EFFECTIVE AND LOW-COST TREATMENT OF ACID MINE DRAINAGE USING CHITIN AS A FRACTIONAL AMENDMENT TO COMPOST

A Thesis in
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by
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Abstract

This work presents the suitability of a mixture of two abundant, sustainable waste products of industry to support the remediation of acid mine drainage (AMD), an environmental pollutant that plagues mining areas all over the world. Low-maintenance, passive treatment systems hold the most promise for remediating AMD at a low cost relative to other treatment options. The choice of substrate in these systems is very important for successful remediation. Recently, our laboratory has shown that crab-shell chitin, a waste product of the shellfish industry, is capable of neutralizing acidity, reducing sulfate, and thoroughly removing aluminum, iron, manganese, and zinc from AMD. Despite its remarkable effectiveness, the relatively high cost of crab-shell chitin ($0.60/lb) compared to the leading substrate, spent mushroom compost substrate (SMS; $0.025/lb), may preclude its use in many systems. To facilitate the development of an effective but cost-efficient approach for AMD remediation, crab-shell chitin was used as a fractional amendment to SMS in this study.

Two batch tests and a continuous-flow column test were used to evaluate different chitin-SMS mixtures for their ability to support AMD treatment. Natural AMD containing aluminum, iron, manganese, and zinc was used in these experiments, and aqueous samples were collected regularly for water quality analysis. In the batch tests, substrates containing only 5% chitin successfully generated alkalinity and partially removed metals from AMD. In the column experiment, the treatment capacity (as L AMD treated per kg of substrate used) for four different substrate mixtures was determined. Treatment capacities were: 36.7 L/kg for 10% limestone + 90% SMS; 40.1 L/kg for 5% chitin + 95% SMS; 162 L/kg for 50% chitin + 50% SMS; and 428 L/kg for 100% chitin. The associated substrate costs per 1000 L of AMD treated are: $1.38/1000 L, $2.95/1000 L, $4.25/1000 L, and $3.09/1000 L, respectively. These results suggest that a small fraction of chitin (5%) does not provide a significant benefit over traditional limestone and compost substrates, but that larger fractions of chitin (50-100%) are significantly more efficient than traditional SMS substrates, especially for the removal of metals.
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<th>Notation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMD</td>
<td>acid mine drainage</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Search Alignment Tool</td>
</tr>
<tr>
<td>BDL</td>
<td>below detection limit</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>chitin</td>
<td>when describing the present research, refers to SC-20 ChitoRem™ Chitin Complex, a crab-shell chitin product</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DO</td>
<td>dissolved oxygen</td>
</tr>
<tr>
<td>DOC</td>
<td>dissolved organic carbon</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatograph</td>
</tr>
<tr>
<td>HRT</td>
<td>hydraulic retention/residence time</td>
</tr>
<tr>
<td>IC</td>
<td>ion chromatograph</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>inductively coupled plasma-atomic emission spectrometry</td>
</tr>
<tr>
<td>MDL</td>
<td>method detection limit</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PV</td>
<td>pore volume</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SMS</td>
<td>spent mushroom compost substrate</td>
</tr>
<tr>
<td>SRB</td>
<td>sulfate-reducing bacteria</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VFA</td>
<td>volatile fatty acid</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate-EDTA</td>
</tr>
</tbody>
</table>
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1. Introduction

“Acid mine drainage is considered the most important and widespread mining industry related pollution problem around the world” (Neculita et al. 2007). Acid mine drainage (AMD) results from the oxidation of pyrite to form sulfuric acid, creating an acidic discharge which also contains high levels of dissolved metals such as iron, aluminum, and manganese. The environmental need for continued developments in AMD treatment is clear—it is estimated that AMD impacts 15,000-24,000 km of streams in the United States alone (EPA 1994). Acidity and metal content endanger the health of natural ecosystems, humans, and structures downstream of AMD sources. Restoration of affected waters will rely on the development and construction of low-cost and low-maintenance passive treatment systems.

Passive treatment systems are preferred for their reduced operating costs in comparison to other systems, but they do have limitations (Johnson and Hallberg 2005a). In most cases, passive treatment relies on anaerobic sulfate-reducing bacteria (SRB) to reduce sulfate to sulfide, which facilitates the precipitation of metal sulfides. Passive treatment systems include an organic substrate to provide carbon, nutrients, and an attachment surface for the SRB (Webb 1998). SRB do not consume complex organic materials such as cellulose, so these systems require a diverse community of anaerobic cellulolytic and fermentative bacteria to break down the polymeric substrate into usable organic monomers (Neculita et al. 2007; Pruden et al. 2007). Such systems are not consistently successful (Pruden et al. 2007), and are often deficient in nitrogen (Waybrant et al. 2002), suggesting a failure of the substrate to support the diverse bacterial community necessary for SRB to thrive. One of the most commonly used substrates for AMD remediation is spent mushroom compost substrate (SMS) mixed with limestone (calcium carbonate; Hedin 2005; Dietz 2006). However, SMS contains only about 1.8-2.4% nitrogen (Stewart et al. 1998; Ntougias et al. 2004).

Recent studies have shown that chitin (poly-N-acetylglucosamine) is an outstanding organic substrate and nutrient source for enhancing the activity of SRB in passive AMD treatment systems (Daubert and Brennan 2007; Robinson-Lora and Brennan 2009a). Chitin is available commercially in the form of crab shells, which are
sustainable waste products of the shellfish industry. Chitin is attractive as a long-term, slow-release electron donor source to support bacterial growth (Vera et al. 2001; Brennan et al. 2006a), and it contains 6-7% nitrogen, giving it a nearly ideal carbon to nitrogen ratio for use by microorganisms (Harkness et al. 2003). The least refined crab-shell chitin product also contains approximately 40% calcium carbonate by weight, which increases alkalinity and buffers AMD waters. In addition to serving as a bacterial substrate and a source of chemical buffering, chitin can remove metallic pollutants through physical adsorption (Felse and Panda 1999).

The multifaceted capabilities of chitin make it attractive for use in passive remediation systems; however, economic considerations are also important. Unrefined crab-shell chitin is a relatively inexpensive microbial substrate, retailing at $0.60 per pound, but it is expensive in comparison to SMS, which costs only $0.025/lb. An economic benefit may be realized by adding fractional amounts of crab-shell chitin to SMS in order to improve the efficiency of the substrate with little, if any, added cost. This research investigates the ability of substrate mixtures of crab-shell chitin and SMS to support AMD remediation.

It was anticipated that SMS enhanced with chitin would support a more diverse community of bacteria with a greater population of SRB, yielding faster sulfate and metal removal rates. In addition, it should be possible to use chitin as a fractional amendment to less expensive SMS in order to make the widespread application of chitin in AMD systems economically feasible, without sacrificing the ability to support a diverse bacterial community for efficient remediation. In order to evaluate this hypothesis, batch and continuous-flow experiments were conducted using SMS and chitin. Water quality data was collected throughout the experiments to assess the level of remediation under each test condition. Microbial community analysis was also attempted in order to provide greater insight on the effectiveness of the substrates.
2. Literature Review

2.1. AMD: A Widespread Environmental Challenge

AMD, characterized by low pH and high concentrations of dissolved sulfate and metals, is a ubiquitous problem in mining areas. In 1994, the EPA estimated that AMD impacts 15,000-24,000 km of streams in the United States alone. A legacy of Pennsylvania’s coal mining history, AMD is considered the state’s primary cause of water pollution and has degraded 3,680 km of streams (Rossman et al. 1997). While the acidity of AMD is certainly a problem, the metals in the discharges are an even greater concern (Sheoran and Sheoran 2006). The most common metals in AMD are iron, aluminum, and manganese; however, arsenic and heavy metals such as cadmium, chromium, cobalt, copper, lead, and zinc may also be present depending on the local geology (Johnson and Hallberg 2005a; Sheoran and Sheoran 2006). The cost of treating AMD is a major issue, and has limited the ability of government agencies and others to remediate the discharges (Milavec).

AMD is produced by the oxidation of pyritic (FeS$_2$) minerals upon exposure to air and water. Although water is pumped from active mines, abandoned mines are allowed to flood, providing ideal conditions for AMD formation (Equation 2.1, Johnson and Hallberg 2005a). The ferric iron produced in this reaction also acts as an oxidizing agent, thereby accelerating the process of AMD generation. Iron-oxidizing bacteria are very important in facilitating the reaction, especially at low pH (Johnson and Hallberg 2005a).

\[
4\text{FeS}_2 + 15\text{O}_2 + 14\text{H}_2\text{O} \rightarrow 4\text{Fe(OH)}_3 + 8\text{SO}_4^{2-} + 16\text{H}^+
\]

2.2. Principles of AMD Treatment

Acid mine drainage treatment options are categorized as active or passive (Figure 2-1). The most widely-used method for treating acidic discharges is an active treatment method whereby a chemical neutralizing agent is continuously added to the discharge, along with aeration or addition of a chemical oxidizing agent (Johnson and Hallberg, 2005a). This process neutralizes AMD and promotes the precipitation of metals, which will settle out of the drainage to form a sludge blanket. A major disadvantage of active
treatment is the cost of continuously replenishing chemical supplies and removing sludge (Johnson and Hallberg, 2005a).

Due to the high maintenance costs of active treatment systems, as well as accessibility issues at remote sites, passive treatment systems have gained popularity. Passive treatment systems allow the AMD to flow through a solid reactive mixture which requires only periodic maintenance and replenishment. Passive treatment systems may be aerobic or anaerobic and rely on chemical and/or biological processes for remediation (Figure 2-1). Passive treatment systems that achieve remediation mainly through chemical means are limestone drains and limestone channels. The calcium carbonate in limestone contributes alkalinity to the AMD, thereby raising the pH and causing metals to precipitate (Neculita et al. 2007). Unfortunately, limestone-based treatment systems are susceptible to armoring, or passivation. Passivation occurs due to precipitate formation on the surface of the limestone or deposition of sediments and precipitates on the surface of the limestone bed, which inhibit the dissolution of calcium carbonate and render the limestone ineffective (Green et al. 2008; Soler et al. 2008).

**Figure 2-1.** AMD treatment options, adapted from Johnson and Hallberg (2005a) and Neculita et al. (2007).
Biological treatment is usually achieved in anaerobic systems containing an organic substrate to support the growth of SRB. SRB reduce sulfate and produce alkalinity as described by Equation 2.2, where \( \text{CH}_2\text{O} \) represents a simple carbon source (Johnson and Hallberg 2005a). Since SRB do not consume complex organic materials such as cellulose, these systems require a diverse community of anaerobic cellulolytic and fermentative bacteria to break down the polymeric substrate into usable organic monomers (Neculita et al. 2007; Pruden et al. 2007).

\[
\text{SO}_4^{2-} + 2\text{CH}_2\text{O} + 2\text{H}^+ \rightarrow \text{H}_2\text{S} + 2\text{H}_2\text{CO}_3
\]

2.2

2.3. Organic Substrates for Passive Treatment

A wide variety of slow-degrading solid substrates have been tested to support SRB and remediate AMD, with varying success. Wood chips, sawdust, manure, various types of compost, and hay have all been investigated, sometimes mixed with limestone to increase alkalinity (Gibert et al. 2003; Johnson and Hallberg 2005b; Neculita and Zagury 2008). Some studies have concluded that a mixed substrate provides the best conditions for remediation of AMD through sulfate-reducing processes (Waybrant et al. 1998; Neculita and Zagury 2008). However, nitrogen availability has been cited as a concern when using solid organic substrates to support SRB growth (Waybrant et al. 1998; Waybrant et al. 2002; Coetser et al. 2006).

Substrate experiments have been conducted using widely varying test conditions, making it difficult to assess their comparable efficiency for AMD remediation. In addition, many laboratory studies have supplemented the solid substrates to enhance sulfate reduction, including acetate as a readily available carbon source (Gibert et al. 2004), ammonium or urea as a nitrogen source (Chang et al. 2000; Cocos et al. 2002), and carbonate solutions as a buffering agent (Cocos et al. 2002; Waybrant et al. 2002). Such supplementation is undesirable for field applications since these materials would require frequent reapplication, which would lead to increased costs. Some continuous-flow studies have relied upon long hydraulic retention times for successful AMD treatment (e.g. 20 days, Chang et al. 2000). A very long incubation period (10 months) was required to allow a sulfate-reducing microbial community to become established.
before commencing continuous-flow treatment of AMD in a typical compost bioreactor (Johnson and Hallberg 2005b).

Spent mushroom compost substrate (SMS) is used widely for AMD remediation. It is an abundant waste product of the mushroom-growing industry, and reuse applications provide an attractive alternative to landfilling (Chiu et al. 2000). SMS is a heterogeneous material typically containing straw, peat, animal manure, gypsum, and limestone (Dvorak et al. 1992; Ntougias et al. 2004). It is used for AMD treatment because it provides a source of carbon and other nutrients (Jordan et al. 2008), it is highly porous (Chiu et al. 2000), and it is widely available and inexpensive (Hedin 2005; Dietz 2006). The capacity of SMS to adsorb metals such as iron has also been noted (Manyin et al. 1997). Studies using SMS to support sulfate-reducing processes in AMD treatment have had varying degrees of success, requiring long retention times for the best results (Dvorak et al. 1992; Stark et al. 1994; Stark et al. 1995). Some researchers have reported poor removal of metals during treatment with SMS (Vile and Wieder 1993; Tarutis and Unz 1995). Furthermore, undesirable increased sulfate concentrations in treated effluent are possible from the dissolution of gypsum (CaSO₄•2H₂O) in SMS (Dvorak et al. 1992; Stark et al. 1995). Bacterial sulfate reduction has been inhibited by the limited availability of suitable carbon substrates for SRB, due to the slow decomposition rate of SMS (Dvorak et al. 1992). However, it has been shown that adding a source of calcium carbonate like limestone to SMS can boost alkalinity and improve its performance for AMD treatment (Stark et al. 1996).

2.4. Evaluation of Microbial Communities in AMD Treatment Systems

In anaerobic passive treatment of acid mine drainage, successful remediation requires an active microbial consortium to effectively degrade the solid substrate and support sulfate reduction. Researchers have frequently noted the importance of providing favorable conditions for the establishment of a sulfate-reducing community in these systems (Logan et al. 2005; Johnson and Hallberg 2005b; Pruden et al. 2007). In the past, cell cultures have been used to enrich and evaluate the presence of SRB and other bacteria in passive treatment systems (Webb et al. 1998; Chang et al. 2000; Hiibel et al. 2008). Recent technologies provide a more complete characterization of the microbial community in environmental samples through culture-independent methods (Nocker et al.
Microbial community analyses in AMD treatment environments have employed techniques such as denaturing gradient gel electrophoresis (DGGE), real-time quantitative polymerase chain reaction (Q-PCR), fluorescent in situ hybridization, and capillary electrophoresis single-strand conformation polymorphism (Icgen et al. 2007; Hong et al. 2007; Pruden et al. 2007). Most of these studies have targeted the 16S rDNA gene of bacteria. Another useful target is the \textit{apsA} gene, which is associated with SRB (Hiibel et al. 2008). Previous work has shown that successful communities are dominated by three main groups of bacteria: cellulose degraders, fermenters, and SRB (Hong et al. 2007; Pruden et al. 2007; Hiibel et al. 2008). The activity of cellulose degraders and fermenters is especially important to provide an appropriate electron donor source for SRB (Figure 2-2) and to facilitate reducing conditions within the substrate (Clarke et al. 2004).

