EVALUATION OF CHITINOUS MATERIALS AS A MULTIFUNCTIONAL SUBSTRATE FOR THE REMEDIATION OF MINE IMPACTED WATER

A Dissertation in
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by
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ABSTRACT

The generation of mine impacted waters is one of the most serious environmental problems originated by the mining industry. For the remediation of these metal-laden and often acidic streams, several passive treatment technologies have been applied. The success of these technologies greatly depends on the selection of the substrate. This study evaluates the ability of crab-shell chitin (SC-20) as a multifunctional substrate for MIW treatment. Both biologically active and abiotic tests were conducted to 1) assess the contributions of each of the components of SC-20 (chitin, proteins, and minerals) to the observed changes, and 2) compare the performance of crab-shell chitin to other commonly used materials.

In biologically active microcosm tests, SC-20 demonstrated a superior performance in comparison to spent mushroom compost and sodium lactate. The addition of SC-20 increased the pH from 3.0 – 3.5 to near neutral in 2 – 3 days, steadily generated alkalinity at a rate of 26.5 – 40.3 mg CaCO$_3$ /L-d, and supported the activity of sulfate reducing bacteria (SRB) with a lag period of 7 – 9 d and sulfate reduction rates of 11.9 – 17.8 mg SO$_4^{2-}$/L-d. While no major changes were observed using spent mushroom compost as a sole substrate, alkalinity generation and sulfate reduction rates promoted by sodium lactate (30.3 mg CaCO$_3$ /L-d and 24.8 mg SO$_4^{2-}$/L-d) were comparable to those obtained with SC-20, but such changes only occurred after 27 days of incubation. Al and Fe removal was observed with all three materials, but it was much faster with SC-20. The latter was the only substrate able to partially remove manganese (>73%).

In biologically active columns using SC-20, a hydraulic retention time of 11.2 h was enough to raise the pH from 3.5 to ~7.5. Alkalinity increased at a rate of 50 ± 20 mg CaCO$_3$/day, and lasted throughout the duration of the test (125 days or 268 pore volumes (PV)) without showing signs of exhaustion. Metals (Al, Fe, and Mn) were completely removed for 171 PV. Manganese and iron breakthroughs occurred after 174 and 234 PV, respectively, whereas aluminum breakthrough was never observed. The steady generation of alkalinity in SC-20-treated systems was attributed to the dissolution of chitin-associated carbonates (mainly calcite), while the prompt onset of the SRB activity
was supported by the fermentation of chitin and its associated proteins. Results from thermodynamic geochemical modeling using PHREEQC indicate that Al removal was likely due to the precipitation of hydroxides and/or alunite (\( \text{KAl}_3(\text{SO}_4)_2(\text{OH})_6 \)). Iron removal appeared to be driven by precipitation of ferric oxides at the beginning of the test, and by iron sulfide precipitation once the SRB became active. The partial removal of manganese could be explained by the formation of rhodochrosite (\( \text{MnCO}_3 \)), although other mechanisms like sorption or precipitation of other minerals cannot be discarded.

Abiotic and anoxic tests were conducted to isolate the chemical and physical treatment mechanisms from those driven by biological activity, using SC-20 with different grades of purity. In particular, the generation of alkalinity and the removal of manganese due to mineral dissolution and precipitation were evaluated and compared to those obtained using limestone in closed-system and kinetic tests. In closed systems with a contact time of 72 h, manganese removal \( \geq 95\% \) (initial concentration = 10 mg/L) was obtained using only 5 g/L of SC-20 (raw or deproteinized); the pH was increased from 3 to 9.2-10.2; and 83-187 mg \( \text{CaCO}_3/\text{L} \) of alkalinity was generated. In contrast, 5-125 g-limestone/L only raised the pH to 7.8-8.3, leading to lower alkalinity levels (56-63 mg \( \text{CaCO}_3/\text{L} \)) and poor metal removal efficiencies (\( \leq 85\% \)). Results from kinetic tests indicated that removal of \( \geq 95\% \) of the initial Mn load by SC-20 was achieved after 48 h. Geochemical calculations (PHREEQC) indicate that limestone-treated systems were close to equilibrium with calcite, while octacalcium phosphate (\( \text{Ca}_4\text{H(PO}_4\text{)}_3 \)) appears to be the controlling phase in systems treated with SC-20. The removal of manganese could be attributed to the precipitation of rhodochrosite (\( \text{MnCO}_3 \)) and/or MnHPO\(_4\). The faster changes observed with the two grades of SC-20 compared to limestone could be attributed to their larger surface area and their distinct composition, including phosphates and soluble organic compounds.

The sorption of manganese onto the organic components of SC-20 (chitin and proteins) was evaluated using two purities of SC-20 (demineralized (chitin + proteins) and demineralized/deproteinized (“pure” chitin)) under different pH conditions by means of kinetic tests and sorption isotherms. The kinetics of manganese adsorption onto both types of solids was well described by the pseudo-second order model, with faster changes occurring under alkaline conditions and with “pure” chitin. The equilibrium of
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To Cecilia and Benoît,
my love and my strength.
CHAPTER 1
INTRODUCTION

1.1. Introduction

Mine impacted waters (MIW) or acid mine drainage (AMD) are the result of the exposure of sulfide minerals to oxygen and water. These metal-laden and often acidic waters constitute a threat to the biodiversity and health of the ecosystems of streams and rivers. Therefore, the generation of MIW is considered to be the most serious environmental effect of the mining activity and it is a problem that affects many countries around the world. It is estimated that in the eastern United States only about 20,000 km of streams and rivers can be classified as MIW (Ziemkiewicz et al., 2003).

In an effort to control, minimize, and mitigate the deleterious effects of MIW, many treatment technologies have been developed over the past three decades. Treatment approaches have been often classified as “active” and “passive” (Johnson and Hallberg, 2005). Active treatment technologies require the continuous addition of an alkaline agent and/or sulfide to promote the precipitation of the metals as hydroxides, carbonates, or sulfides. In contrast, by taking advantage of naturally occurring chemical and/or biological processes, passive treatments offer an alternative, low cost, and demonstrated effective solution (Johnson and Hallberg, 2005; Gibert et al., 2002; Ziemkiewicz et al., 2003). Another advantage of these passive technologies is the lower demand for monitoring and maintenance, allowing them to be implemented in remote sites. The success of these technologies, however, depends greatly on the selection of the substrate. Extensive research has been carried out to assess the suitability of many waste materials and it has been reported that a substrate consisting of a mixture of various materials leads to better results (Neculita et al., 2007; Gibert et al., 2004). The resulting mixed substrate often consists of a variety of waste materials such as animal manure, wood chips, and different types of compost, amended with limestone or calcite as an additional alkalinity source (Batty and Younger, 2004; Chang et al., 2000; Cocos et al., 2002; Gibert et al., 2004; Neculita et al., 2007; Waybrant et al., 1998; Zagury et al., 2006).
In a preliminary study, Daubert and Brennan (2007) reported promising results for the treatment of MIW using raw crab-shell chitin as a sole substrate. After nine days of incubation, pH increased from 3.2 to 6.8 and 235 mg/L of alkalinity were generated. This substrate also sustained the activity of sulfate reducing bacteria (SRB), leading to a sulfate removal of 37%. Significant removal of metals was also observed: >99% for iron and aluminum, and 81% for manganese. In particular, the high removal of manganese is of great significance, since previous studies have shown poor removal of this metal under reducing conditions.

The aim of this study is to further evaluate the ability of a multifunctional substrate, raw-crab-shell chitin, to support MIW remediation and refine the understanding of the mechanisms involved in this process. This material offers a unique opportunity to study separately the effect of each of its components (chitin, proteins, and minerals) on the overall removal of metals and acidity. Microcosms and column tests were conducted to assess the ability of crab-shell chitin to support the activity of SRB and promote the removal of acidity and metals. Its performance was also compared to other commonly used substrates under biologically active (sodium acetate and spent mushroom compost) and abiotic conditions (limestone). Three additional grades of purity, obtained by acid and/or alkaline treatment of the raw crab shells, were tested under abiotic conditions to evaluate the contribution of each of the previously mentioned components to mineral precipitation and manganese sorption.

1.2. Hypothesis and Objectives

It was hypothesized that the use of a complex material such as raw-crab-shell chitin for the remediation MIW will result not only in the enhancement of SRB activity, but also in important changes of the water chemistry due to the rapid dissolution its associated minerals and the sorption capacity of its organic components.

The following are the objectives that guided this study.

- Quantify the remediation rates (i.e. the removal of metals, sulfate, and acidity) using crab-shell chitin;
• Compare remediation rates promoted by crab-shell chitin as a sole substrate in biologically active systems to those obtained with other commonly used substrates (i.e. spent mushroom compost and sodium lactate);

• Compare crab-shell chitin and limestone as buffering agents for the removal of acidity and metals (Al, Fe, and Mn) under abiotic conditions;

• Determine the individual contribution of chitin-associated proteins and minerals on the removal of metals and the generation of alkalinity;

• Quantify the sorption capacity of chitin and its associated proteins for the removal of Mn.

1.3. Dissertation layout

This dissertation is composed by four manuscripts that discuss the performance of crab-shell chitin as a substrate for the remediation of MIW and the role that each of its components play in the observed removal of contaminants. The dissertation is divided into the following chapters:

• CHAPTER 2: “Efficient metal removal and neutralization of acid mine drainage by crab-shell chitin under batch and continuous-flow conditions”


• CHAPTER 3: “Chitin Complex for the Remediation of Mine Impacted Water: Geochemistry of Metal Removal and Comparison with other Common Substrates”

  Material presented in this chapter in under revision for publication in *Applied Geochemistry*.

• CHAPTER 4: “Manganese Removal from Mine Impacted Water Using Crab-Shell Chitin: I. Role of Associated Minerals in Precipitation”

• CHAPTER 5: “Manganese Removal from Mine Impacted Water Using Crab-Shell Chitin: II. Role of Chitin and Associated Proteins in Biosorption”

  Material presented in chapters 4 and 5 will be submitted for publication as companion articles in *Water Research*. 
References


CHAPTER 2
Efficient metal removal and neutralization of acid mine drainage by crab-shell chitin under batch and continuous-flow conditions

2.1. Abstract

Crab-shell chitin was evaluated as a multifunctional substrate for treating acid mine drainage (AMD) in both batch-microcosms and continuous-flow column tests. In microcosms, crab-shell chitin was able to treat AMD from three different sites with similar results: pH increased from 3.5 to ~7.5 within 2 days; alkalinity increased at a rate of 37.9 ± 2.2 mg CaCO$_3$/L day; and sulfate was reduced at a rate of -13.6 ± 2.6 mg SO$_4^{2-}$/L day. In columns, a hydraulic retention time of 11.2 h was enough to raise the pH from 3.5 to ~7.5. Alkalinity increased at a rate of 50 ± 20 mg CaCO$_3$/day, and lasted throughout the duration of the test (125 days, 268 pore volumes (PV)) without showing signs of exhaustion. Metals (Al, Fe, and Mn) were completely removed for 171 PV, and geochemical modeling indicates that they likely precipitated as insoluble hydr(oxides), sulfides, and carbonates. Manganese and iron breakthroughs occurred after 174 and 234 PV, respectively, whereas aluminum breakthrough was never observed. These results demonstrate for the first time that crab-shell chitin can completely remove metals and neutralize the pH of AMD under continuous-flow conditions.

