especially sulfate-reducing bacteria (SRB). Under favorable conditions, SRB can convert $\text{SO}_4^{2-}$ into hydrogen sulfide ($\text{H}_2\text{S}$) and increase alkalinity in the presence of carbon and nitrogen sources. As a result, the precipitation of dissolved metals as metallic sulfides can take place.

AMD is generally deficient in dissolved carbon and nitrogen; therefore, organic substrates are typically added to the treatment systems for successful remediation. Spent mushroom compost (SMC), a mixture of cellulosic and organic wastes conditioned with gypsum and limestone (Dvorak et al., 1992), is one of the most commonly used organic substrates for AMD treatment, but has had varying degree of success (Benner et al., 1999; Blowes et al., 2000; Johnson and Hallberg, 2002). Besides SMC, an alternative sustainable waste product, crab shell (CS), has been evaluated by our research team for its ability to remediate AMD-impacted watersheds. Crab shells are primarily composed of chitin (poly-N-acetylglucosamine), protein, and $\text{CaCO}_3$. Recent studies have shown that CS is a promising multifunctional substrate for AMD treatment because of its ability to serve as a slow-release carbon and nitrogen source and neutralizing agent (Daubert and Brennan, 2007; Robinson-Lora and Brennan, 2009).

Since many AMD passive treatment systems are microbiologically driven, a comprehensive understanding of the microbial community is of great importance for exploring both theoretical mechanisms and long-term performance. Since the 1990s, various studies have been conducted to investigate the microbial population in sulfate-reducing treatment systems for mine drainage by using culture-based methods (Hallberg and Johnson, 2005; Hard et al., 1997). More recently, the microbial compositions in these
systems have been evaluated qualitatively and quantitatively through culture-independent molecular biological techniques such as PCR, cloning, denaturing gradient gel electrophoresis (DGGE), fluorescent *in situ* hybridization (FISH), qPCR, etc. (Chandler et al., 2006; Clarke et al., 2004; Labrenz and Banfield, 2004; Pereyra et al., 2008; Pruden et al., 2007). Until now, most studies on AMD treatment have focused primarily on systems containing traditional substrates, such as SMC and limestone.

In AMD treatment systems fed with SMC, cellulose degradation has been recognized as the rate-limiting step of the entire carbon flow (Logan et al., 2005). In addition, only a very small fraction of the substrate is readily available to SRB (Benner et al., 1999) because SRB typically require short chain organic carbon compounds (alcohols, organic acids, or H₂) for growth (Widdel, 1988). Others have compared the compositions and behaviors of microbial inocula with respect to their ability to remediate mine drainage, and pointed out that the cooperation within and/or among different microbial communities in certain cultures was critical for the performance of the entire treatment system (Pereyra et al., 2008). By comparing the performance of different simple and complex organic substrate, it was demonstrated that microbial population in treatment systems for mine drainage were qualitatively and quantitatively affected by both substrate compositions and environmental stress (Hiibel et al., 2011). To date, no one has compared the microbial communities in AMD treatment systems amended with CS.

Our research group previously evaluated different CS-SMC mixtures for their ability to support AMD treatment in a series of microcosm batch tests and continuous-flow laboratory columns. The results suggested that larger fractions of CS (50-100%)
provided a significant benefit over traditional limestone and SMC substrates, especially for the generation of alkalinity, the reduction of sulfate, and the removal of metals (Newcombe and Brennan, 2010). To better explain the biological mechanisms of treatment and to comprehensively evaluate CS as a substrate amendment, both qualitative (clone library construction) and quantitative (qPCR) comparisons of microbial compositions and key functional groups were carried out in this work. This is the first comparative study of microbial communities in AMD remediation systems containing crab shell, and may help to comprehensively understand the interactions between microbial community members and serve as a reference to guide future remediation strategies.

2.3. Materials and Methods

2.3.1. Column test setup and sampling

In this study, microbial communities in continuous-flow laboratory columns containing varying mixtures of CS and SMC were examined. The experimental setup and water quality results are described elsewhere (Newcombe and Brennan, 2010). In brief, five columns were packed with a mixture of approximately 1260 g silica sand and 50 g of substrate composed of 0 to 100% crab shell and SMC as described below (percentages listed are by mass of substrate):

a. “Control”: silica sand only, no substrate;

b. “10% LS”: 10% limestone and 90% SMC;

c. “5% CS”: 5% crab shell and 95% SMC;
d. “50% CS”: 50% crab shell and 50% SMC;
e. “100% CS”: substrate is 100% crab shell.

Argon-purged AMD from Kittanning Run in Altoona, Pennsylvania, was continuously pumped into the columns for 148 days (approximately 428 pore volumes). At the conclusion of the experiment, samples of packing materials were collected through a sampling port at the effluent end of each column using a flame-sterilized spatula, and preserved at -20°C for later microbial analysis.

2.3.2. DNA extraction

DNA extractions were carried out on the five preserved column samples using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s protocol with two extra steps after the addition of Solution C1:

a. To each tube, 20 µl of 100 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO) and 10 µl of 25 mg/ml achromopeptidase (Sigma-Aldrich, St. Louis, MO) were added, and then incubated in a 37°C water bath for 1 hour;

b. To each tube, 45 µl of 20 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) was added, followed by incubation in a 55°C water bath for 1 hour.

The purpose of these steps was to maximize the extraction output by assisting in the lysis process of gram-positive bacteria (lysozyme and achromopeptidase), or to remove impurities from DNA preparations by digesting protein molecules (proteinase K). All five DNA samples were used for the clone library construction, quantitative polymerase reactions, and statistical analyses. Approximately 0.26 g of column packing
material was used per extraction, and the DNA preparations were stored at -20°C before downstream analysis.

2.3.3. Polymerase chain reaction (PCR)

16S rRNA genes were PCR-amplified with corresponding primers set to target bacteria and archaea, and the 18S rRNA gene was used for fungal PCR reactions (Table 2.1). The optimized PCR reaction mixture contained 1X PCR buffer (QIAGEN Inc., Valencia, CA); 3.0 mM (bacteria) or 2.5 mM (archaea and fungi) MgCl₂ (QIAGEN Inc., Valencia, CA); 0.4 mg/ml (bacteria) or 0.5 mg/ml (archaea and fungi) Bovine Serum Albumin (BSA) solution (Sigma-Aldrich, St. Louis, MO); 200 µM each (bacteria and fungi) or 300 µM each (archaea) dNTP mix (QIAGEN Inc., Valencia, CA); 0.5 µM (bacteria), 0.7 µM (archaea), or 0.4 µM (fungi) each primer; 0.5 U (bacteria) or 1.0 U (archaea and fungi) of HotStarTaq DNA polymerase (QIAGEN Inc., Valencia, CA); 3 µl of 10-fold diluted DNA template; and sterile nuclease-free water to a final volume of 20 µl.

The thermal cycling program consisted of an initial 15-min activation of the polymerase at 95°C before 30 (bacteria), 40 (archaea), or 35 (fungi) cycles consisting of 1 min (bacteria and fungi) or 40 s (archaea) of denaturation at 94°C, 1 min of annealing at specified temperature (Table 2.1), and 2 min (bacteria) or 1 min (archaea and fungi) of elongation at 72°C; finally, a 10-min extension at 72°C was performed.

Amplification of PCR products of the proper size was confirmed by electrophoresis through a 0.8% (w/v) agarose gel stained with SYBR® Safe DNA Gel
Stain (Invitrogen, Carlsbad, CA) in 1X TBE buffer, and viewed under a gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA).

2.3.4. Cloning reaction and plasmid isolation

The PCR products of the five column samples were cloned with a TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Plasmid DNA isolation of the selected clones was performed using an E-Z 96™ Fastfilter Plasmid Kit (Omega Bio-Tek, Inc., Norcross, GA) following the manufacturer’s Spin Protocol except 50 µl of sterile nuclease-free water was used for DNA elution instead of elution buffer, since the buffer is known to interfere with sequencing. For each domain, a total of 48 clones from each column sample were randomly picked for sequencing in the first round. An additional 48 clones per sample were sequenced for samples in which the constructed rarefaction curves still showed an exponential increase of operational taxonomic units (OTUs).

2.3.5. Gene quantification by qPCR

In DNA samples collected from the five columns treating AMD, the 16S rRNA genes of total bacteria and total archaea, the 18S rRNA gene of total fungi, and four key functional groups—cellulose degraders, fermenters, sulfate reducers, and chitin degraders were targeted using real-time quantitative PCR (qPCR) as previously described (Manter and Vivanco, 2007; Nadkarni et al., 2002; Pereyra et al., 2010; Takai and Horikoshi, 2000; Yergeau et al., 2007). To access to a wider range of phylogenetic groups in anaerobic cellulose degraders, cel5 genes were selected as primary targets instead of
*cel48* genes which were recognized as highly divergent (Henrissat, 1991; Pereyra et al., 2010). For fermenters, *hydA* genes represent a highly conserved region in all iron hydrogenases (Vignais et al., 2001), and proved to have broader coverage of fermenters in different phylogenetic groups in previous qPCR runs (Henrissat, 1991; Pereyra et al., 2010). The qPCR primer set targeting *dsrA* genes ensures broad specificity for SRB while avoiding matches for sulfur-oxidizing bacteria (SOB) (Pereyra et al., 2010). The *chiA* genes, highly abundant in nature, were selected to be used in qPCR runs for chitin degraders due to the extensive characterization of these genes in recent studies (Li and Greene, 2010; Metcalfe et al., 2002). The absolute abundance of each gene of interest was obtained after every qPCR run.

To prepare microbial standards, the designated standard microbes were PCR amplified with corresponding primer sets (Table 2.1), and then the products were purified using a QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). Except for qPCR runs targeting total bacteria and archaea in which Taqman assays were used, SYBR green assays were established for other targets. For each gene target, a triplicate four- to six-point standard curve was constructed (*R^2* ≥ 0.99). In every qPCR run, serial dilutions at a factor of 1:10 and 1:100 were prepared for DNA template from each column, and triplicates were run for all samples. Immediately before qPCR, 0.5 µl of 0.3% BSA was added into each reaction (to reach a total volume of 10 µl) to enhance amplification yields by preventing the inhibitors from interacting with Taq polymerase. All qPCR analyses were performed using a StepOnePlus™ Real-Time PCR System (Life Technologies, Grand Island, NY), and samples below the detection limit of quantification
(approx. 100 gene copies) were not included in the analysis. Final values are expressed in copy numbers per gram of packing material (copies/g).

2.3.6. Sequence analyses

Sequencing of the clones from the five columns was performed by the Penn State Genomics Core Facility (The Pennsylvania State University, University Park, PA). Chimera check was carried out on all sequence files through DECIPHER’s Find Chimeras web tool for 16S rRNA genes (Wright et al., 2012) or KeyDNATools package for 18S rRNA genes (http://KeyDNAtools.com). Sequences that were contaminated or identified to be chimeras were removed from downstream analysis. The best matches of DNA sequences to known microorganisms were determined by the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/blast/), and were verified using the SEQMATCH tool of Ribosomal Database Project (RDP) (Cole et al., 2009). Meanwhile, a literature study was carried out to investigate different functional properties of the microorganisms with the highest similarity to cloned sequences with respect to substrate utilization and sulfate reduction. Not all cloned sequences were assigned a function, particularly those identified as “uncultured clone” in the GenBank database.

To investigate sampling richness and microbial diversity, Good’s coverage and Shannon diversity indices were calculated as previously described (Bianchi and Bianchi, 1982; Good, 1953). In addition, Mothur software was used to assign distance matrix, OTUs with 95% confidence interval, and to construct rarefaction curves for the microbial community of each domain in all five columns (Hughes et al., 2001; Schloss et al., 2009).
The SINA tool by ARB-SILVA was used for the alignment of the sequences from each column (Pruesse et al., 2012). The aligned sequences were then checked using the “cd-hit-est” command on the web server of CD-HIT program (Huang et al., 2010) with the sequence identity cut-off value of 95% to remove highly similar or identical sequences in each column sample. The processed sequences from all columns were then re-aligned using SINA and inserted into an existing Parsimony tree (SILVA SSURef Release 111, July 2012) in the ARB software package (Quast et al., 2013). To compare microbial communities in different columns, the phylogenetic trees were exported for UniFrac analyses including the UniFrac significance tests, cluster environments, principle coordinate analysis (PCoA), and lineage-specific analysis (Lozupone et al., 2006; Lozupone and Knight, 2005; Lozupone et al., 2007). Statistical significance tests were applied to microbial sequences in AMD column samples using overall and pair-wise comparisons, where raw p-values ≤ 0.05 were reported to be statistically significant.

2.3.7. Nucleotide sequence accession numbers

All sequences obtained in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the accession numbers KF581198 to KF581670 for bacteria, KF581671 to KF581830 for archaea, and KF581831 to KF582037 for fungi.

2.4. Results

2.4.1. Microbial diversity
For bacterial clone libraries, on average 84% of the total species richness was reached in each column as indicated by Good’s coverage (Table 2.2). Shannon Diversity indices of bacterial communities in the 10% LS, 5% CS, and 100% CS columns were relatively higher, and no obvious difference was observed (3.77±0.09); the diversity in control column without substrate was the lowest with the value of 1.91. These trends are consistent with the number of OTUs observed in each column.

As shown in Table 2.2, Good’s coverage indices of archaeal communities in all five columns were above 80%, indicating satisfactory sampling coverage. The three columns with crab shell amendment featured higher archaeal diversity in Shannon Diversity tests.

Through fungal clone libraries, an average of 42 fungal sequences were collected from each of the five columns treating AMD, with an average Good’s value of 81% indicating satisfactory sampling coverage (Table 2.2). An inverse, but linear correlation was obtained ($R^2=0.9889$), indicating that as the fraction of crab shell amendment increases, the diversity of fungal community decreases (Fig. 2.1). The number of fungal OTUs (=3) in the 100% CS column was the lowest among all columns. This was not unexpected when considering the higher amount of cellulosic materials in the SMC columns readily available to fungi.

2.4.2. Microbial composition and phylogenetic characterization

The 16S and 18S rRNA gene sequences of clones were compared with the GenBank database to assign reference sequences with the best matches to the query sequences. Table 2.3 summarizes the representative bacteria with the maximum identity
 (> 97%) in the continuous-flow columns. The abundance of core bacterial phyla in the five column samples was calculated as shown in Fig. 2.2. A parsimony tree containing all distinct sequences from bacterial 16S rRNA gene clone libraries of the five columns is depicted in Fig. 2.3.

At the bacterial phylum level, except for the control column in which the most abundant phylum was Proteobacteria, the four active columns with substrates were observed to be predominated by members in Firmicutes (Fig. 2.2). Abundance of Proteobacteria in the 100% CS column was twice as high as that in the 10% LS and 5% CS column. Although Proteobacteria was relatively abundant in both the control and 100% CS columns, bacterial species under this phylum in the two columns mainly fell into different classes: γ-Proteobacteria for the control column; and δ-Proteobacteria (harboring the largest group of SRB) for the 100% CS column.

Four bacterial phyla including Proteobacteria, Bacteroidetes, Acidobacteria, and Firmicutes were abundant across the phylogenetic tree with Firmicutes as a largest group in all column samples (Fig. 2.3). Of the 48 bacterial clones sequenced from the control column (without substrate), almost all were identified to be background bacteria (Lysobacter spp., Alicyclobacillus sp.ZJ-6) which could have been introduced into the system by packing materials and AMD. With respect to the 10% LS, 5% CS, and 50% CS columns, the majority of the bacterial clones were identified to be members of the genera Clostridium and Bacillus; therefore, bacteria in these columns were presumably more active in polysaccharide degradation and fermentation. In addition, a limited number of sulfate-reducing bacteria (SRB), and/or sulfur-reducing bacteria were detected in these three columns.
In the bacterial phylogenetic tree, two clones recovered separately in 10% LS and 5% CS columns were located near a branch representing an arsenic-contaminated mine drainage metagenome (CABM01000005). Remarkably, 19 sequences from 50% CS and 100% CS columns were clearly clustered together to form a deep branch (CAMD17), and the sequences were closely related to *Clostridium lituseburense* (M59107). Moreover, three bacterial sequences in the 100% CS column were inserted into the tree under a specific Blvii28 wastewater-sludge group, and were more similar to a species of *Bacteroidetes* bacterium RL-C (AB611036). Out of the 124 sequenced clones in the 100% CS columns, 53 different bacterial species of the highest match were identified. The most functionally diverse bacterial community was observed in the 100% CS column, with notably, the most varieties of SRB from 8 different genera. As expected, bacterial genera capable of chitin degradation were mainly detected in the 5% CS, 50% CS, and 100% CS columns since chitin is a key component of crab shell.

The majority of the archaeal clones belonged to one metabolic group—methanogens, a group of microorganisms that produce methane (CH$_4$) as a metabolic byproduct in under anoxic conditions. There were two representative archaeal genera associated with all five columns: *Methanocella* and *Methanosarcina*. For the 50% CS and 100% CS columns, three extra methanogenic genera were detected: *Methanomethylovorans*, *Methanobacterium*, and *Methanolobus*. Among them, the species *Methanomethylovorans hollandica*, is known to be able to grow on dimethyl sulfide (Lomans et al., 1999), a metabolic byproduct from the decomposition of organic sulfur compounds.
The leading genera of fungal communities in the columns were identified to be *Candida*, *Scheffersomyces*, *Cladosporium*, *Malassezia*, *Myceliophthora*, and *Penicillium*. Among them, *Candida* spp., which assisted with the fermentation of carbohydrate monomers, was the only fungal genera present in all five columns, while fungi capable of polysaccharide degradation were only detected in the columns containing SMC. This is probably because fungi are the main producers of cellulase and hemicellulase in nature, and therefore have an important role in the decomposition of cellulosic materials which are the major components of SMC.

2.4.3. Statistical significance tests and principal coordinate analysis

UniFrac statistical significance tests were applied to determine the uniqueness or difference of the microbial communities in each AMD column. When comparing all column samples together, only the bacterial communities were statistically different ($p = 0.02$), which might be due to the higher diversity of bacteria than archaea or fungi as indicated by BLAST searches of the cloned sequences. In pair-wise comparisons of each column, UniFrac significance tests demonstrated that bacterial communities in 50% CS and 100% CS were significantly different from other column samples (Table 2.4). This is consistent with the ARB tree of bacterial sequences in that there was a deep branch accommodating the clones only from 50% CS and 100% CS columns. When clustering the bacterial sequences from all columns by Unifrac, the 50% CS and 100% CS column samples were again clustered together. The lineage-specific analysis revealed that the main differences in the column samples containing 50% and 100% CS compared to the other substrates was the presence of species similar to *C. lituseburensis* (NCBI Accession
Number M59107) and an uncultured bacterium (EU775307). Nevertheless, as indicated by Significance and Clustering tests of the UniFrac algorithm, bacterial samples from 10% LS and 5% CS were more similar to each other than were any pair of column samples, probably due to the higher fraction of different species within the genera Bacillus and Clostridium. All clusters were randomly re-sampled and re-clustered using Jackknife Environment Clusters in UniFrac and proved to be well-supported (>90%).

The UniFrac significance test was then applied to archaeal and fungal sequences separately in each of the AMD column samples in the form of pair-wise comparisons. The results showed that the archaeal populations in 50% CS and 100% CS were different from those in control and 10% LS columns (Table 2.4). According to the lineage-specific analysis, the dominant archaeal species were, to some extent, related to the percentage of crab shell in the substrate. Methanosarcina spp. were abundant in 50% CS and 100% CS, and Methanocella paludicola were dominant in the two columns without crab shell amendment.

With respect to fungal significance tests, only sequences in the control column were significantly different from those in the 5% CS and 100% CS columns. This is reasonable when considering the sharp drop of Shannon diversity values from 2.17 in the control to 0.41 in the 100% CS column. The differences among the three columns were mainly caused by the dominance of species similar to Chaetosphaeria ciliate and Malassezia restricta in the control column, Candida spp. in the 5% CS column, and an uncultured fungus clone (EU162634) in the 100% CS column.

PCoA analysis was performed to find similarities or distribution patterns among different column samples. No clear grouping of the bacterial, archaeal or fungal
communities was identified by PCoA (Fig. 2.4). However, bacterial sequences from the 10% LS and 5% CS columns were loosely clustered together in the PCoA plot, indicating that they were more similar to each other than other column samples. This is also the case for the fungal population in these two columns. The first two principal coordinates explained more than 60% of the total variance in PCoA tests of the bacterial, archaeal, and fungal communities. As compared to other column samples, the difference of bacterial or archaeal composition in the control was captured by the first principal coordinate, while the bacterial population in 10% LS and 5% CS columns was mainly separated from that in the 50% and 100% CS columns by the second coordinate. Bacterial communities in the 50% and 100% CS samples exhibited similarity to some degrees along the first principal coordinate, but showed certain differences along the second coordinate. In the fungal PCoA plot, the uniqueness of the sequences from 100% CS column was explained by the first principal coordinate.

2.4.4. Quantification of functional groups

Analyses of clone sequences brought us a broad view of the microbial compositions and functional groups in the five columns treating acid mine drainage. To better understand and compare the role and quantity of each functional group in the system, qPCR was performed to target domain specific 16S rRNA (total bacteria and total archaea) or ITS (total fungi) gene copies and five functional genes including cellulose degraders (*cel5*), fermenters (*hydA*), sulfate reducers (*dsrA*), chitin degraders (*chiA*), and methanogens (*mcrA*). Generally, a clear trend was observed only for total
bacteria in the five columns: copy numbers increased with the fraction of crab shell in the substrate.

Interestingly, the highest copy numbers for total bacteria ($4.76 \times 10^8$ copies/g) and fungi ($5.60 \times 10^6$ copies/g) were detected in the 100% CS column (Fig. 2.5 a), while the 10% LS column accommodated the largest archaeal population ($1.67 \times 10^6$ copies/g). A comparison of the gene copies from the three domains within each column showed that the largest population size was observed for total bacteria, followed by total fungi, and then total archaea.

The highest functional gene copies of fermenters, SRB, and chitin degraders were detected in the 100% CS column with values of $9.92 \times 10^6$ copies/g, $4.19 \times 10^6$ copies/g, and $4.62 \times 10^7$ copies/g, respectively. Analysis for mcrA genes yielded levels below the detection limit for all column samples, as was the case for dsrA and chiA genes in the control column. Comparing the copy numbers of different functional genes in samples from the 100% CS column versus the 10% LS column revealed that only cel5 gene copies were higher in 10% LS (Fig. 2.5 b). This is undoubtedly due to the relatively large supply of cellulosic materials in the substrate (SMC) of this column which could be readily utilized by cellulose degraders. In the 100% CS column, the concentrations of the hydA, dsrA, and chiA genes were approximately 2.37, 3.91, and 3.24 times higher, respectively, than those in 10% LS column. This indicates that the columns amended with higher fractions of crab shell tend to facilitate the growth of fermenters, SRB, chitin degraders, thus enhancing the system performance of AMD treatment. Although not as dramatic of an improvement, lower fractions of CS (5% and 50%) also increased the
copy numbers of all functional genes except cel5 relative to the traditional substrate (10% LS column).

2.5. Discussion

2.5.1. Impact of substrate amendment on microbial composition and key functional groups

Sequencing of the clones revealed the identity of members of the bacterial communities that developed inside the five columns and gave insight into their putative functions, including cellulose degradation, fermentation, sulfur reduction, sulfate reduction, and chitin decomposition. With the exception of chitin decomposition, these findings are consistent with those of other mine drainage passive treatment systems and sulfate-reducing bioreactors (Logan et al., 2005; Pereyra et al., 2008), although the diversity of species within the systems is different.

Specifically in this study, all of the columns with substrates yielded clones corresponding to cellulose-degrading and fermentative microorganisms (Table 2.5). Additionally, a few clones from the control column and the three columns amended with CS were associated to chitin decomposition. Thiosulfate- and sulfur-reducers were detected in the 5% CS and 50% CS columns. Furthermore, sulfate-reducing capabilities were associated with sequences obtained from the 10% LS, 5% CS, and 100% CS columns, but were detected the most frequently in the 100% CS column.

Several cellulose degraders from the genus Clostridium were detected in the three columns containing SMC. In addition, the specialized cellulolytic bacteria Cellulomonas
composti, Acetivibrio cellulolyticus, and Bacteroides cellulosolvens were found in the 5% CS, 50% CS, and 100% CS columns, respectively. Cellulose hydrolysis is the first step of substrate decomposition inside AMD treatment systems fed traditional complex organic substrates like SMC. The presence of cellulolytic microbes is crucial for the success of the whole treatment system because their metabolic products are subsequently degraded into small organic molecules which act as the major substrates for SRB.

Microorganisms capable of degrading organic polymers other than cellulose, such as starch, protein, and chitin, were also discovered in some columns. Typical species of starch hydrolysis in the 10% LS column were Bacillus firmus, Planifilum fimeticola, and Thermoaetinomyces vulgaris, and Clostridium phytotermentans was identified in the 5% CS column. Bacteria capable of degrading certain polypeptides or amino acids were found in the SMC and 5% CS columns: for example, P. fimeticola for casein decomposition (Obst et al., 2005), and Sedimentibacter hongkongensis for cyanophycin degradation (Obst et al., 2005). Whereas AMD treatment systems with primarily cellulosic substrates support the growth of polysaccharide-degrading organisms (Logan et al., 2005; Pereyra et al., 2008; Pruden et al., 2007), our systems were found to support the growth of the microorganisms capable of degrading not only polysaccharides such as cellulose, starch, and pectin, but also other biopolymers such as proteins, polypeptides, and chitin. In the present study, it is not surprising that Clostridium paraputrificum, a chitinolytic microorganism (Simunek et al., 2002), was commonly present in the 5%, 50%, and 100% CS columns which contained chitin. In addition, another specialized chitin-fermenting bacterium, Cellulomonas chitinilytica, was detected in the 100% CS column. To our best knowledge, this is the first time that chitin degrading
microorganisms have been detected in AMD bioremediation systems. The fermentation of chitin results in the production of gases (H₂ and CO₂) and the formation of simple organic acids (acetate, lactate, etc.) which can be selectively utilized downstream by SRB or methanogens, if present.