![Figure 2-2. Degradation pathway of cellulose-based substrates in sulfate-reducing bioreactors (Logan et al. 2005).](image)

DGGE was selected as the tool for microbial community analysis in this research. For DGGE, PCR products of about 200-700 bp are generated using a primer set that includes a GC clamp (about 40 bp) on one of the primers. The PCR products undergo
electrophoresis on an acrylamide gel which contains a gradient of a chemical denaturant solution (Nocker et al. 2007). The double-stranded PCR products begin to separate, or melt, upon exposure to the denaturant during electrophoresis. As the strands separate, their migration through the gel is hindered. Melting is affected by the sequence of the DNA fragment, as the bonds between GC base pairs are more difficult to break than AT bonds. The GC clamp on one end of the double-stranded PCR product ensures that the strands will not separate completely and migrate off the end of the gel. The result of DGGE is a set of discrete bands which represent unique sequences of DNA, giving an indication of the composition of the microbial community. However, co-migration of bands is possible, and in some cases multiple bands may originate from the same species. After electrophoresis, bands of DNA may be excised and re-amplified for sequencing or other applications. DGGE has been used to identify species which represent as little as 1% of the total microbial population in a sample (Muyzer et al. 1993).

Figure 2-3. Conceptual diagram of the DGGE process. PCR products denature at different points in the gradient, depending upon the DNA sequence. The GC clamp prevents the double-stranded PCR products from separating entirely, due to the high melting threshold of GC base pairs.

2.5. Chitin: A Multifunctional Substrate for Remediation

Chitin (poly-N-acetylglucosamine; C₈H₁₃NO₅; Figure 2-4) is the second most abundant biopolymer on earth, after cellulose (Harkness et al. 2003). Chitin is produced naturally in the exoskeletons of insects and crustaceans and in the cell walls of bacteria and fungi (Felse and Panda 1999). Chitin is an ideal substrate for bioremediation applications because it adsorbs metals (Felse and Panda 1999), is easily fermented into soluble carbon substrates (Vera et al. 2001), and contains enough nitrogen to support bacterial growth (Harkness et al. 2003). Unrefined chitin is available as an inexpensive waste product of the shellfish industry. Previous studies have shown that chitin is an
excellent substrate to support reductive dechlorination of tetrachloroethene and biological denitrification (Brennan et al. 2006a,b; Robinson-Lora and Brennan 2009b). Recently, chitin has been used to remediate AMD in laboratory and pilot-scale studies (Daubert and Brennan 2007; Venot et al. 2008; Robinson-Lora and Brennan 2009a).

**Figure 2-4.** Part of a chitin molecule, showing two monomers of N-acetylglucosamine joined by a beta-1,4 linkage.
3. **Materials and Methods**

3.1. **Environmental Samples: AMD and Sediments**

AMD for all experiments was collected from Kittanning Run in Altoona, Pennsylvania. Samples were taken from a utility bridge crossing the stream adjacent to the municipal water reservoirs and Kittanning Point Road, and about 0.9 km east of Horseshoe Curve, a railroad landmark. AMD was collected in high-density polyethylene containers and capped with minimal headspace.

Since Kittanning Run is diverted through a concrete channel at the sampling location, sediment to be used as a bacterial inoculum in the experiments was collected elsewhere. Sediment for the batch tests was collected from Scotch Gap Run in Altoona, adjacent to Kittanning Point Road and approximately 1.2 km east of the AMD collection location. Sediment for the column test was collected from Shaver’s Creek, within the Stone Valley Recreation Area of the Penn State Experimental Forest.

3.2. **Substrates**

The substrates used to promote AMD remediation in the experiments were ChitoRem® Chitin Complex (chitin; grade SC-20, JRW Bioremediation, Lenexa, KS), SMS (Mushroom Test Demonstration Facility, The Pennsylvania State University), and limestone (0.35-1.0 mm chips, 49.6% CaCO$_3$, Prairie Central, IL). The chitin product is derived from Dungeness crab-shells and contains approximately 20% chitin, 40% calcium carbonate, and 40% protein by weight. The chitin was used as received from the distributor. Before addition to test microcosms, SMS was processed in a coffee grinder in order to obtain a more homogenous material. This was necessary due to the very small mass of SMS added to the microcosms (0.25 g or less). In the column experiment, much greater amounts of substrate were used, so the SMS was applied without any further processing. The particle size distribution of the organic substrates is provided in Table 3-1. Additionally, silica sand (16-20 mesh, Badger Mining Corp., Berlin, WI) was used as an inert packing material in the column study. Sand was washed overnight in 0.25 M nitric acid, thoroughly rinsed with deionized water, and dried at 105 °C before use. The
The purpose of this acid-wash was to prevent any iron residues on the sand from leaching into the AMD during the column study.

**Table 3-1.** Particle size distribution of chitin used in batch and column tests, SMS used in the column test, and ground SMS used in the batch tests.

<table>
<thead>
<tr>
<th>Particle size range (mm)</th>
<th>Chitin</th>
<th>SMS</th>
<th>Ground SMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;2.80</td>
<td>17.0%</td>
<td>40.7%</td>
<td>0.9%</td>
</tr>
<tr>
<td>0.850-2.80</td>
<td>32.1%</td>
<td>41.6%</td>
<td>44.7%</td>
</tr>
<tr>
<td>0.425-0.850</td>
<td>28.1%</td>
<td>11.2%</td>
<td>23.8%</td>
</tr>
<tr>
<td>0.106-0.425</td>
<td>21.9%</td>
<td>6.3%</td>
<td>25.9%</td>
</tr>
<tr>
<td>&lt;0.106</td>
<td>0.9%</td>
<td>0.3%</td>
<td>4.7%</td>
</tr>
</tbody>
</table>

**3.3. Preliminary Batch Study**

The purpose of the preliminary batch study was to test chitin as a fractional amendment to SMS, and to identify the range of chitin-to-SMS ratios that were effective and warranted further investigation. Ten different substrate conditions were evaluated in duplicate microcosms (Table 3-2, Figure 3-1). All microcosms contained 100 mL AMD. Microcosms 1 and 5-10 contained 0.5 g stream sediment as a bacterial inoculum. Microcosms 2-10 contained 0.25 g total substrate, in the proportions indicated in Table 2-1. Microcosm 1, as a negative control, did not contain any substrate. Microcosms 2-4, the uninoculated controls, did not contain stream sediment, and substrates for these microcosms were treated for 15 minutes with a UV lamp in order to minimize biological activity.

**Table 3-2.** Contents of microcosms for the preliminary batch study.

<table>
<thead>
<tr>
<th>Type of microcosm</th>
<th>Negative control</th>
<th>Uninoculated controls</th>
<th>Active microcosms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcosm name</td>
<td>1</td>
<td>2 3 4 5 6 7 8 9 10</td>
<td></td>
</tr>
<tr>
<td>Chitin (% total substrate mass)</td>
<td>n/a</td>
<td>100 0 0 0 5 25 50 100</td>
<td></td>
</tr>
<tr>
<td>Limestone (% total substrate mass)</td>
<td>n/a</td>
<td>0 0 100 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>SMS (% total substrate mass)</td>
<td>n/a</td>
<td>0 100 0 100 95 95 75 50 0</td>
<td></td>
</tr>
<tr>
<td>Total substrate mass (g)</td>
<td>0 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stream sediment (g)</td>
<td>0.5 0 0 0 0.5 0.5 0.5 0.5 0.5 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMD (mL)</td>
<td>100 100 100 100 100 100 100 100 100 100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Each microcosm was assembled by first adding the appropriate amounts of sediment and substrates to an acid-washed 160 mL glass serum bottle. Bottles were then purged with ultra-high purity nitrogen gas to eliminate oxygen. Meanwhile, AMD was also purged with nitrogen for at least one hour to minimize the dissolved oxygen (DO) content. After measuring the DO concentration of the purged AMD, 100 mL was transferred to each anoxic serum bottle. The headspace was purged with nitrogen for an additional 5 minutes, then the microcosms were sealed with butyl rubber stoppers and aluminum crimp tops. Microcosms were incubated upside down in the dark at 20 °C with continuous agitation on an orbital shaker. Samples of the initial AMD and each substrate were taken for comparison with samples at the end of the experiment.

After 4 weeks, the microcosms were opened and treated AMD was withdrawn. Aliquots were set aside for same-day determinations of pH, alkalinity, acidity, and ammonium. The remaining aqueous sample was filtered (0.45 µm) and stored under low temperature conditions for further analysis. Samples were preserved at 4 °C at pH<2 for dissolved metals and at -20 °C for anions, volatile fatty acids (VFAs), and dissolved organic carbon (DOC). Microcosm sediments were stored in sterile centrifuge tubes at -20 °C for analysis of the associated microbial community. Molecular biology work on
these samples included EDTA washing to remove inhibitors of downstream applications, DNA extraction, and PCR amplification, as described in Section 3.7.

3.4. Extended Batch Study

The purpose of the extended batch study was to capture initial changes and long-term performance of different chitin-to-SMS substrate ratios for AMD treatment. Microcosm reference labels and contents are described in Table 3-3, with 0.5 g of sediment and 100 mL AMD in all microcosms, and 0.25 g substrate for all test conditions except the negative control. One hundred microcosms, 20 for each of 5 substrate conditions, were established as described in Section 3.3. The microcosms were incubated in the dark at 20-22 °C, and shaken by hand once per day to ensure uniform contact between substrate, sediment, and AMD. Portions of the initial AMD and each substrate were preserved for comparison with later sampling points.

Table 3-3. Contents of microcosms for extended batch study.

<table>
<thead>
<tr>
<th>Microcosm contents</th>
<th>Control</th>
<th>5%</th>
<th>15%</th>
<th>25%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin (% total substrate mass)</td>
<td>n/a</td>
<td>5</td>
<td>15</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>SMS (% total substrate mass)</td>
<td>n/a</td>
<td>95</td>
<td>85</td>
<td>75</td>
<td>50</td>
</tr>
<tr>
<td>Total substrate mass (g)</td>
<td>0</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Stream sediment (g)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>AMD (mL)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Microcosms were sampled on days 1, 3, 5, 7, 10, 14, 21, 28, 42, and 63. Duplicate microcosms of each test condition were sacrificed for every sampling point. Treated AMD was withdrawn and aliquots set aside for same-day determinations of pH, alkalinity, acidity, and ammonium. The remaining AMD was filtered (0.45 μm) and stored for further analysis (separate aliquots with additions of acid at 4 °C for dissolved metals and VFAs; 4 °C for anions and DOC). Microcosm sediments were stored in sterile centrifuge tubes at -20 °C for microbial analysis. Molecular biology work on these samples included DNA extraction, PCR amplification, DGGE, and DNA sequencing.

3.5. Continuous-Flow Column Study

The purpose of the column test was to determine the treatment efficiency and longevity of different chitin-to-SMS substrate ratios. Column contents are described in
Table 3-4. The fractions of chitin tested were 5% and 50% (remainder was SMS), as well as 100%. A column containing 10% limestone (remainder SMS) was established to represent a typical substrate traditionally used for passive treatment of AMD. Thus, the results for treatment with SMS + chitin could be compared to a conventional substrate (10% limestone + 90% SMS), no substrate (negative control) and 100% chitin substrate (positive control).

Table 3-4. Packing materials for continuous-flow columns.

<table>
<thead>
<tr>
<th>Column contents</th>
<th>Control</th>
<th>10% Limestone</th>
<th>5% Chitin</th>
<th>50% Chitin</th>
<th>100% Chitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin (% total substrate mass)</td>
<td>n/a</td>
<td>0</td>
<td>5</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Limestone (% total substrate mass)</td>
<td>n/a</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SMS (% total substrate mass)</td>
<td>n/a</td>
<td>90</td>
<td>95</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Total substrate mass (g)</td>
<td>0</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Stream sediment (g)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Sand (g)</td>
<td>1,282</td>
<td>1,254</td>
<td>1,247</td>
<td>1,257</td>
<td>1,278</td>
</tr>
</tbody>
</table>

Columns (Figure 3-2) were constructed of 2 ft lengths of PVC pipe with end-caps of the same material (Harvel Clear™ Schedule 40 PVC pipe and fittings, 1-½”, United States Plastic Corp.). Prior to assembly and packing, 3-way polycarbonate stopcocks with luer fittings (Cole-Parmer) were attached to the end-caps and 2-way stopcocks were attached every 6 in along the length of the columns to allow for continuous flow and sampling at various hydraulic residence times (HRT). All column components were washed with Liquinox detergent and tap water, rinsed with deionized water, and air dried prior to experimentation. To pack the columns, end-caps were first attached to the bottom of the columns and sealed with plastic pipe cement. Solids were wet-packed into the columns in approximately 1 inch lifts with free-standing anoxic AMD in the following order, from bottom to top: 1) 30 g sand (to prevent loss of substrate through the bottom sampling port); 2) 50 g stream sediment (as a bacterial inoculum) mixed with 50 g substrate (where applicable) and enough sand to fill the remaining space in the column, in order to provide sufficient hydraulic conductivity and the desired residence time; 3) enough sand to fill any gaps at the top of the column. After packing, another end-cap was attached to seal the column. A sample cell consisting of a 6 in length of PVC pipe and end-caps (Harvel Clear™ Schedule 40 PVC pipe and fittings, ¾”, United
States Plastic Corp.) was placed at the top of the column so that it could be drained readily for effluent sampling. Columns were allowed to incubate in batch mode for 8 days before starting continuous-flow operation.

AMD was stored in a 50 L reservoir continuously purged with argon gas to maintain anoxic conditions. AMD was conveyed through a stopcock at the bottom of the reservoir through Tygon® tubing (1/4” and 1/8”, Saint-Gobain Performance Plastics), and then diverted through 5 separate lines (Masterflex Tygon® lab L/S® 13, Cole-Parmer) for delivery by a peristaltic pump consisting of a digital drive and a 4-roller cartridge pump head (Masterflex L/S, Cole-Parmer). Columns were operated in continuous, up-flow mode for 148 days. Nominal flow rates for the columns were increased periodically, from 0.25 mL/min (day 0-50) to 0.5 mL/min (day 51-106) to 1 mL/min (day 107-148). Since flow rates varied slightly between columns, actual flow rates were determined by measuring the effluent from each column at regular intervals. The columns and AMD reservoir were covered with opaque black plastic to prevent the growth of phototrophic organisms.

Columns were sampled every 2-7 days during continuous flow treatment of AMD. Samples were taken from the influent line, effluent sample cells, and the sampling port in the center of each column. Samples of column influent and effluent were processed for determinations of acidity, ammonium, alkalinity, DOC, dissolved metals, pH, and VFAs. The additional samples were analyzed only for acidity, alkalinity, dissolved metals, and pH. Samples were either analyzed on the sampling day or preserved, as in the batch experiments (Sections 3.3 and 3.4). After 148 days (an average of 416 pore volumes), column flow was stopped. Samples of packing material were removed through the sampling ports using a flame-sterilized spatula and stored in sterile centrifuge tubes at -20 °C for future microbial community analysis.
Figure 3-2. Column experiment setup.
3.5.2. **Conservative Tracer Tests**

Tracer tests using sodium chloride were performed to determine the HRT and pore volume of each column. The approximate mass of chloride to be used in the slug \( (m_{\text{tracer}}, \text{mg}) \) was calculated using Equation 3.1, where \( t_r \) is the estimated HRT (min), \( Q_L \) is the column flow rate (L/min), and MDL is the method detection limit (mg/L).

\[
m_{\text{tracer}} = 100 t_r Q_L \text{MDL}
\]  \hspace{1cm} 3.1

Using an anticipated retention time of 12 hr, nominal flow rate of 0.0005 L/min, and MDL for chloride of 1 mg/L, the calculated tracer mass was 36 mg chloride. The tracer was prepared by dissolving 200 mg sodium chloride in 2 mL deionized water, and 0.5 mL of this solution was injected through the influent port of the column. Effluent samples were automatically collected every 0.5 hr for 48 hr (Spectra/Chrom® IS-95 Interval Sampler, Spectrum Chromatography), then filtered and preserved at 4 °C for determination of chloride concentration.