2.2. Introduction

One of the most serious environmental concerns of mining activity is the production of mine impacted water (also known as acid mine drainage (AMD)). This is a recurring problem around the world, especially in some specific areas of United States, including Pennsylvania (PADEP, 2000). AMD occurs when pyrite and other sulfide minerals are exposed to air and water. Atmospheric oxygen rapidly oxidizes pyrite releasing large amounts of sulfuric acid and ferric iron, which precipitates as ferric hydroxide or “yellow boy” (Eq. 2.1).

$$4\text{FeS}_2 (s) + 15\text{O}_2 (aq) + 14\text{H}_2\text{O (l)} \rightarrow 4\text{Fe(OH)}_3 (s)↓ + 8\text{SO}_4^{2-} (aq) + 16\text{H}^{+}(aq)$$ (2.1)
The typical high acidity of AMD can also promote the dissolution of metals such as Zn, Cu, Cd, As, Ni, and Pb, from other minerals (Cocos et al., 2002; Gibert et al., 2003). The resulting acidic and metal contaminated streams can adversely impact humans and wildlife.

Remediation of AMD requires three different processes: the addition of a neutralizing agent, the reduction of sulfate concentrations, and the removal of dissolved metals. Traditional treatment of AMD involves ex-situ chemical processes by the addition of an alkaline agent and/or sulfide to promote the precipitation of the metals as hydroxides, carbonates, or sulfides. Due to the very high cost of this type of treatment, alternative, in situ and low-tech treatments have been investigated. They include the use of limestone channels and drains, aerobic wetlands, vertical flow wetlands, and permeable reactive barriers (Johnson and Hallberg, 2005; Gibert et al., 2002). Anaerobic processes offer the advantage of allowing the three necessary processes for AMD remediation to occur almost simultaneously. They rely on the activity of sulfate reducing bacteria (SRB, Eq. 2.2 and 2.3):

\[
\begin{align*}
\text{CH}_2\text{O}(s) + \text{SO}_4^{2-}(aq) + 6\text{H}^+(aq) & \rightarrow \text{H}_2\text{S}(aq) + \text{CO}_2(aq) + 3\text{H}_2\text{O} \\
\text{Me}^{2+}(aq) + \text{H}_2\text{S}(aq) & \rightarrow \text{MeS}(s)\downarrow + 2\text{H}^+(aq)
\end{align*}
\]

(2.2) (2.3)

where CH$_2$O represents an organic substrate, and Me$^{2+}$ represents a divalent metal. The selection of an appropriate organic substrate to support SRB has important impacts on both operational costs and the overall biological performance (Gibert et al., 2002). Numerous fermentable waste materials have been used as substrates with good results. Researchers have noticed that a mixture of a variety of materials leads to better performance (Neculita et al.; 2007, Gibert et al., 2002). It has been also reported that low nitrogen availability can limit the growth of SRB and thereby decrease sulfate reduction rates (Waybrant et al., 1998; El Bayoumy et al. 1999). A C:N ratio between 10 and 16 has been proposed as optimum for SRB activity (Neculita et al., 2007; Cocos et al., 2002), with a maximum of 45:1 – 120:1 recommended to prevent a significant decrease in SRB activity due to growth limitations in a nitrogen-starved environment (Gibert et al., 2004). Excessively high nitrogen concentrations (>600 mg/L as N) are to be avoided, however, as they can have a toxic effect on the SRB community (El Bayoumy et al., 1999). In addition, SRB require an environmental pH between 5 and 8 for efficient
metabolism (Willow and Cohen, 2003). Although the sulfate reduction reaction consumes some acidity, limestone chips are traditionally added in both laboratory and field studies to fully satisfy this pH requirement (Neculita et al., 2007; Gibert et al., 2002). Other practical considerations that need to be addressed in the selection of the substrate include the use of a bulky material with stable porosity to prevent major head losses and/or clogging, and slow degradability to ensure acceptable longevity (Johnson and Hallberg, 2005). In summary, an ideal substrate for passive AMD bioremediation will need to possess several characteristics to support SRB activity: provision of required nutrients, buffering capacity, and long-lasting supply of electron donor.

Chitin (a linear polysaccharide of N-acetylglucosamine) is, after cellulose, the second most abundant biopolymer in nature, with an estimated yearly production of several gigatons (Beaney et al., 2005; Howard et al. 2003; Percot et al., 2003). It is naturally produced by arthropods (insects and crustaceans), mollusks, and fungi. For commercial use, chitin is mainly extracted from crustacean shells, especially crab and shrimp, which are waste products of the seafood industry. Crab-shell chitin can be purchased for $0.20/lb (dewatered) – $0.60/lb (dried) (JRW Bioremediation). In crustaceans, chitin is associated with high levels of calcium carbonate (CaCO$_3$, the same mineral found in limestone), which enhances the strength of their shells (Percot et al., 2003). The nitrogen content of chitin is also quite high (C$_8$H$_{13}$NO$_5$, indicating a C:N ratio of 6.9 on mass basis). Recent studies have shown that the degradation of chitin creates reducing conditions that can promote and sustain anaerobic, reductive processes (Vera et al., 2001; Brennan et al., 2006; Robinson-Lora and Brennan, 2009). The fermentation of crab-shell chitin has been shown to produce volatile fatty acids (VFAs: predominantly acetate, with lesser amounts of butyrate, propionate, and formate), some alcohols, and ammonium, resulting in an overall bioavailable C:N of 5 – 20. The short chain organic compounds can be easily used by SRB as electron donors, while the nitrogen supply falls within the optimum range for SRB activity. This makes chitin a suitable organic substrate for the growth of SRB, and suggests that it could be used as a fractional amendment to other, nitrogen-poor substrates to enhance their effectiveness. Additionally, the presence of CaCO$_3$ in the crab shell can provide the required buffering capacity for the rapid recovery of alkalinity in acidic waters, without the traditional addition of limestone. The solid
nature of crab-shell chitin makes it easy to be delivered in situ, while its particle size and non-swelling nature help to maintain porosity and prevent clogging in continuous-flow systems (Brennan, 2003).

It has been shown that chitinous materials can be effectively used for AMD remediation (Daubert and Brennan, 2007), and this is likely due to its ability to simultaneously serve as an electron donor source (VFAs), nitrogen source (ammonium), and neutralizing agent (CaCO$_3$) to sustain SRB activity. The aim of this study is to further evaluate the characteristics and performance of raw crab-shell chitin for the remediation of AMD. Specifically, remediation rates (i.e. the removal of metals, sulfate, and acidity, and the generation of alkalinity) using crab-shell chitin were quantified under different raw water characteristics from three AMD sites (microcosm tests) and under continuous-flow conditions (column test).

2.3.Methods

2.3.1. Chemicals

All chemicals used in this study were reagent grade or better. Ultra High Purity nitrogen gas (UHPNG) and Argon gas were provided by MG Industries (Malvern, PA). ChitoRem$^\text{®}$ SC-20 (minimally processed crab shell), derived from Dungeness crab (JRW Bioremediation, LLC, Lenexa, KS), was used as the chitin source. To evaluate its composition, demineralization and deproteinization of SC-20 were conducted based on protocols described in previous studies (Beaney et al., 2005; Cira et al., 2002; Percot et al., 2003). Results indicate that SC-20 contains ~10% chitin (C$_8$H$_{13}$NO$_5$), ~12% protein (C$_{16}$H$_{24}$O$_5$N$_4$), and ~78% mineral matter (35% as CaO). Before experiments, the SC-20 chitin was dry- and then wet-sieved (with deionized water) using a 40 mesh sieve to remove fine particles. The remaining chitin was dried overnight at 50 °C. The particle size of the resulting material ranged between 5 and 0.425 mm. Silica sand (16 – 20 mesh, Badger Mining Corp., Berlin, WI) was used as supplementary packing material in the column test. Extractable levels of aluminum, iron, and manganese from the packing sand corresponded to 16.2, 12.0, and 1.6 mg/kg, respectively (Mehlich 3 extraction, Agricultural Analytical Services Laboratory at The Pennsylvania State University).
2.3.2. Water and sediment sources

The AMD samples and associated benthic sediments (microbial source) that were used in the microcosm test were obtained from three different streams within the Snow Shoe area (Centre County, PA): Beech Creek (BC), North Fork (NF), and Cherry Run (CR) (Table 2.1). Sediments were collected in sterile centrifuge tubes, transported at 4 °C, and used the same day of sampling. Influent water for the column test was obtained from Cherry Run and from Kittanning Run in Altoona, PA. The source of column influent water was changed from Cherry Run to Kittanning Run at t = 102 days due to inclement weather. Before use, the water was purged with UHPNG for 2 h to ensure a final DO \( \leq 0.5 \) mg/L. Anoxic water was sampled and analyzed for redox potential (ORP), pH, electrical conductivity (EC), chloride, sulfate, ammonium, dissolved metals, and volatile fatty acids (VFAs). Sediments were drained to remove excess water and obtain a more solid material, and then measured for water content (Table 2.1).