The fermentation of cellulolytic products and other simple molecules plays a key role in microbial processes since it is located mid-way in the fermentation pathway. Fermentation byproducts, including ethanol, lactate, acetate, and H₂, are immediate electron donors utilized by various SRB. While all cellulose and other polysaccharide degraders are capable of fermentation, there were still certain fermenters detected in all columns, except the control column, that could only ferment simpler compounds like di- and/or monosaccharides. Most of these fermenters belonged to the genera of Clostridium, Bacillus, and Bacteroides. These fermentative microbes can form close associations with cellulolytic bacteria, thus benefiting the process of cellulose decomposition by keeping the products of cellulose hydrolysis at a relatively low concentration (Leschine, 1995). The fermentative bacteria observed here have been also reported in other AMD treatment systems (Pereyra et al., 2008; Pruden et al., 2007).

Although previous research has shown that sulfate reducers account for only a small proportion of total microbial communities inside bioreactors treating mine drainage (Logan et al., 2005; Morales et al., 2005; Pereyra et al., 2008), they are no doubt key players in the system because the activity of SRB contributes to the generation of alkalinity, reduction of sulfur species, and precipitation of metal sulfides. Specifically, no more than 5% of the total sequenced clones were found to be SRB in each of the columns fed SMC and 5% CS, as compared to approximately 18% of the clones featuring 8
different species of SRB in the 100% CS column. Representatives of SRB were within the genera of *Desulfotomaculum*, *Desulfovibrio*, *Desulfosporosinus*, *Desulfotrhaphidus*, *Desulfomicrobium*, and *Thermodesulfobium*. Of these, only a few species in the genus *Desulfovibrio* were previously confirmed to be present in some AMD bioremediation systems with traditional limestone and SMC substrates (Pereyra et al., 2008; Pruden et al., 2007). In contrast, the continuous-flow columns treating AMD amended with crab shell in our study accommodated more varieties of SRB, which might be responsible for the improved system performance that we observed (Newcombe and Brennan, 2010). In addition, thiosulfate- and sulfur-reducers were detected in the 5% and 50% CS columns, while a sulfite-reducing bacterium, *Desulfitobacterium* spp., was found in the 100% CS column. This might be an indication of the sulfate-reducing pathway in each of these columns, which would require validation in future work.

Although present in smaller quantities than the bacterial population, archaea and fungi may also play functional roles in AMD treatment systems. While methanogenic archaea may weakly compete with SRB for simple organic substrates in these systems (Geets et al., 2006; Logan et al., 2005), they can also utilize $\text{H}_2/\text{CO}_2$ in their catabolism (Madigan et al., 2009), wherein CO$_2$ is a byproduct of sulfate reduction by SRB. In addition, certain methanogenic species have been shown to grow on products from crab shell and protein fermentation, keeping the microbial community in the whole treatment system more functionally diverse. In addition to archaea, fungal species can also play an important part in assisting with the hydrolysis or degradation of polymers and monomers.

2.5.2. Microbial diversity and column performance
Since AMD passive treatment systems rely on biological processes, system longevity and performance can relate to microbial diversity, especially the abundance of sulfate reducers—key players in the treatment systems. It has been previously noted that systems exhibiting the best AMD treatment performance contain more diverse microbial communities, including the capacity for polysaccharide hydrolysis, fermentation of hydrolysis products, acetogenesis, and sulfate reduction (Pereyra et al., 2008), as was the case in the 100% CS column in our study. In contrast, in our control column without substrate, only waterborne or airborne microorganisms were present, thereby, no bioremediation behavior was detected, and the effluent quality was similar to that of the influent.

The previous characterization of system performance of the five columns suggested that larger fractions of crab shell (50-100%) showed a benefit over traditional limestone and SMC substrates, especially for the removal of acidity and metals (Newcombe and Brennan, 2010). This trend can be explained from two perspectives. From the chemical point of view, the absence of chitinous materials in the 10% limestone-90% SMC column contributed to the rapid depletion of carbon and nitrogen sources, as well as alkalinity. With respect to microbial analysis, the majority (>90%) of the clones sequenced in the 10% limestone-90% SMC column were related to microbes that degrade polysaccharides and the downstream products—a relatively homogeneous microbial community compared to that of the 100% CS column. The performance data also indicated that the column using crab shell as the sole substrate showed the greatest increase in the maximum alkalinity reached, up to 6700 mg/L CaCO$_3$ (Fig. 2.6). Meanwhile, time for complete iron and manganese breakthrough to influence levels
(Newcombe and Brennan, 2010) had positive linear correlations with crab shell proportions in the four columns containing substrates, the $R^2$ values of which were 0.9883 and 0.9950, respectively (Fig. 2.6). Correspondingly, the 100% CS column supported the growth of the highest copies of dsrA genes representing sulfate reducers (Fig. 2.5) and 53 different species spanning all functional levels (Table 2.5), which indicated the most diverse microbial community among the five columns. Although the 5% CS column also possessed nearly all functional groups of the microorganisms stated above, the much lower chitin content and lower copy numbers of dsrA genes (Fig. 2.5) resulted in a shorter lifetime and poorer performance than the 100% CS column.

Another observation of microbial community composition may be explained by column performance: it was noted that air was accidentally pumped into the columns on day 26 after the AMD influent line became dislodged (Newcombe and Brennan, 2010). Interestingly, Desulfosporosinus spp., a spore-forming SRB (Stackebrandt et al., 2003), were found to exist in the 10% LS, 5% CS, and 100% CS columns, suggesting habitual stress, perhaps from oxygen exposure.

Finally, in the five continuous-flow columns treating AMD, the proportion of crab shell in the substrate was observed to have generally positive correlations with the diversities of both archaeal clones (Table 2.2) and SRB, but somewhat negative correlation with the copy numbers of total archaea (Fig. 2.5 a). By comparing the theoretical Gibbs free energies for hydrogen-consuming reactions, it is clear that SRB have a thermodynamic advantage over methanogens if sulfate is provided as the final electron acceptor. Moreover, methanogens are more sensitive to low temperatures than SRB (Neculita et al., 2007), indicating that they may not be able to withstand the harsh
environmental conditions that these treatment systems often face in winter. Therefore, it is possible that the predominant growth of SRB in the 100% CS column suppressed the reproduction of methanogens, resulting in the lower copies number of total archaea than that in 10% LS. This finding also has implications for the bioremediation of organic compounds (ex., chlorinated solvents), when practitioners aim to increase the population of contaminant-degrading SRB while reducing substrate loss to methanogenic organisms (McElhoe and Brennan, 2012). Besides, from the standpoint of metabolic diversity, it seems that the SRB and methanogens in the systems featuring a higher fraction of crab shell may actually use different substrates. Protein and chitin in the crab shell materials can be converted to methylamines, methanethiol, and/or dimethyl sulfide through fermentation, and selectively utilized by certain genera of methanogens. For example, Methanosarcina spp. and Methanolobus spp. have been found to be able to grow on methylamines and methanethiol (Madigan et al., 2009), and M. hollandica can utilize dimethyl sulfide (Lomans et al., 1999; Lyimo et al., 2000). Interestingly, all these archaeal genera were detected in the 100% CS column. The increasing percentage of CS may have provided an increasing variety of alternative substrates which could be utilized by methanogens, but not by SRB, which in turn, presumably increased the archaeal diversity in 100% CS column relative to the 10% LS column, although the quantity of total archaea was lower.

All observations of crab shell impact on microbial composition and diversity in this study provided insight into the functioning of passive AMD treatment systems which are microbiologically driven, while in the mean time, had implications for the design of future remediation strategies by utilizing cost-efficient substrate combinations.
2.6. Conclusions

Through clone library construction and qPCR, crab shell amendments were observed to have a significant impact on microbial communities in sulfate-reducing columns treating AMD. The major conclusions of this study were:

a. Columns with larger fractions of crab shell possessed higher diversities for bacterial and archaeal communities, but lower diversity for fungal communities;

b. The 100% CS column supported the growth of microbial populations from all key functional levels in the system, especially SRB from eight different genera;

c. In bacterial ARB tree, a deep branch (CAMD17) consisting of 19 sequences from 50% CS and 100% CS columns was observed, and the sequences were closely related to *Clostridium lituseburense* (M59107);

d. Based on Unifrac tests, bacterial and archaeal communities in columns featuring larger fractions of CS were more likely to cluster together, indicating structural differences from those in 5% CS, SMC and control columns;

e. Abundance of the functional genes representing fermenters, sulfate reducers, and chitin degraders had positive correlation with the proportions of CS.

These observations agree well with the chemical performance data, further validating that crab shell is a promising multifunctional substrate, capable of improving both system longevity and performance. By supporting the growth and activity of diverse microbial communities, chitinous substrates may provide benefits for improving the treatment of not only AMD but also other environmental contaminants which are amenable to anaerobic bioremediation.
2.7. Acknowledgements

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2.8. References


**TABLE 2.1. Gene primer sets used in PCR and qPCR.**

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>Gene target</th>
<th>Primer Probe</th>
<th>Strain used to generate standard</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>PCR Bacterial 16S rRNA</td>
<td>27F/1492R</td>
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<td>50</td>
<td>(Anderson and Haygood, 2007)</td>
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<tr>
<td>Archaeal 16S rRNA</td>
<td>A571F/UA1204R</td>
<td>N/A</td>
<td>59</td>
<td>(Baker and Banfield, 2003)</td>
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<tr>
<td>Fungal 18S rRNA</td>
<td>nu-SSU-0817-5'/nu-SSU-1536-3'</td>
<td>N/A</td>
<td>56</td>
<td>(Borneman and Hartin, 2000)</td>
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</tr>
<tr>
<td>qPCR Total bacteria, 16S rRNA</td>
<td>BAC331F/ BAC797R</td>
<td>Geobacter sulfurreducens DSM 12127</td>
<td>60</td>
<td>(Nadkarni et al., 2002)</td>
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<tr>
<td>Total archaea, 16S rRNA</td>
<td>Arch349F/Arch806R</td>
<td>Methanosarcina barkeri DSM 800</td>
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<td>(Takai and Horikoshi, 2000)</td>
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<td>Total fungi, ITS</td>
<td>ITS1F/ITS4</td>
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<td>(Manter and Vivanco, 2007)</td>
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<td>Cellulose degraders, cel5</td>
<td>cel5_392F/ cel5_754R</td>
<td>Clostridium cellulovorans DSM 3052</td>
<td>52</td>
<td>(Pereyra et al., 2010)</td>
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<td>Fermenters, hydA</td>
<td>hydA_1290F/ hydA_1538R</td>
<td>Clostridium acetobutylicum ATCC 824</td>
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<td>(Pereyra et al., 2010)</td>
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</tr>
<tr>
<td>Sulfate reducers, dsrA</td>
<td>dsrA_290F/ dsrA_660R</td>
<td>Desulfovibrio vulgaris DSM 644</td>
<td>60</td>
<td>(Pereyra et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>Chitin degraders, chiA</td>
<td>chi_A1F/chi_A1R</td>
<td>Serratia marcescens DSM 30121</td>
<td>55</td>
<td>(Yergeau et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Methanogens, mcrA</td>
<td>mcrA_1035F/ mcrA_1530R</td>
<td>Methanosarcina acetivorans C2A</td>
<td>56</td>
<td>(Pereyra et al., 2010)</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2.2. Good’s Coverage and Shannon Diversity of the bacterial community associated with sulfate-reducing columns after treating a continuous supply of AMD for 148 days. The substrate in the columns contained crab shell (CS) and limestone (LS) as indicated, with the balance composed of spent mushroom compost (SMC).

<table>
<thead>
<tr>
<th>Type of clone libraries</th>
<th>Column</th>
<th>No. of sequences</th>
<th>No. of OTUs (95% confidence interval)</th>
<th>Good’s coverage (%)</th>
<th>Shannon diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Control</td>
<td>38</td>
<td>10</td>
<td>87</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td>10% LS</td>
<td>105</td>
<td>54</td>
<td>78</td>
<td>3.77</td>
</tr>
<tr>
<td></td>
<td>5% CS</td>
<td>103</td>
<td>52</td>
<td>81</td>
<td>3.86</td>
</tr>
<tr>
<td></td>
<td>50% CS</td>
<td>126</td>
<td>27</td>
<td>94</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td>100% CS</td>
<td>102</td>
<td>51</td>
<td>82</td>
<td>3.68</td>
</tr>
<tr>
<td>Archaea</td>
<td>Control</td>
<td>38</td>
<td>6</td>
<td>92</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>10% LS</td>
<td>39</td>
<td>7</td>
<td>87</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>5% CS</td>
<td>35</td>
<td>6</td>
<td>86</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>50% CS</td>
<td>24</td>
<td>6</td>
<td>80</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>100% CS</td>
<td>30</td>
<td>10</td>
<td>83</td>
<td>1.64</td>
</tr>
<tr>
<td>Fungi</td>
<td>Control</td>
<td>44</td>
<td>9</td>
<td>73</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td>10% LS</td>
<td>38</td>
<td>7</td>
<td>76</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>5% CS</td>
<td>43</td>
<td>7</td>
<td>79</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>50% CS</td>
<td>45</td>
<td>6</td>
<td>84</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>100% CS</td>
<td>42</td>
<td>3</td>
<td>93</td>
<td>0.41</td>
</tr>
</tbody>
</table>
TABLE 2.3. Representative bacteria in five continuous-flow columns treating AMD with different substrate mixtures of crab shell (CS) and limestone (LS) as indicated, with the balance composed of spent mushroom compost (SMC).

<table>
<thead>
<tr>
<th>Representative bacteria of the best matches (≥ 97%)</th>
<th>GenBank accession number</th>
<th>Possible function</th>
<th>Control</th>
<th>10% LS</th>
<th>5% CS</th>
<th>50% CS</th>
<th>100% CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetivibrio cellulolyticus</td>
<td>NR_025917</td>
<td>Cellulose degradation</td>
<td>-a</td>
<td>-</td>
<td>-</td>
<td>+b</td>
<td>-</td>
</tr>
<tr>
<td>Alicyclobacillus sp. ZJ-6</td>
<td>FM179383</td>
<td>Ferrous and sulfur oxidation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacteroides cellulosolvens</td>
<td>NR_025918</td>
<td>Cellulose degradation</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus firmus</td>
<td>HQ285922</td>
<td>Lipid hydrolysis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus massiliensis</td>
<td>AB299159</td>
<td>Polyester degradation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cellulomonas chitinilytica</td>
<td>AB268586</td>
<td>Chitin degradation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cellulomonas composti</td>
<td>AB166887</td>
<td>Chitin degradation</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium beijerinckii</td>
<td>CP000721</td>
<td>Fermentation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium bifermentans</td>
<td>AF320283</td>
<td>Fermentation of carbohydrates and amino acids</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium botulinum</td>
<td>X68171</td>
<td>Saccharide degradation and fermentation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium chromoreductans</td>
<td>CP001056</td>
<td>Cr(VI) reduction</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium collagenovorans</td>
<td>NR_029246</td>
<td>Collagen decomposition</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium paraputrificum</td>
<td>AB536771</td>
<td>Chitin and polysaccharide degradation</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium straminisolvens</td>
<td>AR24829</td>
<td>Cellulose degradation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium thiosulfurodicans</td>
<td>AF317650</td>
<td>Thiosulfate and sulfur reduction</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Desulfotobacterium sp. Viet-1</td>
<td>AF357919</td>
<td>Sulfite and As(V) reduction; dechlorination</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Desulfurhabdus sp. DDT</td>
<td>EF424978</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Desulfofumarium baculatum</td>
<td>CP001629</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Desulfofumarium auripigmenti</td>
<td>NR_025551</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desulfofumarium orientis</td>
<td>NR_026411</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desulfofumarium sp. 44a-T3a</td>
<td>AR082482</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Desulfofumarium sp. Ox39</td>
<td>AB577273</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Desulfofumarium sp.</td>
<td>AJ133797</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lysobacter spp.</td>
<td>AB429529; AB429528; AB429072; FI845030</td>
<td>Antibiotics production; chitin degradation</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methylocapsa sp. KYG</td>
<td>FN433469</td>
<td>Methanotroph, nitrogen fixation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Paenibacillus barengoltzii</td>
<td>GQ284356</td>
<td>Saccharide fermentation</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sedimentibacter hongkongensis</td>
<td>AY571338</td>
<td>Cyanophycin degradation</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thermoactinomycetes vulgaris</td>
<td>EU430570</td>
<td>Polysaccharide hydrolysis and fermentation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thermodesulfobium narugense</td>
<td>NR_024789</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Uncultured Desulfofumaricaceae</td>
<td>FJ516880</td>
<td>Sulfate reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

a "-" means a representative bacterial species was absent from the column
b "+" means a representative bacterial species was detected in the column
**TABLE 2.4.** *p*-values from UniFrac significance tests on the 16S rRNA gene clone libraries in pair-wise comparisons of column substrate mixtures containing limestone (LS) and crab shell (CS) as indicated, with the balance composed of spent mushroom compost.

<table>
<thead>
<tr>
<th>Column</th>
<th>Bacterial clone libraries</th>
<th>Archaeal clone libraries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10% LS</td>
<td>5% CS</td>
</tr>
<tr>
<td>Control</td>
<td>0.15</td>
<td>0.05*</td>
</tr>
<tr>
<td>10% LS</td>
<td>0.92</td>
<td>0.00</td>
</tr>
<tr>
<td>5% CS</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>50% CS</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Shaded values indicate that the two microbial communities are significantly different (*P*-value < 0.05).
TABLE 2.5. Presence/absence of major functions assigned to bacterial 16S rRNA clones in sulfate-reducing columns treating AMD with substrates containing limestone (LS) and crab shell (CS) as indicated, with the balance composed of spent mushroom compost (SMC).

<table>
<thead>
<tr>
<th>Functions</th>
<th>Control</th>
<th>10% LS</th>
<th>5% CS</th>
<th>50% CS</th>
<th>100% CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose Degradation</td>
<td>-(^a)</td>
<td>+(^b)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fermentation</td>
<td>-</td>
<td>++(^c)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Sulfur Reduction</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulfate reduction</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Chitin Decomposition</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) “-” means a specific function was absent from the column
\(^b\) “+” means < 10% of total sequenced clones in a column were identified to possess a specific function
\(^c\) “++” means ≥ 10% of total sequenced clones in a column were identified to possess a specific function
FIG. 2.1. Preliminary Shannon Diversity Indices of fungal communities within the four sulfate-reducing columns treating AMD (control column is not included).
FIG. 2.2. Abundance of core bacterial phyla in 5 column samples featuring different substrate compositions as revealed by Sanger sequencing. The abundance is presented as a percentage in total effective bacterial clone reads in a sample.
FIG. 2.3. Maximum parsimony tree of bacterial 16S rRNA gene sequences from all sampled columns containing limestone (LS) and crab shell (CS) as indicated, with the balance spent mushroom compost (SMC). The label of each group (a total of 23) is given by the group name and the specific column(s) from which the sequences were obtained, followed by the number of the sequences in the corresponding column.
FIG. 2.4. Principal coordinate analysis (PCoA) of (a) total bacterial community and (b) total archaeal community based on 16S rRNA gene sequences, and (c) total fungal community based on 18S rRNA gene sequences in the five columns featuring different substrate compositions of limestone (LS) and crab shell (CS) as indicated, with the balance composed of spent mushroom compost (SMC). The Control contained no substrate.
FIG. 2.5. (a) Quantification of total bacteria, total archaea, and total fungi in the five sulfate-reducing columns featuring different substrate compositions by qPCR; (b) Comparison of copy numbers of different functional genes (*cel5*, *hydA*, *dsrA*, and *chiA*) in samples from columns containing 100% crab shell (CS) versus 10% limestone (LS) with the balance spent mushroom compost as determined by qPCR.
FIG. 2.6. Correlation of different substrate compositions with the copy numbers of *dsrA* genes as quantified by qPCR (copies/g), maximum alkalinity reached (mg/L as CaCO$_3$), and time prior to iron (Fe) and manganese (Mn) breakthrough (pore volumes) in the sulfate-reducing columns treating AMD. The 0% crab shell (CS) column refers to the 10% limestone (LS) column with 90% spent mushroom compost (SMC) in the substrate layer.
CHAPTER 3

Effect of Substrate Compositions and Varying Environmental Conditions on the Treatment Efficiency of Pilot-scale Field Reactors Remediating High Strength Acid Mine Drainage

3.1. Abstract

Crab shell (CS) amendments have previously been shown to enhance both the efficiency and longevity of AMD treatment in laboratory-scale systems. However, the effect of scale-up and environmental conditions on the performance and stability of AMD treatment systems amended with CS has not been fully evaluated at the pilot or field scale. In this study, four pilot-scale vertical flow pond (VFP) systems featuring different substrate combinations of CS and spent mushroom compost (SMC) and different underdrain materials were constructed and operated for 633 days at the Klondike-1 site in Cambria County, PA. Regular monitoring of the chemical performance data showed that the influent pH rapidly increased from < 3 to above 6.0 and was maintained circum-neutral by all VFPs. As compared to a control reactor containing SMC, the reactors containing CS promoted a higher and steadier alkalinity level of 428 ± 54 mg/L as CaCO₃ before the exhaustion. Furthermore, more thorough metal removal was generally observed in reactors containing CS than SMC: >94% for the removal of iron (Fe) and aluminum (Al) and >57% for manganese (Mn) with CS, compared to <93% removal of Fe and Al, and <28% for Mn with SMC. Geochemical modeling indicated that possible
mineral phases of precipitation were goethite and mackinawite for Fe, gibbsite for Al, and rhodochrosite for Mn. Sorption or absorption onto the surface of Fe and/or Al minerals could be another mechanism for metal removal. Noticeably, a reactor with 70% CS and 30% SMC displayed comparable treatment efficiency to a reactor with 100% CS, and more importantly, maintained steadier pH and metals removal rates throughout the seasonal changes of environmental conditions at the field site. This pilot study provides further evidence of the advantages of crab shell as a substrate amendment to promote more efficient and stable performance in passive AMD treatment systems.

3.2. Introduction

Acid mine drainage (AMD) is a common environmental problem caused by the exposure of reactive surfaces of rocks and minerals during or after mining activities. The release of large amounts of hydrogen ions (H⁺), sulfate (SO₄²⁻), and dissolved metals greatly impairs the quality of receiving watersheds and even public health. Upon investigation, AMD impacts between 15,000 and 23,000 kilometers (km) of streams in the United States (U.S.) (USEPA, 1994) and more than 8,850 km in the Pennsylvania alone (PA DEP, 2010). In central Pennsylvania, AMD from the abandoned Klondike mine in northeastern Cambria County adversely impacts Little Laurel Run, which eventually drains into the Chesapeake Bay via Clearfield Creek and the Susquehanna River. The contaminated Little Laurel Run has been classified as “not attaining” its quality as a Cold Water Fishery (Rose, 2005). Acidic discharge from The Klondike-1 mine (located at 40° 33’ N, 78° 30’ W) emerges from an abandoned strip mine on the
Clarion, Lower Kittanning, and Middle Kittanning coal seams with an average flow rate of 15 gallons per minute (gpm), acidity of 417 mg/L as CaCO$_3$, pH 3.4, 141 mg/L iron (Fe), 4 mg/L aluminum (Al), and 30 mg/L manganese (Mn) (Rose, 2008). Based on the risk classification categories for mine impacted water (MIW) established by the U.S. Office of Surface Mining and the Pennsylvania Department of Environmental Protection, the Klondike-1 discharge falls into the category of “high risk” due to its high concentration (> 50 mg/L) of combined metals (PA DEP, 2008).

The remediation of AMD involves three major processes: the neutralization of acidity, the reduction of sulfate, and the removal of dissolved metals. Current remediation techniques for AMD can be divided into two different approaches: ex-situ chemical treatment (active treatment) and in-situ biological treatment (passive treatment). Several studies have shown that passive treatment systems for AMD can be more cost-efficient than other treatment options due to their low operation and maintenance requirements as well as minimal production of metal-containing sludge (Berghorn and Hunzeker, 2001; Waybrant et al., 2002). Biological passive treatment remediates AMD in part by relying on the activity of sulfate-reducing bacteria (SRB) which can convert SO$_4^{2-}$ into hydrogen sulfide (H$_2$S), increase alkalinity, and as a result, precipitate dissolved metals as sulfides. Successful remediation of AMD requires the addition of organic carbon and nitrogen sources, but the most commonly used substrate—spent mushroom compost (SMC)—has sometimes shown compromised system longevity and performance (Benner et al., 1999; Blowes et al., 2000; Johnson and Hallberg, 2002). Recent studies have evaluated crab shell (CS), primarily composed of chitin (poly-N-acetylglucosamine), protein, and CaCO$_3$, for its ability to remediate AMD-impacted watersheds. It was demonstrated that
CS is a promising substrate amendment serving as a slow-release carbon and nitrogen source and neutralizing agent, and when combined with SMC in an optimal proportion, both high treatment efficiency and low cost can be achieved in AMD passive treatment systems (Daubert and Brennan, 2007; Grembi, 2011; Robinson-Lora and Brennan, 2009). In addition, treatment systems amended with crab shell have shown a remarkably high rate of removal for manganese (Daubert and Brennan, 2007; Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2009), an otherwise recalcitrant metal in AMD (Venot et al., 2008).