3.6. **Analytical Methods**

pH and ammonium were measured using electrodes (Thermo-ORION) connected to a pH/mV meter (Accumet® Basic AB15, Fisher Scientific). Acidity and alkalinity were measured using the titration methods described in *Standard Methods for the Examination of Water and Wastewater* (Methods 2310 and 2320; APHA 1998). The endpoint for alkalinity titrations was pH 4.5 and the endpoint for acidity titrations was 8.3. Anion analyses (chloride, nitrate, phosphate, and sulfate) for the batch studies and column tracer tests were accomplished using an ion chromatograph (IC; Dionex DX-100). Dissolved metals were measured using inductively coupled plasma-atomic emission spectrometry (ICP-AES; Leeman Labs PS3000UV) by the Materials Characterization Laboratory at The Pennsylvania State University. VFAs were analyzed by high performance liquid chromatography (HPLC; Waters 2695). During the column test, select aqueous samples were analyzed for total iron by the nitric acid digestion method and for sulfide by the methylene blue method, according to Standard Methods 3030 and 4500-S\(^2\), respectively (APHA 1998). DOC was analyzed using a total organic carbon analyzer (TOC-V CSN, Shimadzu). Substrate analyses for total carbon and nitrogen
(combustion method) and extractable metals (Mehlich 3 method) were performed by Agricultural Analytical Services Laboratory at The Pennsylvania State University.

For the batch tests, sulfate was measured using the IC. However, after the discovery of pH-related interferences with sulfate detection by IC (Robinson-Lora and Brennan 2009a), sulfate concentrations were determined by ICP-AES. ICP-AES detects total elemental sulfur, so this method requires that the presence of other sulfur species is negligible. Samples which had been preserved for ICP-AES analysis (filtered and adjusted with nitric acid to pH<2) were purged using compressed air to strip out any hydrogen sulfide gas prior to the analysis. Sulfate concentrations were calculated based on the stoichiometric ratio between sulfur and sulfate (Appendix A).

3.7. Molecular Biology Methods

Initial substrates and solids from sacrificed microcosms and columns were preserved at -20 °C until extraction of microbial DNA using a commercial kit (UltraClean™ Soil DNA Kit, MoBio Laboratories, Inc.). The DNA extraction procedure recommended by the kit was followed, with the addition of an incubation step at 70 °C for 10 minutes after the addition of Solution S1 and prior to the addition of Solution IRS. Samples from the first batch test were pretreated with EDTA to remove PCR inhibitors associated with AMD sediments (Hao et al. 2002; Nicomrat et al. 2006); however, subsequent tests without EDTA showed that this step had negatively affected DNA yields and it was discarded in future experiments. DNA was amplified by PCR using universal bacterial primers I-341fGC and I-533r targeting the 16S rDNA gene and a touchdown thermocycler program (thermocycler: Techne Flexigene; procedure: Watanabe et al. 2001). Optimization of PCR reagent concentrations was required for successful amplification of template DNA (Appendix B). The final composition of each 50 μL reaction mixture for PCR was informed by the results of the optimization test (Table 3-5).
Table 3-5. Composition of PCR reaction mixture.

<table>
<thead>
<tr>
<th>Component (supplier)</th>
<th>Volume (µL)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Colorless GoTaq® Flexi Buffer (Promega Corp.)</td>
<td>10</td>
<td>1X</td>
</tr>
<tr>
<td>MgCl₂, 25 mM Solution (Promega Corp.)</td>
<td>6</td>
<td>3 mM</td>
</tr>
<tr>
<td>PCR Nucleotide Mix, 10 mM each (Promega Corp.)</td>
<td>1</td>
<td>200 µM each</td>
</tr>
<tr>
<td>GoTaq DNA Polymerase, 5 units/µL (Promega Corp.)</td>
<td>0.25</td>
<td>1.25 units/50 µL</td>
</tr>
<tr>
<td>Bovine serum albumin, acetylated, 10 µg/µL (Promega Corp.)</td>
<td>2</td>
<td>0.4 µg/µL</td>
</tr>
<tr>
<td>Primer I-341fGC, 10 µM (Biosearch Technologies)</td>
<td>2.5</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Primer I-533r, 10 µM (Biosearch Technologies)</td>
<td>2.5</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Template DNA (user-supplied)</td>
<td>1</td>
<td>varies</td>
</tr>
<tr>
<td>Sterile water (EMD Chemicals Inc)</td>
<td>24.75</td>
<td>49.5%</td>
</tr>
</tbody>
</table>

PCR products were confirmed on agarose gels (0.8% agarose in 1X TBE buffer) stained with ethidium bromide (1 µg/mL) and viewed using a gel imaging system (Bio-Rad). DGGE was employed to create a visual community fingerprint of bacterial DNA. PCR products were loaded onto an 8% acrylamide gel with a denaturing gradient of 30-60%. Gels were run in a DCode Universal Mutation Detection System (Bio-Rad) at 75 V for 14 hr. After electrophoresis, gels were fixed by silver staining and imaged on a scanner (UMAX PowerLook 2100X). Representative bands were excised from the gel using sterile razor blades, mixed with 20 µL sterile PCR water in 0.2 mL centrifuge tubes, and incubated for 18-36 hours at 4 °C to allow elution of DNA in the gel fragments.

The eluted DNA was then used as a template for a second PCR reaction. This step used the same conditions as the first PCR, except that the forward primer used was I-341f instead of I-341fGC (Watanabe et al. 2001). This is the same primer used in the first PCR, but without the GC clamp. The long sequence of G and C bases is only needed when PCR products will be used in DGGE. PCR products were verified on an agarose gel and purified with a QIAquick® PCR Purification Kit (QIAGEN Inc.) before DNA sequencing (Nucleic Acid Facility, The Pennsylvania State University) with an ABI Hitachi 3730XL DNA Analyzer (Applied Biosystems). Sequencing results were viewed using Mega 4.0.2 software (Tamura et al. 2007), and sequences were evaluated using the Basic Local Alignment Search Tool (BLAST).
4. Results

4.1. Preliminary Batch Study

The preliminary batch study tested ten different substrate conditions in microcosms containing 100 mL AMD. Active microcosms contained 0.5 g stream sediment as a bacterial inoculum and 0.25 g substrate. Negative control microcosms did not contain substrate. Uninoculated controls did not contain stream sediment, and substrates for these microcosms were treated with a UV lamp. All microcosms were allowed to incubate for 4 weeks. Full results of this experiment are tabulated in Appendix C.

4.1.1. Characterization of Starting Materials

The raw AMD was analyzed to determine the starting point for treatment (Table 4-1), and the carbon and nitrogen content of the stream sediment, chitin, and SMS were also determined (Table 4-2). As expected, chitin had the highest nitrogen content. After completion of the experiment, possible contributions of the SMS and sediment to dissolved iron concentrations in the microcosms became a concern. The Mehlich 3 test was used to quantify the amount of extractable iron in these solids. The SMS was found to contain 106 mg extractable Fe/kg dry SMS. Sediment collected from the same stream as the microcosm inoculum contained 587 mg extractable Fe/kg dry sediment.

Table 4-1. Analysis of raw AMD for the preliminary batch study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.07</td>
</tr>
<tr>
<td>Acidity</td>
<td>166.4 mg/L as CaCO$_3$</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>0.00 mg/L as CaCO$_3$</td>
</tr>
<tr>
<td>Ammonium</td>
<td>BDL*</td>
</tr>
<tr>
<td>Dissolved aluminum</td>
<td>13.0 ppm</td>
</tr>
<tr>
<td>Dissolved iron</td>
<td>29.0 ppm</td>
</tr>
<tr>
<td>Dissolved manganese</td>
<td>19.0 ppm</td>
</tr>
<tr>
<td>Dissolved zinc</td>
<td>0.73 ppm</td>
</tr>
<tr>
<td>Sulfate</td>
<td>543 mg/L</td>
</tr>
<tr>
<td>Dissolved oxygen (after purging with nitrogen gas)</td>
<td>&lt;0.5 mg/L</td>
</tr>
</tbody>
</table>

*BDL—below detection limit. Detection limit for ammonium was 0.5 mg/L NH$_4$+-N.
Table 4-2. Carbon and nitrogen content of solid materials used in preliminary batch study.

<table>
<thead>
<tr>
<th>Material</th>
<th>Nitrogen (by dry mass)</th>
<th>Carbon (by dry mass)</th>
<th>C:N ratio (dry material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment</td>
<td>0.08%</td>
<td>2.56%</td>
<td>31.2:1</td>
</tr>
<tr>
<td>SMS</td>
<td>2.15%</td>
<td>33.74%</td>
<td>15.7:1</td>
</tr>
<tr>
<td>Chitin</td>
<td>3.92%</td>
<td>20.24%</td>
<td>5.2:1</td>
</tr>
</tbody>
</table>

4.1.2. Final Microcosm Chemistry

Results of the preliminary batch study show a snapshot of AMD treatment at a single time point, as all microcosms were sacrificed after 4 weeks. All substrate conditions resulted in increased pH of the AMD after the 4 week incubation period (Figure 4-1). The pH of the negative control microcosm, which did not contain any substrate, increased only slightly from pH 2.89 to 3.10. In the uninoculated control microcosm containing only SMS as a substrate, pH increased to 5.88. Final pH in all other microcosms was above 6.25. All substrates also contributed to decreased acidity and increased alkalinity (Figure 4-1).

All reported metal concentrations are for dissolved species (<0.45 μm). Aluminum and zinc were effectively removed by a greater variety of substrate combinations than iron and manganese (Figure 4-1). Aluminum, with an initial concentration of 13.0 ppm in the raw AMD, had final concentrations of 0.70 ppm or less in all microcosms (excluding the negative control). Seven substrate conditions yielded complete removal of aluminum below the method detection limit of 0.01 ppm. Zinc concentrations decreased from 0.73 ppm in untreated AMD to below the detection limit of 0.01 ppm under 6 of the 10 substrate conditions. Manganese concentrations varied widely among AMD treated with different substrates, with the greatest removal (up to 75%) by substrates containing chitin, and little removal (21% or less) by other substrates.

Complete iron removal was observed with the 100% chitin substrate (inoculated active microcosm). However, some treatments resulted in increased concentrations of dissolved iron up to 50 ppm above the initial concentration in the raw AMD. The stream sediment and SMS were the source of the high concentrations of iron observed in these microcosms, although the total amount of available iron in the solids is somewhat difficult to assess. The possible contribution of the SMS and stream sediment was
calculated using the extractable iron content determined by the Mehlich 3 test (Table 4-3).
From this calculation, it is clear that much of the increase in dissolved iron cannot be
attributed to the Mehlich 3 extractable iron measured in the microcosm solids. However,
the Mehlich 3 test may not have measured all of the available iron in the solids, since
reducing conditions in the microcosms could be expected to allow further release of iron.
Total iron content of the solids was not measured in this experiment, but has been
measured for these materials in a previous experiment (Robinson-Lora, unpublished data;
Table 4-3). The amount of total iron in the solids is probably much greater than the
amount that could dissolve even under reducing conditions; however, these data show
that significant amounts of iron were introduced into the microcosms as a result of adding
stream sediment and SMS. In contrast to the elevated iron levels observed in microcosms
containing substrate, iron removal occurred in microcosms without an organic substrate
(sediment only and uninoculated limestone test conditions). Oxidizing conditions
prevailed in these microcosms due to the initial presence of oxygen and the lack of an
organic substrate to facilitate the establishment of reducing conditions through microbial
processes. Although the microcosms were purged with nitrogen during set-up, it was
impossible to completely eliminate oxygen, and removal of iron through oxidation and
precipitation likely occurred.

Table 4-3. Maximum possible contribution of SMS and stream sediment to dissolved
iron levels in selected preliminary batch test microcosms, based on the
mass of the solids used, the volume of AMD in each microcosm, and the
amount of iron in the solids measured by two different methods. Mehlich
3 extractable iron levels associated with the solids were 106 mg Fe/kg dry
SMS and 587 mg Fe/kg dry sediment. Total iron measured for these
solids in a previous experiment were 7,922 mg Fe/kg dry SMS and 82,375
mg Fe/kg dry sediment.

<table>
<thead>
<tr>
<th>Test condition</th>
<th>Total dissolved iron in microcosms</th>
<th>Based on Mehlich 3 extractable iron</th>
<th>Based on total iron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ppm)</td>
<td>(ppm)</td>
<td>(ppm)</td>
</tr>
<tr>
<td>Initial AMD</td>
<td>29.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>100% SMS</td>
<td>78.0 ± 18.4</td>
<td>2.41</td>
<td>432</td>
</tr>
<tr>
<td>95% SMS, 5% limestone</td>
<td>75.5 ± 0.7</td>
<td>2.40</td>
<td>431</td>
</tr>
<tr>
<td>95% SMS, 5% chitin</td>
<td>79.0 ± 9.9</td>
<td>2.40</td>
<td>431</td>
</tr>
<tr>
<td>75% SMS, 25% chitin</td>
<td>44.5 ± 24.7</td>
<td>2.34</td>
<td>427</td>
</tr>
</tbody>
</table>
Figure 4-1. Neutralization and removal of metals in AMD by chitin, limestone, SMS, and mixed substrates after 4 weeks of treatment in the preliminary batch study. Values are the average of duplicate microcosms; error bars represent 1 standard deviation.

Decreases in sulfate concentrations after 4 weeks of incubation were observed only in microcosms containing at least 25% chitin in the substrate composition. Black precipitates indicating sulfate reduction to sulfide were observed in additional microcosms, even though sulfate concentrations increased relative to the raw AMD (Table 4-4). The greatest increase (242 ± 12 % ppm $\text{SO}_4^{2-}$) was observed in the negative controls containing only AMD and stream sediment.
Table 4-4. Sulfate concentrations in untreated AMD water and microcosms after 4 week incubation period (duplicate average). Observation of black precipitates indicates sulfate reduction to sulfide. Initial sulfate concentration was 543 mg/L.