2.3.3. Microcosm test setup

For each AMD site, a set of 40 serum bottles (160 mL capacity, non-sterile) was prepared. All bottles were supplied with 1.0 g sediment (wet weight). To half of the bottles, 0.5 g pre-washed SC-20 chitin was added. The bottles with SC-20 chitin were labeled as “actives”, and those without chitin were labeled as “controls”. The bottles were purged for 10 min with UHPNG before and after adding 120 ml anoxic AMD. The bottles were sealed with Teflon stoppers and aluminum crimp tops, manually shaken to ensure homogeneity, and incubated in dark at room temperature (20 ± 1 °C) for 20 days. During the incubation period, all microcosms were shaken by hand about once daily and duplicate bottles of “controls” and “actives” were sacrificed every day during the first 5 days and then every 2 – 5 days depending on the observed rate of treatment. After each bottle was opened, samples were promptly tested for ORP, pH, electrical conductivity (<0.5 h), alkalinity, acidity, and sulfide (<4 h). Another portion of the sample was filtered (0.20 µm) before being stored for future analyses (4 °C for less than a week for anions, and ammonium, 4 °C with 0.2ml/L conc. HNO\(_3\) for dissolved metals, and -10 °C for VFAs).
2.3.4. Column test setup

Three identical columns were constructed using 3.81 cm diameter by 121.9 cm long clear PVC tubes with PVC caps affixed to both ends. Each column was equipped with three lateral sampling ports spaced evenly along its length (every 30.48 cm), as well as an effluent port, to enable the discrete measurement of chemical degradation profiles. All parts were washed with liquid detergent (Liqui-Nox®) and tap water, and then rinsed with milli-Q water and air dried before assembling. Influent AMD was stored in a 50-L plastic carboy (Nalgene) and continuously bubbled with Argon gas to keep it under anoxic conditions. Before packing, the columns were also flushed with Argon gas. Using a wet packing procedure with anoxic AMD, the first quarter of the columns (30.48 cm) was packed as follows. About 30 g sand were placed in the bottom of each column to serve as a support for the packing material. For the two replicate active columns, the packing material consisted of a mixture of 25 g of pre-washed SC-20, 50 g of sediment, and approximately 500 g of sand. For the control column, the packing material consisted of a mixture of 50 g sediment and approximately 565 g of sand. After the packing procedure, the columns were left to incubate stagnant for 8 days. During this incubation period, samples (~25 ml) were taken from the first lateral port and tested for sulfate to monitor the SRB activity. Following the incubation period, the empty portion of the columns was packed with sand only, by means of wet packing procedure with anoxic AMD. Influent water was then continuously pumped through the columns at a flow rate of 0.25 ml/min, equivalent to a Darcy velocity of 0.32 m/day, by means of a peristaltic pump consisting of a digital drive and a 4-roller cartridge pump head (Masterflex L/S, Cole Parmer). The approximate pore volume in each column was 540 ml. Sodium chloride tracer tests were conducted to determine the hydraulic characteristics in all columns. Results indicate that the three columns had very similar characteristics, behaving as plug-flow reactors with low dispersion (d < 0.015) and an average retention time of 44.8 ± 1.6 h. The columns were operated for 125 days. Samples (~20 ml) were taken periodically from the influent reservoir, the lateral ports (at 30.48 and 60.96 cm from the influent), and the effluent port using a 20-ml plastic syringe. After the pH was measured, a portion of the sample (7 ml for the active columns and 10 ml for the control column) was reserved for alkalinity and acidity triturations and the remaining was filtered
(0.20 µm) before being stored for future analyses (4 °C for less than a week for anions and ammonium, 4 °C with 0.2ml/L conc. HNO₃ for dissolved metals, and -10° C for VFAs). Periodically, independent samples for sulfide were taken from the effluent port and immediately analyzed.

2.3.5. Analytical methods

Electrodes were used to measure ORP (platinum electrode, ORION 9778 BN), pH (Accumet® BASIC, AB15 connected to a Thermo-ORION pH probe), EC (Thermo-ORION 105A+), and ammonium concentrations (ISE ORION 9512). Alkalinity and acidity were measured by titrations with 0.02 N H₂SO₄ and NaOH, respectively, according to the procedure described in Standard Methods (APHA, 2005). The titration end points were 4.5 and 8.3 for alkalinity and acidity, respectively. Chloride and sulfate ions were measured using an Ion Chromatograph (IC, Dionex DX-100), the analytical procedure for which is described elsewhere (APHA, 2005). Dissolved metal concentrations were measured by inductively coupled plasma emission spectrometry (ICP, Leeman Labs PS3000UV) at the Materials Characterization Laboratory at The Pennsylvania State University. Volatile fatty acids (VFAs) were determined by high performance liquid chromatography (HPLC, Waters 2695) as described by Robinson-Lora and Brennan (2009). The methylene blue spectrophotometric method (APHA, 2005) was used to measure the concentration of sulfide. Moisture content in sediments was determined gravimetrically, according to the procedure described in Standard Methods (APHA, 2005).

The SRB population in the sediment inoculum was estimated following the most probable number (MPN) method by culturing samples in Modified Baar’s Medium (Atlas, 2005), using acetate (not lactate) as substrate (for details, see Appendix). The use of acetate ensured the enumeration of SRB capable of utilizing this compound, which is the main product of chitin degradation.

Statistical analyses of the collected data were performed using MINITAB® statistical software (Minitab Inc., State College, PA). The geochemical computer program PHREEQC (Parkhurst and Appelo, 1999) was used to estimate the saturation indexes (SI) of several aluminum, iron, and manganese phases.
2.4. Results

2.4.1. Microcosm test

A very rapid pH increase was observed in all chitin-amended bottles (actives, Fig. 2.1A). Circumneutral pH was achieved after only two days of treatment, and continued to increase until it reached a plateau around pH 7.5 after seven days. No significant differences in pH were observed among the three treated AMD sets (α = 0.05). In the control bottles, pH remained unchanged throughout the experiment, except for the site CR, for which pH increased by one unit on average.

Beginning on the first day of treatment, alkalinity was steadily generated while acidity was removed over time in bottles containing chitin (Fig. 2.1B and C). No significant differences were found in these parameters among the three AMD sets at each sacrificial event (α = 0.05). Average rates for alkalinity production and acidity removal corresponded to 37.9 and -27.5 mg CaCO$_3$/L-d, respectively. Parallel to alkalinity, a steady increase in calcium concentration at a rate of 34.5 mg/L-d was observed during the first nine days of treatment, when a plateau of 217 mg/L was reached (Fig. 2.2A). Electrical conductivity also steadily increased in active microcosms, reaching a maximum of 1632 ± 115 µS/cm by the end of the test (Fig. 2.2B). No significant differences were found in the final levels of these four parameters among the three AMD sets (α = 0.05).

Based on triplicate MPN analysis, average estimates of the initial acetate-utilizing SRB population in the sediment used as a microbial source was $14 \times 10^3$, $12 \times 10^3$, and $18 \times 10^4$ cells per gram (on a dry basis) for sites BC, NF and CR, respectively. Changes in sulfate concentration were insignificant during the first seven days of treatment (coefficient of variance < 10%). The activity of the sulfate reducing bacteria started to be evident after this period (Fig. 2.1D). Sulfate was almost completely removed in two of the three evaluated sites (>90% in NF and CR), while in the other site (BC) about 60% of the initial sulfate was removed. Sulfate reduction rates, calculated using data from day 9 to day 20, varied from -11.9 to -16.5 mg SO$_4^{2-}$/L-d (p ≤ 0.01). Based on the mass of substrate, these sulfate reduction rates were -23.7 to -31.1 mg SO$_4^{2-}$/L-d-g chitin. Similarly, sulfide accumulation started to be evident after seven days of treatment. In addition to the characteristic rotten-egg smell of sulfide, the formation of black
precipitates (possibly FeS) was observed. By the end of the test, accumulated dissolved sulfide concentrations ranged between 6.5 and 18.5 mg/L (Fig. 2.2C). No significant changes were observed in the concentration of sulfur species in the microcosms used as controls (data not shown). The development of reducing conditions in active microcosms was also associated with a decrease in ORP values. During the first five days of treatment, ORP rapidly decreased from $387 \pm 87$ mV to $-148 \pm 33$ mV (Fig. 2.2D). Later changes were slower, reaching a minimum of $-271 \pm 36$ mV by the end of the experiment.

While no ammonium or fatty acids were detected in the controls, important quantities of these species were observed to accumulate in the active bottles containing crab-shell chitin. The release of these species followed a very similar pattern in all the evaluated sites. Ammonium accumulation started to be evident after 2 days of treatment, and then steadily increased, reaching values between 48 and 68 mg N/L by the end of the test (Fig. 2.2E). A rapid release of formate was observed at the beginning of the test, ranging in concentration from 9.5 to 13 mg/L after only one day of treatment. This compound, however, rapidly disappeared after few days, and was replaced with a continuous accumulation of acetate, which was the predominant fatty acid throughout the remainder of the test. Concentrations of acetate at the end of the microcosm experiment ranged from 4.1 to 5.8 mM (250 to 350 mg/L). Other acids, mainly butyrate and propionate, were also detected, but their concentrations were significantly lower than those observed for acetate (0.2 – 0.4 mM or 10 – 35 mg/L).

Metal (Fe, Mn, Al) analyses were also performed during this microcosm test. However results did not show a specific removal pattern (data not shown). It is possible that micro-oxic conditions (due to the presence of trace DO) in the bottles led to iron oxidation and precipitation, which may have also promoted the removal of other metals. Deposition of orange particles was observed on the walls of the bottles, supporting this hypothesis.

2.4.2. Column test

During the initial stagnant incubation period, a rapid increase in pH was observed in all columns. After four days of incubation, the pH increased from 3.5 to 7.5 in active
columns, while in the control column, the pH reached a maximum value of 6.3 (Fig. 2.3). A significant (> 50%) decrease in sulfate concentrations was observed in all columns during this initial stage of incubation, however this observation is attributed to low-pH interferences with the analytical procedure (discussion follows), rather than to biological activity. After 5 days of incubation sulfate reduction was detected only in the active columns, and sulfate was completely removed by 8 days of incubation (Fig. 2.3). In active columns, a rapid accumulation of ammonium and VFAs, as well as alkalinity generation and acidity removal, were also observed during this period. Maximum concentrations of 840 mg NH$_4^+$-N/L, 2,000 mg C/L from VFAs, and 4,610 mg/L alkalinity as CaCO$_3$ were reached at the end of the incubation period (8 days). No ammonium, VFAs, or alkalinity were detected in samples from the control column (Fig. 2.3), indicating a lack of microbial activity.

At the beginning of continuous flow operation, values of all measured parameters in the two active columns were very similar. It was also observed that samples taken from the three different sampling ports along the length of each column (actives and control) had very similar characteristics. This was an indication that a retention time of only 11.2 h was necessary to produce the observed changes in water characteristics, likely due to the fact that the electron donor source (SC-20 chitin) was placed only within the first section of each column. For this reason, only data from the first sampling port will be presented here.

After 25 days of continuous operation, a significant decrease in the flow rate in one of the active columns (Column 2) was detected. The new flow rate was 50% lower than the original and kept slowly decreasing over time. The presence of gas accumulation was also observed, preventing sampling in some occasions. After 81 days (174 pore volumes, PV) of operation, flow through Column 2 stopped completely and this column was taken out of service.