The original passive treatment system designed to remediate the Klondike-1 discharge consisted of a primary precipitation pond, a vertical flow pond (VFP), an aerobic settling pond, and a wetland. However, according to the monitoring data of the Clearfield Creek Watershed Association (CCWA), the VFP at the Klondike-1 site clogged within 9 months of operation due to the formation of iron precipitates on top of the organic substrate layer. Even after the removal of the Fe precipitates and the construction of two new oxidation ditches upgradient of the VFP, the system still does not meet effluent requirements for acidity and iron which were as high as 66 mg/L as CaCO$_3$ and 18 mg/L, respectively. The failure of the Klondike-1 system at treating high-risk acidic discharge is mainly attributed to high iron loadings and the shallow compost and limestone layer in VFP due to budgetary constraints. Nevertheless, when considering the outstanding treatment capacities of CS in lab-scale experiments, a possible solution for the field system may be to incorporate CS as an amendment to the existing VFP in proper proportions with SMC to ensure higher efficiency and longevity, while maintaining reasonable cost within the existing system footprint.
The aims of this study were to validate laboratory data which indicated that certain mixtures of CS and SMC would be effective at treating the high-strength AMD at the Klondike-1 site (Grembi, 2011), and to determine the effects of changing environmental conditions on treatment efficiency. For these purposes, a pilot-scale study was initiated by our research group at the Klondike-1 site in 2010 through the construction and monitoring of four VFP systems filled with different substrate and underdrain materials. Performance data over the course of the 633-day test were collected for each VFP system, including standard water quality electrode measurements, as well as alkalinity, acidity, and metals concentrations. Analysis of the resulting data should indicate the best combination of SMC and CS to be used in the substrate layer of VFP systems for treatment of high-risk AMD.

3.3. Materials and Methods

3.3.1. Sources of water and packing materials

The design, construction, sampling and measurement of chemical performance data were done by former researchers in Dr. Brennan’s group, and were described previously (Grembi, 2011). AMD Influent to the pilot reactors was drawn directly from the existing Klondike-1 primary precipitation pond at a distance of approximately 16 inches below the water surface through individual piping networks (Fig. 3.1). Onsite measurements were carried out to determine temperature, pH, conductivity, and oxidation-reduction potential (ORP) of the reactor influent during each sampling event. Samples were also collected and transported on ice to the laboratory for determination of
acidity, alkalinity, and ammonium within 4 hours, and preserved later for dissolved metal analyses. The analysis methods are provided in detail in section 3.3.4.

The materials used in the substrate layers of the pilot reactors were SC-20 CS (JRW Bioremediation), SMC and limestone (LS) chips (from the construction of the original field treatment system at the Klondike site), white silica sand (Seymore Brothers, Inc., Altoona, PA), and pea gravel (Somogyi’s Route 22 Supply, Ebensburg, PA). In reactors containing CS, white silica sand was added into the substrate layer to increase the permeability and to maintain a proper hydraulic retention time (HRT), while pea gravel was added to the top of the substrate layer to retain CS (density lower than water) inside the reactor. Two different underdrain materials used in the construction of the pilot reactors: limestone rock (AASHTO#1, 99.3% CaCO$_3$, New Enterprise Stone and Lime Company, Tyrone, PA), and sandstone rock (#4 Sandstone, 0% CaCO$_3$, Kinkead Aggregates, Homer City, PA) (Fig. I.1). Analyses of packing materials were conducted to determine the mass of total carbon and nitrogen and extractable metals (Table 3.2).

3.3.2. Pilot system design and setup

In this pilot study, four treatments featuring different compositions of substrate and underdrain materials were established on Aug. 27, 2010, as described below (percentages listed are by mass of packing materials):

a. “1-100CS”: 100% CS in substrate, LS rock underdrain;
b. “2-70CS”: 70% CS and 30% SMC in substrate, LS rock underdrain;
c. “3-70CS (SS)” : 70% CS and 30% SMC in substrate, SS rock underdrain;
d. “4-SMC”: 90% SMC and 10% LS chip in substrate, LS rock underdrain;
Each treatment consisted of a pilot VFP simulated by a 1000-gallon (3,785 L) polyethylene septic tank reactor fitted with an underdrain piping network, and two subsequent 350-gallon (1,135 L) aerobic settling ponds arranged in series. There were two manholes at the top of the septic tank of both sides to mimic the field VFP of AMD treatment system. From top to bottom, each pilot reactor was packed with a layer of pea gravel (≈ 7.6 cm thick), a substrate layer (≈ 91 cm thick), and an underdrain layer (≈ 61 cm thick) (Fig. 3.2). The pilot reactors in the four treatments contained three different SMC and CS combinations in the substrate layers, and two different underdrain materials (Table 3.1).

To facilitate the characterization of microbial communities within the pilot systems, special 3 inch × 3 inch extruded nylon mesh bags were made (Fig. I.2), filled with approximately 10 g of the substrate materials used in each tank, sealed with 10 lb. Triline® fishing line, and positioned approximately 8-10 inches below the surface of the organic substrate layer in each tank. A total of 32 microbial sample bags were placed into each reactor, and were periodically pulled out and transferred into sterile 50-mL tubes during most sampling events. The microbial samples were transported back to the lab on ice and frozen at -80 °C for later microbial analysis (Chapter 4).

After the construction of the pilot systems, the reactors were filled with water from the Klondike-1 discharge and flushed with roughly 4,300 gallons of water to remove fines in the packing materials. The systems were then incubated under static conditions for one week to allow acclimatization of microbial populations before introducing continuous flow. On Aug. 27, 2010, the regular operation of the pilot systems was initiated, and the influent flow rate set to 0.2 gpm (0.757 L/min) via an orifice to give
a design HRT in the substrate layer of 16 hours. The AMD water was piped into the reactor, distributed into 7.9 inches of free standing water, then flowed vertically downward through the pea gravel, substrate, and underdrain layers before being collected by an underdrain piping system at the bottom of the reactor. The underdrain piping system was constructed of PVC and consisted of a main header pipe in the center of the reactor with five perpendicular, 60 inch long branches spaced at approximately 46 cm intervals (Fig. 3.2). After exiting the underdrain system, the flow was directed through an open-channel, corrugated plastic pipe (to encourage aeration) into the first aeration pond (design HRT = 17.5 hrs), and then flowed from the first to the second aeration pond (design HRT = 25 hrs) before being discharged via a piping network into the original constructed wetland at the Klondike-1 site. On day 643 (May 31, 2012), all regular sampling and monitoring of the pilot systems were stopped due to declining performance and the observed exhaustion of substrate materials.

3.3.3. Sampling of pilot systems

Sampling of the pilot systems was carried out weekly for the first month (Sept. 2010), biweekly for the second month (Oct. 2010), and then monthly thereafter over the test period. Three locations were sampled from each pilot reactor system at every sampling event: influent to the VFP, effluent from the VFP, and effluent from the second aeration pond. Electrodes were used to measure pH, ORP, conductivity, and water temperature in the field. Additional water samples were transported on ice to the laboratory, and processed for acidity, alkalinity, dissolved metals, and other cations as described in section 3.3.4.
3.3.4. Analytical methods

Field measurements of pH, conductivity, and temperature were determined using an Oakton® Multi-Parameter Tester 35, while ORP was determined separately with an Oakton® Waterproof ORPTestr 10. During laboratory analyses, electrodes connected to a pH/mV meter (Accumet® Basic AB15, Fisher Scientific) were used to measure pH (Thermo-ORION), and ammonium (ISE ORION 9512) by comparing to 1 mg/L and 10 mg/L ammonium standards. Alkalinity and acidity were determined within 4 hours of collection by titrations according to the standard methods of APHA (APHA et al., 1998). The titration endpoints were 4.5 for alkalinity and 8.3 for acidity. Samples for dissolved metal analysis were filtered with a 0.45μm filter, acidified to pH < 2 with 60-70% HNO₃, and then sparged with lab air in a hood through a 25 gauge needle for 5 minutes to drive off hydrogen sulfide. Finally, dissolved metals in the pretreated samples were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) by the Materials Characterization Laboratory at The Pennsylvania State University.

Geochemical modeling of saturation indices for various mineral forms of metal precipitates under specific scenarios was processed through Visual MINTEQ (ver. 3.0, beta) based on the analysis results of chemical performance data (Appendix G). The Debye–Hückel equation was used to calculate the activity coefficient for ionic activities, and the charge difference was adjusted to lower than 10% by varying the SO₄²⁻/HS⁻ ratio of each scenario. In the computation of mass balance, Python Programming Language (ver. 2.7.5) was introduced to integrate the area under the curves of selected parameters via trapezoidal algorithm.
3.4. Results

3.4.1. Influent AMD and packing materials of pilot systems

The water quality of the influent AMD to the pilot systems at the Klondike-1 site was regularly monitored during each sampling event, and was characterized by low pH (2.89±0.24), positive ORP (503±51 mV), elevated acidity (241 ± 44 mg/L as CaCO₃), and high sulfate loading (1060±132 mg/L) (Table 3.3). Component and extractable metal analyses of packing materials revealed that crab shell had noticeably lower carbon to nitrogen ratio and higher percentage of calcium carbonate equivalence than SMC.

3.4.2. Pore volume estimation

Conservative tracer tests with sodium chloride were conducted at the beginning of system operation but failed due to the high concentration of background salts coming out of the pilot VFPs. Due to variable flow rates within each reactor over time and among the four reactors, an estimation of pore volumes (PV) was still necessary to compare the data more accurately based on the amount of AMD processed among different treatments. As previously demonstrated, one of the goals of this pilot study was to validate the results of the laboratory column study with the optimal substrate composition, and examine its performance in a field-scale pilot system; therefore, the pore volume of the pilot VFPs were calculated using the normalized effective porosities of the substrate mixtures from the column study. Assuming the porosity of the substrate layer in a specific pilot VFP was identical to that of the continuous-flow column with the same substrate composition, and the porosity of limestone or sandstone rocks was 0.45, theoretical effective pore
volumes and HRTs were calculated for the different pilot reactors (Table 3.4). The average HRT in the substrate layer of the three CS pilot reactors at the design flow rate of 0.2 gpm was 19.26 hrs. However, fluctuations in flow rate due to freezing temperatures in winter and clogging problems with iron oxides, the actual HRT in all reactors experienced changes of varying degrees over the entire test. To simulate the conventional VFP in the previously clogged, full-scale treatment system onsite, no silica sand was packed into the substrate layer of the SMC reactor, so the effective pore volume of the reactor (1204 L) was lower than the three containing CS, resulting in a relatively smaller HRT of 11.57 hrs in the substrate layer.

3.4.3. Flow rate and temperature
As affected by changing environmental conditions (seasonal temperature swings and variable flow rates), all four pilot-scale field systems at the Klondike-1 site experienced fluctuations in influent water quality over the course of the experiment. The temperature within the four pilot VFPs fluctuated with different seasons, up to 27 °C in summer while down to below zero in winter. During the winter months (December to February), the flow rates of all reactors decreased significantly, and stopped completely at times under freezing conditions (Fig. 3.3). In the summer months, flow into the reactors was also somewhat variable, and was slower than designed starting at day 258. Upon arrival for sampling on day 286 (Jun. 9, 2013) there was no flow into the four reactors, presumably due to iron oxide precipitation clogging the mesh fabric that was wrapped around the inlet hoses that were placed in the primary settling pond of the full-scale system. After
removal of the mesh fabric and installation of a new intake manifold on Jul. 29, 2013, the flow into all of the reactors resumed the design rate (0.2 gpm each).

The regular operation of the 3-70CS (SS) reactor with sandstone underdrain was on a 3-week (21 day) lag behind the other reactors due to clogging problems during the flushing and incubation phases.

Although the ORP of the influent AMD was above 500 mV, ORPs measured in the effluent of the CS reactors were generally below -200 mV during the entire course of the experiment, indicating the formation of a strong reducing environment inside the reactors that might be beneficial for the activity of SRB. In contrast, the reactor with SMC alone was observed to maintain ORPs above -100 mV in most sampling events, increasing 59 mV at day 643.

3.4.4. pH, alkalinity, and acidity

The chemical data of the four pilot VFPs reflected the effectiveness of different substrate compositions over the period of the test. Maximum alkalinity generation was observed in all pilot reactors immediately after incubation (0 PV), providing sufficient alkalinity to completely neutralize the influent acidity of 241 mg/L as CaCO$_3$ on average (Fig. 3.4). Noticeably, the maximum alkalinity in the two CS reactors with the limestone underdrain reached more than 5000 mg/L as CaCO$_3$ as compared to 260 mg/L as CaCO$_3$ in the SMC reactor. Meanwhile, it was observed that the start-up alkalinity after incubation in the 3-70CS (SS) reactor with SS rock underdrain was 1710 mg/L as CaCO$_3$, still more than 6.5 times higher than that of the SMC reactor with LS rock. Together, these observations further demonstrated the strong capacity of crab shell as a neutralizing
agent. After the incubation phase, the alkalinity of all reactors decreased and was maintained around 300 or 150 mg/L as CaCO₃, with or without crab shell respectively, until a sharp increase was observed at day 286 (between 100 and 150 PV in different reactors), due to a lack of flow caused by clogging of the influent lines. This clogging created a lower dilution ratio of alkalinity with fresh influent AMD, resulting in higher alkalinity concentrations inside the reactors. After flow was restored at 150 PV, the alkalinity in the three CS reactors gradually decreased and approached to zero by the end of the tests. In comparison, the exhaustion of alkalinity in the SMC reactor began at around 200 PV (due to the shorter HRT of this reactor) but was approximately equivalent in terms of total time to exhaustion as the CS reactors. The trend of acidity variation during regular operation of the four pilot VFPs mimicked that of alkalinity (Fig. 3.4).

The influent pH of 2.89 ± 0.21 was increased to above 6.0 by all pilot treatments, and stabilized at a circum-neutral level during the entire test (Fig. 3.4). The pH in the CS reactors was generally between 7 and 8, a little higher than the SMC reactor (pH 6.5 on average). Seasonal changes of influent water chemistry did not result in significant impact on the pH or alkalinity in the four pilot VFPs at the Klondike-1 site.

3.4.5. Metal removal

Besides influent data, concentrations of dissolved metals were determined at two sampling locations for each treatment: in the effluent of each pilot VFP, and after passive aeration and settling. All metals data below the detection limit (BDL) were assigned the value of the corresponding detection limit. Breakthrough curves showing normalized
concentrations of dissolved metals \( \frac{\text{Effluent concentration of dissolved metal (c)}}{\text{Influent concentration of dissolved metal (c_0)}} \) versus pore volumes were constructed to account for inconsistent influent quality and to enable an easier understanding of metal removal rates in different pilot treatment units at different sampling points. A normalized concentration of 0 means complete removal of the specific metal by the treatment unit, while a value of 1 indicates 0% treatment efficiency of dissolved metals. A normalized concentration of 0.5 represents the breakthrough point of the dissolved metal, which is defined as an effluent concentration exceeding 50% of the influent concentration. As water chemistry inside the pilot reactor changed over time, desorption and/or re-dissolution of certain metal precipitates might occur, which would render the normalized concentration of the metal larger than 1.

All of the CS reactors were able to promote complete removal of dissolved Fe (>99%) for almost 400 days (151 to 166 PV), and no breakthrough was detected over the entire test (Fig. 3.5). The SMC reactor accomplished >95% of Fe removal during most sampling events, but experienced a breakthrough at 157 PV (day 286) when there was no flow, suggesting a change in the water chemistry under the flow shock. This unstable performance of the SMC reactor potentially reflected an unstable activity of microorganisms inside the reactor since the pilot VFPs were partially microbiological driven. The Fe data after passive aeration showed that all aeration ponds achieved complete Fe removal (>99.9%) during the course of the tests except for the pond located downstream of the 1-100CS reactor at 180 PV when the Fe removal rate was slightly lower (≈ 98.8%) (Fig. D.1). The relatively lower Fe removal was also observed in the effluent of this reactor at the same sampling time (180 PV), indicating that the strong
reducing conditions in the effluent of the reactor probably impaired the establishment of an oxidizing environment preferred for Fe precipitation in the aeration pond. A similar inhibition of Fe removal in the effluent of a column containing 100% CS was also observed previously (Grembi, 2011).

The removal of Al in the first two weeks of reactor operation was almost complete (>99%), then decreased to around 85% from day 14 to day 230 (10 to 140 PV), and finally approached complete removal again at day 398 (Fig. D.2). Highly similar breakthrough curves were observed before and after passive aeration, revealing that contact with oxygen in the passive aeration and settling ponds did not promote the removal of dissolved Al, as would be expected due to the formation of Al(OH)$_3$ within the reactors (Appendix G). Zn removal was somewhat stable before 100 PV (>90%) in all pilot VFPs, but fluctuated thereafter (Fig. D.4). Nevertheless, there was no breakthrough of Zn in any of the reactors at any sampling point.

The efficiency of Mn removal in the pilot VFPs was closely related to the proportion of CS in the substrate layer and alkalinity generation, which was consistent with the observations of previous studies (Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2009). An average of 80% Mn removal was sustained for more than 100 PV during the pseudo-steady state of the three CS reactors, and breakthrough only occurred around 30 PV and after 150 PV where sharp decreases in net alkalinity were observed (Fig. 3.5). Interestingly, the maximum Mn removal (>90%) in the reactors containing CS took place at 0 PV and around 120 PV, corresponding to the two peak values in the alkalinity curves at the same pore volumes. In contrast, the removal of Mn in the SMC reactor was not as good with an average removal of <40% during regular
operation. The breakthrough of Mn above 50% of the influent level occurred only after 21 days (23 PV) in the SMC reactor, meanwhile, the normalized Mn concentration \(\left(\frac{\text{Mn}}{\text{Mn}_0}\right)\) exceeded 1.0 from 83 to 140 PV and again after 261 PV, indicating that the reactor was actually adding dissolved Mn into the effluent due to desorption or re-suspension of Mn precipitates. No obvious contribution of passive aeration to Mn removal was detected over the course of the pilot tests.

3.4.6. Other cations, phosphorus, and nitrogen

Extractable metal analyses of substrate and underdrain materials revealed the presence of a considerable amount of cations in these commercial materials used to pack the pilot VFPs at the Klondike-1 site. Although some cations such as calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na) are trace elements essential to support the growth of living organisms, the presence of these cations above toxic levels in the effluent of the pilot treatments would impair the water quality of the receiving stream and pose threats to aquatic life. In order to comprehensively evaluate the risk associated with the cations and nutrients, continuous monitoring of Ca, Mg, K, Na, phosphorus (P), and ammonium was carried out in the effluent before and after passive aeration over the entire length of the pilot tests.

The maximum concentrations of all cations measured in the four pilot VFPs were achieved immediately after the incubation phase, and gradually stabilized after 5 PV. Although it started at a lower level (350 mg/L), the average concentration of dissolved Ca in the effluent of SMC reactor (270 mg/L) during pseudo-steady state was comparable to that of the three CS reactors (283 to 296 mg/L) (Fig. E.1). The maximum concentration
of dissolved Mg during incubation in SMC reactor (139 mg/L) was only about one third that of the 1-100CS and 2-70CS reactors. Moreover, the relatively high concentrations of Mg in the effluent of the four pilot reactors were mainly attributed to the high influent Mg level (Fig. E.3). The Na and K concentrations in all pilot VFPs was relatively stable during the tests (Fig. E.2 and Fig. E.4).

Phosphorus and ammonium levels in the pilot reactors fluctuated over time. The average P concentration of the 1-100CS reactor (12.10 mg/L) was over 4 times higher than that of the 4-SMC reactor (2.85 mg/L). A similar phenomenon was observed for ammonium concentrations: the three CS reactors achieved values of 19.2 to 26.4 mg/L \(\text{NH}_4^+\) nearly 2 times higher than that achieved in the 4-SMC reactor (12.3 mg/L \(\text{NH}_4^+\)) (Fig. 3.6). This was probably due to the inherently higher nitrogen content of CS materials than SMC. During the initial incubation phase, high concentrations of nutrients ensured the rapid development of microbial communities and thus decreased the duration of lag phase. Nevertheless, the potential impact of treated AMD containing high concentrations of cations and nutrients on the receiving watershed should be evaluated when designing a full-scale bioremediation system with CS.

3.5. Discussion

3.5.1. Alkalinity generation and AMD neutralization

Generation of sufficient alkalinity is very critical for a better performance of AMD bioremediation system in terms of acidity neutralization, as well as sulfate and metal removal. Alkalinity can be generated through two basic mechanisms in pilot-scale
VFPs treating AMD: abiotic dissolution of calcium carbonate from the substrate matrix, and biotic pathways of VFA-producing fermentation and sulfate reduction (Robinson-Lora and Brennan, 2009). In order to evaluate the potential of different substrate matrices for alkalinity production, mass balances were carried out on the theoretical quantity of CaCO$_3$ contained in the packing materials and the experimental mass of CaCO$_3$ calculated from the measured alkalinity and acidity data in each pilot VFP (calculation details in Appendix F).

Table 3.5 summarizes the results of the CaCO$_3$ mass balance. First of all, when looking at the data in the three pilot reactors with a LS underdrain, the actual amount of CaCO$_3$ generated only accounted for 7.8%, 6.9%, and 2.5% of total theoretical CaCO$_3$ mass in the 1-100CS, 2-70CS, and 4-SMC reactors, respectively. Specifically, the limestone rock underdrain of these reactors contributed >92% of total theoretical quantity of CaCO$_3$; however, its potential for alkalinity generation during AMD treatment was questionable considering the high bulk density (around 1.6 mg/L) and low surface area (Ziemkiewicz et al., 1997). Moreover, it has been noticed that metal precipitation on the surface of limestone rocks could make it more difficult for the CaCO$_3$ to contact with water, thus possibly blocking its further dissolution (Ziemkiewicz et al., 1997). Noticeably, the carbonates associated with CS were observed to be more accessible and reactive than those in SMC and limestone chips due to the higher surface area of crab shell and its biogenic character (Robinson-Lora and Brennan, 2011; Robinson-Lora and Brennan, 2009). Indeed, a strong correlation ($R^2=0.98$) between alkalinity generation and the proportion of CS in the substrate layer was obtained in this pilot test, the same as observed in previous research (Grembi, 2011; Newcombe and Brennan, 2010).
Secondly, when comparing the two 70% CS reactors with different underdrain materials, the actual contribution of limestone rock to alkalinity generation can be easily evaluated (Table 3.5). Based on experimental data, the 3-70CS (SS) reactor with the sandstone rock underdrain produced a total amount of 158 kg CaCO$_3$ during the entire pilot test (85% of theoretical mass), only 19 kg less than that in the 2-70CS reactor with limestone rock (177 kg). This provides another line of evidence that although the CaCO$_3$ equivalence of limestone rock was extremely high (2396 kg), it contributed very little of the alkalinity levels in the pilot VFPs. As previously demonstrated, the chemical performance of the 3-70CS reactor with sandstone mimicked the 2-70CS reactor with LS underdrain. Therefore, if the unit price of limestone rocks were higher, the sandstone rocks could be considered as fractional substitutes to ensure similar treatment efficiency and, at the same time, lower the construction costs of passive AMD treatment systems.

Finally, it has been pointed out that biological processes of fermentation and sulfate reduction also contributed to the total alkalinity in the treatment system but to varying degrees (Grembi, 2011; Robinson-Lora and Brennan, 2009). Since previous microbial studies showed that a higher abundance of fermenters and SRB were present in columns featuring larger fractions of CS (Chapter 2), the proportion of alkalinity generated through microbial metabolism should be higher in the three CS reactors. If proven true at the field scale, this would make CS even more advantageous than the traditional SMC and limestone substrate in its potential to maximize alkalinity generation through the dissolution of integral carbonate content of the substrate, while at the same time promoting fermentative and sulfate-reducing activities.
3.5.2. Metal precipitation and sulfate reduction

One of the major goals for AMD remediation is the removal of dissolved metals, especially of primary metals such as Fe, Al, and Mn which are usually present in a considerable amount in AMD. Previous studies showed that metal removal capacity in the AMD treatment units containing >50% CS was higher than that in the treatment with traditional SMC and limestone substrate (Grembi, 2011; Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2009). To better understand the proportions of dissolved metals retained in each pilot VFPs, mass balances were performed on the total mass of influent and effluent metals; the difference of the two denoted the mass of a specific metal being retained in the pilot reactor (details are in Appendix H). Removal of Fe, Al, and Zn in all pilot VFPs achieved comparable rates. Specifically, Fe removal was quite thorough with >99% retained in the three CS reactors and 95% in the 4-SMC reactor (Fig. 3.7). In addition, more than 90% of Al was retained in the four pilot VFPs, followed by Zn of approximately 70%. The Mn removal varied among different substrate compositions with the lowest removal rate of 19% in the 4-SMC reactor. Noticeably, the percentage of Mn retained in the 2-70CS reactor was more than 2X that of the 1-100CS reactor. This observation indicated that the performance of the AMD-treating reactor with 70% CS and 30% SMC as substrates might be comparable to or even more efficient than the 100% CS reactor in terms of metal removal.

Based on the chemical performance data, the operational process of the pilot tests could be classified into four stages: start-up (day 0 to 42), pseudo-steady state (day 56 to 312), declining (day 340 to 475), and end (day 633) phases (Table 3.6). Each phase exhibited different characteristics with regard to alkalinity generation, metals removal,
and nutrients production. For the purpose of investigating different mineral forms of metal precipitates, geochemical modeling by Visual MINTEQ was carried out using the given experimental data in the four pilot VFPs under the three selected phases of system operation: immediately after incubation (day 0), pseudo-steady state (day 56 to 312), and declining phase (day 340 to 475). The SO$_4^{2-}$/HS$^{-}$ ratios were used to adjust the charge balance to less than 10% during modeling so that an approximate sulfate removal rate could be predicted for different scenarios in different pilot reactors. Details of the scenario definitions and geochemical modeling work are provided in the Appendix G.