<table>
<thead>
<tr>
<th>Test condition</th>
<th>Black precipitate observed?</th>
<th>Sulfate (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment only (negative control)</td>
<td>No</td>
<td>1,314 ± 66</td>
</tr>
<tr>
<td>Chitin only (uninoculated)</td>
<td>Yes</td>
<td>448 ± 61</td>
</tr>
<tr>
<td>SMS only (uninoculated)</td>
<td>Yes</td>
<td>697 ± 29</td>
</tr>
<tr>
<td>Limestone only (uninoculated)</td>
<td>No</td>
<td>637 ± 1</td>
</tr>
<tr>
<td>100% SMS</td>
<td>Yes</td>
<td>933 ± 195</td>
</tr>
<tr>
<td>95% SMS, 5% limestone</td>
<td>No</td>
<td>1,013 ± 64</td>
</tr>
<tr>
<td>95% SMS, 5% chitin</td>
<td>Yes</td>
<td>795 ± 192</td>
</tr>
<tr>
<td>25% SMS, 75% chitin</td>
<td>Yes</td>
<td>479 ± 67</td>
</tr>
<tr>
<td>50% SMS, 50% chitin</td>
<td>Yes</td>
<td>298 ± 42</td>
</tr>
<tr>
<td>100% chitin</td>
<td>Yes</td>
<td>135 ± 56</td>
</tr>
</tbody>
</table>

4.1.3. Effect of Chitin as a Fractional Amendment to SMS

The effect of chitin as a fractional amendment was evaluated by comparing substrates with various percentages of chitin in SMS to a 100% SMS substrate (0% chitin). Alkalinity was generated by all substrates. pH>6.5 was achieved with all substrates containing chitin, while the pH only reached 5.88 with SMS alone. Thus, all substrates containing at least 5% chitin effectively neutralized the raw AMD (Figure 4-2).

Removal of common AMD metals (aluminum, iron, manganese, and zinc) was improved by fractional amendment of chitin to SMS (Figure 4-2). All fractions of chitin successfully removed dissolved aluminum and zinc below their detection limit of 0.01 ppm. A linear relationship was observed between the fraction of chitin in the substrate and the amount of manganese removed, with additional removal of 0.12 ppm manganese per 1% increase in chitin fraction ($R^2=0.98$; Figure 4-3). Iron removal was observed only in microcosms containing 50-100% chitin, although removal in these cases was very good (99% or better).
Figure 4-2. Effect of fractional amendment of SMS with chitin on (a) neutralization, (b) removal of metals, (c) anions, (d) carbon and nitrogen in AMD after 4 weeks of treatment. Portion of substrate that is not chitin is SMS. Values are averages of duplicate microcosms; error bars represent 1 standard deviation. For initial values in raw AMD refer to Tables C-1 through C-4.
Figure 4-3. Manganese removal was correlated to the amount of chitin in the substrate in the preliminary microcosm study. Concentrations shown are duplicate averages after 4 weeks of treatment in microcosms; error bars represent 1 standard deviation. Substrate is SMS amended with chitin.

IC analysis of anions showed that removal of sulfate increased with increased fractions of chitin (Figure 4-2). While sulfate reduction was evident in the 100% SMS microcosms as well as all microcosms containing chitin (due to formation of black precipitates and hydrogen sulfide odors), sulfate removal below initial AMD levels was achieved only with substrates containing at least 25% chitin. Chloride and phosphate, and to a lesser extent, nitrate, were more concentrated in microcosms containing more chitin, due to dissolution of salts associated with the crab-shell material.

Production of ammonium, DOC, and VFAs occurred in microcosms containing a substrate of only SMS as well as with substrates containing chitin (Figure 4-2). Ammonium production was low (1.6 mg/L NH$_4^+$-N) for the SMS-only substrate, and increased steadily with addition of chitin. Ammonium production is greater with chitin because it contains more nitrogen than SMS (Table 4-2), and the nitrogen associated with chitin may be more bioavailable. DOC concentrations were not significantly different for substrates containing 100% SMS, 5% chitin, or 25% chitin; however, greater DOC concentrations were observed with 50% and 100% chitin. Isobutyrate was the only VFA detected in the 100% SMS microcosms, whereas formate, acetate, propionate, butyrate,
isobutyrate, isovalerate, valerate, and isocaproate were detected in microcosms containing chitin.

4.1.4. Molecular Biology

PCR amplification of DNA extracted from preliminary batch test microcosm solids was very poor. No further work was done with these samples.

4.2. Extended Batch Study

The extended batch study allowed monitoring of the performance of different chitin-to-SMS substrate ratios over 63 days of AMD treatment. Microcosms contained 0.5 g of sediment, 0.25 g substrate, and 100 mL AMD. Substrates consisted of SMS amended with 5%, 15%, 30%, or 50% chitin. The negative control did not contain substrate. Microcosms were sacrificed every 2 days at the beginning of the experiment and then with less frequency as the experiment progressed and chemical conditions stabilized.

4.2.1. Characterization of Starting Materials

Although collected from the same location, the raw AMD used in this test was somewhat less severe than the AMD used in the preliminary batch test, as the metals concentrations, acidity and sulfate were all lower (Table 4-5), presumably due to seasonal fluctuations in flow rate. Carbon and nitrogen content of microcosm solids were very similar to the previous experiment (Table 4-6). SMS was also tested for extractable iron due to the variable iron concentrations observed in the preliminary microcosm test. Mehlich 3 test extractable iron in the SMS was 38.0 mg/kg. A comparison of initial and final water quality data is tabulated in Appendix D.
Table 4-5. Analysis of raw AMD for the extended batch study.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.13</td>
</tr>
<tr>
<td>Acidity</td>
<td>94.0 mg/L as CaCO$_3$</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>0.00 mg/L as CaCO$_3$</td>
</tr>
<tr>
<td>Ammonium</td>
<td>BDL*</td>
</tr>
<tr>
<td>Dissolved aluminum</td>
<td>8.09 ppm</td>
</tr>
<tr>
<td>Dissolved iron</td>
<td>15.0 ppm</td>
</tr>
<tr>
<td>Dissolved manganese</td>
<td>10.4 ppm</td>
</tr>
<tr>
<td>Dissolved zinc</td>
<td>0.53 ppm</td>
</tr>
<tr>
<td>Sulfate</td>
<td>366 mg/L</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>1.20 ± 0.25 mg/L</td>
</tr>
</tbody>
</table>

*BDL—below detection limit. Detection limit for ammonium was 0.5 mg/L NH$_4^+$-N.

Table 4-6. Carbon and nitrogen content of solid materials used in the extended batch study.

<table>
<thead>
<tr>
<th>Material</th>
<th>Nitrogen (by dry mass)</th>
<th>Carbon (by dry mass)</th>
<th>C:N ratio (dry material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment</td>
<td>0.09%</td>
<td>2.46%</td>
<td>28.8:1</td>
</tr>
<tr>
<td>SMS</td>
<td>1.45%</td>
<td>31.45%</td>
<td>21.7:1</td>
</tr>
<tr>
<td>Chitin</td>
<td>4.09%</td>
<td>21.13%</td>
<td>5.2:1</td>
</tr>
</tbody>
</table>

4.2.2. Microcosm Chemistry during Treatment

Results of the extended batch study show the effectiveness of substrates containing various fractions of chitin (5-50%) in SMS over a period of 63 days. Neutralization was faster in microcosms containing more chitin, with a 50% chitin substrate treating the AMD to pH 6.32 within 3 days (Figure 4-4). In comparison, it took 10 days for pH to reach 6.29 with the 5% chitin substrate. Final pH in the active microcosms was in the range of 6.5-7.5. Generation of alkalinity and reduction in acidity occurred as the pH increased, and was clearly related to the amount of chitin in the substrate (Figure 4-4). Final alkalinity was 55.2-343 mg/L as CaCO$_3$. 

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Figure 4-4. pH, acidity, and alkalinity in the extended batch study. Points are averaged values from duplicate microcosms; error bars represent 1 standard deviation.
Aluminum and zinc were successfully removed by all substrates (Figure 4-5). The rate of removal depended upon the proportion of chitin in the substrate. All substrate treatments resulted in near-complete removal of aluminum (from 8.1 ppm to <1 ppm) within 3-5 days. Zinc removal occurred more slowly, with removal from 0.5 ppm to the detection limit of 0.01 ppm occurring within 5-28 days. Manganese removal was fastest in the first 5 days of the experiment, with rates of 0.26-0.80 mg/L-d for active microcosms during this time period (Figure 4-5). Removal after 63 days was as high as 60 ± 20 % (50% chitin substrate). The substrates containing 5% and 15% chitin effected the least removal of manganese (27 ± 12 % and 24 ± 10 %; no significant difference).

Iron was not reliably removed in this experiment (Figure 4-5). Dissolved iron concentrations fluctuated throughout the treatment period, and duplicate microcosms had dissimilar iron measurements, resulting in large standard deviations. During the first 5 days of the experiment, iron concentrations decreased in all microcosms. Initially, conditions in the microcosms were oxidizing due to residual DO present in the AMD even after purging with nitrogen, allowing iron to oxidize and precipitate from the solution. In the control microcosms, which had no organic substrate to stimulate the establishment of reducing conditions, dissolved iron levels continued to decrease and remained low for the rest of the experiment. As was the case in the preliminary microcosm study, extractable iron in substrates and sediment detected by the Mehlich 3 test cannot account for the high dissolved iron concentrations measured during the course of the experiment.

Sustained removal of sulfate was observed only during treatment with the substrate containing 50% chitin (Figure 4-6). Sulfate concentrations increased in all active microcosms at the beginning of the experiment, and remained elevated in the 5% and 15% chitin test conditions. Chloride, nitrate, and phosphate were also measured at each sampling point (Figure 4-6). Chloride concentrations increased most rapidly during the first week of the experiment and were higher in microcosms containing more chitin. The dissolution of chloride salts from the surface of the crab-shell particles is expected to be the main source of chloride beyond the background level in the raw AMD, as previously reported (Robinson-Lora and Brennan 2009b). Phosphate and nitrate concentrations were similar among all test conditions.
Figure 4-5. Removal of metals in the extended batch study. Points represent averaged values from duplicate microcosms; error bars represent 1 standard deviation.
Figure 4-6. Anion concentrations in the extended batch study. Points represent averaged values from duplicate microcosms; error bars represent 1 standard deviation.
Production of ammonium, DOC, and VFAs increased for the first 7-14 days of the experiment for all substrates (Figure 4-7). For each analyte, production increased with increasing fractions of chitin, and was much greater for substrates containing 30% and 50% chitin than for other substrates. In the 50% chitin test condition, DOC and VFAs increased rapidly in the first 10 days (to 35.8 ± 9.1 and 24.5 ± 6.4 mg/L C, respectively), and then began to decline. Ammonium concentrations did not decline, suggesting that as microbes consumed the available carbon substrates, their growth was not limited by nitrogen availability. A similar trend is noted for the 30% chitin test condition. Of the VFAs detected, acetate and valerate were detected in the highest concentrations (peak concentrations of 0.03-0.28 mM and 0.03-0.14 mM, respectively). Formate was detected in concentrations of 0.06 mM or less in microcosms containing at least 15% chitin substrates, usually during the first week of the experiment. Propionate, isobutyrate, butyrate, and isovalerate were also detected, but at lower concentrations (0.04 mM or less). Concentrations of all VFAs detected increased with increasing fractions of chitin in the substrate.
Figure 4-7. Ammonium, DOC, and VFAs in the extended batch study. Points represent averaged values from duplicate microcosms.
4.2.3. Molecular Biology

DNA was extracted from representative samples in the extended batch study. Samples were from microcosms under each substrate condition sacrificed after 1, 5, 28, and 63 days. DNA was amplified and applied to DGGE gels (Figure 4-8). Given the number and intensity of the bands in each lane, it appears that samples with at least 15% chitin had more diverse bacterial communities. Based on the appearance and disappearance of bands, the communities also appear to have changed over time. For the 50% and 100% chitin substrates, it appears that microbial community diversity may have begun to decline between days 5 and 28. Prominent DGGE bands were excised for DNA sequencing; however, the sequencing was unsuccessful, so bands in the gels cannot be correlated to specific populations.

Figure 4-8. DGGE gels from extended batch study. DNA was extracted from microcosm solids after the indicated incubation period.

DNA from the initial AMD, stream sediment, chitin, and SMS used in the extended batch study was also applied to DGGE gels. Multiple bands appeared for each of these components (Figure 4-9), which indicates that bacteria were introduced into the microcosms with the AMD and organic substrates, in addition to those associated with the sediment inoculum. Since there are significant populations of bacteria associated with the AMD and substrates, it may not be necessary to add a bacterial inoculum in future laboratory studies.
4.3. Continuous-Flow Column Study

Continuous-flow columns were utilized to evaluate the treatment efficiency and longevity of different chitin-to-SMS substrate ratios. The fractions of chitin tested were 5% and 50% (remainder was SMS). A typical substrate traditionally used for passive treatment of AMD (10% limestone, 90% SMS) was also tested for comparison, along with a negative control (no substrate) and positive control (100% chitin substrate). All columns were inoculated with stream sediment. The flow rate for the columns was increased periodically, nominally ranging from 0.25 mL/min to 1 mL/min over the course of the experiment. Actual flow rates were slightly different for each column, and were measured directly by collecting the column effluent. In the following text and figures, columns are referred to by their substrates:

- “Sediment only” or “control”: no substrate
- “10% limestone”: substrate is 10% limestone, 90% SMS
- “5% chitin”: substrate is 5% chitin, 95% SMS
- “50% chitin”: substrate is 50% chitin, 50% SMS
- “100% chitin”: substrate is entirely chitin

![DGGE gel for initial AMD and solids added to microcosms in the extended batch study.](image-url)
4.3.1. Characterization of Starting Materials

The raw AMD and solids used to pack the columns were analyzed in order to characterize the inputs into the treatment system. Water chemistry of the AMD was similar to AMD previously collected from Kittanning Run (Table 4-7). The carbon and nitrogen content of the chitin and SMS were similar to the substrates used in the batch experiments (Table 4-8). The sediment was collected in a different location and contained less carbon than the sediment previously used. The Mehlich 3 test was used to determine the extractable amounts of selected elements in all solids used in the columns (Table 4-9).

After packing, the columns were allowed to incubate for 8 days. The incubation period allowed the bacterial community to become established, thereby eliminating lag time for remediation once continuous-flow operation began. During incubation, alkalinity in the active columns increased dramatically, with a corresponding increase in pH and decrease in acidity. Ammonium and VFAs were also produced. Water quality data for all columns following the incubation period is included in Appendix E.

Table 4-7. Analysis of raw AMD used to wet-pack columns for the continuous-flow study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2.80</td>
</tr>
<tr>
<td>Acidity</td>
<td>176.8 mg/L as CaCO$_3$</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>0.00 mg/L as CaCO$_3$</td>
</tr>
<tr>
<td>Ammonium</td>
<td>BDL*</td>
</tr>
<tr>
<td>Dissolved aluminum</td>
<td>10.3 ppm</td>
</tr>
<tr>
<td>Dissolved iron</td>
<td>16.4 ppm</td>
</tr>
<tr>
<td>Dissolved manganese</td>
<td>16.4 ppm</td>
</tr>
<tr>
<td>Dissolved zinc</td>
<td>0.56 ppm</td>
</tr>
<tr>
<td>Sulfate</td>
<td>646 mg/L</td>
</tr>
<tr>
<td>Dissolved oxygen (after purging with nitrogen gas)</td>
<td>0.49 ± 0.01 mg/L</td>
</tr>
</tbody>
</table>

*BDL—below detection limit. Detection limit for ammonium was 0.5 mg/L NH$_4^+$-N.
Table 4-8.  Carbon and nitrogen content of solid materials used in the column study.