During continuous flow operation, the pH of the active columns remained slightly alkaline (between pH 7.3 to 8.0). In the control column, pH values remained between 6.1 and 6.3 during the first 30 days; then rapidly decreased to a value of 4.5 by 45 days, and then slowly decreased to a pH close to that of the influent (pH 3.3) by the end of the experiment (Fig. 2.4A).
Accumulated alkalinity from the incubation period in the active columns was rapidly flushed out at the beginning of the continuous operation. After 21 days of flow (45 PV), the alkalinity decreased by more than 95% (from 4,610 to 210 mg/L as CaCO$_3$). Thereafter, alkalinity continued to decrease with time but at a much slower rate, resulting in an average alkalinity of 120 mg/L as CaCO$_3$ from day 21 – 102. At t = 102 d, alkalinity levels slightly increased again, when the source of influent water was changed to a more acidic source of AMD which induced a higher dissolution of calcium carbonate minerals (Fig. 2.4B). By the end of the experiment (t = 125 d, 268 PV) a residual alkalinity of 105 mg/L CaCO$_3$ was still remaining in the active column.

Due to the detected interference of high hydronium ions with the measurement of sulfate by IC, charge balance calculations with PHREEQC were used to estimate the concentration of this anion in acidic samples (influent and control column). These corrected values showed that there was no sulfate removal occurring in the control column (Fig. 2.4C). In the active columns, about 80 – 90% of the influent sulfate was removed at the beginning of continuous flow operation, and decreased with time. The effluent concentration of sulfate in Column 1 equaled that of the influent by the end of the test, while in Column 2, removal efficiency varied between 55 to 85% until it was taken out of service.

No dissolved metals were detected in the active columns at the beginning of continuous flow operation. Complete (100%) removal lasted for almost 80 days (171 PV). Manganese breakthrough occurred first at t = 81 d (174 PV), followed by iron breakthrough at t = 109 days (234 PV), and aluminum breakthrough was never detected (Fig. 2.4D, E, and F). Initially, aluminum removal was detected in the control column as well, but it only lasted for 40 days; after this period, levels of this metal were higher than those measured in the influent. No iron or manganese removal was observed in the control column. Instead, concentrations of iron in the effluent of the control column were higher than those measured in the influent water. It is likely that the iron coating of the silica sand used as packing material was leached by the acidic influent water.

Ammonium and VFA concentrations rapidly decreased after the incubation period. Ammonium levels were already below 10 mg/L after 25 days and dropped below 2 mg/L after 80 days (171 PV) of continuous flow (data not shown). Acetate was the main VFA
released by the chitinous material during the test, at an average concentration of 88 mg/L (1.49 mM) for $t = 4 - 45$ d. Although butyrate, propionate, and isovalerate had accumulated during the incubation period, their concentrations dropped below 0.1 mM after the first week of continuous operation. After 25 days (54 PV), acetate was the only VFA detected, but at low concentrations (< 0.5 mM = 12 mg C/L, data not shown).

2.5. Discussion

2.5.1. Microcosm test

When converted to meq/L-d, the generation rates for alkalinity and calcium are extremely similar (0.82 and 0.86 meq/L-d, respectively). This indicates that the dissolution of the chitin-associated carbonates present on crab-shell chitin particles is the primary mechanism responsible for the initial chemical changes in the three sets of microcosms. Furthermore, the dissolution rates of calcium carbonates (calcite, dolomite) are pH dependent: dissolution is very fast at low pH and decreases about an order of magnitude with every unit increase in pH (Stumm and Morgan, 1996). Under acidic conditions, dissolution is mass transport limited, while under near-neutral pH, the rate is limited by surface area. The present microcosm test was run under semi-stagnant conditions, with infrequent agitation (about once a day) and consequently limited exposure of the surface of the chitinous materials. Therefore, it is likely that the observed alkalinity generation and acidity removal rates are lower than those that could be obtained under well-mixed or continuous-flow operation.

Previous AMD remediation studies have augmented different substrate materials with an additional source of alkalinity, such as limestone (Gibert et al., 2002). The presence of an alternative alkalinity source, like chitin-associated carbonates, can produce similar changes in an AMD treatment system. As the pH increases, the water chemistry changes, affecting the equilibrium of the dissolved species, thereby allowing some metals to precipitate as the solubility limits of their hydroxides or carbonates are reached. The rapid recovery of pH and alkalinity obtained in the present study shows that chitinous materials are a promising and efficient source of neutralizing power for AMD remediation, and eliminate the need for an additional buffering agent. In these systems, there were three significant sources of alkalinity: calcium carbonate dissolution from the
crab shell; volatile fatty acid production from chitin and protein fermentation; and sulfate reduction by sulfate reducing bacteria (SRB). The relative contribution of each of these to the alkalinity in the microcosms can be estimated by assuming that during the first 7 – 9 days of incubation, the increase in alkalinity (~350 mg/L as CaCO$_3$) corresponded mainly to the dissolution of chitin-associated CaCO$_3$. This assumption is supported by observed plateau reached by calcium concentration after nine days. At later times, substrate fermentation became the predominant source of alkalinity from the production of volatile fatty acids, corresponding to ~200 mg/L as CaCO$_3$. Sulfate reducing bacteria contributed a total alkalinity of 160 – 180 mg/L as CaCO$_3$ from the reduction of 150 – 170 mg/L sulfate. Since the final alkalinity in the microcosm test was around 700 mg/L, it can be concluded that half of the produced alkalinity was due to calcium carbonate dissolution, and that VFA production and sulfate reduction each contributed to ~¼ of the total.

In the microcosms, the acclimation of SRB took about 7 days. The fact that the pH rapidly reached suitable values for SRB activity before this suggests this lag period is due to other limiting factors. SRB usually need to be in a consortium with fermentative bacteria to degrade complex substrates and provide simple, short-chain organics suitable for their growth. Therefore, it is likely that carbon and nutrient limitations at the beginning of the tests (before chitin fermentation occurred) played a major role on the duration of the lag period. When comparing the differences in the initial population of acetate-utilizing SRB added to each microcosm with the change in sulfate concentrations over time, it is apparent that the SRB activity was different for each site. Microcosms for the CR site had an initial acetate-utilizing SRB inoculum about an order of magnitude higher than the other two sites; however, the reduction rates obtained were relatively comparable (-13.6 mg/L-d ± 2.6). In previous batch studies by others, sulfate removal rates ranged between 3 to 109 mg/L-d, with an acclimation time of around a week (Gibert et al., 2002). Waybrant et al. (1998) calculated a sulfate reduction rate on a substrate mass basis ranging from -0.14 to -4.23 mg SO$_4^{2-}$/L-d-g. Although these rates appear to be relatively similar to the ones we obtained in this study, the experiments were run under different conditions, and therefore cannot be directly compared. It is also important to note that the sulfate reduction rates reported in this study may be very different from those that could be obtained in situ. In the field, lower water temperatures would be
expected to decrease sulfate reduction rates significantly from those reported here. In addition, differences in the solid-to-solution ratio, hydraulic retention time, as well as changes in the substrate characteristics over time can all affect the acclimation time and sulfate reduction rates (Johnson and Hallberg, 2005; Neculita et al., 2007).

2.5.2. Column test

Acclimation of SRB and complete sulfate removal was achieved more rapidly during the column test than in the microcosm test. In the packed columns, the chitin-to-sediment inoculum ratio was the same as used for the microcosm test (1:2), but the liquid-to-solid ratio was much lower (packed columns had an approximated porosity of 0.38). Therefore, a higher concentration of electron donors (VFAs) and nutrients rapidly accumulated in the columns, boosting the microbial activity and leading to a shorter acclimation time. There were also more microorganisms per mass of sulfate, increasing the overall sulfate removal during the incubation period. In addition, the rapid increase in pH may have also contributed to the rapid development of a healthy fermentative and sulfate-reducing microbial community. As carbon and nitrogen supplies decreased over the course of the experiment, sulfate removal efficiencies also decreased. However, the concentration of fermentation products increased and higher sulfate removal was observed as the flow rate in Column 2 decreased due to gas-clogging, indicating that greater sulfate removal may be obtained in systems with a longer (> 11.2 hr) retention time in the substrate.

Complete and efficient removal of the three most abundant metals in the AMD used (Al, Fe, and Mn) was observed during the first 80 days of operation of the active columns. To identify the most likely mechanisms for the observed changes in metal concentrations and alkalinity, saturation indices (SI) were calculated using PHREEQC (Fig. 2.5). Aluminum solubility is controlled by pH: in both active and control columns, the pH rose very fast, reaching values at which Al solubility is very low, and allowing it to be removed as a hydroxide precipitate from the beginning of the continuous flow operation. As the very limited alkalinity source in the control column was exhausted and the pH dropped, aluminum breakthrough rapidly occurred. The higher effluent concentrations of aluminum after breakthrough indicate that acidic conditions caused a
leaching process of the initially retained aluminum hydroxides. Contrasting, since pH in the active column was maintained near neutral, aluminum was always retained. Calculated saturation indexes (SI > 0, Fig. 2.5) indicate that the removal of aluminum in the active columns was due to precipitation of hydroxide phases (gibbsite, diaspore, boehmite).

In spite of all efforts to maintain anoxic conditions, some iron precipitation occurred in the influent reservoir. However, it is clear that iron removal occurred in the active columns. Positive SI values were obtained for both ferrous sulfide (FeS, mackinawite, pyrite) and ferric (hydr)oxide (ferrihydrite, hematite, goethite) solid phases. Phases like FeS and ferrihydrite could be considered as transient and precursors of the more stable minerals like pyrite and goethite. Therefore the later were chosen as representatives of where the system would be driven to, after reaching equilibrium (Fig. 2.5). More work must be done to determine the exact removal mechanism for iron in AMD treatment systems supported by crab-shell chitin.

Removal of manganese often represents a major challenge for the remediation of AMD. This requires an increase in pH to promote the (abiotic or biological) oxidation of Mn (II), followed by the precipitation of insoluble Mn (IV) oxides (Hallberg and Johnson, 2005). Relatively high alkaline conditions are usually necessary to ensure appropriate abiotic oxidation rates since manganese oxidation is very slow at pH < 8 (Stumm and Morgan, 1996). Under reductive conditions, the removal of this metal appears to be driven by the precipitation of carbonate phases (Benner et al., 1999). Calculated SI values for this experiment (Fig. 2.5) indicate that the column system was highly supersaturated with respect to calcium carbonate minerals and suggest rhodocrosite precipitation as the main mechanism for manganese removal, consistent with previous reports. The removal of manganese at relatively low pH (~7.5), compared to the high pH commonly required in conventional Mn-removal processes, may represent a major advantage of crab-shell chitin over alternative substrates. After breakthrough, effluent manganese concentrations remained around 1 mg/L for more 30 PV and then rapidly increased, reaching influent concentrations within 64 pore volumes.