Complete and efficient removal of the two most abundance metals, Fe and Al, occurred immediately after incubation (0 PV), and was sustained in all pilot VFPs over the entire experiment. Results of geochemical modeling revealed no significant difference of saturation indices (SI) of metal minerals relative to variable input of experimental data in different pilot VFPs under the same operational stage. Therefore, the 2-70CS reactor was selected as a representative to illustrate the changes in mineral precipitation under the three operational phases (Fig. 3.8).

In general, the SI of different Fe and Al minerals varied as pH changed. Two main mechanisms can contribute to Fe removal in the pilot VFPs: precipitation as iron sulfides or (oxy)hydroxides (Larese-Casanova and Scherer, 2007; Majzlan and Myneni, 2005; Sanchez-Espana et al., 2011; Silvester et al., 2005), and sorption onto the surface of Al or Fe minerals (Jong and Parry, 2004). Visual MINTEQ predicted the formation of goethite (FeOOH) and mackinawite (FeS) starting at around pH 3.0 and 5.5, respectively, during start-up, steady, and declining phases. Interestingly, goethite, a stable mineral phase, reached positive SI values even at pH 3.0 given the experimental conditions,
probably due to the high alkalinity level detected in the pilot tests. Similar observations of goethite formation have been made by others at pH 4.0 and pH 12 during the storage of ferrihydrite in aqueous suspensions at 24 °C (Schwertmann and Murad, 1983). In addition, positive SI values were observed for other ferric (hydr)oxides such as jarosite (KFe$_3$(SO$_4$)$_2$(OH)$_6$), ferrihydrite, and hematite (Fe$_2$O$_3$). It was also reported that in acid waters rich in Fe(III), sulfate, and K/Na, precipitation of jarosite occurs at pH < 3, followed by schwertmannite with intermediate pH, and then ferrihydrite at higher pH (> 6); moreover, metastable schwertmannite tends to transform to goethite or hematite over time (Majzlan and Myneni, 2005). This corresponded with the observation that only the more stable Fe minerals, like goethite and mackinawite, were predicted by geochemical modeling to precipitate in our pilot system. Some ferrous sulfides like FeS and mackinawite have been shown to be transient and precursors of more stable phases like pyrite (FeS$_2$). In the CS reactors featuring higher sulfate removal rates and lower ORP (<150 mV), mackinawite was predicted to start precipitating at lower pH values (~5.2) due to higher concentrations of sulfide and ferrous iron under this more reducing environment.

As previously noted, the removal of Al in the four treatments was nearly complete (>90%) even though the influent Al concentration was relatively low (2.80 ppm). The SI curves of Al minerals such as alunite (KAl$_3$(SO$_4$)$_2$(OH)$_6$), amorphous basaluminite (Al$_4$(OH)$_{10}$SO$_4$), and gibbsite (Al(OH)$_3$) had strong correlations with pH. The geochemical model reported the precipitation of alunite from pH 4.3 to 5.2, followed by gibbsite at pH 5.5 through above 8.5. Throughout the entire duration of the pilot tests, pH
levels were above 6.0 regardless of the exhaustion of alkalinity after 150 PV, making gibbsite the most likely mineral phase in the reactors.

In mixing zones exhibiting a strong pH gradient (e.g. from acidic to circum-neutral), Fe and Al minerals can precipitate fast and promote a significant decrease in metal concentrations (Sanchez-Espana et al., 2011). Furthermore, due to their inherent characteristics of low crystallinity, small particle size, and large specific surface area, these minerals are efficient absorbents of dissolved compounds such as metallic cations (Cu$^{2+}$, Pb$^{2+}$, Zn$^{2+}$, etc.), anions (SO$_4^{2-}$, PO$_4^{3-}$, AsO$_4^{3-}$, etc.), and/or organic acids. However, the absorption process was shown to be largely dependent on pH, temperature, ORP, and solution compositions (Majzlan and Myneni, 2005; Sanchez-Espana et al., 2011). Indeed, sorption and absorption were recognized as possible mechanisms for the removal of Mn and Zn in previous batch and column tests treating AMD (Grembi, 2011; Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2011; Robinson-Lora and Brennan, 2009). Although positive SI of Zn minerals such as sphalerite ((Zn,Fe)S) was obtained above pH 3.3 and its maximum value occurred at around pH 5.8, no precipitation form of Zn was predicted by geochemical modeling under the given experimental conditions in the pilot tests. Therefore, a possible mechanism for Zn removal observed in the pilot VFPs might be sorption or absorption onto the surface of Fe and/or Al minerals.

Mn represents the most recalcitrant metal in this system, and the removal of Mn has often been recognized as a major challenge in AMD treatment systems (Bamforth et al., 2006; Sikora et al., 2000; Steed et al., 2000; Willow and Cohen, 2003). It has been previously demonstrated that Mn removal rates have a positive correlation with alkalinity generation during AMD bioremediation process (Newcombe and Brennan, 2010;
Similarly, in this pilot test, the highest removal rates of Mn were observed whenever alkalinity was maintained at relatively higher levels (Fig. 3.5). According to the results of mass balance calculations, percentages of Mn retained in the pilot VFPs containing CS were generally higher than that in the 4-SMC reactor. Geochemical modeling indicated that when alkalinity in the 2-70CS reactor dropped over time from 5,095 mg/L as CaCO$_3$ to 322 mg/L as CaCO$_3$ to 18 mg/L as CaCO$_3$, the pH at which rhodochrosite (MnCO$_3$) started to form changed from 6.4 to 7.9 to 8.5, respectively. Recent studies have indicated that dissolved Mn was removed from the liquid phase through sorption and/or co-precipitation in biological passive AMD treatment systems amended with crab shell (Robinson-Lora and Brennan, 2011; Robinson-Lora and Brennan, 2010), which could be another mechanism for the observed removal rate of Mn in our pilot treatments.

In order to approximate sulfate removal rates in different pilot reactors at different operational phases, SO$_4^{2-}$/HS$^-$ ratios were used to adjust charge balance to less than 10% during geochemical modeling by Visual MINTEQ. The results showed that during pseudo-steady state (day 56-312), the highest sulfate removal rate occurred in the 1-100CS reactor (~35%) whereas the lowest occurred in the 4-SMC reactor (~15%). Comparable removal rates of sulfate have been reported previously in anaerobic AMD treatment systems containing crab shell (Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2009). This agrees well with the observation of a recent microbial study that the sulfate-reducing column with 100% CS as substrate supported the growth of more diverse SRB, and the copy number of SRB in this column was 3.91 times higher than that in the column with traditional SMC and limestone substrate (Chapter 2).
Remarkably, at day 0 (immediately after incubation), 75% of sulfate removal was predicted by geochemical modeling in the 2-70CS reactor, the highest among all pilot reactors. Besides biological sulfate reduction, sorption and/or absorption onto Fe and/or Al (hydr)oxides might be alternative removal mechanisms for sulfate in anaerobic AMD treatment systems (Christensen et al., 1996; Rose and Elliott, 2000; Sanchez-Espana et al., 2011).

3.5.3. Impact of cations and nutrients

Release of nutrients and cations from the pilot VFPs could potentially damage water quality and aquatic life in the receiving stream through negative effects caused by eutrophication and/or toxicity. This is especially a risk with CS, in which mass of C, N, P, Ca, K, Mg, and Na is higher than SMC and limestone as revealed by compost and extractable metal analyses (Table 3.3). However, it should be noticed that higher content of nutrients and trace metals in crab shell could accelerate the establishment of a healthier and more diverse microbial community, and shorten the lag phase during incubation, which would be beneficial for overall system performance and stability. Moreover, through the initial flushing before operation, most mineral fines were washed out of the pilot systems, and the initial high concentration of nutrients and trace metals were lowered to a stable state as the continuous-flow condition was established. In order to get a basic idea of how much nutrients and trace metals in the effluent of the pilot VFPs were released from the packing materials, mass balances were carried out on both influent and effluent concentrations of ammonium, P, Ca, K, Mg, and Na (Appendix H).
Mass balance of cations in the pilot systems indicated that small portions of K, Mg, and Na in the effluent originated from the dissolution or degradation of the packing materials (Table 3.7). It was observed that dissolved Mg in the effluent from all four treatments was above the tolerance limit of 15 mg/L for fish culture as recommended by Meade (1989) throughout the entire experiment, but this was mainly attributed to the comparably high concentration of Mg in the influent AMD (Fig. E.3). More than 50% of effluent Ca mass was released by the substrate and underdrain layers, and the percentages were even higher for P (> 90%) and ammonium (>70%) in the CS reactors. However, it was suggested that the formation of soluble metal-ligand complexes in aqueous phases could diminish the negative effects of metal species on aquatic life, so active free ion concentrations are more suitable to evaluate the actual toxicity and impact of cations (Viadero et al., 2004). For this purpose, the speciation of cations was performed through Visual MINTEQ given the experimental data during the pseudo-steady state phase (day 56 to 312) at fixed average pH and alkalinity specific to each pilot VFPs. Results of predicted average free ion and average active free ion concentrations are provided in Table 3.8.

Among all cations, Mg and P were the only two species of which the average active free ion concentrations in the effluent of all pilot VFPs exceeded the fish tolerance limit of 15 mg/L and the EPA criterion of 0.05 mg/L, respectively. The difference was that the relatively high concentration of active free Mg\(^{2+}\) was a result of the high concentration in the influent AMD, whereas the high concentration of active free P mainly came from the release of packing materials.
Finally, ammonium levels of all pilot VFPs during the tests (Fig. 3.6) were above the chronic toxicity criterion for aquatic life of 1.9 mg/L as total ammonia nitrogen (TAN) (USEPA, 2013). The three CS reactors reached ammonium concentrations higher than the acute toxicity criterion of 17 mg TAN/L before 10 PV and again after 100 PV. It was observed that the packing materials contributed greatly to the overall ammonium loads in the effluent of the pilot VFPs (Table 3.7). This was especially the case for the CS reactors in which the percentage of total nitrogen was almost 3 times higher than that of SMC. However, it should be noticed that the 2013 recommended aquatic life criteria for ammonia by EPA were based on the most sensitive endpoints (aquatic lives) known at the time. Moreover, free ammonia is known to be more toxic to aquatic life than the ionized ammonium, and their speciation is greatly dependent on the pH and temperature. Both chronic and acute criteria by the EPA are normalized concentrations at pH 7.0 and 20 °C; therefore, the actual toxic effects of ammonium in the four reactors need to be re-evaluated for specific pH, temperature, and most probable fish species present in the receiving watersheds.

3.5.4. Treatment efficiency and stability

Although net alkalinity in all pilot VFPs was gradually approaching zero, acidity neutralization was still in progress and pH was still sustained above 6.0 at the end of regular operation and monitoring (day 633). It was speculated that the pilot systems were still functioning after day 633 in terms of alkalinity generation, acidity neutralization, and metal removal; therefore, the true longevity of the pilot tests is not known. It was observed that compared to flow rate, the seasonal changes of ORP, salinity, and
temperature in the influent AMD had negligible impact on the performance of the pilot VFPs at the Klondike-1 site. All pilot reactors sustained a reducing environment with temperatures above 5 °C even in the coldest months. In contrast, the fluctuation of flow rates during the operation of the pilot tests greatly affected the reactor performance with regard to alkalinity generation, acidity neutralization, and the removal of some metals like Fe and Mn in specific reactors.

At day 286, the flow rate in all pilot VFPs was zero due to the clogging of inlet hoses with iron oxides, and consequently all reactors experienced high peak values in alkalinity generation, subsequently resulting in an increased Mn removal rate. Specifically, the 4-SMC reactor exhibited compromised treatment efficiency in terms of lower alkalinity, earlier exhaustion of alkalinity, and lower removal capacity of Fe, Mn, and sulfate over the entire test. In addition, although the 1-100 CS had advantages in the total amount of alkalinity generated and stronger reducing environment created, the 2-70CS reactor still displayed comparable treatment efficiency, and more importantly a more stable pH and removal of metals throughout the seasonal changes of environmental conditions at the field site.

3.5.5. Effects of scale-up on system performance

This pilot test was initiated in part to evaluate the effects of scale-up on the treatment efficiency of high-strength AMD at the field site by comparing to the experimental data of the previous column study (Grembi, 2011) with the same AMD influent source and organic substrate compositions. To last through several seasons, yet still remain within the budgeted project lifetime, the pilot systems were designed to
exhaust within 1.5 to 2 years (assuming that 1 g CS treats 1 L AMD), whereas the columns were designed to exhaust within approximately one semester (i.e., 4 to 6 months). To ensure proper hydraulic residence times, silica sand was added to both systems as an inert filler at a mass ratio of 9:1 of sand to organic substrate in the columns, versus nearly 1:1 in the pilot VFPs. Since the total effective PV of each pilot VFP was over 5500 times higher than that of each column reactor, the total mass of substrate in each pilot VFP was at least 1043 times higher than in the column test. To account for the high scale-up factors and differences in system design, the performance of the two systems at each sampling event was compared based on normalized treatment capacity as cumulative AMD volume treated per mass of organic substrate (mL/g).

A strong positive correlation between alkalinity generation and the proportion of CS in the substrate layer was obtained in the pilot test, similar to the findings of the previous column study (Grembi, 2011). However, alkalinity in the three pilot reactors containing CS was maintained above 200 mg/L as CaCO₃ longer than in the column test: 220 mL/g in the pilot VFPs, as compared to less than 150 mL/g in the 100% CS column and 200 mL/g in the 70% CS column (Grembi, 2011). Even with the traditional 90% SMC and 10% limestone as substrates, the 4-SMC pilot VFP was able to sustain an alkalinity level of > 100 mg/L as CaCO₃ for approximately twice as long as the column test (based on normalized treatment capacity). The high alkalinity level maintained in the pilot VFPs greatly benefited the abiotic processes of acidity neutralization and metal removal, as well as the biologically mediated processes of biopolymer decomposition, fermentation, and sulfate reduction. All pilot VFPs continued to show buffering capacity even at the end of the tests: the pH was above 6.0 and the acidity was well below the
influent level. In contrast, the breakthroughs of acidity to the influent level and pH below 5.0 were observed around 700 mL/g in the previously constructed columns.

Higher total metal removal was observed in the pilot VFP, especially for Fe and Mn, as compared to the columns with the same substrate composition (Grembi, 2011). All of the CS reactors were able to promote complete removal of dissolved Fe (>99%) for almost 400 days (291 to 349 mL/g), and no breakthrough (to 50% of influent concentration) was detected over the entire test. This was not the case for the three columns with 100% CS, 70% CS, and 90% SMC as substrates: near complete removal of Fe was observed only before 150 mL/g, and breakthrough occurred after 300 mL/g (Grembi, 2011). Moreover, more efficient and stable Mn removal was detected in the pilot systems containing CS with an average removal rate of 80% for a period of 220 mL/g on average. Based on a metals mass balance, the percentages of Fe, Mn, Al, and Zn retained in the pilot reactors were much higher than those in the continuous-flow columns. Apart from the higher alkalinity and pH levels, the better performance of the pilot reactors can also be a result of longer HRT calculated from actual flow rate. The average operational HRT in the substrate layer of the four pilot VFPs during the entire tests was 24 hrs, approximately 1.5 times higher than the average HRT value of the three previously constructed columns. This clearly validates the importance of maintaining an appropriate HRT to promote effective metal removal. Nevertheless, similar metal removal mechanisms including sorption and precipitation, as well as similar contribution of substrate materials to cations and nutrient levels were obtained in both pilot and column tests. Overall, the four pilot VFPs in this study exhibited better performance in
their capabilities of sustaining a longer period of net alkalinity generation and acidity neutralization, and more thorough metal removal rates, especially for Fe and Mn.

As can be seen in this study, CS has many attractive characteristics for use as an alternative substrate in passive AMD treatment systems, such as higher nitrogen content, larger specific surface area, and higher reactivity of chitin-associated CaCO$_3$ (Robinson-Lora and Brennan, 2009). All these outstanding features make CS more advantageous in sustaining a strongly reducing and highly buffered environment to support a healthier and functionally diverse microbial community, thus enhancing the performance and stability of anaerobic AMD treatment systems. Moreover, since CS can be obtained from the waste products of the seafood industry, given the considerable number of mines located near coastal areas all over the world (Fig. K1), the application of CS to the bioremediation of mine sites could be economically beneficial due to the reduced expenses for CS processing and transportation. On the other hand, CS has some drawbacks such as a higher content of cations and nutrients, and higher unit price as compared to traditional cellulosic substrate like SMC. Therefore, it may be more feasible and economical to use CS as a fractional amendment instead of as a sole substrate, while still improving the efficiency and stability of field systems treating AMD.

3.6. Conclusions

The findings of this study indicated that the pilot VFPs containing CS sustained more efficient and stable treatment of AMD under changing environmental conditions at
the Klondike-1 site. To summarize, the three crab shell reactors (1-100CS, 2-70CS, and 3-70CS (SS)):

a. Sustained higher alkalinity (around 300 mg/L as CaCO$_3$), slower alkalinity exhaustion (after day 442), and steady acidity neutralization as compared to the 4-SMC reactor;

b. Rapidly increased the pH of the influent AMD to above 6.5 and maintained it at the circum-neutral level over the entire test;

c. Promoted more complete metals removal and retained a higher mass of dissolved metals during the operation of the pilot tests. No breakthrough was observed for Fe, Al, and Zn. Geochemical modeling indicated possible phases of precipitation were goethite and mackinawite for Fe, gibbsite for Al, and rhodochrosite for Mn. A possible mechanism for Zn removal was sorption or absorption onto the surface of Fe and/or Al minerals.

d. Achieved higher sulfate removal rates (>20%) under pseudo-steady state as predicted by geochemical modeling.

These observations make CS an advantageous substrate for use in passive AMD treatment systems due to its ability to simultaneously serve as slow-releasing electron donor and neutralizing agent. In addition, the 70CS pilot VFP was shown to maintain a more stable performance while possessing comparable treatment efficiency to the 100CS reactor over varying environmental and operational conditions during the pilot study. Therefore, the use of CS as a fractional amendment to the substrate mixture in passive treatment systems for AMD or other environmental contaminants will be more attractive when considering both the costs and system performance. Microbial studies documented
in a companion paper (Chapter 4) characterize the changes of the microbial communities in the different pilot VFPs under field conditions to further aid in the understanding of reactor performance and to potentially serve as guidance for future design and operation strategies.

3.7. Acknowledgements

This work was supported in part by the National Science Foundation, CAREER Award No. CBET-0644983. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. The Foundation for Pennsylvania Watersheds is also thanked for their support of pilot system materials and construction.
3.8. References


Grembi, J.A. 2011. Remediation of high-strength mine impacted water with crab shell substrate mixtures: laboratory column and field pilot tests. in: *Civil and*

University park.


**TABLE 3.1.** Compositions and masses of substrate layer components in pilot-scale VFPs treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Reactor name</th>
<th>Substrate composition</th>
<th>Underdrain</th>
<th>Mass of substrate layer components (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>100% CS</td>
<td>LS</td>
<td>Crab shell (CS) 685</td>
</tr>
<tr>
<td>#2</td>
<td>70% CS/30% SMC</td>
<td>LS</td>
<td>577</td>
</tr>
<tr>
<td>#3</td>
<td>70% CS/30% SMC</td>
<td>SS</td>
<td>577</td>
</tr>
<tr>
<td>#4</td>
<td>90% SMC/10% LS</td>
<td>LS</td>
<td>0</td>
</tr>
</tbody>
</table>
**TABLE 3.2.** Compost and extractable metal analyses of packing materials in pilot-scale VFPs treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Substrate</th>
<th>SMC</th>
<th>LS</th>
<th>Sand</th>
<th>LS rock</th>
<th>Sandstone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen (%)</td>
<td>Crab shell</td>
<td>4.41</td>
<td>1.39</td>
<td>&lt;0.1</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Carbon (%)</td>
<td>Crab shell</td>
<td>21.83</td>
<td>15.38</td>
<td>11.33</td>
<td>0.02</td>
<td>10.06</td>
</tr>
<tr>
<td>Carbon:Nitrogen ratio</td>
<td>Crab shell</td>
<td>5</td>
<td>11</td>
<td>N/A</td>
<td>2</td>
<td>N/A</td>
</tr>
<tr>
<td>Calcium carbonate equivalence (%)</td>
<td>Crab shell</td>
<td>27</td>
<td>6.8</td>
<td>91</td>
<td>2.1</td>
<td>99.3</td>
</tr>
<tr>
<td>Aluminum (mg/kg)</td>
<td>Crab shell</td>
<td>1.68</td>
<td>11.58</td>
<td>BDL</td>
<td>28.59</td>
<td>BDL</td>
</tr>
<tr>
<td>Calcium (mg/kg)</td>
<td>Crab shell</td>
<td>28423</td>
<td>12280</td>
<td>1815</td>
<td>364</td>
<td>20883</td>
</tr>
<tr>
<td>Cobalt (mg/kg)</td>
<td>Crab shell</td>
<td>BDL</td>
<td>BDL</td>
<td>0.033</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>Iron (mg/kg)</td>
<td>Crab shell</td>
<td>2.18</td>
<td>234.02</td>
<td>103.58</td>
<td>7.74</td>
<td>4.62</td>
</tr>
<tr>
<td>Magnesium (mg/kg)</td>
<td>Crab shell</td>
<td>2444</td>
<td>686</td>
<td>967</td>
<td>33.50</td>
<td>212</td>
</tr>
<tr>
<td>Manganese (mg/kg)</td>
<td>Crab shell</td>
<td>2.07</td>
<td>42.26</td>
<td>1.18</td>
<td>0.66</td>
<td>0.94</td>
</tr>
<tr>
<td>Phosphorus (mg/kg)</td>
<td>Crab shell</td>
<td>2211</td>
<td>1231</td>
<td>11.34</td>
<td>55</td>
<td>0.40</td>
</tr>
<tr>
<td>Potassium (mg/kg)</td>
<td>Crab shell</td>
<td>1338</td>
<td>492</td>
<td>11.61</td>
<td>12.64</td>
<td>5.84</td>
</tr>
<tr>
<td>Sodium (mg/kg)</td>
<td>Crab shell</td>
<td>2641</td>
<td>69</td>
<td>27.35</td>
<td>66</td>
<td>16.82</td>
</tr>
<tr>
<td>Sulfur (mg/kg)</td>
<td>Crab shell</td>
<td>735</td>
<td>897</td>
<td>16.65</td>
<td>9.36</td>
<td>5.66</td>
</tr>
<tr>
<td>Zinc (mg/kg)</td>
<td>Crab shell</td>
<td>4.91</td>
<td>75.58</td>
<td>0.31</td>
<td>0.35</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*a “N/A”: not applicable  
*b “BDL”: below detection limit
TABLE 3.3. Water quality analyses of influent AMD (average and SD of influent during regular sampling events) for pilot-scale VFPs treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Influent AMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2.89 ± 0.24</td>
</tr>
<tr>
<td>Conductivity (µS/cm)</td>
<td>2.10 ± 0.41</td>
</tr>
<tr>
<td>ORP (mV)</td>
<td>503 ± 51</td>
</tr>
<tr>
<td>Acidity (mg/L as CaCO$_3$)</td>
<td>241 ± 44</td>
</tr>
<tr>
<td>Alkalinity (mg/L as CaCO$_3$)</td>
<td>0.00</td>
</tr>
<tr>
<td>Aluminum (ppm)</td>
<td>2.80 ± 0.33</td>
</tr>
<tr>
<td>Iron (ppm)</td>
<td>61.90 ± 19.13</td>
</tr>
<tr>
<td>Manganese (ppm)</td>
<td>27.00 ± 34.19</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>0.60 ± 0.26</td>
</tr>
<tr>
<td>Calcium (ppm)</td>
<td>147 ± 54</td>
</tr>
<tr>
<td>Potassium (ppm)</td>
<td>4.90 ± 0.68</td>
</tr>
<tr>
<td>Magnesium (ppm)</td>
<td>132 ± 25</td>
</tr>
<tr>
<td>Sodium (pm)</td>
<td>8.08 ± 1.52</td>
</tr>
<tr>
<td>Sulfate (mg/L)</td>
<td>1060 ± 132</td>
</tr>
</tbody>
</table>
TABLE 3.4. Hydraulic parameters during the operation of pilot systems treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Reactor name</th>
<th>Calculated effective pore volume$^a$ (L)</th>
<th>Average flow rate (mL/min)</th>
<th>HRT at designed flow rate$^b$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 100CS</td>
<td>1597</td>
<td>534</td>
<td>11.57 14.94</td>
</tr>
<tr>
<td>#2 70CS</td>
<td>15367</td>
<td>546</td>
<td>18.89 16.60</td>
</tr>
<tr>
<td>#3 70CS (SS)</td>
<td>1612</td>
<td>563</td>
<td>18.89 14.94</td>
</tr>
<tr>
<td>#4 SMC</td>
<td>1204</td>
<td>559</td>
<td>20.00 14.94</td>
</tr>
</tbody>
</table>

$^a$ Effective pore volumes were estimated based on the results of conservative tracer tests conducted in a previous continuous-flow column study (Grembi, 2011).