<table>
<thead>
<tr>
<th>Material</th>
<th>Nitrogen (by dry mass)</th>
<th>Carbon (by dry mass)</th>
<th>C:N ratio (dry material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment</td>
<td>0.09%</td>
<td>0.85%</td>
<td>9.00:1</td>
</tr>
<tr>
<td>SMS</td>
<td>2.26%</td>
<td>34.97%</td>
<td>15.5:1</td>
</tr>
<tr>
<td>Chitin</td>
<td>5.35%</td>
<td>26.23%</td>
<td>4.90:1</td>
</tr>
</tbody>
</table>

Table 4-9.  Extractable elements in column study solids materials as determined by the Mehlich 3 test (mg/kg dry solids).  Note that the Mehlich 3 test may not account for all available elements under reducing conditions established during AMD treatment.

<table>
<thead>
<tr>
<th>Material</th>
<th>Al</th>
<th>Ca</th>
<th>Fe</th>
<th>K</th>
<th>Mg</th>
<th>Mn</th>
<th>Na</th>
<th>P</th>
<th>S</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment</td>
<td>481</td>
<td>828</td>
<td>668</td>
<td>40.0</td>
<td>88.5</td>
<td>269</td>
<td>21.5</td>
<td>4.22</td>
<td>9.56</td>
<td>5.60</td>
</tr>
<tr>
<td>Chitin</td>
<td>0.588</td>
<td>35,000</td>
<td>12.9</td>
<td>3,540</td>
<td>2,250</td>
<td>47.7</td>
<td>18,700</td>
<td>2,740</td>
<td>1,430</td>
<td>45.2</td>
</tr>
<tr>
<td>SMS</td>
<td>39.0</td>
<td>11,200</td>
<td>266</td>
<td>20,200</td>
<td>2,960</td>
<td>98.9</td>
<td>1,910</td>
<td>2,330</td>
<td>9,650</td>
<td>60.6</td>
</tr>
<tr>
<td>Sand</td>
<td>13.5</td>
<td>27.6</td>
<td>2.96</td>
<td>3.91</td>
<td>6.59</td>
<td>0.954</td>
<td>27.2</td>
<td>3.00</td>
<td>5.20</td>
<td>0.237</td>
</tr>
<tr>
<td>Limestone</td>
<td>2.59</td>
<td>1,560</td>
<td>151</td>
<td>13.5</td>
<td>885</td>
<td>7.82</td>
<td>15.4</td>
<td>1.28</td>
<td>4.80</td>
<td>0.645</td>
</tr>
</tbody>
</table>

4.3.2.  Conservative Tracer Tests and Column Flow

Sodium chloride tracers were used during the continuous-flow column test to determine the HRT, effective pore volume, and dispersion coefficient for each column (Table 4-10).  The nominal flow rate during the tracer tests was 0.5 mL/min, although the measured flow rate varied slightly for each column.  The tracer response curves are shown in Figure 4-10.  Calculations for HRT, dispersion numbers, and effective pore volume are described in Appendix A.  HRT ranged from 9.3-11.4 hr, with columns containing chitin having shorter HRTs.  Dispersion numbers ranged from 0.029-0.067.  A dispersion number less than 0.05 indicates a plug-flow reactor with low dispersion, while 0.05-0.25 qualifies as moderate dispersion (Metcalf & Eddy 2003).  Effective pore volume in the columns ranged from 264-337 mL.

Table 4-10.  HRT and dispersion numbers for column test.

<table>
<thead>
<tr>
<th>Column</th>
<th>HRT (hr)</th>
<th>Dispersion number</th>
<th>Flow type</th>
<th>Flow rate during test (mL/min)</th>
<th>Effective pore volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.3</td>
<td>0.029</td>
<td>Low dispersion</td>
<td>0.495</td>
<td>337</td>
</tr>
<tr>
<td>10% Limestone</td>
<td>11.4</td>
<td>0.067</td>
<td>Moderate dispersion</td>
<td>0.489</td>
<td>334</td>
</tr>
<tr>
<td>5% Chitin</td>
<td>10.2</td>
<td>0.054</td>
<td>Moderate dispersion</td>
<td>0.532</td>
<td>325</td>
</tr>
<tr>
<td>50% Chitin</td>
<td>10.1</td>
<td>0.044</td>
<td>Low dispersion</td>
<td>0.435</td>
<td>264</td>
</tr>
<tr>
<td>100% Chitin</td>
<td>9.3</td>
<td>0.065</td>
<td>Moderate dispersion</td>
<td>0.504</td>
<td>282</td>
</tr>
</tbody>
</table>
Column operation was divided into three phases with different flow rates to help determine the minimum HRT required for effective AMD treatment. During phase I, the nominal flow rate was 0.25 mL/min (~22 hr HRT). Nominal flow rates were then increased to 0.5 mL/min (~11 hr HRT) and 1 mL/min (~5 hr HRT) in phases II and III. Variations in flow rate and pore volume between columns and/or phases also resulted in variations in retention time. Table 4-11 summarizes the flow rate and retention time for each column during each phase of the experiment.
Table 4-11. Hydraulic parameters during each phase of the column study. “Average flow rate” is the time-weighted average of flow rates determined periodically during each phase by the rate of effluent collection.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Column</th>
<th>Nominal flow rate (mL/min)</th>
<th>Time range (days)</th>
<th>Cumulative pore volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I</td>
<td>Control</td>
<td>0.25</td>
<td>0-50</td>
<td>0-54</td>
</tr>
<tr>
<td></td>
<td>10% Limestone</td>
<td>0.22</td>
<td>51-108</td>
<td>55-167</td>
</tr>
<tr>
<td></td>
<td>5% Chitin</td>
<td>0.24</td>
<td>108-148</td>
<td>167-416</td>
</tr>
<tr>
<td></td>
<td>50% Chitin</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100% Chitin</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A significant flow interruption occurred between 22 and 26 days of continuous flow operation (approximately 23-27 PV). At some point during this period, the influent line became disconnected from the AMD reservoir, and air was being pumped into the columns. It is not clear at what point during this time the line was disconnected and therefore the exact length of the interruption is unknown. The line was reconnected on day 26. Column flow was interrupted again for about 12 hr on day 101 (approximately 170 PV), when the pump stopped during a power outage.

4.3.3. pH, Alkalinity, and Acidity

During the initial incubation period, pH increased in all of the columns (Figure 4-11). There was only a slight increase in pH in the control column (from initial pH of 2.80 to 3.33) and much greater increases for columns containing substrate, with pH reaching 7.15 in the 100% chitin column. Alkalinity was generated in all columns except the control, with levels as high as 6,700 mg/L as CaCO$_3$ in the 100% chitin column (Figure 4-12). Acidity also decreased in all columns except the control during the incubation period (Figure 4-13). The 5% chitin column was the first active column to exhaust its neutralizing capacity, with pH and alkalinity steeply declining after 33 days (35 PV) of continuous flow and approximately reaching influent pH after 78 days (119 PV). The 10% limestone column was the next to experience a decline in performance, with neutralization dropping off sharply after 64 days (77 PV) and having virtually no effect.
on pH after 120 days (220 PV). The 100% chitin substrate had the best performance for neutralization, maintaining circumneutral pH for 99 days (182 PV).

Figure 4-11. Influent and effluent pH of columns during the continuous-flow experiment.
Figure 4-12. Alkalinity in effluent from columns treating AMD with different substrate mixtures. Alkalinity in influent AMD and control column effluent was zero throughout the experiment.

Figure 4-13. Acidity in influent and effluent of columns treating AMD with different substrate mixtures.
4.3.4. Metal Removal

Dissolved aluminum and zinc were removed by all columns during the incubation period (Figure 4-14 and Figure 4-15). In the control columns, there was an immediate release of these metals once continuous-flow operation began. In the columns containing substrate, removal of aluminum and zinc was sustained during continuous flow. Zinc removal lasted the longest. Complete breakthrough to influent levels did not occur until day 64-127 (87-301 PV), depending on the substrate. Aluminum was also successfully removed for a relatively long period of time, with removal occurring for up to 108 days (207 PV; for 100% chitin). After breakthrough, dissolved concentrations of aluminum and zinc in the column effluent were higher than the influent, suggesting that metals that had been retained in the columns dissolved and were released as the pH within the columns decreased. Average influent levels for aluminum and zinc were 10.4 ± 0.4 ppm and 0.66 ± 0.23 ppm, respectively.

In agreement with the batch studies, manganese and iron removal was less thorough than aluminum and zinc removal. Manganese was the first metal to break through in all of the columns (Figure 4-16). In the 10% limestone and 5% chitin columns, manganese breakthrough above influent levels (16.8 ± 0.8) occurred after only 7 days (6 PV). Partial manganese removal continued for 57 days (76 PV) in the 100% chitin column. In all the active columns, manganese removal was best when the alkalinity was at least 350 mg/L as CaCO₃. A sharp increase in effluent manganese concentrations occurred as alkalinity dropped between 350 and 150 mg/L as CaCO₃. At alkalinity less than approximately 150 mg/L as CaCO₃, manganese removal was not observed. It is interesting to note that while the 10% limestone column generally performed better than the 5% chitin column (in terms of having greater alkalinity, better removal of aluminum, zinc, etc.), the two columns exhibited very similar removal of manganese.

Iron removal did not follow the same pattern as other metals (Figure 4-17). The average influent iron concentration was 7.35 ± 3.0 ppm. In the control column, effluent iron concentrations remained 2-4 ppm lower than the influent throughout most of the experiment. In the 10% limestone column, there was a release of iron after the incubation period, resulting in effluent concentrations as high as 64 ppm. Iron was removed to levels below the influent concentration beginning on day 7 (6 PV), but
breakthrough occurred on day 15 (13 PV). Breakthrough occurred at the same time for the 5% chitin substrate, although there was no initial release of iron after the incubation period. Removal was more sustained in the columns containing 50% and 100% chitin (57 and 92 days, or 79 and 164 PV, respectively).

Since samples had to be filtered before metals analysis, any iron that entered the columns in particulate form is not accounted for by the concentrations plotted in Figure 4-17. Total iron was determined by nitric acid digestion for the AMD used to pack the columns, the influent AMD on day 127, and the influent AMD at the end of the experiment (day 148). The total iron concentrations ranged from 7.7-25.2 ppm, compared to dissolved concentrations of 3.6-16.4 ppm. Dissolution of particulate iron in the influent and iron associated with the solid packing materials contributed to the high concentrations plotted in Figure 4-17.
Figure 4-14. Aluminum in influent and effluent of columns treating AMD with different substrate mixtures.

Figure 4-15. Zinc in influent and effluent of columns treating AMD with different substrate mixtures.
Figure 4-16. Manganese in influent and effluent of columns treating AMD with different substrate mixtures.

Figure 4-17. Iron in influent and effluent of columns treating AMD with different substrate mixtures.
4.3.5. **Sulfate Reduction**

Signs of sulfate reduction (hydrogen sulfide odors and black precipitates) were observed in all active columns after the incubation period (Figure 4-18). Sulfate increased in all active columns during the incubation period. The average influent sulfate concentration during the entire experiment was $670 \pm 60$ ppm. After initial flushing (2-4 days, 2-4 PV), sulfate levels in all active columns were lower in column effluent than the influent (Figure 4-19). Sulfate reduction appeared to continue in all columns until after the flow interruption on day 26, when air was being pumped through the columns (refer to Section 4.3.2). Between day 33-50 (31-59 PV), sulfate concentrations increased in the effluent of all columns. This was especially marked in the 100% chitin column, where the effluent concentration peaked at 1,420 ppm on day 50 (59 PV). It is possible that the introduction of oxygen into the columns resulted in the oxidation of reduced sulfur species, causing a spike in effluent sulfate concentrations until anoxic conditions were reestablished in the columns. In addition, exposure to oxygen inhibits SRB metabolism, although the effect is reversible (Neculita et al. 2007). The 10% limestone and 5% chitin columns did not recover their sulfate-reducing ability after this interruption, and effluent sulfate concentrations generally mirrored the influent. Sulfate reduction continued in the 50% and 100% chitin columns until day 85 and 92 (149-164 PV), respectively. Sulfate removal was moderate in all columns and never exceeded 25% (Table 4-12).

Sulfide concentrations were measured in the effluent during the first 15 days of continuous flow to verify that sulfate was being reduced. Sulfide concentrations were greater in the effluent of columns containing chitin, reaching a maximum of 52 ppm on day 4 (4 PV) for the 100% chitin column, compared to a maximum of only 2.8 ppm for the 10% limestone column.
Figure 4-18. Columns before (a) and after (b) the 8-day incubation period. The order of the columns, from left to right in each picture, is: control (sediment only) column, 10% limestone column, 5% chitin column, 50% chitin column, 100% chitin column. Black precipitates visible in the active columns (b) likely indicate sulfate reduction.

Table 4-12. Sulfate reduction in continuous flow experiment before and after a disturbance in the anoxic treatment environment. Since the effects of the flow interruption became apparent in each column at slightly different times, influent concentrations were averaged separately for each column encompassing the appropriate time period.

<table>
<thead>
<tr>
<th>Column</th>
<th>Time until breakthrough [day (PV)]</th>
<th>Type of sample</th>
<th>Before disturbance</th>
<th>Maximum value during disturbance</th>
<th>After disturbance, before breakthrough</th>
<th>After breakthrough</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>x</td>
<td>Influent</td>
<td>674 ± 60</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effluent</td>
<td>661 ± 38</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>10% Limestone</td>
<td>52 (52)</td>
<td>Influent</td>
<td>656 ± 24</td>
<td>700</td>
<td>x</td>
<td>687 ± 59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effluent</td>
<td>634 ± 18</td>
<td></td>
<td>x</td>
<td>673 ± 36</td>
</tr>
<tr>
<td>5% Chitin</td>
<td>40 (43)</td>
<td>Influent</td>
<td>647 ± 16</td>
<td>794</td>
<td>x</td>
<td>687 ± 75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effluent</td>
<td>590 ± 52</td>
<td></td>
<td>x</td>
<td>666 ± 30</td>
</tr>
<tr>
<td>50% Chitin</td>
<td>85 (149)</td>
<td>Influent</td>
<td>656 ± 24</td>
<td>676</td>
<td>695 ± 3</td>
<td>690 ± 96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effluent</td>
<td>584 ± 32</td>
<td></td>
<td>657 ± 16</td>
<td>688 ± 57</td>
</tr>
<tr>
<td>100% Chitin</td>
<td>92 (164)</td>
<td>Influent</td>
<td>640 ± 16</td>
<td>1,420</td>
<td>680 ± 19</td>
<td>692 ± 101</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effluent</td>
<td>573 ± 56</td>
<td></td>
<td>634 ± 15</td>
<td>682 ± 44</td>
</tr>
</tbody>
</table>
Figure 4-19. Sulfate in column influent and effluent. The spike in effluent concentrations around 50 PV occurred after oxygen infiltrated the columns.

4.3.6. Carbon and Nitrogen Species

Ammonium concentrations were very high in the columns containing chitin after the incubation period, ranging from 135 mg/L NH$_4^+$-N (for the 5% chitin column) to 681 mg/L NH$_4^+$-N (for the 100% chitin column). In comparison, the ammonium concentration in the 10% limestone column was only 19.8 mg/L NH$_4^+$-N after the incubation period, suggesting that most of the excess ammonium production was due to chitin and not SMS in columns containing both substrates. Ammonium concentrations decreased rapidly once continuous-flow operation began, although ammonium production was observed for as long as 225 PV (100% chitin column; Figure 4-20). No ammonium was detected in influent AMD (detection limit was 0.5 mg/L NH$_4^+$-N).