Another possible mechanism that could explain (at least partially) the removal of metals in this experiment is adsorption. This has previously been identified as an
important method of metal sequestration, especially under moderately acidic conditions. Sorption of dissolved metals onto organic substrates or onto Al-Fe-(oxy)hydroxides has been observed in laboratory and field studies (Webb et al, 1998; Willow and Cohen, 2003; Gibert et al, 2005; Neculita et al, 2007). Recent studies have evaluated the potential of chitin and chitosan (its deacetylated derivative) as biosorptive agents for the removal of industrially-relevant metals like zinc, copper, chromium, cadmium, uranium, and lead (Yang and Zall, 1984; Boukhlifi and Bencheikh, 2000; Benguella and Benaissa, 2002; Maruca et al., 2003; Karthikeyan, et al., 2005). However, the competitive biosorption of metals commonly found in AMD (such as aluminum, iron, and manganese) has not yet been quantified. Biosorption is a complex process that is not well understood since it can involve several simultaneous mechanisms such as complexation, chelation, ion exchange, physical adsorption. Therefore, although sorption is likely contributing toward the removal of metals in this study, more research is needed to quantify the metal removal capacity of crab-shell chitin in AMD systems, which we will address in future work.

The sustained generation of alkalinity throughout the column test demonstrates the potential of crab-shell chitin to serve as a continuous, long-term source of neutralizing power for AMD remediation. Indeed, the columns were packed with 25 g of SC-20 chitin (35% CaO, or 625 mg/g as CaCO₃) giving a carbonate-derived neutralization capacity of more than 15,600 mg as CaCO₃. In addition, the chitin and protein in the crab-shell can be converted into alkalinity through microbial degradation, translating into an additional (theoretical) neutralization capacity of 23,500 mg as CaCO₃ in each column. Based on the measured acidity of the influent, the total acidity input over the course of the column test was 2,750 mg, whereas the net alkalinity released (based on effluent titrations) corresponded to 10,300 mg as CaCO₃, with a contribution of 1,930 mg from biological sulfate reduction (1,853 mg of sulfate were removed). This calculation shows that, by the end of the test, the potential neutralization capacity of the SC-20 chitin was far from being exhausted. However, it is important to point out that not all of the calcium content of the SC-20 would necessarily be converted into alkalinity, even under longer tests, due to the structural complexity of the material. In addition, it should be noted that the theoretical neutralization capacity derived from the organic carbon was
calculated assuming that every organic carbon is converted into carbonate alkalinity; in reality, an incomplete oxidation of these complex molecules can be expected. Also, a fraction of the available (organic) carbon will be used for biomass synthesis, and the rate of biological alkalinity generation will depend on the activity of the fermentative-SRB consortium.

The generation of high amounts of ammonium and organic carbon during the initial stages of incubation may denote a potential drawback in the use of chitinous materials for passive treatment of AMD. However, a rapid nutrient delivery at the beginning of the process could be beneficial to stimulate bacterial activity and decrease the duration of the lag period. Furthermore, those initial high nutrient levels were rapidly flushed out as continuous operation proceeded. One potential solution to high nutrient levels would be to mix crab-shell chitin as an amendment with other solid substrates (for example, to spent mushroom compost). This alternative simultaneously takes advantage of the abundant nutrient and alkalinity provision from chitin, while limiting the release of nitrogen and carbon to the system, and maximizing cost effectiveness.

The use of crab-shell chitin as a fractional amendment, rather than as the sole substrate, may be the most economically practical solution at the field scale, since it is considerably more expensive on a mass-basis than other currently available substrates ($0.20 – 60/lb for SC-20 crab-shell chitin vs. $50/ton for spent mushroom compost and $20 – 30/ton for limestone). A fractional amount of crab-shell chitin may be all that is required in some cases to increase the activity of SRB, and thereby decrease the size and cost of passive AMD treatment systems. There may be some applications, however, where the use of crab-shell chitin as the sole substrate is warranted both in terms of treatment capacity and cost. As a neutralizing agent, crab-shell chitin exceeds that of traditional limestone. The micro-porous structure of chitinous material provides a surface area that is several orders of magnitude higher than that of limestone powder (14 vs. <0.5 m$^2$/g). To achieve the same surface area as crab-shell chitin, limestone addition would cost approximately ten times more just in terms of materials alone. Furthermore, chitin-associated CaCO$_3$ appears to have a higher reactivity than that of limestone, leading to higher dissolution rates and therefore faster changes in pH and alkalinity. The higher chemical reactivity of its surface may explain why crab-shell chitin has not been
observed to armor with metal precipitates as limestone often does. Together, these properties make crab-shell chitin an attractive alternative for treating acidic waste streams containing particularly recalcitrant compounds (such as manganese), where the cost of continuous chemical addition would be significantly greater than that of passive treatment. The U.S. Environmental Protection Agency is currently field-testing crab-shell chitin at two Superfund sites in Colorado: one for general AMD treatment, and the other specifically for manganese removal. Continued monitoring of these field sites and further testing of crab-shell chitin as a fractional amendment will provide additional insight into the practical advantages of using this multifunctional substrate.

2.6. Conclusions

The findings of this study demonstrate that chitinous materials can be used as an alternative substrate to support the remediation of acidic and metal-laden waters. The obtained results indicate that, beyond its capacity to release electron donors for microbial activity, this material can play a major role in the neutralization of acidic streams and the removal of metal contaminants. In summary, in this study, the addition of raw crab-shell chitin:

- Rapidly increased the pH of mine impacted waters from pH ~3 to near-neutral values in less than three days.
- Rapidly stimulated the activity of SRB, sustaining removal rates of -13.6 mg/L-d ± 2.6 mg SO$_4^{2-}$/L-d.
- Promoted steady alkalinity generation and acidity removal for over 260 pore volumes of continuous flow, due to a combination of chitin-associated carbonate mineral dissolution, substrate fermentation, and sulfate reduction.
- Under continuous flow, promoted complete (100%) removal of metals for 171 pore volumes. Metals were likely removed by precipitation as insoluble phases: aluminum hydroxides, manganese carbonate (rhodochrosite), and iron hydroxides and/or sulfides.

Additional tests are currently underway to evaluate the use of crab-shell chitin as a fractional amendment to other solid substrates to enhance overall treatment effectiveness.
while reducing cost, as well as to evaluate the mechanisms of metal removal induced by the addition of this alternative material (i.e. precipitation and adsorption).

2.7. Acknowledgements

Funding for this project was generously provided by the US Geological Survey through the Pennsylvania Water Resources Research Institute. Two anonymous reviewers are thanked for their helpful comments to improve the manuscript.
2.8. References


Fig. 2.1. Changes in pH (A), alkalinity (B), acidity (C), and sulfate (D), over time in active (closed symbols, with crab-shell chitin) and control (open symbols, no chitin) microcosms containing AMD and sediment from three different locations in central Pennsylvania (BC = Beech Creek; NF = North Fork; CR = Cherry Run). Data points represent duplicate average measurements; error bars represent 1 standard deviation.
Fig. 2.2. Changes in calcium (A), electric conductivity (B), sulfide (C), redox potential (D), and ammonium (E) over time in active (closed symbols, with crab-shell chitin) and control (open symbols, no chitin) microcosms containing MIW and sediment from three different locations in central Pennsylvania (BC = Beech Creek; NF = North Fork; CR = Cherry Run). Data points represent duplicate average measurements; error bars represent 1 standard deviation.
Fig. 2.3. Changes in sulfate, ammonium, total carbon from VFAs, and pH for active columns 1 (A) and 2 (B), and the control column (C), during the pre-run incubation period. Negative values on the x-axis correspond to the number of days prior to the beginning of continuous flow.
Fig. 2.4. Changes in pH (A), alkalinity (as CaCO$_3$, B), sulfate (C), aluminum (D), iron (E), and manganese (F) in active (with chitin) and control (no chitin) columns during continuous flow operation. The arrow indicates the point at which the source of influent water was changed.
Fig. 2.5. Calculated saturation indices for representative mineral phases of aluminum (gibbsite, Al(OH)$_3$), iron (pyrite, FeS$_2$, and goethite, FeOOH), manganese (rhodochrosite, MnCO$_3$), and calcium (calcite, CaCO$_3$) in active (with crab-shell chitin) Column 1 during continuous flow operation.
Table 2.1. Sampling locations and initial characteristics of AMD and sediments used in the microcosm test.

<table>
<thead>
<tr>
<th>Site name:</th>
<th>Beech Creek (BC)</th>
<th>North Fork (NF)</th>
<th>Cherry Run (CR)</th>
<th>Kittanning Run</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approximate sampling location (GPS)</td>
<td>41.08598°, -77.86725°</td>
<td>41.051015°, -77.96397°</td>
<td>41.05428°, -77.95562°</td>
<td>40.49781°, -78.47633°</td>
</tr>
</tbody>
</table>

**Water**

| ORP (mV) | 286 | 438 | 437 | ND |
| pH | 3.51 | 3.5 | 3.25 | 2.95 |
| EC (µS) | 788 | 382 | 483 | ND |
| Alkalinity (mg/L CaCO₃) | 0 | 0 | 0 | ND |
| Acidity (mg/L CaCO₃) | 68 | 30 | 60 | 153 |
| Sulfate (mg/L) | 393 | 181 | 293 | 570⁺ |
| Chloride (mg/L) | 8.9 | 15.2 | 6.2 | ND |
| Al (ppm, MCL = 2) | 1.7 | 2.9 | 1.6 | 10 |
| Fe (ppm, MCL = 0.3) | 9.3 | 7.4 | 1.2 | 10 |
| Mn (ppm, MCL = 0.05) | 5.8 | 3.5 | 2.3 | 15 |

**Sediment**

| Moisture content (%) | 27.7 | 51 | 39 | 31.9ᵇ |

⁺Estimated value based charge balance calculations (PHREEQC)  
ᵇApproximate sampling location for sediment: 40.49660°,-78.46230°
CHAPTER 3

Chitin Complex for the Remediation of Mine Impacted Water: Geochemistry of Metal Removal and Comparison with other Common Substrates

3.1. Abstract

Remediation of mine impacted water (MIW) generally requires decreasing the acidity and concentrations of dissolved and/or particulate contaminants (sulfates, metals, and metalloids). By fulfilling these requirements in both laboratory and field trials, the sustainable composite waste material, crab-shell chitin complex (CC) has proven to be a promising substrate for MIW remediation, but has not yet been directly compared with other substrates under controlled conditions. In this study, remediation rates and metal removal mechanisms promoted by CC were evaluated and compared to the more commonly used lactate and spent mushroom compost (SMC) using sacrificial batch microcosms and geochemical modeling.