$^b$ Hydraulic retention time (HRT) estimated using the calculated effective pore volume and designed flow rate of 757 mL/min in the pilot VFPs.
TABLE 3.5. Theoretical and experimental mass of CaCO₃ in the pilot-scale VFPs treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Pilot reactor</th>
<th>Theoretical mass of CaCO₃ in packing materials (kg)</th>
<th>CaCO₃ calculated from experimental data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate</td>
<td>Underdrain</td>
</tr>
<tr>
<td>1-100CS</td>
<td>199</td>
<td>2396</td>
</tr>
<tr>
<td>2-70CS</td>
<td>185</td>
<td>2396</td>
</tr>
<tr>
<td>3-70CS (SS)</td>
<td>185</td>
<td>0</td>
</tr>
<tr>
<td>4-SMC</td>
<td>93</td>
<td>2396</td>
</tr>
</tbody>
</table>
TABLE 3.6. Different operational stages identified based on the chemical performance of the four pilot VFPs treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Operational stages</th>
<th>1-100CS</th>
<th>2-70CS</th>
<th>3-70CS (SS)</th>
<th>4-SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (days)</td>
<td>Pore volumes</td>
<td>Time (days)</td>
<td>Pore volumes</td>
</tr>
<tr>
<td>Start-up</td>
<td>0-42</td>
<td>0-33</td>
<td>0-42</td>
<td>0-31</td>
</tr>
<tr>
<td>Pseudo-steady</td>
<td>56-312</td>
<td>42-114</td>
<td>56-312</td>
<td>40-125</td>
</tr>
<tr>
<td>End</td>
<td>633</td>
<td>252</td>
<td>633</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 3.7. Mass balance of cations and nutrients in the pilot VFPs treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Cations and nutrients</th>
<th>1-100CS</th>
<th>2-70CS</th>
<th>3-70CS (SS)</th>
<th>4-SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>mass of Ca (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m_in</td>
<td>45.59</td>
<td>47.76</td>
<td>46.45</td>
<td>49.33</td>
</tr>
<tr>
<td>m_out</td>
<td>97.50</td>
<td>101.37</td>
<td>102.27</td>
<td>90.75</td>
</tr>
<tr>
<td>m_released</td>
<td>51.91</td>
<td>53.60</td>
<td>55.81</td>
<td>41.42</td>
</tr>
<tr>
<td>% released</td>
<td>53.24</td>
<td>52.88</td>
<td>54.58</td>
<td>45.64</td>
</tr>
<tr>
<td>mass of K (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m_in</td>
<td>1.50</td>
<td>1.55</td>
<td>1.46</td>
<td>1.61</td>
</tr>
<tr>
<td>m_out</td>
<td>1.54</td>
<td>1.76</td>
<td>1.56</td>
<td>1.90</td>
</tr>
<tr>
<td>m_released</td>
<td>0.04</td>
<td>0.21</td>
<td>0.10</td>
<td>0.29</td>
</tr>
<tr>
<td>% released</td>
<td>2.70</td>
<td>12.18</td>
<td>6.46</td>
<td>15.44</td>
</tr>
<tr>
<td>mass of Mg (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m_in</td>
<td>41.69</td>
<td>43.34</td>
<td>41.65</td>
<td>44.80</td>
</tr>
<tr>
<td>m_out</td>
<td>43.24</td>
<td>46.99</td>
<td>44.41</td>
<td>52.04</td>
</tr>
<tr>
<td>m_released</td>
<td>1.55</td>
<td>3.65</td>
<td>2.75</td>
<td>7.24</td>
</tr>
<tr>
<td>% released</td>
<td>3.59</td>
<td>7.77</td>
<td>6.20</td>
<td>13.92</td>
</tr>
<tr>
<td>mass of Na (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m_in</td>
<td>2.48</td>
<td>2.58</td>
<td>2.47</td>
<td>2.66</td>
</tr>
<tr>
<td>m_out</td>
<td>2.99</td>
<td>3.22</td>
<td>3.14</td>
<td>2.84</td>
</tr>
<tr>
<td>m_released</td>
<td>0.51</td>
<td>0.64</td>
<td>0.67</td>
<td>0.18</td>
</tr>
<tr>
<td>% released</td>
<td>17.15</td>
<td>19.95</td>
<td>21.27</td>
<td>6.32</td>
</tr>
<tr>
<td>mass of P (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m_in</td>
<td>0.06</td>
<td>0.07</td>
<td>0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>m_out</td>
<td>3.23</td>
<td>2.49</td>
<td>2.01</td>
<td>0.84</td>
</tr>
<tr>
<td>m_released</td>
<td>3.17</td>
<td>2.43</td>
<td>1.95</td>
<td>0.77</td>
</tr>
<tr>
<td>% released</td>
<td>98.06</td>
<td>97.39</td>
<td>96.92</td>
<td>91.98</td>
</tr>
<tr>
<td>mass of ammonium (kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>as NH₄⁺-N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m_in</td>
<td>2.24</td>
<td>2.41</td>
<td>1.93</td>
<td>2.43</td>
</tr>
<tr>
<td>m_out</td>
<td>11.65</td>
<td>10.38</td>
<td>6.52</td>
<td>4.85</td>
</tr>
<tr>
<td>m_released</td>
<td>9.40</td>
<td>7.97</td>
<td>4.59</td>
<td>2.42</td>
</tr>
<tr>
<td>% released</td>
<td>80.75</td>
<td>76.79</td>
<td>70.37</td>
<td>49.86</td>
</tr>
</tbody>
</table>
**TABLE 3.8.** Average measured concentrations, free ion, and active free ion concentrations (day 56 to 312) of cations from the pilot VFPs treating AMD at the Klondike-1 site, and their corresponding tolerance limits.

<table>
<thead>
<tr>
<th>Average concentration of cations (mg/L)</th>
<th>Measured concentration</th>
<th>1-100CS</th>
<th>2-70CS</th>
<th>3-70CS (SS)</th>
<th>4-SMC</th>
<th>Water quality criterion (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>259.7</td>
<td>280.0</td>
<td>283.2</td>
<td>223.5</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>1.8</td>
<td>5.2</td>
<td>5.1</td>
<td>4.9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>110.7</td>
<td>138.8</td>
<td>126.7</td>
<td>137.8</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>7.9</td>
<td>9.9</td>
<td>8.7</td>
<td>7.2</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>10.6</td>
<td>10.7</td>
<td>6.4</td>
<td>2.1</td>
<td>0.05</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Free ion concentration</th>
<th>Ca</th>
<th>194.8</th>
<th>206.6</th>
<th>206.0</th>
<th>159.8</th>
<th>160</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
<td>1.7</td>
<td>5.1</td>
<td>5.0</td>
<td>4.8</td>
<td>5</td>
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<tr>
<td></td>
<td>Mg</td>
<td>86.3</td>
<td>106.6</td>
<td>96.4</td>
<td>103.5</td>
<td>15</td>
</tr>
<tr>
<td></td>
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<td>7.7</td>
<td>9.7</td>
<td>8.5</td>
<td>7.1</td>
<td>75</td>
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<td>P</td>
<td>6.7</td>
<td>6.5</td>
<td>4.0</td>
<td>1.3</td>
<td>0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Active free ion concentration</th>
<th>Ca</th>
<th>107.7</th>
<th>111.4</th>
<th>111.4</th>
<th>87.7</th>
<th>160</th>
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<tr>
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<td>K</td>
<td>1.5</td>
<td>4.3</td>
<td>4.2</td>
<td>4.0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mg</td>
<td>48.4</td>
<td>58.4</td>
<td>53.0</td>
<td>57.7</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Na</td>
<td>6.6</td>
<td>8.2</td>
<td>7.2</td>
<td>6.0</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>4.6</td>
<td>4.5</td>
<td>2.7</td>
<td>0.9</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Free ion and active free ion concentrations were predicted by geochemical modeling using Visual MINTEQ (ver. 3.0, beta) given the average experimental data during the pseudo-steady phase (day 56 to 312) at fixed pH and alkalinity specific to each pilot VFPs.

*For Ca, K, Mg and Na, recommended tolerance limits for fish were used to represent water quality criteria (Meade, 1989); for P, the EPA water quality criteria of 0.05 mg/L (USEPA, 1986) were used for streams finally discharging into lakes or reservoirs.
FIG. 3.1. Schematic of the location of pilot-scale systems in the previously constructed full-scale AMD treatment system at the Klondike-1 site.
FIG. 3.2. Schematic of the pilot VFP constructed at the Klondike-1 site.
FIG. 3.3. Flow rate fluctuation of the four pilot treatments at the Klondike-1 site over the time of tests (643 days).
FIG. 3.4. Alkalinity generation (a), acidity (b) and pH data (c) of the influent and within the four pilot VFPs featuring different substrate compositions at the Klondike-1 site.
FIG. 3.5. Breakthrough curves of dissolved iron (Fe) (a) and dissolved manganese (Mn) (b) in the effluent of pilot VFPs at the Klondike-1 site.
FIG. 3.6. Concentration of ammonium in the effluent of pilot VFPs treating AMD at the Klondike-1 site. The two red dashed lines refer to the acute (17 mg TAN/L) and chronic (1.9 mg TAN/L) criteria for aquatic lives normalized to pH 7.0 and 20 °C (U.S. EPA, 2013).
FIG. 3.7. Percentages of metals (Fe, Al, Mn, and Zn) retained in the pilot VFPs treating AMD at the Klondike-1 site.
FIG. 3.8. Saturation indices (SI) of metal minerals versus pH at day 0 (a), from day 84 to 258 (b), and from day 340 to 442 (c) in the 2-70CS reactor at the Klondike-1 site. SI values were predicted by geochemical modeling via Visual MINTEQ using the measured experimental conditions at specific scenarios.
CHAPTER 4
Development and Adaptability of Microbial Communities in Pilot-scale Field Reactors Treating Acid Mine Drainage Amended with Crab Shell

4.1. Abstract

Although passive treatment systems for acid mine drainage (AMD) have been extensively studied, little research has addressed the effect of changing environmental conditions on microbial communities associated with different substrate materials. In this study, 454 sequencing and qPCR were used to characterize the microbial communities in the three pilot-scale vertical flow ponds (VFPs) with different substrate combinations of crab shell (CS) and spent mushroom compost (SMC) for treating high risk AMD in central Pennsylvania. Results showed that the two pilot VFPs containing CS substrates (100% CS and 70% CS/30% SMC) accommodated microbial communities of increasing core phyla abundance over different operational phases. Cluster analyses showed that two clusters based on operational taxonomic units (OTUs) at 3% cutoff level in the 70CS and 100CS reactors were more closely related to each other in terms of distance matrix and the number of shared OTUs. Interestingly, functionally more diverse bacterial communities, especially SRB, were observed in the two CS reactors, as compared to the relative homogeneous community with large populations of hydrolytic and fermentative bacteria in the SMC reactor. Correspondingly, copy numbers of genes representing total bacteria, fermenters, and sulfate reducers were higher in the pilot reactors containing CS than in the SMC reactor. When flow temporarily ceased due to clogging, the relative
abundance of the core phyla shifted in all pilot reactors, but the smallest changes in functional gene copies were observed in the 70CS/30SMC reactor. This agrees well with the chemical performance data of the pilot systems, providing further justification on the beneficial combination of CS with cellulosic materials at optimal ratios to enhance the treatment efficiency and longevity of passive AMD treatment systems.

4.2. Introduction

Acid mine drainage (AMD) is a world-wide environmental concern because it contaminates receiving watersheds and thus poses potential threats to aquatic life and even human health. This is especially the case for high-risk AMD characterized by extremely low pH, high concentrations of combined metals, and/or high flow rate (PA DEP, 2008). The most desirable treatment systems for AMD are those with high treatment efficiency, reasonable construction costs, and minimal maintenance and post-treatment needs (Berghorn and Hunzeker, 2001; Waybrant et al., 2002). Therefore, biological passive AMD treatment with external substrate to supply organic carbon and nitrogen has been commonly applied at contaminated sites and has proven to be successful in some cases (Benner et al., 1997; Whitehead and Prior, 2005). Since the primary driving force of most passive AMD treatment systems is the microbial population, the selection of substrate materials is crucial to support their growth and activity. Traditionally, substrates for these systems have included simple organic acids or alcohols and cellulosic waste materials like spent mushroom compost (SMC) coupled with limestone, but recently, a new multifunctional substrate—crab shell (CS) was shown
to be able to simultaneously serve as an electron donor source (organic content), neutralizing agent (CaCO$_3$), and nitrogen source (ammonium from protein and chitin decomposition) (Daubert and Brennan, 2007; Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2009).

In order to maximize system performance, longevity, and reliability, the establishment of a healthy and diverse microbial community in AMD bioremediation systems is of great importance. In general, the native microorganisms existing in AMD or in watersheds contaminated by it are mainly extremophiles, such as acidophiles and halophiles. Previous reports pointed out that the formation of AMD involves primarily chemical and microbial reactions with sulfide-containing minerals, and that iron oxidation rates can be increased by several orders of magnitude by iron- or sulfur-oxidizing bacteria and archaea, such as Acidithiobacillus ferrooxidans, Leptospirillum ferrooxidans, and Gallionella ferruginea (Baker and Banfield, 2003; Fowler et al., 1999; Johnson et al., 2005; Lear et al., 2009). During the bioremediation of AMD-impacted sites, however, increases in pH and alkalinity, and decreased loadings of dissolved metals encourage the formation of more diverse microbial communities of different compositions and functions.

Among all metabolic groups involved in AMD treatment, the key microorganisms have been identified to be sulfate-reducing bacteria (SRB), the activity of which can promote metal precipitation as metallic sulfides and the generation of net alkalinity. At field sites, the nature of the substrate and environmental conditions during the operation of AMD treatment systems can greatly affect the metabolism and behavior of SRB, and hence also affect system performance (Hiibel et al., 2011; Pruden et al., 2007; Waybrant et al., 1998; Weber et al., 2010). It was previously reported that a mixture of complex
organic substrate materials led to better performance than single or simple organic compounds (Gibert et al., 2002; Neculita et al., 2007), and a carbon to nitrogen ratio between 10 to 16 was optimal for SRB activity (Cocos et al., 2002; Neculita et al., 2007; Rabus et al., 2006). Better growth of SRB has been observed at pH 7.0 to 7.8 and temperature between 28 °C and 38 °C (mesophilic), but most SRB can tolerate pH from 5.5 to 9.0, and thermophilic SRB species grow optimally at 54 °C to 70 °C (Rabus et al., 2006; Widdel, 1993). In terms of oxidation-reduction potential (ORP), the ceiling value of -100 mV was suggested for the cultivation of SRB, with the optimal ORP ranging from -150 mV to -200 mV (Leung, 1998; Rabus et al., 2006).

The growth of SRB requires simple organic compounds, thus, the development of a healthy microbial community composed of upstream biopolymer degraders and fermenters is needed in AMD treatment systems containing complex organic substrate materials (Hiibel et al., 2011; Logan et al., 2005; Pereyra et al., 2010). By applying molecular biological techniques, the composition and diversity of microbial communities in field bioremediation systems for mine-impacted water (MIW) have been investigated (Hiibel et al., 2011; Johnson and Hallberg, 2005; Neculita et al., 2007; Pruden et al., 2007). It was demonstrated that the complexity and abundance of the microbial community impacts AMD reactor performance in several aspects including initial start-up stage, as well as metal removal and sulfate reduction processes (Christensen et al., 1996; Pereyra et al., 2008; Weber et al., 2010). However, the composition and adaptability of microbial communities under changing environmental conditions of temperature and flow in field AMD treatment systems amended with crab shell has not been fully
documented. Furthermore, the potential benefit to the microbial community of utilizing substrate mixtures containing CS under these conditions has not been explored.

The objective of this study was to evaluate the establishment, development, and adaptability of microbial communities in three pilot-scale field reactors utilizing different substrate mixtures to treat a high-risk AMD discharge throughout seasonal temperature and flow variations. The three pilot reactors were packed with different substrate mixtures of SMC and CS (90% SMC/10% limestone; 70% CS/30% SMC; and 100% CS), and were fed a continuous flow of AMD influent from the existing Klondike-1 primary precipitation pond for almost 643 days. The influent water was first piped into each pilot VFP individually, then flowed into two subsequent passive aeration ponds to induce metal oxidation and precipitation, and was finally discharged into a constructed wetland on site. Regular operation and monitoring of the pilot treatments were started on Aug. 27, 2010 for approximately 643 days. Chemical performance data indicated prominent advantages of alkalinity generation and metal removal in the pilot reactors containing CS as compared to the one containing only the traditional SMC/limestone mixture. In order to comprehensively understand the roles of microorganisms and their cooperation associated with different reactor efficiencies under varying environmental conditions, 454 sequencing of 16S rRNA genes of bacterial communities and quantitative PCR (qPCR) targeting different functional groups were carried out. This should provide additional justification for the best combination of SMC and CS to be used in the substrate layer of passive AMD treatment systems. More importantly, insights into the microbiological mechanisms controlling the performance of CS-based AMD treatment systems may
benefit future design and operation strategies for field bioremediation of other environmental contaminants.

4.3. Materials and Methods

4.3.1. Pilot test setup and sampling

In this study, the establishment and adaptability of microbial communities in pilot-scale vertical flow ponds (VFPs) featuring different substrate components were evaluated over varying environmental conditions during the course of regular operations at the Klondike-1 site in Cambria County, Pennsylvania. The design, system setup, construction, and influent quality data have been described elsewhere (Chapter 3). In brief, three pilot VFPs were packed with different CS and SMC mixtures (90% SMC/10% limestone; 70%CS/30% SMC; or 100% CS) in the substrate layer and limestone (LS) rocks in the underdrain. An additional reactor with 70%CS/30% SMC substrate was established with a sandstone (SS) underdrain for comparison of alkalinity generation (Table 4.1).

It was observed that the treatment efficiency of the 70% CS reactor with SS underdrain mimicked that of the 70% CS reactor with LS underdrain over the entire test. Therefore, only substrate samples from the three pilot VFPs with LS underdrain (100CS, 70CS, and SMC) were selected for microbial study to ensure parallel comparison of microbial communities associated with different substrate compositions at the same sampling event. The chemical performance data indicated different phases of reactor operation: start-up (day 0 to 42), pseudo-steady state (day 56 to 312), declining (day 340
to 475), and end (day 633) phases. Each phase featured different treatment efficiencies with regard to alkalinity generation, metal removal, and nutrients levels. In order to evaluate the development of microbial communities over the course of the tests, microbial samples at six sampling events for each of the three pilot VFPs were chosen to represent the different operational phases: day 28 (Sept. 24, 2010) for start-up, day 84 (Nov. 19, 2010) and 258 (May 12, 2011) for pseudo-steady state, day 286 (Jun. 9, 2011) for no flow, day 340 (Aug. 2, 2011) for declining, and day 643 (May 31, 2012) for end phase. A total of 18 samples from the three selected pilot VFPs at six sampling events were included in the microbial study.

In order to facilitate the collection of microbial samples within the pilot VFPs, special 3 inch × 3 inch microbial sample bags (Fig. I.2), each filled with approximately 10 g of the substrate materials used in the target reactor, were made and positioned approximately 8-10 inches below the surface of the organic substrate layer in each reactor. At each sampling event, two sample bags were pulled out from each reactor and transferred into sterile 50-mL tubes. Then, the microbial samples were transported back to the lab on ice, and frozen at -80°C for later microbial analysis.

4.3.2. DNA extraction and purification

After the completion of the last sampling round on day 643, DNA extractions were carried out on the 18 preserved samples from the three selected pilot VFPs using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). A flame-sterilized spatula was used to transfer some substrate material from a microbial sample
bag to a bead tube, and then the extraction work was performed according to the manufacturer’s protocol with two extra steps after the addition of Solution C1:

a. To each bead tube, 20 µl of 100 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO) and 10 µl of 25 mg/ml achromopeptidase (Sigma-Aldrich, St. Louis, MO) were added, followed by incubation in a 37°C water bath for 1 hour;

b. To each bead tube, 45 µl of 20 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) was added, and then incubated in a 55°C water bath for 1 hour.

These two extra steps were introduced to maximize the extraction output (lysozyme and achromopeptidase), and to remove impurities from DNA preparations (proteinase K). Approximately 0.45 g of the substrate materials were used per extraction, and the newly extracted DNA samples were purified by a PowerClean® DNA Clean-Up Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the protocol provided by the manufacturer except that in step 17, 65µl of PCR-grade DNase- and RNase-free water was added to each spin filter to elute DNA from the filter membrane. The DNA purification was necessary to ensure the quality of downstream analyses through the removal of PCR inhibiting substances, particularly humics and polyphenols mainly associated with cellulosic materials in the substrate layer of the 70CS and SMC reactors. The Purified DNA preparations were then aliquoted (10µl per tube) and preserved at -20°C until further analyses.

4.3.3. 454 sequencing

In this study, a metagenomics approach by means of the “One-Way Reads” Amplicon sequencing of 16S ribosomal RNA (rRNA) gene was introduced to obtain
long reads from a common primer starting point and to identify bacterial species in
the DNA samples from the pilot VFPs at the Klondike-1 site. The aliquots of purified
DNA samples were submitted to the Penn State Genomics Core Facility (The
Pennsylvania State University, University Park, PA) for PCR library construction and
454 sequencing. In detail, one-way read amplicons (Lib-L) were prepared using bar-
coded fusion forward and reverse primers

CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID- 27F and
CCTATCCCTGTGTGCCTTGGCAGTCTCAG-907R, respectively. To target the
bacterial 16S rRNA gene specifically, the forward primer 27F (5’-
AGAGTTTGATCMTGGCTCAG
-3’) and reverse primer 907R (5’-
CCCCGTCAATTCTTTTGGTTT -3’) were used. PCR reactions for the 16S rRNA
gene amplicons contained 5 pmoles of each primer, 5 nmoles of dNTP, 0.25 µl of TAQ
(Fast Start High Fidelity PCR system, Roche, Indianapolis, IN), 2.5 µl of 10X buffer
supplied with the enzyme, 10 to 40 ng of double-strand (ds) DNA template, and sterile
nuclease-free water to a final volume of 25 µl. Samples were denatured at 94°C for 3 min,
then cycled (Gene AMP PCR System 9700, Applied Biosystems, Foster City, CA) 27 to
35 times of 94°C for 15 sec, 55°C for 45 sec, and 72°C for 60 sec, with a final extension
at 72°C for 8 min.

The PCR products (approximately 700 to 1000 bps) were purified using the
Agencourt AMPure technology (Beckman Coulter, Brea, CA) as described in 454
Technical Bulletin #2011-002, Short Fragment Removal Procedure. After clean-up, the
products were quantified by both Qubit (Lifetech, Carlsbad, CA) and qPCR using the
KAPA Biosystems Library Quantification Kit (KapaBiosystems, Woburn, MA). Products
of satisfactory quantities were then pooled based on molar amounts, run on a 1% agarose gel and extracted. After clean-up with a QIAquick PCR Purification kit (Qiagen, Valencia, CA), sample quality and quantity were assessed using a DNA 7500LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit quantification, respectively. A total of two sequencing runs were performed, each using a quarter PTP plate on a 454 Life Sciences Genome Sequencer FLX+ (Roche Diagnostics, Indianapolis, IN) at the Penn State Genomics Core Facility.

4.3.5. Gene quantification by qPCR

Real-time quantitative PCR (qPCR) was performed on the 18 DNA samples from the pilot VFPs by targeting the 16S rRNA genes of total bacteria and total archaea, 18S rRNA gene of total fungi, and three functional genes—cel5 (cellulose degraders), dsrA (SRB), and hydA (fermenters)—as previously described (Manter and Vivanco, 2007; Nadkarni et al., 2002; Pereyra et al., 2010; Takai and Horikoshi, 2000; Yergeau et al., 2007).

Microbial standards were prepared through the PCR amplifications of the designated standard microbes with corresponding primer sets (Table 4.2). The PCR products were then purified using a QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). Except for qPCR runs targeting total bacteria and archaea in which Taqman assays were used, SYBR green assays were established for other targets. For each gene target, a triplicate four- to six-point standard curve was constructed ($R^2 \geq 0.99$). In every qPCR run, serial dilutions of DNA template were prepared at factors of 1:10 and 1:100 for each pilot VFP, and triplicates were run for all samples. All qPCR
analyses were performed using a StepOnePlus™ Real-Time PCR System (Life Technologies, Grand Island, NY), and samples below the detection limit of quantification (approx. 100 gene copies) were excluded from the analysis. Final values are expressed in copy numbers per gram of substrate (copies/g).

4.3.6. Post-sequencing analyses

The two sequencing files containing all the raw reads (435,337 sequences) from 454 sequencing were combined together, and treated with the 454 Data Pipeline in Mothur (v.1.31.2) platform (Hughes et al., 2001; Schloss et al., 2009). Data analyses were carried out by cooperating with the research group of Dr. Istvan Albert in the Department of Biochemistry and Molecular Biology (The Pennsylvania State University, University Park, PA). An initial quality check was performed to trim the primer sequences and barcodes with the ‘trim.seqs’ command. A sliding window of 50 bp was used and the sequences were trimmed when the average quality score over that window dropped below 35.

The denoised sequences were then aligned to the Mothur version of the SILVA-based bacterial reference, and pre-clustered to merge the sequence counts that were within 2 bp of a more abundant sequence. PCR chimeras were detected using the ‘chimera.uchime’ command in Mothur and subsequently removed from downstream analysis. The average length of all bacterial sequences without primers and barcodes was 570 bp. Taxonomic classification was done based on the Ribosomal Database Project (RDP) naïve Bayesian rRNA Classifier version 2.5 trained on 16S rRNA training set 9 consisting of high-quality 9665 bacterial and 384 archeal 16S rRNA sequences (Wang et
al., 2007). Heat maps of selected bacterial phyla or genera were drawn with dendrograms added to the left and to the top using the R Stats Package (version 2.15.2).