DOC was elevated in all of the columns after the incubation period, ranging from 414 mg/L (no substrate) to 6,000 mg/L (100% chitin column). DOC steadily decreased once continuous-flow operation began, eventually reaching influent levels (4.8 ± 3.4 mg/L) in all of the columns by about day 60 (70 PV). Before reaching influent levels,
DOC concentrations were very similar for the 10% limestone and 5% chitin columns, but significantly higher for the columns containing more chitin (Figure 4-21).

VFAs were produced in all active columns during the incubation period, and production was sustained for 4-85 days (3-147 PV; Figure 4-22). VFA production was generally higher for columns containing more chitin, although there was little difference between the columns containing 10% limestone and 5% chitin. Valerate was detected by the HPLC in column influent and in the effluent of the control column; however, VFAs are expected only in AMD samples treated with an organic substrate. A carbon balance between DOC and total VFAs confirmed that the observed concentrations of valerate in these samples were impossible, and the measurement is attributed to an artifact detected by the HPLC at the normal elution time for valerate. In the effluent of active columns, acetate was the dominant VFA. Columns containing more chitin produced greater concentrations and a greater variety of VFA compounds. Individual VFAs measured in the 100% chitin column demonstrate the relative variety and abundance of VFAs produced with a chitin substrate (Figure 4-23).
Figure 4-20. Ammonium in column effluent. Ammonium was not detected in influent AMD or in effluent of the control column at any point during the experiment. The detection limit for ammonium was 0.5 mg/L NH$_4^+$-N; any sample for which ammonium was not detected is plotted above as 0.5 mg/L NH$_4^+$-N.

Figure 4-21. DOC in influent and effluent of columns treating AMD with different substrate mixtures.
Figure 4-22. VFAs in effluent of columns treating AMD with different substrate mixtures. There were no VFAs in column influent or in the effluent of the sediment only column. The detection limit of the individual VFAs is 0.24-2.4 mg/L as C; any sample for which no VFAs were detected is plotted as 0.24 mg/L as C (location of the x-axis).

Figure 4-23. Individual VFAs in the effluent of the 100% chitin column. The detection limit of each VFA is 0.02 mM (location of the x-axis).
5. Discussion

5.1. AMD Neutralization

In the batch tests, all active microcosms with substrate combinations containing SMS with chitin or limestone (or chitin alone) achieved circumneutral pH and produced alkalinity. In the extended microcosm study, SMS substrates containing 5-50% chitin had alkalinity generation rates of 1.7-14.3 mg as CaCO$_3$/L-d during the period of rapid alkalinity generation during the first 10 days of the experiment. In a study using 100% chitin as the substrate, with comparable experimental conditions (Daubert and Brennan 2007), microcosms produced alkalinity at a rate of 26.1 mg as CaCO$_3$/L-d during 9 days of incubation. As the rate of alkalinity generation with a substrate containing 50% chitin was approximately half the rate of a 100% chitin substrate, it appears that alkalinity generation during this initial period is almost solely influenced by the amount of chitin present. SMS does contain a small amount of limestone which is added during the mushroom-growing process, and the SMS-only substrate in the preliminary batch test did increase the pH (from 2.89 to 5.88) and generate measurable alkalinity. However, since chitin contains approximately 40% calcium carbonate by mass, it has a much greater role in alkalinity generation in a mixed substrate.

All of the columns treated AMD to neutral pH and produced measurable alkalinity during the incubation period or within the first week of continuous flow operation. Alkalinity production lasted for 40-106 days (43-198 PV), with production continuing the longest in the column containing the 100% chitin substrate. Results from a previous column experiment indicate that alkalinity production can be sustained 29% longer if a longer HRT is maintained (Robinson-Lora and Brennan 2009a). In the previous experiment, a comparable experimental design testing 25 g of 100% chitin substrate at an HRT of 11.2 hours (0.25 ml/min) continued to produce alkalinity for 278 PV. While the 5% chitin substrate in the current study ceased to produce alkalinity before the transition from Phase I to Phase II (HRT transition from ~22 hr to ~11 hr), alkalinity production in the other columns was reduced after the change in flow rate and HRT. The 100% chitin column was the only column still producing alkalinity at the end of Phase II (HRT ~11 hr), but there was no alkalinity in the effluent after the transition to
Phase III (HRT ~5 hr). The periodic increases in flow rate may have contributed to the
termination of alkalinity production in the columns. It is possible that the substrates were
not completely exhausted in their ability to produce alkalinity but were unable to
contribute measurable alkalinity during the relatively short HRT in phases II and III.

5.2. **Removal of Metals**

The metals of concern in the AMD used in these experiments were aluminum,
iron, manganese, and zinc. Removal of all metals began more quickly, lasted longer, and
was more complete as the proportion of chitin in the substrate increased.

Aluminum and zinc were the most successfully removed metals in the batch and
column studies. Aluminum concentrations in the raw AMD from Kittanning run were
moderate at 8.1-13.0 ppm. All substrates containing chitin effected at least 99% removal
of aluminum, in 5-7 days in batch tests, or within 4 days (3 PV) of continuous flow
column operation. SMS amended with limestone was similarly successful for aluminum
removal. Aluminum removal is controlled by pH, and the increase in pH caused by any
of these substrates allowed the precipitation of aluminum hydroxide. Excellent removal
efficiencies for aluminum have been reported in passive treatment studies using a variety
of organic substrates for AMD remediation (Benner et al. 1999; Christensen et al. 1996;
Dvorak et al. 1992; Robinson-Lora and Brennan 2009a). In the column experiment, the
concentration of aluminum in the effluent dropped sharply when pH rose above 4.3. The
experimental data indicate that treated AMD is supersaturated with respect to gibbsite
(chemical formula: Al(OH)₃), but is also in good agreement with the saturation index for
amorphous Al(OH)₃ (Figure 5-1).
Figure 5-1. Concentration of dissolved aluminum vs. pH of AMD treated in continuous-flow columns. The saturation index for gibbsite (calculated using Visual MINTEQ ver. 2.53; gibbsite: Al(OH)₃) and the influent aluminum concentration are shown for reference. Aluminum concentrations above the influent level reflect the release of aluminum from exhausted columns.

Zinc concentrations in the Kittanning Run AMD were low (0.53-0.73 ppm), and removal was generally complete with all of the active substrate conditions tested. Zinc removal is usually very good under sulfate-reducing conditions, as long as sulfide production exceeds the zinc loading rate (Willow and Cohen 2003; Zagury et al. 2006). The expected removal mechanism under these conditions is precipitation of sphalerite or amorphous ZnS (Benner et al. 1999; Waybrant et al. 2002). With low starting concentrations, adsorption onto the substrate or co-precipitation may also play a significant role in removal (Benner et al. 1999). Adsorption of zinc onto typical AMD precipitates becomes significant at pH greater than 5-6 (Figure 5-2; Micera et al. 1986; Pokrovsky et al. 2005).
In both the batch and column studies, manganese removal was largely dependent on the proportion of chitin in the substrate. In the columns, substrates containing chitin achieved more thorough removal of manganese over longer periods of time. However, manganese was the most difficult metal to remove. In the preliminary batch test, active microcosms containing 100% chitin removed 76% of the initial manganese, compared to removal below the detection limit of aluminum, iron, and zinc under the same conditions. In the column test, manganese in the effluent of the 100% chitin column reached influent concentrations after 52 days (64 PV). In all columns, manganese reached influent levels before the other metals of concern.

Manganese is notoriously difficult to remove from AMD, in both aerobic and anaerobic remediation systems (Sikora et al. 2000; Steed et al. 2000; Willow and Cohen 2003; Bamforth et al. 2006). While zinc and iron may be removed through precipitation
with sulfide, the formation of manganese sulfide is unlikely in passive treatment of AMD. Manganese sulfide phases are unstable due to their high solubility as compared to manganese carbonate phases, the relatively high concentration of sulfide required for precipitation of manganese sulfide, and the favorability of iron sulfide precipitation, which tends to scavenge the available sulfide, making Mn(II) sulfide precipitation impossible (Benner et al. 1999; Edenborn and Brickett 2002; Bamforth et al. 2006). As alkalinity is added to the AMD during treatment, the formation of manganese carbonate is generally cited as the most significant mechanism for manganese removal (Benner et al. 1999; Edenborn and Brickett 2002; Bamforth et al. 2006; Robinson-Lora and Brennan 2009a). Calculation of saturation indices for rhodochrosite (MnCO$_3$) in the column experiment indicates that manganese carbonate precipitation is responsible for the removal of manganese in treatment using SMS and chitin as well (Figure 5-3).

Figure 5-3. Concentration of dissolved manganese vs. alkalinity of AMD treated in continuous-flow columns. The saturation index for rhodochrosite (calculated using Visual MINTEQ ver. 2.53; rhodochrosite: MnCO$_3$) and the influent manganese concentration are shown for reference. Manganese concentrations above the influent level reflect the release of manganese from exhausted columns.

While manganese was the most difficult metal to treat in the AMD, removal increased with larger fractions of chitin in the substrate. Manganese removal observed in
the present column experiment (up to 96% for the 50% and 100% chitin substrates) was quite thorough compared to studies using other substrates at comparable HRT (0-88%; Benner et al. 1999; Willow and Cohen 2003; Hallberg and Johnson 2005; Venot et al. 2008). Based on the results of the preliminary batch study and the column study, chitin was more effective than limestone or SMS in increasing alkalinity and removing manganese. Alkalinity in reactors containing chitin was produced mainly as a result of dissolution of calcium carbonate from the chitin. Alkalinity is also produced by microbial activity in the substrate. The much greater surface area of chitin compared to limestone (14 vs. <0.5 m²/g; Robinson-Lora and Brennan 2009a) contributes to a higher rate of dissolution of calcium carbonate from the chitin, and may also help prevent armoring and subsequent inactivation of the material, which has been cited as a reason for decreased alkalinity production in limestone systems (Johnson and Hallberg 2005a). Increased biological activity in substrates containing chitin could also contribute to higher alkalinity.

Iron removal was less thorough than removal of aluminum and zinc. This behavior has been observed in many studies using passive treatment of AMD under sulfate-reducing conditions (Benner et al. 1999; Waybrant et al. 2002). In the extended batch study, iron concentrations decreased initially, probably due to the precipitation of iron hydroxides under the slightly oxic conditions in the microcosms. These precipitates tended to re-dissolve once biological activity created reducing conditions in the microcosms containing active substrates. In the microcosms containing at least 30% chitin, additional removal of iron was observed, most likely as a ferrous sulfide phase (Robinson-Lora and Brennan 2009a). In the columns, reducing conditions were established during the incubation period, and removal began immediately or within one week of continuous flow. After breakthrough, the amount of iron released from the columns often exceeded the amount of dissolved iron removed from the influent during treatment. The possible contributions to these high breakthrough concentrations of dissolved iron are: dissolution of precipitates formed during treatment, dissolution of ferric hydroxide precipitates present in the column influent, and release of iron from solid materials in the columns (most likely the sand, stream sediment, and SMS). Relatively constant dissolved iron concentrations in the effluent of the control column show some
removal of iron due to the oxic conditions in the absence of an organic substrate and the filtration effect of running the AMD through a column full of sand.

The release of dissolved iron from sulfate-reducing reactors is not necessarily problematic in a field-scale treatment system. Many systems incorporate both anoxic and oxic treatment steps (Johnson and Hallberg 2005a; Neculita et al. 2007). In these systems, it is preferable to reduce iron and allow it to escape from the anoxic treatment step in order to reduce clogging in the organic substrate. In the oxic treatment step, iron will precipitate and can be retained without clogging the treatment system (e.g. in a settling pond).

Johnson and Hallberg (2005b) have reported soluble iron concentrations in the effluent of compost bioreactors that are higher than influent concentrations. They attributed this difference to biological reduction and dissolution of particulate ferric iron minerals that entered the reactors. This study and others have noted the tendency of ferrous sulfide ($K_{sp}=6 \times 10^{2}$) to remain soluble at higher pH than zinc sulfide ($K_{sp}=6 \times 10^{-4}$-$6 \times 10^{-2}$), explaining why zinc removal may continue to be effective even when iron is not being removed (Christensen et al. 1996; Zagury et al. 2006; CRC Press 2008).

5.3. Sulfate Reduction

In the preliminary batch study, sulfate concentrations in AMD decreased only in microcosms containing a substrate of at least 25% chitin. In the extended batch study, 50% chitin was required to remove sulfate. Black precipitates indicating sulfate reduction to sulfide were observed for some test conditions where sulfate concentrations increased in the treated AMD relative to the raw AMD.

The high sulfate concentrations observed after treatment, even in reactors showing evidence of sulfate reduction, are not unprecedented. Dvorak et al. (1992) reported sulfate leaching from SMS in a passive treatment system for AMD. The facility that provided the SMS for these experiments adds gypsum (chemical formula CaSO$_4$•2H$_2$O) to their SMS mixture, which explains the higher sulfate concentrations observed when SMS was used. The release of sulfate sorbed onto ferric oxyhydroxide precipitates in AMD has also been reported (Christensen et al. 1996, Rose and Elliott 2000). In the present experiments, such precipitates may have been suspended in the AMD or present in stream sediments used to inoculate the reactors.
5.4. **Carbon and Nitrogen Species**

Ammonium concentrations in the batch tests were as high as 42 mg/L NH\(_4^+\)-N in the batch tests and as high as 681 mg/L NH\(_4^+\)-N in the columns after the incubation period. While ammonium concentrations were much lower when less chitin was used in the substrate, SMS contributed some ammonium to the reactors as well. Ammonium is the byproduct of degradation of organic nitrogen under reducing conditions, and its production has been reported during the fermentation of chitin (Robinson-Lora and Brennan 2009a,b), SMS (Stewart et al. 1998), and other organics substrates used in passive treatment of AMD (Zagury et al. 2006). Ammonium production provides a suitable source of nitrogen for microbial growth, which is desirable in sulfate-reducing treatment systems. However, excessive releases of ammonium into waterways should be avoided because surplus nitrogen can lead to eutrophication of aquatic ecosystems. In addition, ammonium partitioning to ammonia, which is toxic to aquatic life, may be significant depending upon the pH and temperature of the water.

As with ammonium, DOC and VFAs were produced at higher rates with chitin than with other substrates, indicating that chitin provides a more bioavailable carbon source and is more apt to support microbial activity than SMS. This agrees with previous results showing greater VFA production during fermentation of chitin compared to plant-derived substrates (Vera et al. 2001).

5.5. **Substrate Treatment Capacity and Cost**

Treatment capacities were calculated from the continuous-flow study based on the amount of AMD treated by a given mass of substrate before breakthrough of acidity, dissolved metals, or sulfate above influent levels. For all substrates, manganese was the first parameter to reach breakthrough, and therefore was the controlling factor in determining substrate treatment capacities. Based on cost estimates of $0.60/lb for chitin, $0.025/lb for SMS, and $0.006/lb for limestone, the cost to treat a given volume of AMD with each mixture was also calculated (Table 5-1). The chitin substrates are cost-competitive with the traditional limestone and SMS mixture, especially as a treatment system using chitin (50% chitin and 50% SMS or 100% chitin) would have a much smaller volume than a limestone and SMS system, as less substrate would be required to
treat the same amount of AMD. Also, the chitin substrates provided better treatment of constituents that could not be entirely removed from the AMD (e.g. manganese and sulfate). The substrate containing only 5% chitin did not provide as much of a benefit as the more chitin-rich substrates. Thus, we recommend using chitin alone or SMS mixed with a large proportion of chitin (at least 50%). In field applications, a pure chitin substrate must be mixed 1:1 by volume with sand in order to maintain sufficient hydraulic conductivity, whereas the SMS would provide enough permeability in a 50% chitin substrate that sand could be omitted. A more detailed cost analysis should be done on a project-specific basis to identify the most effective and economically viable substrate.