Under comparable conditions with equivalent mass of substrate to water ratios, increases in pH were much faster in the microcosms containing CC than with the other substrates: CC increased the pH from pH 3.0 to near neutral in 3 days. In microcosms containing CC, steady alkalinity generation and acidity removal were observed at average rates of 26.5 and -25.2 mg CaCO$_3$/L-d, respectively. The activity of sulfate reducing bacteria was evident after 9 days of incubation, with average reduction rates of -17.8 mg SO$_4^{2-}$/L-d. Similar changes in alkalinity, acidity, and sulfate were also observed in lactate-containing microcosms, but only after a 27 day lag period. No alkalinity generation or sulfate reduction activity was observed in bottles containing SMC. Aluminum removal (100 %) was eventually observed with all substrates, but occurred much faster with CC. Results from thermodynamic geochemical modeling indicate that Al removal was consistent with the precipitation of hydroxides and/or alunite. Iron removal was consistent with precipitation of ferric oxides and ferrous sulfides, as well as sorption onto CC and SMC. The addition of sodium lactate interfered with such mechanisms due to complexation effects. Chitin complex was the only substrate able to
partially remove manganese (>73%), likely due to the formation of rhodochrosite. The results of this study indicate that CC is an attractive substrate for treating metal-laden waste streams, especially those which are high in manganese.

3.2. Introduction

The generation of mine impacted water (MIW, also known as acid mine drainage) is a recurring problem around the world. These often acidic, metal-contaminated discharges can cause serious adverse effects to the environment (Johnson and Hallberg, 2005; McCauley et al., 2009). In general, remediation of MIW requires three different processes: the addition of a neutralizing agent; a decrease in sulfate concentrations; and the removal of other dissolved and/or particulate contaminants (metals and/or metalloids).

Numerous active and passive treatment technologies have been suggested to remediate affected streams, each with its own advantages and disadvantages. Typically, abiotic, active treatment systems require a constant input of an alkaline material like lime, caustic soda, soda ash, or ammonia to neutralize the acidic streams. Although effective, their constant demand for supplies, energy, and maintenance make these systems extremely expensive and usually impractical, especially for remote sites (Ziemkiewicz et al., 2003). As an alternative, a variety of passive treatment systems (abiotic and biological) have been developed over the last 30 years. Common technologies used now include limestone channels and drains, aerobic wetlands, vertical flow wetlands, and permeable reactive barriers (Gibert et al., 2002; Johnson and Hallberg, 2005; Skousen and Ziemkiewicz, 2005). Among these, biological/anaerobic systems (such as wetlands and permeable reactive barriers) rely on the activity of sulfate reducing bacteria (SRB), which are sustained by the provision of a suitable organic substrate and trace nutrients. These microorganisms can only metabolize simple, short-chain organic compounds (acetate, lactate, ethanol, glucose, etc.) of which lactate has been shown to be a superior substrate as compared to other organic compounds (Neculita et al., 2007). For the passive treatment of MIW, however, long-lasting, solid substrates are needed. Numerous studies have been conducted evaluating the use of a wide variety of fermentable waste materials, such as animal manure, wood chips, and different types of compost (Batty and
Younger, 2004; Chang et al., 2000; Cocos et al., 2002; Gibert et al., 2004; Neculita et al., 2007; Waybrant et al., 1998; Zagury et al., 2006). The selection of the organic substrate has important impacts on both operational costs and overall biological performance.

Spent mushroom compost (SMC) is a by-product of the mushroom industry, and is composed mainly of agricultural materials (cereal straw and poultry or horse manure) and gypsum (Ntougias et al., 2004). Due to its relative abundance and low cost, SMC has been commonly and successfully used in the field as a substrate for the passive remediation of MIW in anaerobic and vertical flow wetlands (Ziemkiewicz et al., 2003). Systems containing SMC are not always successful in stimulating biological sulfate reduction, however (Pruden et al., 2007), possibly due to a deficiency in available nitrogen (Waybrant et al., 2002).

Crustacean shells (such as crab and shrimp) are composed of a complex solid matrix of chitin (poly-N-acetylg glucosamine), protein, and calcium carbonate, and provide a sustainable, slow-release source of carbon, nitrogen, and alkalinity in one substrate. Successful results have been obtained using crab-shell chitin complex (CC) for anaerobic dechlorination (Brennan et al. 2006), denitrification (Robinson-Lora and Brennan, 2009a), and MIW remediation (Daubert and Brennan, 2007; Venot et al., 2008; Robinson-Lora and Brennan, 2009b). In batch laboratory tests with soft-rock MIW, the addition of CC resulted in fast increases in pH (from 3.2 – 3.5 to circumneutral in 2 – 5 days), rapid increases in alkalinity (26 – 38 mg CaCO$_3$/L day), efficient removal of metals (as high as 99 % for Fe and Al, and 81% for Mn), as well as biological sulfate reduction (Daubert and Brennan, 2007; Robinson-Lora and Brennan, 2009b). In small-scale field studies with hard-rock MIW, CC also promoted the activity of SRB, the generation of significant amounts of alkalinity, and the removal of metals (Venot et al., 2008). In addition, other aquatic species containing chitin have also been successfully used for MIW remediation. Under continuous-flow conditions, McCauley and co-workers (2009) observed a more effective removal of sulfate and metals using mussel shells than limestone. Although CC has proven to be a promising substrate for MIW remediation in both laboratory and field trials, it has not yet been directly compared with other commonly used organic substrates under controlled laboratory conditions, nor the mechanisms of its effectiveness thoroughly explained.
The aim of the present study was to further evaluate the characteristics and performance of crab-shell CC for the remediation of MIW in comparison to other commonly used substrates. Under controlled conditions, remediation rates using CC as a sole substrate were quantified and compared with those obtained using either SMC or sodium lactate. The probable mechanisms for metal removal were evaluated by means of geochemical modeling calculations (PHREEQC).

3.3. Methods

3.3.1. Chemicals

All chemicals used in this study were reagent grade or better. Ultra High Purity nitrogen gas (UHPNG, MG Industries, Malvern, PA) was used to purge samples. Minimally processed (dried and crushed) crab shell flakes, derived from Dungeness crab (ChitoRem® SC-20, JRW Bioremediation, LLC, Lenexa, KS), were used as an example of chitin complex. Demineralization and deproteinization, based on protocols described in previous studies (Percot et al., 2003), were conducted to determine its composition. Results indicate that SC-20 contains 10% chitin, 12% protein, and 78% mineral matter (35% as CaO). Sodium L-lactate (~98% purity, Sigma-Aldrich) was used as a source of lactate. SMC was obtained from the Mushroom Test Demonstration Facility at The Pennsylvania State University (University Park, PA), which had been composted according to the procedure described by Royse et al., (2008). According to the provider, the original components of this SMC were straw-bedded horse manure, switch grass straw, poultry litter, distiller's grain, and gypsum. Samples of CC and SMC were analyzed for their extractable elements and their total C and N content (Table 3.1).

3.3.2. Water and sediment sources

MIW was obtained from Kittanning Run in Altoona, PA. Benthic sediments, used as microbial source, were collected in sterile centrifuge tubes from an adjacent marsh, transported at 4 °C, and used the same day of sampling. Before use, the water was purged with UHPNG for 4 hours to remove trace oxygen (final DO ≤ 0.5 mg/L). The resulting anoxic water was sampled, filtered (0.20 µm), and analyzed for pH, dissolved metals, anions, ammonium, and dissolved organic carbon (DOC) (Table 3.1). Sediments
were first drained to remove excess water, and then measured for final water content and concentration of extractable elements (Table 3.1).

3.3.3. Setup of microcosms

For each evaluated substrate, a set of 30 serum bottles (160 ml capacity) was prepared. All bottles were supplied with 0.5 g sediment and 0.25 g of the substrate. The bottles were then purged for 10 minutes with UHPNG before adding 100 ml anoxic (unfiltered) MIW and then purged for 10 - 12 minutes more. A subset of 10-bottles per substrate was labeled as “killed controls” and treated with 5 ml of formaldehyde solution (36.5%) to inhibit microbial activity. An additional set of 14 bottles were prepared with sediment only (no substrate or formaldehyde added) and served as active controls. The bottles were sealed with Teflon-lined stoppers and aluminum crimp tops, manually shaken to mix the contents, and incubated in the dark (to prevent the possible occurrence of photosynthesis) at room temperature (20 ± 1°C) for up to 50 days.

During the incubation period, duplicate, randomly-selected bottles of each set of actives or controls were sacrificed periodically. Sampling frequency decreased as total incubation time increased. After each bottle was opened, samples were promptly tested for pH (<0.5 h), alkalinity, and acidity (<4 h). Another portion of the sample was filtered (0.20 µm) before being stored for future analyses (4°C for less than a week for anions and ammonium, 4°C with 0.2 ml/L conc. HNO₃ for dissolved metals, and -10°C for DOC).

3.3.4. Analytical methods

Electrodes were used to measure pH (Accumet® BASIC, AB15 connected to a Thermo-ORION pH probe, daily calibrated with pH 4.01, 7.00, and 10.01 standards) and ammonium concentrations (ISE ORION 9512, daily calibrated with standards of 1, 10, and 100 mg/L N). Alkalinity and acidity were measured by titration with 0.02 N H₂SO₄ and NaOH, respectively, according to the procedure described in the Standard Methods 2310B and 2320B (APHA, 2005). The titration end points were 4.5 and 8.3 for alkalinity and acidity, respectively. The concentration of anions was measured using an Ion Chromatograph (IC, Dionex DX-100), the analytical procedure for which is described elsewhere (APHA Method 4110B, APHA, 2005). Dissolved metal concentrations were
measured by inductively coupled plasma emission spectrometry (ICP, Leeman Labs PS3000UV) at the Materials Characterization Laboratory at The Pennsylvania State University. DOC was measured using a TOC analyzer (Shimadzu TOC-V CSN). At least one blank and one internal standard were analyzed with each set of samples when conducting IC, ICP, and DOC analyses.