In order to ensure a fair comparison of the 18 samples at the same sequencing depth, the sequence number of each sample was normalized to 9563 sequences for OTU-based analyses, the minimum number of total bacterial sequences among the 18 samples. Pairwise distance between aligned DNA sequences was calculated in Mothur at a cutoff value of 0.15, and sequences were then clustered into operational taxonomic units (OTUs) at 97% similarity (3% cutoff). Based on the OTU clusters, rarefaction curves, Good’s coverage, and Shannon diversity indices were calculated in the Mothur platform, and the grouping of different bacterial communities from the 18 samples was conducted using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) by Jaccard calculator (Hughes et al., 2001; Schloss et al., 2009). A Newick-formatted tree describing the dissimilarity among the 18 samples was then generated with the ‘tree.shared’ command in Mothur. To better visualize the dissimilarity of bacterial communities within or among the three pilot VFPs at the OTU level, Venn diagrams, each comparing three different samples, were produced using Mothur. Significance tests were then performed using pair-wise comparisons by weighted-UniFrac algorithm, where raw p-values ≤ 0.05 were reported to be statistically significant. In addition, principal coordinate analyses of bacterial communities in the 18 samples (PCoA) at a 3% cutoff-OTU level were carried out by applying Jaccard calculations. To determine whether there were any OTUs that were differentially represented among the samples from the three pilot reactors, the ‘metastats’ command in Mothur was applied with pair-wise comparisons. The raw sequencing reads produced in this study have been deposited in the Sequence Read
4.4. Results

4.4.1. Estimation of bacterial diversity

Total effective reads for the 18 samples from the pilot VFPs were reduced to 335,388 sequences from the raw data set of 435,337 sequences after trimming primers and barcodes, and were further lowered to 303,456 sequences after pre-clustering and chimera removal. Taxonomic classification and cluster analyses were subsequently conducted on the denoised and cleaned sequences of the three pilot VFPs at six sampling events.

The number of effective sequences per sample varied from the lowest of 9,563 in the 100CS-D84 (100% CS, day 84) sample to the highest of 24,748 in the 70CS-D643 (70% CS, day 643) sample (Table 4.3). Generally, more sequences were obtained in the 6 samples from the SMC reactor by 454 sequencing. Good’s coverage indicated 83% of species richness was achieved on average in each bacterial community. OTU counts in the SMC samples were approximately 5 times higher than those in the 100CS samples, and 4 times higher than those in the 70CS at the same sampling event. This corresponded with the estimation of Shannon diversity indices which were determined to be different among bacterial communities from the 18 samples (4.97±1.53, n=18, P-value <0.05) with the highest diversity of 6.98 on average in the SMC reactor. Nevertheless, no differences were observed in the bacterial diversity of the two CS reactors (3.97±0.57, n=12, P-
value > 0.05), which might be an indication of similar bacterial compositions and clustering styles. Moreover, the highest diversity of bacterial communities of the six samples within the 100CS, 70CS, and SMC reactors were detected at day 258, 643, and 340, respectively.

4.4.2. Classification of bacterial taxa

The top three bacterial phyla were identified to be *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* in all 18 samples with the sum of reads accounting for 54% to 91% of total effective bacterial sequences (Fig. 4.1). However, the most dominant phylum and the changes in its abundance over time were different in different pilot reactors. Specifically, in the 100CS reactor, *Firmicutes* prevailed over other bacterial phyla at day 28, 258 and 286 (no flow) after which its top place was gradually replaced by *Bacteroidetes*. Similarly, the top two phyla in the 70CS reactor were *Firmicutes* and *Bacteroidetes*, and the abundance of both exhibited slight fluctuations but the percentage of *Bacteroidetes* reads was highest (50% on average) during the entire course of the test. The case was totally different with the SMC reactor in which *Proteobacteria* predominated all the time during regular operation.

Apart from the three dominant phyla, other major phyla (abundance > 0.5%) such as *OD1* and *Spirochaetes* were more frequently observed in the two crab shell reactors (100CS and 70CS), whereas relatively higher percentages of bacterial reads were classified to be members of *Actinobacteria*, *Acidobacteria*, *Chlorofexi*, *Planctomycetes*, *Verrucomicrobia*, and *Gemmamimonadetes* in the SMC reactor (Fig. 4.2). Obviously, the abundance of the *OD1* phylum increased significantly after day 286 in the two CS
reactors. This was especially true with the 100CS reactor in which the abundance of \textit{ODI} at the last sampling event (day 643) reached to 22\%. The last major phyla, \textit{Chlorobi}, was distributed evenly among all reactors and sampling events. The rest of the 12 bacterial phyla were grouped under the category of minor phyla, referring to those with their maximum abundance lower than 0.5\% in any sample. The percentage of unclassified phyla in total effective bacterial sequences ranged from 8\% to 22\%.

As shared by all three pilot reactors, \textit{Proteobacteria} represents one of the most diverse phyla in terms of various bacterial metabolic groups and functions. Therefore, as the substrate composition changed, the dominant classes in \textit{Proteobacteria} varied among the different reactors (Fig. 4.2). The abundance of $\delta$-\textit{Proteobacteria} in total \textit{Proteobacteria} reads (76\% on average) was prevailing among other classes in the two CS reactors, followed by $\gamma$- (11\% on average), $\beta$- (5\% on average), $\alpha$-\textit{Proteobacteria} (4\% on average). Noticeably, the most taxonomically and functional diverse SRB were present in the class of $\delta$-\textit{Proteobacteria}, which might be an indicator of advantageous reactor performance in sulfate removal in the 70CS and 100CS reactors. The distribution of classes in \textit{Proteobacteria} was quite different in the SMC reactor with $\alpha$-\textit{Proteobacteria} (51\% on average) as the dominant one. Species in the class of $\varepsilon$-\textit{Proteobacteria} were also detected in all reactors but with a much lower average abundance value of 0.13\%.

Other dominant bacterial classes (abundance > 0.5\%) in the 18 samples were determined to be \textit{Clostridia}, \textit{Bacilli}, \textit{Flavobacteria}, \textit{Sphingobacteria}, \textit{Bacteroidia}, \textit{Actinobacteria}, \textit{Anaerolineae}, \textit{ODI_class_incertae_sedis}, \textit{Negativicutes}, and \textit{Spirochaetes}. In addition, statistical analysis indicated that among a total of 80 assigned bacterial classes, 16 were
shared by all 18 samples (Table 4.4). Moreover, sequences from these 16 classes accounted for 86.7% of the total classified bacterial sequences.

A higher abundance of sequences with similarity to members in the orders of \textit{Clostridiales}, \textit{Flavobacterales}, \textit{Bacteroidales}, and \textit{Rhizobiales} (>5% of total classified sequences) were observed in the 18 pilot VFP samples. A total of 21.28% of the classified orders was shared by the 18 samples, accounting for 82.93% of all effective sequencing reads. In the family level, the top 10 occupying 52.36% of the total classified sequences were identified to be \textit{Flavobacteriaceae}, \textit{Porphyromonadaceae}, \textit{Clostridiaceae\textsubscript{1}}, \textit{Lachnospiraceae}, \textit{OD1\_family\_incertae\_sedis}, \textit{Anaerolineaceae}, \textit{Ruminococcaceae}, \textit{Desulfovibrionaceae}, \textit{Flammeovirgaceae}, and \textit{Bacteroidetes\_incertae\_sedis\_family\_incertae\_sedis}. Of the 622 total assigned families, 53 were shared by the 18 samples (Table 4.4), but still consisted of a considerable part of the total sequencing reads (66.63%). Recall that similar trends were observed in other taxonomic levels which indicated that some rare taxa might not be statistically significant in their contribution to the diversity of microbial functions, although they were included in the Shannon diversity calculations based on OTUs.

4.4.3. Cluster analysis and significance tests

Cluster analysis based on OTUs at a 3% cutoff revealed the grouping patterns and similarity/dissimilarity of bacterial communities in the three pilot VFPs at different sampling events. A total of six groups were classified at the similarity level of 0.58 (Fig. 4.3). Bacterial compositions of the five 100CS samples were highly similar and clustered into Group I, with the exception for one sample collected at the end of the tests (day 643)
which was assigned to Group II. The two samples representing declining and end phases, 70CS-D340 and 70CS-D643, showed less similarity with the other four samples (Group III) from the 70CS reactor, and were separated to form Group IV. For the SMC reactor, the four samples collected on and after day 258 were branched together (Group V), whereas bacterial communities at the first two sampling events (day 28 and day 84) were classified into Group VI based on their distinct OTU levels. Noticeably, samples from each of the three pilot VFPs were on different main branches and tended to form three big clusters with the SMC samples located farther from those of the two CS reactors, indicating the impact of substrate compositions on the development of bacterial communities.

In order to determine the uniqueness or difference of the bacterial communities in each pilot VFP at a specific sampling event, statistical significance tests were applied using weighted UniFrac algorithm. In pair-wise comparisons of each sample, bacterial communities in the SMC-D28 and SMC-D84 samples were observed to be significantly different from the other 17 samples. This agrees well with the dendrogram showing a distinct branch consisting only of the two samples from the SMC reactor (Group VI). In addition, bacterial communities in any pair of samples from the 100CS and/or 70CS reactors at different sampling times were more similar to each other and no significant differences were observed ($P$-value > 0.05), probably due to the higher fraction of shared taxa developed in the organic substrates of similar characteristics.

PCoA analysis was performed to find similarities or distribution patterns among different samples from the three pilot VFPs. Obviously, when plotting the first coordinate (P1) versus the second coordinate (P2), bacterial sequences from each pilot VFP were
closely clustered together (Fig. 4.4), which is consistent with the observation of grouping patterns in the dendrogram (Fig. 4.3). As compared to other reactor samples, the difference of bacterial compositions in the SMC reactor was captured by the first principal coordinate, while the bacterial population in the 70CS reactor was mainly separated from that in the 100CS reactor by the second coordinate. Moreover, bacterial sequences within the same pilot reactor but at different sampling events were spatially distributed along the direction of the third coordinate. In sum, the first three principal coordinates explained approximately 40% of the total variance in PCoA tests of the bacterial communities.

For the purposes of determining whether the spatial separation in the PCoA plots was statistically significant and what was responsible for the difference, a population-level analysis of the three pilot VFPs was conducted using the ‘metastats’ command in Mothur platform. Results indicated that the difference of bacterial communities between the 70CS and 100CS reactors was mainly explained by the three most abundant OTUs classified to be Parabacteroides spp. and Flexibacter spp. in the 70CS reactor, and Blvii28 spp. in the 100CS reactor. Furthermore, the different clustering patterns between the reactors with or without CS were mostly attributed to the dominant OTU species in the genera of Desulfobulbus, Desulfovibrio, Treponema, and Clostridium and the phyla of Bacteroidetes and Firmicutes in the 70CS and 100CS reactors, as well as the genus of Flexibacter and the order of Sphingobacteriales in the SMC reactor.

4.4.4. Core and distinct genera
When increasing the taxonomic classification depth to the genus level, distinct patterns of the distribution of key genera in the 18 samples from different pilot reactors were observed. Among a total of 1442 bacterial genera assigned, 55 were shared by the 18 samples, accounting for 41.32% of total classified bacterial sequences. Specifically, 560 genera were detected in all 6 samples from the SMC reactor, but when adding one sample (70CS-D643) from the 70CS reactor, the number of shared genera in the 7 samples was decreased considerably to 377. This correlates well with another observation that more than half of the 546 rare genera appearing in less than 5 samples were detected only in the SMC reactors.

A heat map of the top 30 genera showing higher abundance in total bacterial reads of the 18 samples demonstrated a clear clustering pattern in terms of the difference in prominent genera and their distribution in the samples from different pilot reactors. The 70CS and 100CS reactors supported the growth of microorganisms identified to be species in a similar group of the leading genera including *Clostridium*, *Desulfovibrio*, *Treponema*, *Coenonia*, *Sedimentibacter*, and *Anaerovorax*. Although detected in both CS reactors, the three genera of *OD1*, *Desulfosporosinus*, and *Sediminibacter* were more predominant in the samples of the 100CS reactor, while species similar to the genera of *Parabacteroides*, and *Dysgonomonas* exhibited preferable growth in the 70CS reactor. Clearly, bacterial communities in the samples of the SMC reactor formed a distinct cluster, separating them from those of the two CS reactors. The top 10 genera in the SMC reactor were classified to be *Desulforhopalus*, *Gp6*, *Bacillus*, *Hyphomicrobium*, *Ohtaekwangia*, *Bellilinea*, *Brevundimonas*, *Geobacillus*, *Pasteuria*, and *Gemmatimonas*.

In addition to the impact of substrate composition, the relative abundance of dominant
genera within the same pilot reactors varied between different operational phases. Although no common trend could be concluded due to different environmental conditions required by the metabolism and activity of different microorganisms, it was observed that SRB genera showed lower abundance at day 286 when there was no flow into the three reactors, whereas the abundance of hydrolytic and fermentative genera fluctuated over the course of the pilot test.

4.4.5. Gene quantification by qPCR

Previous microbial study on the sulfate-reducing columns treating AMD has identified four key functional groups: cellulose degraders, chitin degraders, fermenters, and SRB, the presence and relative quantity of which were closely related to system performance and stability (Chapter 2). Therefore, in order to evaluate the difference in microbial communities among the three pilot reactors at different operational phases both qualitatively and quantitatively, qPCR was performed to target domain specific 16S rRNA (total bacteria and total archaea) or ITS (total fungi) gene copies and three functional genes including cellulose degraders (cel5), fermenters (hydA), and sulfate reducers (dsrA).

Generally, the largest population size within each sample was observed for total bacteria, followed by total fungi, and then total archaea (Fig. 4.6). At the same sampling event, higher copy numbers of total bacteria were detected in the 12 samples from the two CS reactors, while the SMC reactor accommodated the largest fungal and archaeal population. Specifically, in the 100CS reactor, copy numbers of total bacteria increased from $8.54 \times 10^8$ copies/g at day 28 to $1.38 \times 10^{10}$ copies/g at day 286 and then dropped to
2.18×10⁹ copies/g at day 643. No archaeal copies (below the detection limit of 100 copies/μl) were detected at the first two sampling events in the 100CS reactor, and the highest copies of total fungi occurred at day 84 (1.62×10⁷ copies/g). A similar trend of total bacterial copies over time was observed in the 70CS reactor, but the population size of total archaea and fungi fluctuated among different operational phases with the highest at 2.09×10⁷ copies/g and 6.37×10⁷ copies/g at day 643 and 84, respectively. In the SMC reactor, the fungal community decreased over time, while the highest bacterial copies appeared at day 340 (2.96×10⁹ copies/g). In addition, the copy number of total archaea was highest at day 286 (2.50×10⁷ copies/g) in the SMC reactor when flow rate was down to zero, indicating an expansion of methanogens due to diminished SRB activity under limited sources of electron acceptor (SO₄²⁻).

Crab shell amendments increased the copy numbers of hydA and dsrA genes relative to the traditional SMC substrate (Fig. 4.7). Gene copies of fermenters in the three pilot VFPs fluctuated over time until day 286 when an obvious increase was detected (highest of 1.13×10⁸ copies/g in the 100CS-D286 sample), and subsequently decreased to a lower level thereafter. In contrast, SRB copies in each pilot reactor gradually increased before day 286, at which point a drop occurred, and then continued to increase thereafter to the highest quantities at day 340. Specifically, the highest SRB population within the the 100CS reactor was detected to be 9.15×10⁷ copies/g under normal flow conditions (day 340). With regard to cellulose degraders, comparable gene copies were maintained in all samples from the 70CS and SMC reactors, and exhibited slightly higher copy numbers than the 100CS reactor at the same operational phase. This is as expected when
considering the relatively higher supply of cellulosic materials in the 70CS and SMC reactors which could be readily utilized by cellulose degraders.

4.5. Discussion

4.5.1. Development of the microbial community in the pilot tests

In this pilot study, 454 sequencing revealed the evolution and composition of microbial communities in the 18 samples in a more comprehensive way due to its higher sequencing depth as compared to other biological molecular techniques such as cloning and DGGE. Generally, the number of OTUs in the 70CS and 100CS reactors at 3% cutoff level gradually increased from day 28 to 643 when the test ended; however, this was not the case for the SMC reactor in which the OTUs decreased to the start-up level after day 340 when the reactor experienced declined treatment efficiency (Table 4.3).

Microbial communities in the three pilot VFPs exhibited changes in their structure and abundance over different operational phases. Distinct microbial compositions in the three reactors were observed even as early as day 28 during the start-up phase with the dominant phyla of Firmicutes, Bacteroidetes, and Proteobacteria in the 100CS, 70CS, and SMC reactors, respectively (Fig. 4.8). This indicates that the inherent chemical and physical characteristics of CS and SMC created different microbial environments that were preferred by different groups of microorganisms, and suggests that the substrates were likely already colonized with an assortment of microorganisms prior to system start up. Although the microbial population in the SMC reactor was dominated by members in Proteobacteria, the class δ-Proteobacteria accounted for less than 12% of the total
Proteobacteria reads, and the most abundant class was α-Proteobacteria. It should be noticed that the largest groups of SRB—key players in passive treatment systems—were found in the δ-Proteobacteria which was determined to be the dominant Proteobacteria class in the two CS reactors at all sampling events by 454 sequencing. Moreover, as revealed by qPCR, copy numbers of total bacteria, fermenters, and SRB in the two CS reactors at day 28 were almost an order of magnitude higher than those in the SMC reactor (Fig. 4.6 and 4.7), further validating the ability of CS to stimulate bacterial growth and as a result, decrease the duration of the lag period during the initial start-up phase of passive AMD treatment systems (Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2009).

From day 84 to day 258 during pseudo-steady state, microbial communities in the two CS reactors continued to evolve in terms of new and dominant species as well as increase in population size. In contrast, few changes were observed either qualitatively or quantitatively in the SMC reactor (Fig. 4.8), likely demonstrating the limited capacity of SMC to provide long-lasting nutrients or a favorable environment for the growth and development of microorganisms. A comparison of shared OTUs at the 3% cutoff level between any two pilot VFPs revealed that only 18 OTUs were shared between groups 100CS and SMC while the number increased to 107 between the two CS groups (Fig. 4.9). This again suggests that the two substrate materials, CS and SMC, supported the growth and activity of microbial communities of different compositions.

At day 286, no flow was detected in any pilot VFPs upon arrival for sampling. The selection of this sampling point was necessary to evaluate the stability of the pilot treatments in terms of both treatment efficiency and microbial compositions over varying
environmental conditions which are faced by almost all field AMD treatment systems. Microbial populations in the three reactors shifted to varying degrees under no flow. The abundance of *Bacteroidetes*, *Firmicutes*, and *OD1* phyla increased in the 100CS reactor from day 258 to 286 together with a slight decrease in *Proteobacteria*, and *Spirochaetes*. The case was different for the 70CS reactor in which a sharp decrease and a sharp increase were observed in the abundance of *Bacteroidetes* and *Firmicutes*, respectively. At day 286, the copy numbers of fermenters in the 100CS reactor increased remarkably to more than 4.57 times those at day 258, while a slight decrease was observed for SRB copies (Fig. 4.7). Similar phenomena occurred in the 70CS reactor but with smaller changes. The decreasing copies of SRB may be attributed to the lack of electron acceptors for the energy metabolism of SRB due to a reducing amount of sulfate load as flow ceased. With regard to the SMC reactor, although the abundance of core phyla was generally sustained at similar level around day 286, the gene copies of cellulose degraders, fermenters, and SRB decreased as revealed by qPCR, probably indicating compromised treatment efficiency.

When the performance of the treatments started to decline after day 286 and gradually approached the end phase (day 643), microbial communities in the three pilot VFPs shifted but to a lower degree. At day 643, the abundance of *Proteobacteria* and *OD1* phyla in the CS reactors were higher as compared to their start-up level at day 28, whereas no obvious changes were observed in the abundance of core phyla in the SMC reactor (Fig. 4.8). Nevertheless, the abundance of species in some rare phyla (*Fusobacteria*, *Tenericutes*, *Deferrribacteres*, *OP11*, and *Chlamydiae*) which were the main contributors to the relatively higher Shannon diversity indices in the SMC reactor,
gradually decreased to extinction (Fig. 4.10). Remarkably, qPCR data revealed that SRB copies in the 70CS and 100CS reactors increased to $7.71 \times 10^7$ and $4.91 \times 10^7$ copies/g, respectively, at day 643, more than 56.3 and 27.4 times higher than those at day 28 (Fig. 4.7). This is impressive considering the ability of CS to sustain a continuous supply of nutrients and a satisfactory microbial environment to support the growth of SRB during the entire test. This observation contributed to one of the major reported advantages of CS amendment in the passive AMD treatment systems.

4.5.2 Presence and diversity of key functional groups

Putative functions of core genera revealed five key functional groups including cellulose and other biopolymer degraders, fermenters, sulfur-reducing bacteria, SRB, and chitin degraders. This is consistent with the observations of a previous microbial study of five continuous-flow columns (Chapter 2) and other sulfate-reducing bioreactors and treatment systems (Logan et al., 2005; Pereyra et al., 2008). Specifically in this pilot test, all reactors were observed to accommodate species from the five functional groups, although the diversity and quantity were different. Noticeably, a higher abundance of SRB was associated with sequences obtained from the 70CS and 100CS pilot VFPs, and was the most frequently detected functional group within the two reactors.

Hydrolysis and/or degradation of cellulosic materials and other biopolymers represents the first step of substrate decomposition in biological AMD treatment systems fed with complex substrate materials. It was reported that this process was a possible bottle-neck in the entire carbon and nitrogen flow of the treatment systems because of the inherent complicated chemical structure of biopolymers which renders them hard to be
decomposed. In addition to some common genera (*Clostridium*, *Cellulomonas*, *Bacteroides*, etc.) the most frequently observed for cellulose degradation, *Acetivibrio* spp. were also identified in all pilot VFPs with the highest abundance in the SMC reactor (Table 4.5). In addition, the genus *Ohtaekwangia*, shown to be able to assimilate cellulose (Yoon et al., 2011) was also detected in the two pilot reactors containing different fractions of SMC. Different from the traditional AMD treatment systems filled primarily with cellulosic materials as substrate (Logan et al., 2005; Pereyra et al., 2008; Pruden et al., 2007), pilot reactors with CS amendment tended to accommodate higher varieties of microorganisms capable of degrading biopolymers other than polysaccharides, such as proteins, gelatins, polypeptides, and chitin. Specifically, chitin-degrading genera such as *Chitinilyticum* and *Coenonia* (Euzeby, 2000; Vandamme et al., 1999) were observed to be more abundant in the two CS reactors. Overall, the degradation of biopolymers yields gases and simpler organic compounds which can be selectively utilized by downstream microbes for their growth and activity.

As located mid-way in the entire carbon and nitrogen flow, fermenters not only take advantage of the products from cellulose hydrolysis to support their own activity, but also act as suppliers of simple organic compounds, CO₂, and/or H₂ to downstream microorganisms, especially to SRB. Because of the higher sequencing depth in this pilot study, some fermentative genera which were rarely detected in other AMD treatment systems showed higher abundance in the three reactors. Some representative genera were *Parabacteroides*, *Treponema*, *Dysgonomona*, *Bellilinea*, *Leptolinea*, and *Hydrogenoanaerobacterium*, among them, the last genus was also found to exist in the granular sludge of a laboratory-scale, H₂-producing, up-flow anaerobic sludge blanket.
(UASB) reactor (Song and Dong, 2009). In addition, sequences which were similar to species in the core genera of *Sedimentibacter*, *Anaerovorax*, and *Treponema* were reported to be able to ferment amino acids (Hespell and Canale-Parola, 1971; Matthies et al., 2000; Obst et al., 2005), and were predominately detected in the two CS reactors.

SRB have previously been recognized as key players in passive AMD treatment systems due to their contribution to the reduction of sulfur species, generation of alkalinity, and precipitation of metal sulfides (Logan et al., 2005; Morales et al., 2005; Pereyra et al., 2008). Specifically in our pilot test, a higher abundance of SRB was observed in the 12 samples from the 70CS and 100CS reactors with maximum values of 9.8% and 6.4%, respectively (Fig. 4.11). Correspondingly, the Shannon diversity index calculated for SRB genera in each sample revealed higher SRB diversity in the 70CS (0.89 on average) and 100CS reactors (0.76 on average). A total of 19 SRB genera were detected in the three pilot VFPs, among them, *Desulfosporosinus* and *Desulfovibrio* were shared by all samples over the entire test. In addition, a representative genus of sulfur-reducing bacteria, *Petrimonas*, and sulfite-reducing bacteria, *Desulfitobacterium*, were detected in all three pilot VFPs. Furthermore, it was observed that the *OD1* genus increased noticeably over time, especially in the 100CS reactor. Although no cultivated strain has been obtained within the genus, sequencing of its DNA molecules demonstrated that members in *OD1* might be anaerobic and involved in sulfur cycling (Borrel et al., 2010; Harris et al., 2004). Compared to some AMD bioremediation systems with traditional limestone and SMC substrates (Pereyra et al., 2008; Pruden et al., 2007) which habited only a few SRB species, the two pilot treatments amended with CS in our
study accommodated more varieties of SRB, probably explaining the improved system performance observed.

   Apart from the key putative functions, species of some dominant genera were shown to be able to grow using diverse substrates through various pathways. *Hyphomicrobium* spp. and *Gemmatimonas* spp. are aerobes capable of metabolizing methylated sulfur compounds (Fukushima et al., 2013) and accumulating phosphates (Zhang et al., 2003), respectively, and were exclusively detected in the pilot VFPs containing SMC in their substrate layer. Moreover, genera possessing other functions or metabolic pathways such as nitrate reduction and the degradation of steroidal hormones (*Steroidobacter*), slow-growing oligotrophs (*Gp6*), and anaerobic photoheterotrophs (*Blastochloris*), were more frequently detected in the 70CS and SMC reactors.