Table 5-1. Substrate treatment capacity and associated cost. Cost values assume retail values of $0.60/lb for chitin, $0.025/lb for SMS, and $0.006/lb for limestone.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment capacity (L AMD treated per kg substrate)</th>
<th>Substrate cost (Cost of substrate to treat 1000 L AMD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Limestone</td>
<td>36.7</td>
<td>$1.39</td>
</tr>
<tr>
<td>5% Chitin</td>
<td>40.1</td>
<td>$2.95</td>
</tr>
<tr>
<td>50% Chitin</td>
<td>162</td>
<td>$4.25</td>
</tr>
<tr>
<td>100% Chitin</td>
<td>428</td>
<td>$3.09</td>
</tr>
</tbody>
</table>

For comparison to the calculated treatment capacities in Table 5-1, AMD in the batch tests was applied at 400 L AMD per kg substrate (microcosms contained 100 mL AMD and 0.25 g substrate), and very good treatment was still achieved. Of course, the contact time between the AMD and substrate was much longer in the batch tests. A longer HRT in a continuous-flow treatment system would yield better treatment capacities than those obtained from the present study. Due to the decline in performance noted in the transition between phase I (HRT ~22 hr) and phase II (HRT ~11 hr), a HRT of >11 hr is recommended. In practice, the HRT in anaerobic vertical flow wetlands treating AMD with SMS and limestone ranges from 4 – 24 hr, with 16 hr being typical in successful systems (Dietz, 2006).

Finally, it is apparent that the substrate should be replaced or replenished as soon as effluent alkalinity reaches zero or effluent metal concentrations begin to approach
influent levels. If AMD is allowed to run through the substrate after this point, catastrophic release of metals from the treatment system will occur.

5.6. **Molecular Biology**

The results of DGGE from the extended batch test indicate that chitin helps increase the number and diversity of bacteria. This agrees well with the better sulfate reduction observed in substrates containing more chitin. However, the DNA from these gels needs to be sequenced in order to identify specific bacteria in the samples. Sequencing was unsuccessful because the excised DGGE bands contained multiple DNA sequences. Therefore, it may be possible to obtain sequence data by adjusting the techniques used. For example, DNA from DGGE bands may be cloned, and the subsequent colonies could be sequenced. Optimization of DGGE operating conditions such as denaturant and acrylamide concentrations may also help prevent the appearance of one band for multiple sequences. Finally, using PCR primers targeting specific groups of bacteria like SRB would reduce the total number of sequences in a DGGE gel lane, thereby reducing the likelihood of finding multiple sequences in a single band.

It is expected that DNA sequence analysis will show that chitin supports the growth of a greater number and diversity of fermentative bacteria and SRB. Analysis of DNA from column solids may provide additional insight into the microbial community in each substrate under continuous-flow conditions.
6. Conclusions, Engineering Significance, and Future Work

6.1. Conclusions

Based on the results of batch and continuous-flow experiments using chitin as an amendment to SMS for AMD treatment:

- Substrate treatment capacities (L AMD treated per kg substrate) are: 36.7 for a substrate containing 10% limestone and 90% SMS, 40.1 for a substrate containing 5% chitin and 95% SMS, 162 for a substrate containing 50% chitin and 50% SMS, and 428 for a 100% chitin substrate.
- Substrates containing more chitin are more effective for neutralizing acidity, removing metals, and reducing sulfate in AMD. However, a substrate containing only 5% chitin is not significantly more effective than SMS amended with limestone.
- While all of the substrates tested generated alkalinity and removed aluminum and zinc, the 50% and 100% chitin substrates removed iron and manganese most effectively, and sustained treatment for a longer period of time.
- Preliminary results from DGGE analysis suggest that substrates containing at least 15% chitin supported more diverse microbial communities, which could be a major advantage in passive treatment systems.

6.2. Engineering Significance

The results of these experiments provide guidance for how chitin and SMS substrate mixtures may be applied for treatment of acid mine drainage:

- The use of chitin for passive treatment of AMD is not cost-prohibitive.

Relative costs of different substrate mixtures (cost per 1000 L AMD treated) are: $1.39 for a substrate containing 10% limestone and 90% SMS, $2.95 for a substrate containing 5% chitin and 95% SMS, $4.25 for a substrate containing 50% chitin and 50% SMS, and $3.09 for a 100% chitin substrate. These cost differences are reasonable, and show that the treatment efficiency of chitin compensates for the orders-of-magnitude price gap between 1 lb of chitin ($0.60) vs. 1 lb of SMS ($0.025).
Substrates containing at least 50% chitin should be used because they provide the best balance between cost and treatment efficiency. Greater amounts of chitin provide superior removal of metals and sustain AMD treatment for a longer period of time. Although the substrate cost rises with the amount of chitin used, these costs may be mitigated because the greater efficiency of chitin allows for construction of smaller treatment systems than traditional substrates, and its longevity would contribute to lower maintenance costs.

HRT should be 16 hr or longer.
This HRT is consistent with the treatment times used in other passive treatment systems. A shorter HRT may lead to incomplete treatment of AMD or premature failure of the substrate.

Whether chitin should be applied as the sole substrate or mixed in a proportion of 50% or more by mass with SMS needs to be determined on a project-by-project basis. A detailed cost analysis would help identify what proportion of chitin in the range of 50%-100% is most cost-effective for a specific project. Substrate cost, treatment capacity, size of the treatment system, and the frequency of substrate replacement would all affect this decision.

The substrate should be replaced or replenished before metals are released.
The continuous-flow column experiment showed that metals retained in the treatment system can be released once the treatment capacity of the substrate is exceeded, resulting in high concentrations of metals in the effluent. Based on experimental evidence, manganese is the first metal to be released from the substrate, and release may occur even before effluent pH drops below acceptable levels. Manganese levels should be monitored closely after effluent alkalinity drops below about 300 mg/L as CaCO₃. If manganese levels are increasing, fresh substrate should be applied promptly to avoid release.
6.3. Future Work

Further research would be useful for application of chitin as a fractional amendment to SMS in AMD treatment applications:

- Chitin and SMS substrate mixtures should be tested in the field to determine whether treatment capacities determined in the laboratory are replicated under field conditions and at a larger scale.

- Chitin and SMS substrate mixtures should be tested with AMD of different compositions to determine the extent to which treatment capacity is affected by influent water quality.

- A detailed cost analysis of a vertical flow wetland system with different substrate mixtures should be conducted to compare both capital and operation and maintenance costs over the lifetime of the system (for example, using AMDTreat cost estimating software).

- More work should be done on sediment samples preserved from the batch and column tests to characterize the effect of substrate composition on microbial community development. Sequence data could identify substrate degraders and SRB to give a complete picture of microbial community composition. Since sequencing was unsuccessful due to the presence of multiple sequences in the excised DGGE bands, a different technique should be used to avoid a similar outcome. Possible techniques include cloning, optimization of DGGE operating conditions, and the use of primers that target specific groups of bacteria.
References


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fingerprinting." Journal of Microbiological Methods, 44(3), 253-262.
for use in permeable reactive walls for treatment of mine drainage." 
using permeable reactive barriers: Column experiments." Environmental Science
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Applied Microbiology, 84(2), 240-248.
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Appendix A. Calculations

A.1. Sulfate Calculation

Sulfate concentration is determined by the molecular weight ratio of sulfate to elemental sulfur (Equation A.1).

\[
1 \text{ mg/L of S} = \left( \frac{96.056}{32.06} \right) \text{ mg/L of SO}_4^{2-} = 2.996 \text{ mg/L of SO}_4^{2-} \quad \text{A.1}
\]

A.2. Conservative Tracer Test Calculations

The HRT, variance, and dispersion number were calculated from the tracer test data using Equations A.2-A.5 (Metcalf & Eddy 2003). The dispersion number is the ratio of mass transport due to dispersion and advection. The calculated HRT (\(\bar{t}\)) was used as the estimated retention time (\(\tau\)) to calculate the dispersion number. The HRT and measured flow rate were then used to calculate pore volume.

\[
\bar{t} = \frac{\sum t_i C_i \Delta t_i}{\sum C_i \Delta t_i} \quad \text{A.2}
\]

\[
\sigma^2 = \frac{\sum t_i^2 C_i \Delta t_i}{\sum C_i \Delta t_i} - \left(\bar{t}\right)^2 \quad \text{A.3}
\]

\[
\frac{\sigma^2}{\tau} = 2d + 8d^2 \quad \text{A.4}
\]

\[
\text{PV} = \bar{t} \cdot Q \quad \text{A.5}
\]

where:
\(\bar{t}\) = HRT
\(t_i\) = time at \(i^{th}\) measurement
\(C_i\) = tracer concentration at \(i^{th}\) measurement
\(\Delta t_i\) = time increment about \(C_i\)
\(\sigma^2\) = variance
\(\tau\) = estimated HRT
d = dispersion number
PV = column pore volume
Q = flow rate
Appendix B. PCR Optimization Test

B.1. Optimization Test Setup

Optimization of the PCR reaction mixture was required due to poor amplification of DNA extracted from preliminary batch test samples. The optimization test was performed on solids from a previous AMD batch test left over from another student. Four parameters were tested in the optimization test:

1. **Microcosm substrate.** Solids from microcosms that had been established under different substrate conditions were used to ensure that the selected method would work for all of the samples from these experiments.
   
   - Test conditions/notation:
     - S: Sediment-only (no substrate)
     - L: Limestone substrate
     - C: Chitin substrate

2. **DNA dilution.** Extracted DNA was used for PCR with and without dilution. Diluting DNA also dilutes PCR inhibitors, which can result in better amplification.
   
   - Test conditions/notation:
     - 1: No dilution
     - 5: 5X dilution
     - 10: 10X dilution
     - 50: 50X dilution

3. **Magnesium chloride concentration.** Magnesium chloride is a required catalyst for PCR. Inhibitory compounds in DNA samples may bind the magnesium chloride and hinder amplification. Thus, it is worthwhile to test higher concentrations when inhibition is suspected.
   
   - Test conditions/notation:
     - 2.0: 2.0 mM
     - 2.5: 2.5 mM
     - 3.0: 3.0 mM
     - 3.5: 3.5 mM
4. Absence/presence of bovine serum albumin (BSA). BSA is commonly used to enhance amplification in PCR.
   - Test conditions/notation:
     - (+): BSA used (final concentration 0.4 μg/μL)
     - (-): no BSA

B.2. Optimization Test Results

PCR products were viewed on agarose gels to determine the best reaction mixture. The best condition, based on the results of this test and recommendations from scientific literature and PCR kit manufacturers, was determined to be 10X dilution of DNA with BSA and 2.5 mM magnesium chloride.
Figure B-1. Results of the PCR optimization test. Clear, dark bands represent a successful amplification.
Appendix C. Complete Results of the Preliminary Batch Test: Initial and Final Water Quality
Table C-1. pH, alkalinity, acidity, ammonium, and DOC in the preliminary batch study. Values are for the raw AMD and samples after 4 weeks of treatment under various conditions.

<table>
<thead>
<tr>
<th>Substrate condition</th>
<th>pH</th>
<th>Alkalinity</th>
<th>Acidity</th>
<th>Ammonium</th>
<th>DOC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg</td>
<td>Std. Dev.</td>
<td>Avg. (mg/L as CaCO₃)</td>
<td>Std. Dev. (mg/L as CaCO₃)</td>
<td>Avg. (mg/L as CaCO₃)</td>
</tr>
<tr>
<td>Initial AMD</td>
<td>2.89</td>
<td>0.0</td>
<td>166.4</td>
<td>0.0</td>
<td>BDL</td>
</tr>
<tr>
<td>Sediment only (negative control)</td>
<td>3.10</td>
<td>0.01</td>
<td>0.0</td>
<td>0.0</td>
<td>156.4</td>
</tr>
<tr>
<td>Chitin only (uninoculated)</td>
<td>7.57</td>
<td>0.55</td>
<td>408.6</td>
<td>30.5</td>
<td>-304.6</td>
</tr>
<tr>
<td>SMS only (uninoculated)</td>
<td>6.46</td>
<td>0.07</td>
<td>61.6</td>
<td>10.5</td>
<td>16.9</td>
</tr>
<tr>
<td>Limestone only (uninoculated)</td>
<td>6.61</td>
<td>0.04</td>
<td>35.4</td>
<td>2.0</td>
<td>-7.7</td>
</tr>
<tr>
<td>100% SMS</td>
<td>5.88</td>
<td>0.49</td>
<td>17.3</td>
<td>14.0</td>
<td>126.9</td>
</tr>
<tr>
<td>95% SMS, 5% limestone</td>
<td>6.25</td>
<td>0.09</td>
<td>29.4</td>
<td>2.8</td>
<td>108.6</td>
</tr>
<tr>
<td>95% SMS, 5% chitin</td>
<td>6.50</td>
<td>0.01</td>
<td>57.2</td>
<td>0.3</td>
<td>63.0</td>
</tr>
<tr>
<td>75% SMS, 25% chitin</td>
<td>6.92</td>
<td>0.10</td>
<td>167.5</td>
<td>36.9</td>
<td>-141.4</td>
</tr>
<tr>
<td>50% SMS, 50% chitin</td>
<td>7.93</td>
<td>0.04</td>
<td>458.6</td>
<td>40.7</td>
<td>-434.9</td>
</tr>
<tr>
<td>100% chitin</td>
<td>8.10</td>
<td>0.03</td>
<td>718.4</td>
<td>12.7</td>
<td>-657.9</td>
</tr>
</tbody>
</table>
Table C-2. Anions measured by IC in the preliminary batch study. Values are for the raw AMD and samples after 4 weeks of treatment under various conditions.