Solids (CC, SMC, and sediment) were analyzed for extractable aluminum, iron, manganese, calcium, magnesium, sodium, potassium, and sulfate. Measurements were conducted at the Agricultural Analytical Services Laboratory at The Pennsylvania State University (Mehlich 3 extraction, Table 3.1). Moisture content in the sediment was determined gravimetrically, according to the procedure described elsewhere (APHA Method 2540B, APHA, 2005).

3.3.5. Geochemical modeling

The geochemical computer programs MINTEQA2 (Allison, 1990) and PHREEQC (Parkhurst and Appelo, 1999) were used to simulate the equilibrium in the systems. The saturation indexes (SI) of several aluminum, iron, and manganese phases were calculated with PHREEQC, using the equilibrium constants from the MINTEQA2 database. When considering the occurrence of sulfide minerals, the concentration of S(-II) in the aqueous phase was calculated from the deficit of sulfate in the system, based on previous studies under nearly identical conditions which showed that sulfide was formed (Robinson-Lora and Brennan, 2009b).

3.4. Results

3.4.1. Water neutralization

The addition of CC caused a rapid increase in pH in all microcosms, compared with the other evaluated substrates. In CC-active bottles, pH increased from pH 3.0 to pH 6.5 in 3 days (Fig. 3.1A), while in CC-killed controls, near-neutral pH (6.7) was observed after 8 days of incubation (Fig. 3.1B). Sodium lactate caused an almost immediate increase in pH of about two units (in both active microcosms and killed controls); however, further increases in pH were only observed in active microcosms after 27 days of incubation, reaching a maximum of pH 6.6 by the end of the test (Fig. 3.1A and B).
Changes in bottles containing SMC were much slower: after 37 days of incubation, maximum values of pH 6.2 in active microcosms and pH 5.1 in killed controls were reached. No pH changes were observed in bottles without substrate amendment (sediment only).

Steady alkalinity generation was observed in CC-containing microcosms (Fig. 3.1C and D). Alkalinity was generated at much higher rates in CC-active microcosms than in CC-killed controls, where it reached a maximum of 941 mg/L as CaCO$_3$ by the end of the test. Only minimal changes were observed in killed controls after 14 days of incubation. The addition of sodium lactate caused an almost immediate generation of 90 mg/L as CaCO$_3$ (on average) of alkalinity in both active microcosms and killed controls (Fig. 3.1C and D). Further changes in alkalinity were only observed in lactate-active microcosms after 27 days of incubation. No alkalinity generation was observed in bottles containing SMC or sediment only.

Similar trends were observed for the removal of acidity in active microcosms (Fig. 3.1E). Changes in acidity were almost immediate in the CC-active microcosms, while changes in lactate-active microcosms were only evident after 27 days of incubation. A minimum of -800 mg/L acidity as CaCO$_3$ was measured in CC-active microcosms after 37 days of incubation. In lactate-active microcosms, a minimum of -531 mg/L acidity as CaCO$_3$ was measured at day 44. In addition, a slight acidity removal was observed in active microcosms amended with SMC at the beginning of the test: a minimum value of 13 mg/L acidity as CaCO$_3$ was observed after 27 days of incubation. No acidity was removed in bottles containing sediment only. Changes in acidity for killed controls were not possible to measure since the addition of formaldehyde increased the background level to more than 3000 mg/L as CaCO$_3$ (Fig. 3.1F).

The rates of alkalinity production for CC and lactate were comparable, but the rate of acidity removal with lactate was about 50% of that obtained with CC (Table 3.2).

3.4.2. Sulfate changes and nutrient availability

The accuracy of the water chemistry analyses was verified by means of anion-cation balance. Errors were in general less than 10%, except for the analysis corresponding to the control set with sediment only. In addition, an initial partial removal of sulfate was
observed in all other sets on the first sampling period (Fig. 3.2A). Such a significant decrease in sulfate concentration during the first day of treatment cannot be attributed to SRB activity as previous observations under similar incubation conditions suggest a lag period of at least one week (Robinson-Lora and Brennan, 2009b; Waybrant et al., 1998). These two observations indicated the existence of a systematic error in the analysis of sulfate. The source of error was identified as an increase in the IC detector response at low pH values (pH < 4). Therefore, an estimate range of the true initial sulfate concentration was calculated based on a 5% charge balance error, assuming the measurements for the other major ions were accurate. The estimated initial sulfate concentration lies within the values measured in all microcosms during the lag period, when pH values were greater than pH 4, validating the assumed charge balance calculations. After \( t = 0 \), the pH in all active microcosms rose above pH 4, \( H^+ \)-interferences ceased, and the values measured for sulfate were again accurate. Biological sulfate reduction was evident in active microcosms with CC and lactate (Fig. 3.2A): in addition to the decrease in sulfate concentration, the characteristic rotten-egg smell of hydrogen sulfide and the presence of black precipitates were observed. Comparable sulfate reduction rates, calculated for \( t > t_{\text{lag}} \), were obtained for these two substrates (Table 3.2); however, the extent of the lag period was much shorter with CC (9 days) than with lactate (27 days). Sulfate was completely removed in microcosms containing CC after 37 days of incubation. At the end of the 50 days of incubation, an average of 97 mg SO\(_4^{2-}\)/L was still detected in lactate-containing microcosms. No sulfate reduction activity was observed in bottles containing SMC, sediment only, or in the killed controls.

Significant amounts of ammonium accumulated only in CC-containing active microcosms, presumably from the fermentation of the nitrogen-rich protein and/or chitin in the crab shell. After 15 days of incubation, ammonium concentrations fluctuated around 40 mg/L (Fig. 3.2B). Some ammonium was also quickly released from SMC after one day of incubation; however, its concentration did not exceed 1 mg/L. No ammonium was detected in microcosms containing sodium lactate or sediment only.

DOC concentrations were only measured in active microcosms and sediment-only controls, since the addition of formaldehyde to killed controls increased the background DOC to several thousand mg/L. An average of 250 mg/L of DOC was generated as the
result of the addition of sodium lactate. This concentration remained fairly constant in active microcosms throughout the duration of the test (Fig. 3.2C). DOC in CC-containing active microcosms accumulated up to a maximum of 46 mg/L after 12 days of incubation. Carbon concentrations remained relatively constant during the following 10 days, and then started to decline down to 18 mg/L at the end of the test. In SMC-containing microcosms, DOC concentrations did not exceed 5 mg/L, while in those bottles containing sediment only, carbon concentrations remained close to the initial concentration (0.8 mg/L).

While the expected stoichiometric increase in sodium concentration was observed in the lactate active and control sets, the addition of more complex substrates (i.e., CC and SMC) caused increases in calcium, potassium, and sodium concentrations as well. Changes in sodium and potassium concentrations were almost immediate after the addition of those two substrates (Fig. 3.3A – D). CC increased the concentration of sodium and potassium by 123 and 5 mg/L, respectively, while the SMC increased the concentrations of those elements by 1.3 and 15 mg/L respectively. The increase in calcium concentrations occurred gradually (Fig. 3.3E and F); in CC microcosms its concentration reached a plateau around 235 mg/L after 15 days of incubation, while the maximum concentration of calcium in SMC microcosms was 156 mg/L after 27 days. With minor fluctuations, the magnesium concentrations did not appear to be affected by the addition of any of the evaluated substrates (Fig. 3.3G and H).

3.4.3. Changes in metal concentrations

Aluminum was completely removed (100%) by all substrates in active microcosms (Fig. 3.4A). However, removal was much faster with CC than with SMC or lactate (3, 12, and 37 days, respectively). In killed controls, the addition of CC also led to a rapid, complete removal of aluminum (Fig. 3.4B). Slower aluminum removal was observed in SMC- and lactate-containing killed controls (100 and 30% after 37 days, respectively). Iron was rapidly removed in those sets containing CC or SMC (active and controls): after 3 – 5 days of incubation, iron concentrations decreased between 93 – 97% (Fig. 3.4C and D). However, as the incubation proceeded in the active bottles, iron was partially released back to solution, most likely caused by the reduction of precipitated ferric
oxides. In CC-treated systems though, this secondary iron release was only temporary and lower (up to 20%) than in those treated with SMC (up to 70%). Iron was also removed in the sediment-controls (~96%) by the end of the test, while no iron removal was observed in bottles containing lactate. CC was the only substrate able to partially remove manganese in both active microcosms and killed controls (~73%, Fig. 3.4E and F).

3.4.4. Thermodynamic calculations

The saturation indices of commonly formed minerals were calculated using water quality data from active microcosms. Some representative phases were selected to illustrate the results based on their stability. For example, phases like FeS and ferrihydrite could be considered as transient precursors of more stable minerals like pyrite and goethite. Therefore, the latter were chosen as representatives of where the system would be driven to after reaching equilibrium (Fig. 3.5). Saturation with respect to calcite was only reached by CC-containing microcosms after about 12 days of incubation (Fig. 3.5A), while those treated with lactate or SMC were always undersaturated with respect to this mineral. Results also show that all three microcosm sets were rapidly supersaturated (SI > 0) with respect to aluminum hydroxides (like gibbsite), as well as alunite (KAl$_3$(SO$_4$)$_2$(OH)$_6$). Positive SI for ferric hydroxides were obtained in all systems. However, some delay was observed in CC- and SMC-containing microcosms (3 and 22 days, respectively; Fig. 3.5A and C). At later times, the occurrence of positive saturation indices of ferrous sulfides in CC (t > 9 days) and lactate (t > 37 days) systems is coincident with the onset of biological sulfate reduction. Rhodochrosite supersaturation occurred in CC and lactate systems; however, positive SI values were reached faster with CC (t > 9d) than with lactate (t > 37d).

3.5. Discussion

3.5.1. Water neutralization

In CC-amended microcosms, the dissolution of carbonate minerals present in the crab shells appeared to play a major role in the changes observed at early times during the test. This hypothesis is supported by the fact that calcium and alkalinity concentrations clearly
followed the same pattern during the first 12 days of incubation, with calcium and alkalinity production rates corresponding to 0.50 and 0.71 meq/L-d, respectively (p-value = 0.000). Furthermore, a direct relationship was found between calcium and alkalinity increases, with \( \Delta \text{Alk}/\Delta \text{Ca} = 1.3 \) \( (r^2 = 0.976, \text{p-value} = 0.000) \). Afterwards, the calcium concentration reached a plateau as a consequence of the saturation of the system with respect to calcium carbonate. At this time, sulfate reduction and alkalinity production rates were fairly similar (-0.67 and 0.77 meq/l-d, respectively, p-value ≤ 0.011) and \( \Delta \text{Alk}/\Delta \text{SO}_4^{2-} = -0.96 \) \( (r^2 = 0.688, \text{p-value} = 0.041) \). Therefore, it is likely that the latter increases in alkalinity were due mostly to the sulfate reduction activity that started after nine days of incubation. At both stages, alkalinity production rates were higher than those calculated for calcium release and sulfate reduction. The production of fatty acids (mainly acetate) due to chitin fermentation could have contributed to additional alkalinity generation.