   As determined by qPCR, the population size of total fungi and archaea was more than two magnitudes lower than that of bacteria; still, their roles in assisting with the decomposition or utilization of specific organic compounds were of special interest during AMD bioremediation. It was previously demonstrated that fungal species played an important part in promoting the hydrolysis or degradation of polymers and monomers in sulfate-reducing columns treating AMD. In addition, most archaeal species in the AMD-treating columns were identified to be methanogens (Chapter 2). Interestingly in our pilot study, almost no archaeal species were detected in the 100CS reactor during the first two sampling events (day 28 and 84) (Fig. 4.6). This could be explained through two perspectives. First of all, during the start-up phase, the 100CS reactor maintained a microbial environment of the highest alkalinity (averaging at more than 1200 mg/L as CaCO₃), more negative ORP (< -260 mV), and circum-neutral pH, which favored the
rapid growth of SRB under sufficient sulfate loads. Indeed, copy numbers of the dsrA gene representing SRB in the 100CS reactor were highest among the three pilot reactors at day 28 as determined by qPCR (Fig. 4.7). Secondly, the prevailing growth and activity of SRB kept simple organic substrates less available and accessible to methanogens. When coupled with the inhibition effect of certain sulfides and sulfur-containing compounds (Karhadkar et al., 1987; Kristjansson et al., 1982; Zeleke et al., 2013; Zinder et al., 1984) generated through sulfate reduction and chitin or protein fermentation, the growth of methanogens was clearly suppressed in the 100CS reactor. During pseudo-steady state and thereafter, the archaeal community in the 100CS reactor gradually increased and became detectable but was still the lowest among all pilot reactors.

4.5.3. Relationship of microbial community and system performance

Previous research by others showed a close and positive relationship between functional diversity of microbial communities and treatment efficiency in AMD passive treatment systems driven by biological processes (Pereyra et al., 2008). Specifically in our pilot tests, bacterial communities in the 70CS and 100CS reactors formed two clusters on the same branch in the dendrogram, indicating similar microbial compositions and diversity. Correspondingly, chemical performance of the two CS reactors mimicked each other and showed little difference in terms of alkalinity generation, sulfate reduction, and dissolved metal removal.

It is well-known that SRB abundance and activity are critical to the performance of AMD bioremediation systems in that sulfate reduction produces net alkalinity and promotes the precipitation of metals as sulfides. Obviously, in the pilot VFPs, both the
amount of total alkalinity released and percentages of total metals retained within the reactors were positively correlated with SRB abundance and diversity (Table 4.6). Meanwhile, sulfate removal rates predicted by geochemical modeling were higher in the two CS reactors during the entire test than in the SMC reactor. Although the Shannon diversity indices at the 3% OTU cutoff level in six SMC samples were relatively higher than in the CS reactors, it should be noticed that this was partly due to various species in the rare phyla, most of which disappeared during the operation of the reactor (Fig. 4.10). Gene quantification by qPCR also demonstrated that the population sizes of total bacteria, fermenters, and SRB in the two CS reactors were significantly larger than in the SMC reactor which had comparable copy numbers of cellulose degraders (Fig. 4.12). Moreover, investigation of core phyla and genera demonstrated that the SMC reactor hosted a relatively homogeneous bacterial community containing a higher abundance of polysaccharide degraders and carbohydrate fermenters with less SRB and nearly no protein-, chitin-, or polypeptide-degraders. Additionally, some strict aerobes were detected in the SMC reactor with the highest abundance, indicating an elevated ORP level and thus a more oxidized microbial environment in the pores of the substrate layer which may have prevented most SRB and other strict anaerobic microorganisms from rapid growth. In summary, bacterial communities in the two pilot VFPs containing CS exhibited higher diversities in terms of key microbial functions, thus providing explanations for the enhanced treatment efficiency and longer life-time of the CS reactors in the pilot-scale field test.

Under system flow shock when the flow rate was reduced to zero around day 286, alkalinity concentrations in all pilot VFPs reached to the second peak value. The two
basic mechanisms that contribute to alkalinity generation in passive AMD treatment systems are: 1) abiotic dissolution of calcium carbonate from the substrate matrix; and, 2) biotic pathways of VFA-production, fermentation, and sulfate reduction (Robinson-Lora and Brennan, 2009). At day 286, the abundance of SRB in total effective bacterial reads decreased in the pilot VFPs as revealed by 454 sequencing, and copy numbers of dsrA gene were lower than those at adjacent sampling event (day 258 and 340). Assuming similar activity for each species in the SRB group, it was speculated that the relatively higher alkalinity levels in the three pilot VFPs around day 286 were mainly due to lower dilution ratios with less or no influent water, and the contribution of SRB activity was negligible.

Finally, from the standpoint of chemical performance, the 70CS pilot reactor showed no obvious difference from the 100CS reactor which contained a higher proportion of CS in its substrate layer. Remarkably, the 70CS reactor exhibited a higher sulfate removal rate immediately after incubation, and higher removal of total Fe, Mn, and Zn (Table 4.6) as compared to the pilot reactor with 100% CS. Correspondingly, 454 sequencing of the 18 samples from the three pilot reactors revealed similar microbial compositions, diversities, and abundance of core phyla and genera in the two reactors containing CS. Meanwhile, qPCR data suggested that except for fermenters, average copy numbers of all quantified functional groups including SRB in the 70CS reactor were at least 1.2 times higher than in the 100CS reactor. When combined together, these observations provide justification for the application of a mixture of CS and cellulosic materials at optimal ratios to ensure comparable treatment efficiency while still maintaining reasonable costs.
4.6. Conclusions

The impact of both substrate composition and operational conditions on the composition, diversity, and abundance of microbial communities in three pilot VFPs treating AMD was revealed through 454 sequencing together with qPCR. The major conclusions were:

a. Based on Unifrac significance tests at the 3% OTU cutoff level, no significant difference was indicated for bacterial communities in the 70CS and 100CS pilot VFPs, whereas the bacterial community in the SMC reactor was observed to be significantly different from the two CS reactors at all sampling events;

b. PCoA analyses indicated that bacterial communities in the six samples from each pilot VFP were more likely to form separate clusters, but the two clusters from the 70CS and 100CS reactors were more closely related to each other in terms of distance matrix and shared OTUs;

c. The 70CS and 100CS pilot VFPs supported the growth of microorganisms at increasing abundance level of core phyla over time, and more importantly, also supported a higher diversity of SRB;

d. A relatively homogeneous bacterial community was observed in the SMC reactor composed of a large population of hydrolytic and fermentative bacteria;

e. Copy numbers of genes representing total bacteria, fermenters, and sulfate reducers were higher in the pilot reactors containing CS;
f. At day 286 under no flow, the relative abundance of the core phyla shifted in all pilot reactors, with the smallest changes in functional gene copies observed in the 70CS reactor.

Together with the chemical performance data, the observations of this microbial study further validated the role of CS as a promising multifunctional substrate, and more practically, justified the beneficial application of CS combined with cellulosic materials at optimal ratios in passive AMD treatment systems to enhance treatment efficiency and system longevity.

4.7. Acknowledgements

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4.8. References


**TABLE 4.1.** Substrate compositions and underdrain layers in pilot-scale VFPs treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Reactor code</th>
<th>Substrate layer components (% total substrate mass)</th>
<th>Total substrate mass (kg)</th>
<th>Silica sand (kg)</th>
<th>Total underdrain mass (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 100CS</td>
<td>Crab shell (CS) 100  SMC 0  Limestone (LS) 0</td>
<td>685</td>
<td>680</td>
<td>2413 0</td>
</tr>
<tr>
<td>#2 70CS</td>
<td>Crab shell (CS) 70  SMC 30  Limestone (LS) 0</td>
<td>824</td>
<td>576</td>
<td>2413 0</td>
</tr>
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<td>#3 70CS (SS)</td>
<td>Crab shell (CS) 70  SMC 30  Limestone (LS) 0</td>
<td>824</td>
<td>576</td>
<td>0 2413</td>
</tr>
<tr>
<td>#4 SMC</td>
<td>Crab shell (CS) 0  SMC 90  Limestone (LS) 10</td>
<td>610</td>
<td>0</td>
<td>2413 0</td>
</tr>
</tbody>
</table>
**TABLE 4.2.** Gene primer sets used in qPCR.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer Probe</th>
<th>Strain used to generate standard</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria, 16s rRNA</td>
<td>BAC331F/ BAC797R Tamra probe BAC</td>
<td><em>Geobacter sulfurreducens</em> DSM 12127</td>
<td>60</td>
<td>(Nadkarni et al., 2002)</td>
</tr>
<tr>
<td>Total archaea, 16s rRNA</td>
<td>Arch349F/ Arch806R Tamra probe Arch516</td>
<td><em>Methanosarcina barkeri</em> DSM 800</td>
<td>59</td>
<td>(Takai and Horikoshi, 2000)</td>
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<td>Total fungi, ITS</td>
<td>ITS1F/ ITS4</td>
<td><em>Trametes versicolor</em> WC861</td>
<td>55</td>
<td>(Manter and Vivanco, 2007)</td>
</tr>
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<td>Cellulose degraders, cel5</td>
<td><em>cel5</em>&lt;sub&gt;392F&lt;/sub&gt;/ <em>cel5</em>&lt;sub&gt;754R&lt;/sub&gt;</td>
<td><em>Clostridium cellulovorans</em> DSM 3052</td>
<td>52</td>
<td>(Pereyra et al., 2010)</td>
</tr>
<tr>
<td>Fermenters, hydA</td>
<td><em>hydA</em>&lt;sub&gt;1290F&lt;/sub&gt;/ <em>hydA</em>&lt;sub&gt;1538R&lt;/sub&gt;</td>
<td><em>Clostridium acetobutylicum</em> ATCC 824</td>
<td>59</td>
<td>(Pereyra et al., 2010)</td>
</tr>
<tr>
<td>Sulfate reducers, dsrA</td>
<td><em>dsrA</em>&lt;sub&gt;290F&lt;/sub&gt;/ <em>dsrA</em>&lt;sub&gt;660R&lt;/sub&gt;</td>
<td><em>Desulfovibrio vulgaris</em> DSM 644</td>
<td>60</td>
<td>(Pereyra et al., 2010)</td>
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</tbody>
</table>
TABLE 4.3. Numbers of OTUs, Good’s coverage, and Shannon diversity indices of the 18 samples calculated from 454 sequencing data at 3% cutoff level.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>No. of sequences</th>
<th>No. of OTUs</th>
<th>Good’s coverage (%)</th>
<th>Shannon diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100CS-D28</td>
<td>9576</td>
<td>552</td>
<td>97</td>
<td>3.51</td>
</tr>
<tr>
<td>100CS-D84</td>
<td>9563</td>
<td>561</td>
<td>97</td>
<td>3.78</td>
</tr>
<tr>
<td>100CS-D258</td>
<td>14590</td>
<td>849</td>
<td>97</td>
<td>4.06</td>
</tr>
<tr>
<td>100CS-D286</td>
<td>13611</td>
<td>652</td>
<td>97</td>
<td>3.73</td>
</tr>
<tr>
<td>100CS-D340</td>
<td>16193</td>
<td>726</td>
<td>97</td>
<td>3.72</td>
</tr>
<tr>
<td>100CS-D643</td>
<td>18231</td>
<td>766</td>
<td>97</td>
<td>3.98</td>
</tr>
<tr>
<td>70CS-D28</td>
<td>18233</td>
<td>892</td>
<td>94</td>
<td>4.55</td>
</tr>
<tr>
<td>70CS-D84</td>
<td>13700</td>
<td>684</td>
<td>96</td>
<td>3.69</td>
</tr>
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<td>70CS-D258</td>
<td>20888</td>
<td>871</td>
<td>98</td>
<td>2.87</td>
</tr>
<tr>
<td>70CS-D286</td>
<td>12107</td>
<td>822</td>
<td>95</td>
<td>4.31</td>
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<td>70CS-D340</td>
<td>18592</td>
<td>1178</td>
<td>95</td>
<td>4.29</td>
</tr>
<tr>
<td>70CS-D643</td>
<td>24748</td>
<td>1835</td>
<td>96</td>
<td>5.11</td>
</tr>
<tr>
<td>SMC-D28</td>
<td>15533</td>
<td>3617</td>
<td>86</td>
<td>7.03</td>
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<tr>
<td>SMC-D84</td>
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<td>6.99</td>
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<td>SMC-D258</td>
<td>22212</td>
<td>4184</td>
<td>89</td>
<td>6.84</td>
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<tr>
<td>SMC-D286</td>
<td>20638</td>
<td>4119</td>
<td>89</td>
<td>6.82</td>
</tr>
<tr>
<td>SMC-D340</td>
<td>23446</td>
<td>4803</td>
<td>88</td>
<td>7.21</td>
</tr>
<tr>
<td>SMC-D643</td>
<td>17407</td>
<td>3758</td>
<td>87</td>
<td>7.01</td>
</tr>
</tbody>
</table>
TABLE 4.4. Percentages of shared taxa by the 18 samples in the pilot VFPs.

<table>
<thead>
<tr>
<th>No. of sample</th>
<th>No. shared</th>
<th>% in classified classes</th>
<th>No. shared</th>
<th>% in classified orders</th>
<th>No. shared</th>
<th>% in classified families</th>
<th>No. shared</th>
<th>% in classified genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73</td>
<td>91.25</td>
<td>126</td>
<td>89.36</td>
<td>263</td>
<td>81.68</td>
<td>907</td>
<td>62.90</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>87.50</td>
<td>119</td>
<td>84.40</td>
<td>252</td>
<td>78.26</td>
<td>807</td>
<td>55.96</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>82.50</td>
<td>114</td>
<td>80.85</td>
<td>240</td>
<td>74.53</td>
<td>721</td>
<td>50.00</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>82.50</td>
<td>112</td>
<td>79.43</td>
<td>232</td>
<td>72.05</td>
<td>678</td>
<td>47.02</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>76.25</td>
<td>106</td>
<td>75.18</td>
<td>220</td>
<td>68.32</td>
<td>605</td>
<td>41.96</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>73.75</td>
<td>103</td>
<td>73.05</td>
<td>216</td>
<td>67.08</td>
<td>560</td>
<td>38.83</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>62.50</td>
<td>90</td>
<td>63.83</td>
<td>190</td>
<td>59.01</td>
<td>377</td>
<td>26.14</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>52.50</td>
<td>76</td>
<td>53.90</td>
<td>155</td>
<td>48.14</td>
<td>254</td>
<td>17.61</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>42.50</td>
<td>58</td>
<td>41.13</td>
<td>114</td>
<td>35.40</td>
<td>159</td>
<td>11.03</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>37.50</td>
<td>52</td>
<td>36.88</td>
<td>100</td>
<td>31.06</td>
<td>132</td>
<td>9.15</td>
</tr>
<tr>
<td>11</td>
<td>27</td>
<td>33.75</td>
<td>49</td>
<td>34.75</td>
<td>89</td>
<td>27.64</td>
<td>110</td>
<td>7.63</td>
</tr>
<tr>
<td>12</td>
<td>23</td>
<td>28.75</td>
<td>44</td>
<td>31.21</td>
<td>83</td>
<td>25.78</td>
<td>101</td>
<td>7.00</td>
</tr>
<tr>
<td>13</td>
<td>19</td>
<td>23.75</td>
<td>38</td>
<td>26.95</td>
<td>72</td>
<td>22.36</td>
<td>77</td>
<td>5.34</td>
</tr>
<tr>
<td>14</td>
<td>19</td>
<td>23.75</td>
<td>37</td>
<td>26.24</td>
<td>67</td>
<td>20.81</td>
<td>71</td>
<td>4.92</td>
</tr>
<tr>
<td>15</td>
<td>18</td>
<td>22.50</td>
<td>35</td>
<td>24.82</td>
<td>64</td>
<td>19.88</td>
<td>67</td>
<td>4.65</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>21.25</td>
<td>33</td>
<td>23.40</td>
<td>61</td>
<td>18.94</td>
<td>63</td>
<td>4.37</td>
</tr>
<tr>
<td>17</td>
<td>16</td>
<td>20.00</td>
<td>31</td>
<td>21.99</td>
<td>58</td>
<td>18.01</td>
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<td>3.88</td>
</tr>
<tr>
<td>18</td>
<td>16</td>
<td>20.00</td>
<td>30</td>
<td>21.28</td>
<td>53</td>
<td>16.46</td>
<td>55</td>
<td>3.81</td>
</tr>
</tbody>
</table>

*a Number of pilot reactor samples which shared the taxa in the corresponding columns.

*b Percentage of the number of a shared taxon in the total number of the classified taxon.
TABLE 4.5. Representative bacterial genera in the three pilot VFPs treating AMD with different substrate mixtures of crab shell (CS) and spent mushroom compost (SMC) based on genera abundance as revealed by 454 sequencing.

<table>
<thead>
<tr>
<th>Representative bacterial genera</th>
<th>Possible function</th>
<th>100CS</th>
<th>70CS</th>
<th>SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium_sensu_stricto</em></td>
<td>Hydrolysis; fermentation</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>OD1_genus_incertae_sedis</em></td>
<td>May be involved in sulfur cycling</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>Desulfovibrio</em></td>
<td>Sulfate reduction</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Parabacteroides</em></td>
<td>Fermentation</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Treponema</em></td>
<td>Fermentation of carbohydrates and amino acids</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>Dysgonomonas</em></td>
<td>Glucose fermentation</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Coenia</em></td>
<td>Hydrolysis of sulfate esters and chitin</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Desulfophorococcus</em></td>
<td>Sulfate reduction</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Candidatus</em></td>
<td>Hydrolysis; fermentation</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Sediminibacter</em></td>
<td>Biopolymer degradation</td>
<td>+++</td>
<td>++</td>
<td>d</td>
</tr>
<tr>
<td><em>Desulfosporosinus</em></td>
<td>Sulfate reduction</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>Sedimentibacter</em></td>
<td>Fermentation of amino acids</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Hyphomicrobiium</em></td>
<td>Grows aerobically on methylated sulfate compounds; denitrification</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Ohtaekwangia</em></td>
<td>Cellulose assimilation; hydrolysis</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Bellenula</em></td>
<td>Carbohydrate fermentation</td>
<td>++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td><em>Petrimonas</em></td>
<td>Carbohydrates fermentation; sulfur reduction</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Gemmatimonas</em></td>
<td>Aerobic, polyphosphate-accumulating</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Geobacillus</em></td>
<td>Hydrolysis, fermentation; denitrification</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Anaerovorax</em></td>
<td>Fermentation, often amino acids</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>Opitutus</em></td>
<td>Polysaccharide degradation</td>
<td>++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td><em>Succinispira</em></td>
<td>Fermentation of succinate, amino acids; need reductant</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>Steroidobacter</em></td>
<td>Nitrate reduction; utilize steroidal hormones</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Paludibacter</em></td>
<td>Carbohydrate fermentation</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Desulfosulfitomonas</em></td>
<td>Sulfate reduction</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Leptolinea</em></td>
<td>Fermentation</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td><em>Anaerospirillum</em></td>
<td>Polysaccharide fermentation</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Hydrogenoanaerobacterium</em></td>
<td>Gelatin hydrolysis; glucose fermentation</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>Acetivibrio</em></td>
<td>Cellulose degradation</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Desulfobulbus</em></td>
<td>Sulfate reduction</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Desulfotomaculum</em></td>
<td>Sulfate reduction</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Thermodesulfobium</em></td>
<td>Sulfate reduction</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td><em>Chitinilyticum</em></td>
<td>Chitin degradation, fermentation</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Gp6</em></td>
<td>Slow-growing oligotrophs</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Blastochloris</em></td>
<td>Anaerobic phototroph</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*a”+++” a specific genus was present at top abundance on average among all reactors.

*b”++” a specific genus was present at the second highest abundance on average among all reactors.

*c”+” a specific genus was present at the lowest abundance on average among all reactors.

*d”-” a specific genus was absent in the reactor.
TABLE 4.6. Abundance and diversity of sulfate-reducing bacteria (SRB) together with performance data in the three pilot VFPs at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>100CS</th>
<th>70CS</th>
<th>SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abundance of SRB (%)</td>
<td>3.96</td>
<td>5.40</td>
<td>2.48</td>
</tr>
<tr>
<td>Shannon diversity of SRB</td>
<td>0.76</td>
<td>0.89</td>
<td>0.49</td>
</tr>
<tr>
<td>Total alkalinity released (kg as CaCO$_3$)</td>
<td>203</td>
<td>177</td>
<td>62</td>
</tr>
<tr>
<td>Percentage of Fe retained (%)</td>
<td>99.30</td>
<td>99.48</td>
<td>95.31</td>
</tr>
<tr>
<td>Percentage of Al retained (%)</td>
<td>92.36</td>
<td>92.01</td>
<td>91.99</td>
</tr>
<tr>
<td>Percentage of Mn retained (%)</td>
<td>32.39</td>
<td>79.15</td>
<td>19.47</td>
</tr>
<tr>
<td>Percentage of Zn retained (%)</td>
<td>68.98</td>
<td>69.62</td>
<td>69.01</td>
</tr>
</tbody>
</table>

$^a$ average values were presented for all parameters in the table.

$^b$ Abundance of SRB referred to the percentage of SRB in total effective bacterial reads in each pilot VFP as determined by 454 sequencing.
FIG. 4.1. Abundance of core phyla in 18 samples from three pilot VFPs (Klondike-1 site) at six sampling events as revealed by 454 sequencing. The abundance is presented as a percentage of total effective reads of bacterial 16S rRNA gene in a sample. Taxa presented here stands for >0.5% abundance in at least one sample.
FIG. 4.2. Abundance of different classes of *Proteobacteria* in 18 samples from the three pilot VFPs (Klondike-1 site) at six sampling events as revealed by 454 sequencing. The abundance is presented as a percentage of total effective *Proteobacteria* reads in a sample.
**FIG. 4.3.** Dendrogram based on Jaccard distances of the 18 samples from the pilot VFPs at 3% cutoff-OTU level. The 18 samples were clustered into six groups at 0.58 similarity value as shown by the dotted line across the tree branches.
FIG. 4.4. Principal coordinate analysis (PCoA) of the total bacterial community in 18 samples collected from three pilot VFPs featuring different substrate compositions of CS and SMC, plotted as (a) P1 versus P2 and (b) P2 versus P3. The PCoA plots were constructed based on a 3% cutoff-OTU level using a Jaccard calculator.
FIG. 4.5. Heat map of the top 30 genera with the highest abundance of total effective bacterial reads in 18 samples from three pilot VFPs (Klondike-1 site) at six sampling events as revealed by 454 sequencing. By comparing with the color key at the top left corner, the color intensity in each panel indicates percentages of a genus in a sample.
FIG. 4.6. Quantification of total bacteria, total archaea, and total fungi by qPCR in 18 samples from three pilot VFPs at six sampling events. qPCR results are expressed as the average copies per gram of substrate; error bars represent the standard deviation of the values from a total of nine replicates at three dilution levels for each DNA sample. The copy numbers below detection limit of archaeal qPCR assays were reported as equal to zero.
FIG. 4.7. Quantification of *cel5* (cellulose degraders), *hydA* (fermenters), and *dsrA* (sulfate reducers) genes by qPCR in 18 samples collected from three pilot VFPs at six sampling events. qPCR results are expressed as average copies per gram of substrate. Error bars represent the standard deviation of the values from a total of nine replicates at three dilution levels for each DNA sample.
FIG. 4.8. Abundance of core phyla in the 100CS (a), 70CS (b), and SMC (c) reactors over the course of the pilot study. The abundance is presented as a percentage of a core phylum in total effective bacterial reads in a sample as revealed by 454 sequencing.
FIG. 4.9. Venn diagram showing the number of species shared by three pilot VFPs at an OTU cutoff of 3%.
FIG. 4.10. Abundance of rare phyla in the SMC reactor over the course of the pilot study. The abundance is presented as a percentage of a rare phylum in total effective bacterial reads in a SMC sample as revealed by 454 sequencing.
FIG. 4.11. Abundance of the genera of sulfate-reducing bacteria (SRB) in the three pilot VFPs over the course of the pilot study. The abundance is presented as a percentage of the SRB genus in total effective bacterial reads in a sample as revealed by 454 sequencing.
FIG. 4.12. Comparison of average copy numbers of total bacterial, total archaea, total fungi and different functional genes (cel5, hydA, and dsrA) in samples from the 70CS and the 100CS pilot reactors (a), and from the 70CS and the SMC reactors (b) as determined by qPCR.
CHAPTER 5

CONCLUSIONS, ENGINEERING SIGNIFICANCE, AND FUTURE WORK

5.1. Conclusions

By applying both chemical and biological molecular techniques, this study:

demonstrated the composition, diversity, and functional roles of microbial communities
associated with different substrate combinations of crab shell (CS) and spent mushroom
compost (SMC) in the five sulfate-reducing columns treating acid mine drainage (AMD);
evaluated the effect of scale-up and varying environmental conditions on system
performance of four pilot-scale vertical flow ponds (VFPs) at the Klondike-1 site; and
investigated the establishment and adaptability of microbial communities and their
relationship with AMD treatment efficiency in three of the VFPs over the course of pilot
tests.

The microbial study in the five continuous-flow columns treating AMD
demonstrated the capacity of CS to support the establishment of functionally more
diverse and more abundant microbial communities, in comparison to traditional cellulosic
materials such as SMC. Columns packed with higher proportions of CS accommodated
more diverse bacterial and archaeal communities, but a less diverse fungal community.
Through statistical analyses using the UniFrac algorithm, bacterial and archaeal
communities in columns with higher fraction of CS were similar to each other and more
likely to cluster together in PCoA plots, suggesting structural differences from those in 5%
CS, SMC, and control columns. This was consistent with the sequence distribution patterns of bacterial ARB tree in which a deep branch (CAMD17) consisting of 19 sequences from 50% CS and 100% CS columns was observed. By assigning putative functions to clone sequences, five key functional groups were identified: cellulose degraders, chitin degraders, fermenters, sulfate-reducing bacteria (SRB), and sulfur-reducing bacteria. Among them, SRB were commonly recognized as key players due to their ability to consume sulfate, produce net alkalinity, and promote metal precipitation. The presence of each functional group is critical in bioremediation systems treating AMD, and the diversity and cooperation among different microorganisms in various groups are of great importance to balance organic compounds and to create a favorable microbial environment. Specifically, the 100% CS column supported the growth of microbial populations from all key functional levels in the system, especially SRB from eight different genera. Moreover, it was observed via qPCR that copy numbers of the functional genes representing fermenters, sulfate reducers, and chitin degraders had positive correlation with the proportions of CS. Together with the consistent chemical performance data, CS was further validated in its promising ability of improving both system longevity and performance from the microbiological perspective by supporting the growth and activity of healthier and diverse microbial communities in passive AMD treatment systems.