<table>
<thead>
<tr>
<th>Substrate condition</th>
<th>Chloride</th>
<th>Nitrate</th>
<th>Phosphate</th>
<th>Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg. (mg/L)</td>
<td>Std. Dev. (mg/L)</td>
<td>Avg. (mg/L)</td>
<td>Std. Dev. (mg/L)</td>
</tr>
<tr>
<td>Initial AMD</td>
<td>1.78</td>
<td>2.49</td>
<td>45.74</td>
<td>543.20</td>
</tr>
<tr>
<td>Sediment only (negative control)</td>
<td>2.79</td>
<td>0.25</td>
<td>6.27</td>
<td>1.98</td>
</tr>
<tr>
<td>Chitin only (uninoculated)</td>
<td>136.57</td>
<td>58.89</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>SMS only (uninoculated)</td>
<td>19.05</td>
<td>4.38</td>
<td>0.35</td>
<td>0.01</td>
</tr>
<tr>
<td>Limestone only (uninoculated)</td>
<td>3.01</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>100% SMS</td>
<td>8.21</td>
<td>1.27</td>
<td>0.25</td>
<td>0.11</td>
</tr>
<tr>
<td>95% SMS, 5% limestone</td>
<td>8.84</td>
<td>0.11</td>
<td>0.23</td>
<td>0.03</td>
</tr>
<tr>
<td>95% SMS, 5% chitin</td>
<td>14.30</td>
<td>7.09</td>
<td>0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>75% SMS, 25% chitin</td>
<td>28.28</td>
<td>6.02</td>
<td>0.22</td>
<td>0.00</td>
</tr>
<tr>
<td>50% SMS, 50% chitin</td>
<td>79.78</td>
<td>20.33</td>
<td>0.27</td>
<td>0.11</td>
</tr>
<tr>
<td>100% chitin</td>
<td>99.63</td>
<td>27.88</td>
<td>0.08</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Table C-3. Results of elemental analysis by ICP-AES for the preliminary batch study. Values are for the raw AMD and samples after 4 weeks of treatment under various conditions.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial AMD</td>
<td>13.00</td>
<td>0.00</td>
<td>90.00</td>
<td>0.00</td>
<td>29.00</td>
<td>0.00</td>
<td>89.00</td>
<td>0.00</td>
<td>19.00</td>
<td>0.00</td>
<td>274.76</td>
<td>274.76</td>
<td>0.73</td>
<td>0.00</td>
</tr>
<tr>
<td>Sediment only (negative control)</td>
<td>12.00</td>
<td>0.00</td>
<td>77.50</td>
<td>4.95</td>
<td>13.50</td>
<td>0.71</td>
<td>80.00</td>
<td>1.41</td>
<td>17.00</td>
<td>0.00</td>
<td>236.26</td>
<td>239.84</td>
<td>0.67</td>
<td>0.01</td>
</tr>
<tr>
<td>Chitin only (uninoculated)</td>
<td>0.00</td>
<td>0.00</td>
<td>207.50</td>
<td>21.92</td>
<td>1.75</td>
<td>2.47</td>
<td>89.00</td>
<td>2.83</td>
<td>8.70</td>
<td>1.13</td>
<td>440.16</td>
<td>348.38</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>SMS only (uninoculated)</td>
<td>0.13</td>
<td>0.09</td>
<td>146.00</td>
<td>12.73</td>
<td>14.50</td>
<td>3.54</td>
<td>91.50</td>
<td>4.95</td>
<td>15.00</td>
<td>0.00</td>
<td>256.44</td>
<td>267.42</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Limestone only (uninoculated)</td>
<td>0.00</td>
<td>0.00</td>
<td>117.00</td>
<td>7.07</td>
<td>3.25</td>
<td>0.49</td>
<td>101.00</td>
<td>5.66</td>
<td>17.00</td>
<td>1.41</td>
<td>235.27</td>
<td>246.84</td>
<td>0.49</td>
<td>0.02</td>
</tr>
<tr>
<td>100% SMS</td>
<td>0.70</td>
<td>0.99</td>
<td>116.00</td>
<td>8.49</td>
<td>78.00</td>
<td>18.38</td>
<td>87.00</td>
<td>5.66</td>
<td>16.50</td>
<td>0.71</td>
<td>258.19</td>
<td>278.80</td>
<td>0.10</td>
<td>0.13</td>
</tr>
<tr>
<td>95% SMS, 5% limestone</td>
<td>0.00</td>
<td>0.00</td>
<td>120.50</td>
<td>10.61</td>
<td>75.50</td>
<td>0.71</td>
<td>91.00</td>
<td>5.66</td>
<td>16.50</td>
<td>0.71</td>
<td>268.68</td>
<td>278.93</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>95% SMS, 5% chitin</td>
<td>0.00</td>
<td>0.00</td>
<td>120.50</td>
<td>6.36</td>
<td>79.00</td>
<td>9.99</td>
<td>86.50</td>
<td>3.54</td>
<td>15.50</td>
<td>0.71</td>
<td>264.32</td>
<td>271.81</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>75% SMS, 25% chitin</td>
<td>0.00</td>
<td>0.00</td>
<td>136.00</td>
<td>11.31</td>
<td>44.50</td>
<td>24.75</td>
<td>87.00</td>
<td>4.24</td>
<td>13.50</td>
<td>0.71</td>
<td>213.05</td>
<td>234.02</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>50% SMS, 50% chitin</td>
<td>0.00</td>
<td>0.00</td>
<td>178.50</td>
<td>9.19</td>
<td>0.23</td>
<td>0.23</td>
<td>87.30</td>
<td>4.95</td>
<td>9.13</td>
<td>0.35</td>
<td>163.80</td>
<td>166.24</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>100% chitin</td>
<td>0.00</td>
<td>0.00</td>
<td>205.50</td>
<td>9.19</td>
<td>0.00</td>
<td>0.00</td>
<td>87.30</td>
<td>3.54</td>
<td>4.65</td>
<td>0.21</td>
<td>721.04</td>
<td>519.07</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Table C-4. VFAs in the preliminary batch study. Values are for the raw AMD and samples after 4 weeks of treatment under various conditions.

<table>
<thead>
<tr>
<th>Substrate condition</th>
<th>Formate</th>
<th></th>
<th>Acetate</th>
<th></th>
<th>Propionate</th>
<th></th>
<th>Isobutyrate</th>
<th></th>
<th>Butyrate</th>
<th></th>
<th>Isovalerate</th>
<th></th>
<th>Valerate</th>
<th></th>
<th>Iso-caproate</th>
<th></th>
<th>Enanthate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial AMD</td>
<td>0.00</td>
<td>--</td>
<td>0.00</td>
<td>--</td>
<td>0.00</td>
<td>--</td>
<td>0.02</td>
<td>--</td>
<td>0.00</td>
<td>--</td>
<td>0.00</td>
<td>--</td>
<td>0.00</td>
<td>--</td>
<td>0.00</td>
<td>--</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Sediment only (negative control)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitin only (uninoculated)</td>
<td>0.01</td>
<td>0.01</td>
<td>2.47</td>
<td>1.29</td>
<td>0.36</td>
<td>0.14</td>
<td>0.11</td>
<td>0.04</td>
<td>0.62</td>
<td>0.01</td>
<td>0.15</td>
<td>0.13</td>
<td>2.83</td>
<td>3.11</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMS only (uninoculated)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
<td>0.01</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limestone only (uninoculated)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% SMS</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% SMS, 5% limestone</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% SMS, 5% chitin</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75% SMS, 25% chitin</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% SMS, 50% chitin</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.04</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% chitin</td>
<td>0.28</td>
<td>0.39</td>
<td>0.10</td>
<td>0.14</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.13</td>
<td>0.19</td>
<td>0.06</td>
<td>0.03</td>
<td>0.09</td>
<td>0.08</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix D. Results of the Extended Batch Study: Complete Initial and Final Water Quality
Table D-1. pH, alkalinity, acidity, ammonium, and DOC in the extended batch study. Values are for the raw AMD and final time point after 9 weeks of treatment with various substrates.

<table>
<thead>
<tr>
<th>Substrate condition</th>
<th>pH</th>
<th>Alkalinity</th>
<th>Acidity</th>
<th>Ammonium</th>
<th>DOC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/L as CaCO$_3$)</td>
<td>(mg/L as CaCO$_3$)</td>
<td>(mg/L as CaCO$_3$)</td>
<td>(mg/L NH$_4^+$)</td>
<td>(mg/L C)</td>
</tr>
<tr>
<td>Initial AMD</td>
<td>3.13</td>
<td>---</td>
<td>0.0</td>
<td>---</td>
<td>94.0</td>
</tr>
<tr>
<td>Control (no substrate)</td>
<td>3.33</td>
<td>0.04</td>
<td>0.0</td>
<td>0.0</td>
<td>107.3</td>
</tr>
<tr>
<td>5% chitin, 95% SMS</td>
<td>6.52</td>
<td>0.03</td>
<td>55.2</td>
<td>9.9</td>
<td>-16.0</td>
</tr>
<tr>
<td>15% chitin, 85% SMS</td>
<td>6.71</td>
<td>0.07</td>
<td>104.6</td>
<td>22.6</td>
<td>-66.9</td>
</tr>
<tr>
<td>30% chitin, 75% SMS</td>
<td>7.01</td>
<td>0.01</td>
<td>195.1</td>
<td>15.1</td>
<td>-163.4</td>
</tr>
<tr>
<td>50% chitin, 50% SMS</td>
<td>7.50</td>
<td>0.54</td>
<td>343.1</td>
<td>123.7</td>
<td>-319.5</td>
</tr>
</tbody>
</table>

Table D-2. Anions measured by IC in the extended batch study. Values are for the raw AMD and final time point after 9 weeks of treatment with various substrates.

<table>
<thead>
<tr>
<th>Substrate condition</th>
<th>Chloride</th>
<th>Nitrate</th>
<th>Phosphate</th>
<th>Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/L)</td>
<td>(mg/L)</td>
<td>(mg/L)</td>
<td>(mg/L)</td>
</tr>
<tr>
<td>Initial AMD</td>
<td>15.03</td>
<td>---</td>
<td>11.14</td>
<td>---</td>
</tr>
<tr>
<td>Control (no substrate)</td>
<td>27.15</td>
<td>18.39</td>
<td>24.99</td>
<td>19.94</td>
</tr>
<tr>
<td>5% chitin, 95% SMS</td>
<td>21.19</td>
<td>3.14</td>
<td>11.76</td>
<td>3.24</td>
</tr>
<tr>
<td>15% chitin, 85% SMS</td>
<td>26.11</td>
<td>2.22</td>
<td>9.59</td>
<td>0.17</td>
</tr>
<tr>
<td>30% chitin, 75% SMS</td>
<td>35.87</td>
<td>1.33</td>
<td>9.67</td>
<td>0.28</td>
</tr>
<tr>
<td>50% chitin, 50% SMS</td>
<td>48.29</td>
<td>5.06</td>
<td>9.62</td>
<td>0.21</td>
</tr>
</tbody>
</table>
Table D-3. Results of elemental analysis by ICP for the extended batch study. Values are for the raw AMD and final time point after 9 weeks of treatment with various substrates.

<table>
<thead>
<tr>
<th>Substrate condition</th>
<th>AI</th>
<th>Ca</th>
<th>Fe</th>
<th>Mg</th>
<th>Mn</th>
<th>S</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial AMD</td>
<td>8.09</td>
<td>---</td>
<td>47.82</td>
<td>---</td>
<td>15.03</td>
<td>---</td>
<td>46.68</td>
</tr>
<tr>
<td>Control (no substrate)</td>
<td>9.48</td>
<td>0.07</td>
<td>48.30</td>
<td>0.38</td>
<td>0.50</td>
<td>0.11</td>
<td>45.96</td>
</tr>
<tr>
<td>5% chitin, 95% SMS</td>
<td>0.02</td>
<td>0.02</td>
<td>123.73</td>
<td>4.87</td>
<td>36.99</td>
<td>---</td>
<td>46.88</td>
</tr>
<tr>
<td>15% chitin, 85% SMS</td>
<td>---</td>
<td>---</td>
<td>130.95</td>
<td>9.33</td>
<td>29.67</td>
<td>5.64</td>
<td>47.32</td>
</tr>
<tr>
<td>30% chitin, 75% SMS</td>
<td>---</td>
<td>---</td>
<td>142.40</td>
<td>4.38</td>
<td>19.31</td>
<td>0.10</td>
<td>48.37</td>
</tr>
<tr>
<td>50% chitin, 30% SMS</td>
<td>---</td>
<td>---</td>
<td>161.81</td>
<td>4.07</td>
<td>13.09</td>
<td>17.60</td>
<td>49.84</td>
</tr>
</tbody>
</table>

Table D-4. VFAs in the extended batch study. Values are for the raw AMD and final time point after 9 weeks of treatment with various substrates.

<table>
<thead>
<tr>
<th>Substrate condition</th>
<th>Formate</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Isobutyrate</th>
<th>Butyrate</th>
<th>Isovalerate</th>
<th>Valerate</th>
<th>Isocaproate</th>
<th>Enanthate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial AMD</td>
<td>0.00</td>
<td>---</td>
<td>0.00</td>
<td>---</td>
<td>0.00</td>
<td>---</td>
<td>0.00</td>
<td>---</td>
<td>0.00</td>
</tr>
<tr>
<td>Control (no substrate)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>5% chitin, 95% SMS</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>15% chitin, 85% SMS</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.05</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>30% chitin, 75% SMS</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>50% chitin, 30% SMS</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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Appendix E. Column Water Quality Before and After Incubation

Table E-1. Analysis of AMD before and after 8-day incubation with substrates in columns.

<table>
<thead>
<tr>
<th>General water quality parameters</th>
<th>Initial AMD</th>
<th>After 8-day incubation in packed columns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (no substrate)</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>2.80</td>
<td>3.33</td>
</tr>
<tr>
<td><strong>Alkalinity (mg/L as CaCO₃)</strong></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Acidity (mg/L as CaCO₃)</strong></td>
<td>176.8</td>
<td>139.6</td>
</tr>
<tr>
<td><strong>Ammonium (mg/L as NH₄⁻N)</strong></td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>DOC</strong></td>
<td>1.50</td>
<td>414.87</td>
</tr>
<tr>
<td><strong>Sulfate (mg/L)</strong></td>
<td>645.78</td>
<td>650.54</td>
</tr>
</tbody>
</table>

| Metals and other elements        |             |                           |               |           |           |             |
| **Al (ppm)**                     | 10.28       | 6.34                      | 0.64          | 0.30      | 0.18       | 0.07        |
| **Ca (ppm)**                     | 77.00       | 93.54                     | 379.91        | 560.19    | 1,514.27   | 1,216.99    |
| **Fe (ppm)**                     | 16.35       | 12.11                     | 64.12         | 7.88      | 1.56       | 0.79        |
| **Mg (ppm)**                     | 77.31       | 73.62                     | 157.30        | 183.28    | 302.14     | 231.28      |
| **Mn (ppm)**                     | 16.41       | 26.76                     | 17.58         | 13.09     | 7.85       | 5.60        |
| **Zn (ppm)**                     | 0.56        | 0.77                      | 0.74          | <0.2      | <0.2       | <0.2        |

| Volatile fatty acids              |             |                           |               |           |           |             |
| **Formate (mM)**                  | 0.00        | 0.00                      | 0.07          | 0.08      | 1.51       | 3.18        |
| **Acetate (mM)**                  | 0.00        | 0.00                      | 20.28         | 30.58     | 76.08      | 87.80       |
| **Propionate (mM)**               | 0.00        | 0.00                      | 4.34          | 9.68      | 9.62       | 8.92        |
| **Isobutyrate (mM)**              | 0.00        | 0.00                      | 0.00          | 1.66      | 3.43       | 4.25        |
| **Butyrate (mM)**                 | 0.00        | 0.00                      | 0.11          | 1.11      | 13.05      | 19.64       |
| **Isovalerate (mM)**              | 0.00        | 0.00                      | 0.00          | 0.14      | 4.26       | 5.37        |
| **Valerate (mM)**                 | 0.00        | 0.00                      | 2.17          | 1.21      | 2.72       | 1.29        |
| **Isocaproate (mM)**              | 0.00        | 0.00                      | 0.61          | 0.42      | 0.13       | 0.49        |
| **Ethanate (mM)**                 | 0.00        | 0.00                      | 1.06          | 3.90      | 62.31      | 64.89       |