In the pilot study, performance data suggested that CS amendments had significant impact on treatment efficiency and stability in terms of alkalinity generation, sulfate removal, and metal precipitation. It was observed that total alkalinity generated had a strong positive correlation ($R^2=0.98$) with the proportion of CS in the substrate
layer. Although pH in all pilot reactors increased to circum-neutral immediately after incubation, the CS reactors sustained higher pH values over the entire duration of the pilot tests. Furthermore, higher fractions of CS were able to promote more complete metal removal and retain a higher amount of dissolved metals through precipitation and sorption. No breakthrough was observed for Fe, Al, and Zn even under changing environmental conditions and flow fluctuations, which are typically encountered in the operation of field remediation systems for AMD or other similar contaminants. Finally, the VFP containing a mixture of 70% CS with 30% SMC was shown to maintain a more stable performance while exhibiting comparable treatment efficiency to the 100% CS reactor over varying environmental and operational conditions during the pilot study. Therefore, the use of CS as a fractional amendment in the substrate mixture of passive treatment systems for AMD, and potentially other environmental contaminants, is more attractive from an engineering standpoint when considering both cost and system performance.

The results of the microbial study on the three pilot VFPs with different substrates also supported the observation of enhanced system performance and stability in the CS-containing reactors. First of all, bacterial communities in the pilot VFPs containing 70% and 100% CS were more similar to each other than to communities in the SMC reactor as revealed by UniFrac significance tests and cluster analysis. Secondly, compared to the larger population of hydrolytic and fermentative bacteria in the SMC reactor, the two CS reactors supported the growth of microorganisms in key functional groups at increasing abundance and diversity levels over time, especially more diverse SRB. Moreover, the 70% and 100% CS pilot reactors supported comparable quantities of total bacteria, total fungi,
cellulose degraders, fermenters, and SRB. Under conditions of zero flow, both the diversity and quantity of microbial community in the 70% CS reactors were shown to be the most stable of all the pilot reactors.

5.2. Engineering Significance

More diverse and stable microbial communities coupled with more efficient and reliable system performance provides further justification and confidence for the future application of CS materials as a fractional amendment to substrate mixtures in passive treatment systems for not only AMD but also other environmental contaminants that are degradable under anaerobic conditions.

Overall, this research served as a further step to evaluate the potential advantages of CS as a multifunctional substrate amendment at the field scale and from a microbiological perspective in passive AMD treatment systems. Due to the relatively higher unit price of CS materials, a mixture of crab shell and other complex organic materials such as SMC is recommended in the future design of bioremediation systems to ensure comparable treatment efficiency and longevity while maintaining reasonable cost. This is well supported by the observations from the microbial study. In addition, the data from the pilot tests showed that the amount of total alkalinity released from the two reactors containing 70% CS with different underdrain materials was quite similar, and was attributable to less than 10% of calculated theoretical alkalinity of the packing materials, indicating the compromised buffering capacity of limestone (LS). However, in a previous column study that was run to exhaustion (Grembi, 2011), the alkalinity of the
CS was observed to exhaust first, leaving fermentable carbon remaining in the substrate, and therefore making the addition of limestone to passive treatment systems necessary to sustain the degradation of the remaining substrate after the exhaustion of the CS buffering capacity. Therefore, as long as LS rock is not cost prohibitive as compared to other underdrain materials such as sandstone, the addition of LS is recommended to support the complete fermentation of substrate materials such as CS.

Based on the comparison of treatment efficiency between the pilot test and previous column study (Grembi, 2011), it is of great importance to design and maintain an appropriate HRT to maximize alkalinity generation and metal removal in anaerobic bioremediation systems. In our AMD treatment systems amended with CS, the suggested HRT range is between 16 to 25 hrs depending on the substrate composition and influent water chemistry. Flow fluctuation occurred even during warmer months due to clogging of inlet hoses with iron oxides, and was shown to affect both system performance and microbial structures to varying degrees. Thus, regular maintenance of pipes and connecting points is necessary to maintain a healthy microbial community, and thus sustain more stable treatment efficiency in AMD bioremediation systems subject to frequent metal oxide precipitation.

5.3. Future Research

More research is needed in the future from two standpoints. In the macro-perspective, although the substrate mixture of 70% CS and 30% SMC was shown to be comparably efficient with 100% CS in treating AMD, different substrate combinations
containing lower CS and higher SMC (such as 60% CS combined with 40% SMC),
which would be more economically advantageous, should be investigated to determine if
the performance is significantly different from the 70% CS reactor. In addition, the
treatment efficiency of AMD or other industrial wastewaters containing higher loadings
of primary metals such as Al, Zn, and Mn, should be evaluated with CS-containing
columns or reactors to expand the potential application of this substrate. Moreover,
continuous monitoring of cations and nutrients released from CS-amended AMD
treatment systems is important to estimate the potential environmental risks associated
with this kind of material to receiving watersheds. It would be valuable to evaluate other
complex organic materials possessing characteristics similar to CS (ex., micro-porous,
high buffering capacity, high nutrient content) for their ability to be used as substrates.

Finally, from the perspective of the micro-environment, in order to access real-
time gene expression and the response of microbial community to varying environmental
conditions, RNA work could be selectively carried out. In addition, sequencing of PCR
products targeting key functional genes will provide a broader view on species richness
and diversity in core functional groups.
Appendix A

Estimation of Shannon Diversity and Good’s Coverage

Good’s coverage was used to estimate sampling coverage and species richness in the microbial study of the five continuous-flow columns as specified below (Eq. A.1):

\[
Coverage = \left(1 - \frac{\text{# of genera}}{\text{# of clones}}\right) \times 100\%
\]  

(A.1)

The Shannon Diversity index was used to quantify the diversity of the microbial community using the following equation (Eq. A.2):

\[
H' = -\sum_{i=1}^{S} p_i \ln (p_i)
\]  

(A.2)

Where: 

\[S = \text{species richness}\]

\[p_i = \text{proportion of a phylotype relative to the sum of all phylotypes}\]

\[i = \text{number of phylotypes}\]
Appendix B

Rarefaction Curves of Sequenced Clones in Column Study

Rarefaction curves of all sequenced bacterial, archaeal, and fungal clones are provided here. The curves were constructed via Mothur software based on operational taxonomic units (OTUs) at a cutoff level of 0.05.

FIG. B.1. Rarefaction curve of bacterial clones sequenced in the five sulfate-reducing columns treating AMD.
FIG. B.2. Rarefaction curve of archaeal clones sequenced in the five sulfate-reducing columns treating AMD.
FIG. B.3. Rarefaction curve of fungal clones sequenced in the five sulfate-reducing columns treating AMD.
Appendix C

Pore Volume Estimation in the Four Pilot VFPs

An attempt to perform conservative tracer test failed due to the interference of high salt concentrations in the effluent of the pilot VFPs. Therefore, given the same substrate compositions in previous continuous-flow columns constructed by former researchers (Grembi, 2011), pore volumes (PV) of the pilot VFPs were estimated by applying normalized effective PV based on the results of a conservative tracer test of those columns. The assumption was that the effective porosity was the same in the pilot reactors for the same composition of substrate. The underdrain layer in each pilot VFP was also included in the calculation considering the possible immigration and transport of organic substrate or degradation products from the substrate layer into the underdrain layer.

First, volumes of the substrate and underdrain layers in each reactor were calculated with the following equation by assigning proper bulk densities to the different packing materials (Eq. C.1):

\[
V_R = \frac{m}{\rho_b}
\]  

(C.1)

Where: \( V_R \) = volume of substrate or underdrain layer in a pilot reactor, \( \text{m}^3 \)

\( m \) = total mass of packing material, \( \text{kg} \)

\( \rho_b \) = bulk density of specific packing material, \( \text{kg/m}^3 \)

The assigned bulk density of each packing material is shown in Table C.1.
Based on the plot of effective PV versus percentage of crab shell in the previous column study, normalized effective PV of the columns were estimated, followed by the calculation of effective porosity using equation C.2.

\[
\theta = \frac{V_{PV}}{V_C} \tag{C.2}
\]

Where: \( \theta \) = effective porosity of the substrate mixture in each column

\( V_{PV} \) = effective pore volume of the substrate mixture in each column, L

\( V_C \) = volume of the column in previous column study, L

The results of calculation are provided in Table C.2.
TABLE C.1. Estimated bulk densities of packing materials in the pilot VFPs at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Packing material</th>
<th>Bulk density (kg/m³)</th>
<th>Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crab shell (SC-20)</td>
<td>450</td>
<td>JRW Bioremediation</td>
</tr>
<tr>
<td>Spent mushroom compost (SMC)</td>
<td>340</td>
<td>Penn State report for fresh mushroom compost</td>
</tr>
<tr>
<td>Limestone chip</td>
<td>1105</td>
<td>Bulk density chart-Anval</td>
</tr>
<tr>
<td>Silica sand</td>
<td>1680</td>
<td>Bulk density chart-Tapco</td>
</tr>
<tr>
<td>Limestone rock</td>
<td>1600</td>
<td>NESL calculator</td>
</tr>
<tr>
<td>Sandstone rock</td>
<td>1440</td>
<td>Kinkead Aggregates</td>
</tr>
</tbody>
</table>

TABLE C.2. Estimated pore volume calculations for the four pilot-scale field reactors with different substrates at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Calculated parameter</th>
<th>1-100CS</th>
<th>2-70CS</th>
<th>3-70CS(SS)</th>
<th>4-SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Total mass (kg)</td>
<td>1365</td>
<td>1400</td>
<td>1400</td>
<td>610</td>
</tr>
<tr>
<td></td>
<td>Volume of the layer (L)</td>
<td>1927</td>
<td>2352</td>
<td>2352</td>
<td>1670</td>
</tr>
<tr>
<td></td>
<td>Effective porosity</td>
<td>0.39</td>
<td>0.36</td>
<td>0.36</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Effective pore volumes (L)</td>
<td>908</td>
<td>858</td>
<td>858</td>
<td>526</td>
</tr>
<tr>
<td>Underdrain</td>
<td>Total mass (kg)</td>
<td>2413</td>
<td>2413</td>
<td>2413</td>
<td>2413</td>
</tr>
<tr>
<td></td>
<td>Volume of the layer (L)</td>
<td>1508</td>
<td>1508</td>
<td>1676</td>
<td>1508</td>
</tr>
<tr>
<td></td>
<td>Porosity</td>
<td>0.45</td>
<td>0.45</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Effective pore volumes (L)</td>
<td>679</td>
<td>679</td>
<td>754</td>
<td>679</td>
</tr>
</tbody>
</table>
FIG. C.1. Correlation of effective pore volumes (PV) with proportion of crab shell in the substrate of a previously conducted continuous-flow column study (Grembi, 2011).
Appendix D

Metal Removal Before and After Passive Aeration and Settling

For each pilot treatment at the Klondike-1 site, metals were measured at two sampling points: in the effluent of pilot VFP and after passive aeration and settling. All metals data before and after passive aeration and settling are provided here for comparison.
FIG. D.1. Breakthrough curves of dissolved iron (Fe) in the effluent of pilot VFPs (a) and after passive aeration (b).
FIG. D.2. Breakthrough curves of dissolved aluminum (Al) in the effluent of pilot VFPs (a) and after passive aeration (b).
FIG. D.3. Breakthrough curves of dissolved manganese (Mn) in the effluent of pilot VFPs (a) and after passive aeration (b).
FIG. D.4. Breakthrough curves of dissolved zinc (Zn) in the effluent of pilot VFPs (a) and after passive aeration (b).
Appendix E

Cation Data in the Effluent of Four Pilot VFPs

The full data sets of other ions such as Ca, K, Mg, Na, and P before and after passive aeration and settling are included in this Appendix for completeness.
**FIG. E.1.** Concentration of dissolved calcium (Ca) in the effluent of pilot VFPs (a) and after passive aeration (b) over time.
FIG. E.2. Concentration of dissolved potassium (K) in the effluent of pilot VFPs (a) and after passive aeration (b) over time.
FIG. E.3. Concentration of dissolved magnesium (Mg) in the effluent of pilot VFPs (a) and after passive aeration (b) over time.
FIG. E.4. Concentration of dissolved sodium (Na) in the effluent of pilot VFPs (a) and after passive aeration (b) over time.
FIG. E.5. Concentration of dissolved phosphorus (P) in the effluent of pilot VFPs (a) and after passive aeration (b) over time.
Appendix F

Mass Balance Calculation of Carbonate

The theoretical mass of carbonate supplied by the packing materials in each pilot VFP was calculated using the mass of each packing material and calcium carbonate equivalence (CCE) data, as shown in Eq. F.1.

\[
m_{\text{CaCO}_3} = m_\text{CS} \times CCE_\text{CS} + m_\text{SMC} \times CCE_\text{SMC} + m_\text{LS}\text{-chip} \times CCE_\text{LS}\text{-chip} + m_\text{LS}\text{-rock} \times CCE_\text{LS}\text{-rock}
\]  

(F. 1)

Where: \( m_{\text{CaCO}_3} \) = theoretical mass of carbonate supplied to the system, kg

\( m \) = mass of the packing material, kg

\( CCE \) = calcium carbonate equivalence of the packing material, %

Estimation of the total \( \text{CaCO}_3 \) released from each pilot VFP was performed by integrating the area under the curves for experimental alkalinity and acidity data using Python Programming Language via trapezoidal algorithm based on the following equation (Eq. F.2).

\[
m_{\text{CaCO}_3} = \sum_k c_{\text{Alk}} \times (PV_k - PV_{k-1}) \times V_{PV} \times 10^{-6}
\]

\[
+ \sum_k c_{\text{Acy}} \times (PV_k - PV_{k-1}) \times V_{PV} \times 10^{-6}
\]

(F. 2)

Where: \( m_{\text{CaCO}_3} \) = experimental mass of carbonate released from the system, kg

\( k \) = the sampling event

\( c_{\text{Alk}} \) = alkalinity concentration at the sampling event \((k)\), mg/L as CaCO\(_3\)

\( c_{\text{Acy}} \) = acidity concentration at the sampling event \((k)\), mg/L as CaCO\(_3\)
\[ PV_k = \text{pore volumes at the sampling event } k \text{ in the pilot reactor} \]

\[ PV_{k-1} = \text{pore volumes at the sampling event } k - 1 \text{ in the pilot reactor} \]

\[ V_{PV} = \text{effective pore volume of the pilot reactor, L} \]

Calculation results were provided in Table 3.5 of Chapter 3.
Appendix G

Geochemical Modeling with Visual MINTEQ

Geochemical modeling was carried out with Visual MINTEQ to determine the speciation and saturation indices (SI) of primary metals and other cations in three operational phases: immediately after incubation (day 0), pseudo-steady state (day 56 to 312), and declining phase (day 340 to 475). A total of 12 scenarios were evaluated, 3 for each pilot VFP: 1-100CS, 2-70CS, 3-70CS (SS), and 4-SMC. Sulfate/sulfide ratios were used to balance charge during modeling to make the difference lower than 10%. The average influent sulfate concentration used in this modeling work was 1100 mg/L, as calculated from the regular monitoring data from CCWA. Influent concentrations of primary metals (Fe, Al, Mn, and Zn) were used in each model, while the concentrations in the effluent from each pilot VFP were used for other cations and alkalinity.

Details on scenarios and sulfate/sulfide ratios used are described in Table G.1 and Table G.2. Saturation indices of different mineral phases of metals in the four pilot reactors are provided in Fig. G.1 to Fig. G.4 for comparison purposes. Generally, at the same operational phase, no obvious difference was observed for SI values among the four pilot VFPs. However, SI values of metal minerals (especially rhodochrosite) changed as the reactor went through different operational phases in part due to the decrease of alkalinity.
### TABLE G.1. Geochemical modeling parameters used in each scenario in the pilot test.

<table>
<thead>
<tr>
<th>Scenario</th>
<th>Reactor</th>
<th>Description</th>
<th>Concentration of different parameters (mg/L or mg/L as CaCO$_3$ for alkalinity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>100CS</td>
<td>Day 0 after incubation</td>
<td>Fe</td>
</tr>
<tr>
<td>1-2</td>
<td>70CS</td>
<td>Day 0 after incubation</td>
<td>Fe</td>
</tr>
<tr>
<td>1-3</td>
<td>70CS(SS)</td>
<td>Day 0 after incubation</td>
<td>Fe</td>
</tr>
<tr>
<td>1-4</td>
<td>SMC</td>
<td>Day 56 to 312 for steady</td>
<td>Fe</td>
</tr>
<tr>
<td>2-1</td>
<td>100CS</td>
<td>Day 56 to 312 for steady</td>
<td>Fe</td>
</tr>
<tr>
<td>2-2</td>
<td>70CS</td>
<td>Day 56 to 312 for steady</td>
<td>Fe</td>
</tr>
<tr>
<td>2-3</td>
<td>70CS(SS)</td>
<td>Day 56 to 312 for steady</td>
<td>Fe</td>
</tr>
<tr>
<td>2-4</td>
<td>SMC</td>
<td>Day 340 to 475 for declining</td>
<td>Fe</td>
</tr>
<tr>
<td>3-1</td>
<td>100CS</td>
<td>Day 340 to 475 for declining</td>
<td>Fe</td>
</tr>
<tr>
<td>3-2</td>
<td>70CS</td>
<td>Day 340 to 475 for declining</td>
<td>Fe</td>
</tr>
<tr>
<td>3-3</td>
<td>70CS(SS)</td>
<td>Day 340 to 475 for declining</td>
<td>Fe</td>
</tr>
<tr>
<td>3-4</td>
<td>SMC</td>
<td>Day 340 to 475 for declining</td>
<td>Fe</td>
</tr>
</tbody>
</table>

### TABLE G.2. Different sulfate/sulfide ratios used in the geochemical modeling for the pilot test.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>75%</th>
<th>50%</th>
<th>40%</th>
<th>35%</th>
<th>25%</th>
<th>20%</th>
<th>15%</th>
<th>10%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO$_4^{2-}$ (mg/L)</td>
<td>275</td>
<td>550</td>
<td>660</td>
<td>715</td>
<td>825</td>
<td>880</td>
<td>935</td>
<td>990</td>
<td>1045</td>
</tr>
<tr>
<td>HS$^-$ (mg/L)</td>
<td>284</td>
<td>189</td>
<td>151</td>
<td>132</td>
<td>95</td>
<td>76</td>
<td>57</td>
<td>38</td>
<td>19</td>
</tr>
</tbody>
</table>
FIG. G.1. Saturation indices (SI) of metal minerals versus pH at day 0 (a), from day 56 to 312 (b), and from day 340 to 475 (c) in the 1-100CS reactor at the Klondike-1 site. SI values were predicted by geochemical modeling via Visual MINTEQ using the measured experimental conditions under the specific scenarios (Table G.1).
FIG. G.2. Saturation indices (SI) of metal minerals versus pH at day 0 (a), from day 56 to 312 (b), and from day 340 to 475 (c) in the 2-70CS reactor at the Klondike-1 site. SI values were predicted by geochemical modeling via Visual MINTEQ using the measured experimental conditions under the specific scenarios (Table G.1).
FIG. G.3. Saturation indices (SI) of metal minerals versus pH at day 0 (a), from day 56 to 312 (b), and from day 340 to 475 (c) in the 3-70CS (SS) reactor at the Klondike-1 site. SI values were predicted by geochemical modeling via Visual MINTEQ using the measured experimental conditions under specific scenarios (Table G.1).
**FIG. G.4.** Saturation indices (SI) of metal minerals versus pH at day 0 (a), from day 56 to 312 (b), and from day 340 to 475 (c) in the 4-SMC reactor at the Klondike-1 site. SI values were predicted by geochemical modeling via Visual MINTEQ using the measured experimental conditions under specific scenarios (Table G.1).
Appendix H

Mass Balance Calculations of Metals and Other Cations

Mass balances of metals and cations were conducted for each pilot VFP to determine the overall capacity of reactor packing materials to retain metals or to release other cations to the effluent in the pilot tests. Integration of the area under each curve of a metal or cation was carried out by using Python Programming Language via trapezoidal algorithm. The equations provided below served as the theoretical basis for the integration by Python.

Integration of the total mass of metal or cation entering each reactor over the entire test was estimated based on the equation below (Eq. H.1):

\[
m_{in} = \sum_{k} c_{in} \times (PV_k - PV_{k-1}) \times V_{PV} \times 10^{-6}
\]  

(H. 1)

Where: \( m_{in} \) = calculated mass (mg) of a specific metal or cation entering the pilot VFP, kg

\( k \) = the sampling event

\( c_{in} \) = influent concentration of the metal or cation at the sampling event \( (k) \), mg/L

\( PV_k \) = pore volumes at the sampling event \( k \) in the pilot reactor

\( PV_{k-1} \) = pore volumes at the sampling event \( k-1 \) in the pilot reactor

\( V_{PV} \) = effective pore volume in the pilot reactor, L

The total mass of metal or cation flowing out of each pilot VFP over the entire test was estimated based on the following equation (Eq. H.2):
\[ m_{out} = \sum_k c_{out} \times (PV_k - PV_{k-1}) \times V_{PV} \times 10^{-6} \]  

(H. 2)

Where: \( m_{out} \) = calculated mass (mg) of a specific metal or cation flowing out of the pilot VFP, kg

\( k \) = the sampling event

\( c_{out} \) = effluent concentration of the metal or cation at the sampling event \( (k) \), mg/L

\( PV_k \) = pore volumes at the sampling event \( k \) in the pilot reactor

\( PV_{k-1} \) = pore volumes at the sampling event \( k-1 \) in the pilot reactor

\( V_{PV} \) = effective pore volume in the pilot reactor, L

Calculation results of cations were provided in Table 3.7 of Chapter 3. The results of the mass balance on metals are shown in Table H.1.
### TABLE H.1. Mass balance of primary metals in the pilot VFPs treating AMD at the Klondike-1 site.

<table>
<thead>
<tr>
<th>Metals</th>
<th>1-100CS</th>
<th>2-70CS</th>
<th>3-70CS (SS)</th>
<th>4-SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>mass of Fe (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m_{in})</td>
<td>19.81</td>
<td>20.92</td>
<td>20.22</td>
<td>21.38</td>
</tr>
<tr>
<td>(m_{out})</td>
<td>0.14</td>
<td>0.11</td>
<td>0.05</td>
<td>1.00</td>
</tr>
<tr>
<td>(m_{released})</td>
<td>19.67</td>
<td>20.81</td>
<td>20.17</td>
<td>20.37</td>
</tr>
<tr>
<td>% released</td>
<td>99.30</td>
<td>99.48</td>
<td>99.77</td>
<td>95.31</td>
</tr>
<tr>
<td>mass of Al (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m_{in})</td>
<td>0.86</td>
<td>0.90</td>
<td>0.86</td>
<td>0.94</td>
</tr>
<tr>
<td>(m_{out})</td>
<td>0.07</td>
<td>0.07</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>(m_{released})</td>
<td>0.80</td>
<td>0.83</td>
<td>0.78</td>
<td>0.86</td>
</tr>
<tr>
<td>% released</td>
<td>92.36</td>
<td>92.01</td>
<td>91.21</td>
<td>91.99</td>
</tr>
<tr>
<td>mass of Mn (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m_{in})</td>
<td>6.75</td>
<td>6.67</td>
<td>6.86</td>
<td>6.96</td>
</tr>
<tr>
<td>(m_{out})</td>
<td>4.56</td>
<td>1.39</td>
<td>2.43</td>
<td>5.60</td>
</tr>
<tr>
<td>(m_{released})</td>
<td>2.19</td>
<td>5.28</td>
<td>4.44</td>
<td>1.35</td>
</tr>
<tr>
<td>% released</td>
<td>32.39</td>
<td>79.15</td>
<td>64.67</td>
<td>19.47</td>
</tr>
<tr>
<td>mass of Zn (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m_{in})</td>
<td>0.21</td>
<td>0.21</td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>(m_{out})</td>
<td>0.06</td>
<td>0.07</td>
<td>0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>(m_{released})</td>
<td>0.14</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>% released</td>
<td>68.98</td>
<td>69.62</td>
<td>70.95</td>
<td>69.01</td>
</tr>
</tbody>
</table>
Appendix I

Photos of Packing Materials in Pilot-Scale Field VFPs

FIG. I.1. Limestone (a) and sandstone (b) rocks as underdrain materials, and (c) pea gravel used to retain substrate in pilot-scale VFPs treating AMD at the Klondike-1 site.
FIG. 1.2. Microbial sample bags made of nylon mesh and 10 lb. Triline® fishing line and filled with different substrate materials specific to each treatment.
Appendix J

Rarefaction Curves of Bacterial Sequences in the Pilot Test

Rarefaction curves of bacterial sequences by 454 sequencing in the three pilot VFPs, each at six sampling events, are provided here. The curves were constructed via Mothur software based on operational taxonomic units (OTUs) at a cutoff level of 3%.
FIG. J.1. Rarefaction curves of the 18 samples from the 100CS (a), 70CS (b), and SMC (c) pilot VFPs based on OTUs of 3% cutoff compared at the same depth of sequencing.
Appendix K

Distribution of Mines Around World

FIG. K.1. Distribution of mines around world (a) and the United States of America (b).

CURRICULUM VITA

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EDUCATION

2006 – 2009  M.S. in Environmental Engineering, Xi’an Jiaotong University, China
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PROFESSIONAL EXPERIENCE

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AWARDS AND SCHOLARSHIPS

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SELECTED PUBLICATIONS AND PRESENTATIONS