Contrastingly, the initial pH and alkalinity changes observed in the lactate-containing systems were due to the dissolution of this salt itself. Simple equilibrium calculations performed using MINTEQA2 show that when of 2.5 g/L of sodium lactate are added to a solution of pH 3, the expected new pH corresponds to 5.1. Indeed, the measured values during the first 27 days of incubation varied around pH 5.0. The later changes in pH values with lactate are associated with the beginning of biological sulfate reduction. In fact, when converted to meq/L-d, the calculated rates for sulfate reduction (-29.0 mg \( \text{SO}_4^{2-}/L-d = -0.60 \) meq \( \text{SO}_4^{2-}/L-d \)) and alkalinity production (30.3 mg \( \text{CaCO}_3/L-d = 0.61 \) meq \( \text{CaCO}_3/L-d \)) are in very close agreement, supporting this hypothesis. An inverse relationship between sulfate and alkalinity changes was found with \( \Delta \text{Alk}/\Delta \text{SO}_4^{2-} = -0.99 \) \( (r^2 = 0.997, \text{p-value} = 0.000) \).

Changes in the pH of microcosms containing SMC were much smaller and slower than with the other evaluated substrates. Although a slight decrease in acidity was observed within this set, the buffering capacity of this substrate was not sufficient to generate any measurable alkalinity. This indicates that although different types of composted materials have been shown to be suitable substrates for MIW remediation, their use implies the necessity of an external alkalinity source. Indeed, limestone amendments are commonly used in laboratory trials and field applications (Gibert et al.,
3.5.2. Sulfate changes and nutrient availability

Previous research has shown lactate to be a superior substrate for SRB activity over other carbon sources, such as acetate or ethanol (Neculita et al., 2007). In addition, successful tests using SMC as substrate for MIW remediation have been previously reported (Dvorak et al., 1992). However, in the present study, faster remediation was achieved with CC, the main fermentation product of which is acetate (Robinson-Lora and Brennan, 2009b; Brennan et al., 2006). Analysis of DOC showed that carbon sources were readily available in both CC and lactate microcosms, with much higher concentrations in the latter. However, very little dissolved carbon was released from SMC, which could explain the limited microbial activity observed in those tests. It is likely that the SRB activity was also inhibited by low pH and the lack of a buffering agent (i.e. limestone) in bottles containing either lactate or SMC. The reduced availability of nutrients like nitrogen could also contribute to the delayed activity of SRB in lactate-containing microcosms. It is likely that earlier changes in systems amended with lactate could be obtained by making appropriate pH and nutrient adjustments; however, the soluble nature of this substrate requires a continuous supply, which would increase operating costs for continuous-flow site remediation. On the other hand, SMC is considered a suitable substrate for long-term operation (Chang et al., 2000), but its slow biodegradability suggests the need for an additional, more easily degradable substrate to sustain the establishment of the microbial community at the beginning of the treatment.

Although the sulfate reduction rates reported in this study are promising, it is important to remember that they were measured under controlled-laboratory conditions. The semi-stagnant conditions under which this study was conducted may have limited exposure of the surface of the chitinous materials and the homogeneity of the systems. Therefore, it is likely that the estimated rates of sulfate removal, as well as alkalinity generation, are lower than those that could be obtained under continuous mixing or flow conditions. For the in situ utilization of this substrate, differences in temperature, solid-
to-solution ratio, hydraulic retention time, etc. will induce different acclimation times and reduction rates. In addition, changes in the substrate characteristics over time could also affect the performance of in situ treatments (Johnson and Hallberg, 2005; Neculita et al., 2007).

Compared to the other substrates, CC degradation generated the highest amounts of ammonium. This fact reveals simultaneously a potential advantage and a drawback in the use of chitinous materials for passive treatment of MIW with respect to nutrient availability. A rapid nutrient delivery at the beginning of the process could be beneficial to stimulate bacterial activity and decrease the duration of the lag period. On the other hand, aquatic life can be affected by high concentrations of inorganic reduced nitrogen (N(-III)). This is especially true at high pH values (pH >9.3) when ammonia, the unionized form of N(-III), is the predominant species. Ammonia can also form complexes with many metals, affecting their solubility and mobility. In these tests, however, pH remained < 7.8, so nitrogen was primarily in the form of ammonium (NH_4^+), which is nontoxic, bioavailable, and less likely to form complexes. Nevertheless, to offset concerns of potential ammonia toxicity and to reduce substrate costs, CC could be used as an amendment to other solid substrates like SMC. In this way, the process could benefit from the nutrient and buffering capacity of CC without diminishing the quality of the treated water due to the excessive release of ammonium. Indeed, other researchers have noticed that a mixture of various substrate materials often leads to better performance of passive biological treatment systems (Neculita et al., 2007; Gibert et al., 2002). Such combined substrate studies are currently being completed in our laboratory.

The release of major cations (Na, K, Mg, and Ca) within each substrate system is consistent with the initial composition of the solid material used. Monovalent cations are likely associated with readily soluble salts and were therefore released almost immediately. To prevent the release of excess sodium from the surface of CC in sensitive field locations, a simple tap water rinse could be conducted before emplacement. The release of calcium is likely associated with minerals with slower dissolution kinetics: calcite for CC, and gypsum for SMC. In the case of magnesium, its concentrations in the solid substrates were too low to cause significant changes in the system, relative to the initial concentration in the MIW.
3.5.3. Changes in metal concentrations

As indicated by the positive SI of Al hydroxides and alunite (KAl$_3$(SO$_4$)$_2$(OH)$_6$), the removal of this metal is likely due to the precipitation of these phases. However, very little removal was observed in lactate microcosms during the lag period. This could have been caused by a chelation effect of the aluminum ions due to the presence of the lactate ion (C$_3$H$_5$O$_3^-$). Indeed, thermodynamic calculations show that more than 99% of this metal tended to form (hydroxo) complexes with lactate ions. After the addition of 2.5 g/L sodium lactate, 92.4% of the aluminum originally present in the MIW used is complexed as AlOH(C$_3$H$_5$O$_3$)$_2$, 4.8% as AlOH(C$_3$H$_5$O$_3$)$_3^+$, 1.0% as Al(C$_3$H$_5$O$_3$)$_2$OH, 0.78% as Al(OH)$_2$(C$_3$H$_5$O$_3$)(aq), 0.60% as Al(C$_3$H$_5$O$_3$)$_3$ (aq), and 0.26% as AlC$_3$H$_5$O$_3^{2+}$.

At the beginning of the incubation, CC- and SMC-containing microcosms appeared to be undersaturated with respect to iron hydroxides. The observed early removal could have been caused by a slow precipitation of colloidal iron particles (unfiltered MIW was used in the test) or sorption mechanisms. Metal sorption in MIW remediation systems during early stages of the treatment has been already documented (Gibert et al., 2005; Willow and Cohen, 2003). As incubation proceeded in the CC systems, it is likely that reductive dissolution of iron hydroxides caused this metal to be partially released back to the solution. Once SRB became active, precipitation as iron sulfides promoted the complete removal of this metal from the system. The occurrence of sulfide minerals as a mechanism for metal removal has been already reported in laboratory and field studies (Gibert et al., 2003; Benner et al., 1999; Waybrant et al., 1998). However, while almost all iron was removed from the CC system, no removal was observed in the lactate one. Again, it is likely that lactate could have acted as a chelating agent, preventing iron precipitation. Changes in the solid-liquid distribution of divalent metals driven by the addition of short-chain acids have been already reported. Ahumada et al. (2001) observed that the addition of lactate and citrate to water-soil systems increased the solubility of cadmium and copper.

A remarkable difference between the evaluated substrates is the capacity of CC to remove manganese. Thermodynamic calculations indicate that CC-containing systems reached rhodochrosite supersaturation at very early times, possibly leading to Mn removal. Furthermore, the fact that similar removals were observed in formaldehyde-
killed controls indicates that abiotic, rather than biological interactions are responsible for such observations. Rhodochrosite (MnCO$_3$) precipitation as the mechanism for the removal of this metal has been already reported (Benner et al., 1999; Waybrant et al., 1998). Rhodochrosite supersaturation was also calculated for lactate microcosms after the occurrence of sulfate reduction, but manganese was not removed. The distinctive efficiency of CC for the removal of manganese compared to other liquid and solid substrates has also been observed in recent field trials (Venot et al., 2008). Furthermore, the findings of this study differ from those recently published using other types of chitin-containing materials obtained from aquatic organisms. For example, systems containing mussel shells failed in the removal of manganese, as the shells appeared to be a source of this metal (McCauley et al., 2009). The reason for the unique behavior of CC is likely due to the rapid dissolution of carbonate minerals, and is the subject of ongoing investigations in our laboratory.

3.6. Conclusions

The results of this study show that crab-shell chitin complex is a very promising material to be used as a substrate or amendment for the passive remediation of MIW. While most field and laboratory applications need a mixture of various materials to improve results, chitin complex alone:

- Effectively neutralizes acidic waters, due to the rapid release of its built-in carbonate minerals;
- Supports the activity of sulfate reducing bacteria, thanks to its slowly fermentable nature and its provision of essential nutrients, like nitrogen;
- Facilitates efficient removal of dissolved metals (Fe, Mn, Al).

In contrast, the addition of sodium lactate and SMC showed limited positive results when used alone, reflecting the need of, at least, a supplementary alkalinity source at the onset of treatment to create a more favorable environment for SRB activity. Similar sulfate reduction rates were observed with CC and sodium lactate, but longer acclimation time was needed with the latter, likely due to low pH and nutrient availability. In addition, the chelating character of sodium lactate appeared to inhibit the removal of
dissolved metals. SMC exhibited very slow biodegradability, limiting SRB activity. However, some metal removal was observed, likely due to sorptive effects.

Finally, the high efficiency of chitin complex in removing manganese, in comparison to the other evaluated substrates, makes it a very attractive alternative for sites with high levels of this metal. The mechanism by which manganese removal is accomplished is not clear and is the subject of ongoing research in our laboratory.

3.7. Acknowledgements

Funding for this project was generously provided by the US Geological Survey through the Pennsylvania Water Resources Research Institute, the Penn State Institutes of Energy and the Environment, and JRW Bioremediation, LLC. Special thanks to the Mushroom Test Demonstration Facility at The Pennsylvania State University (University Park, PA) for donating the spent mushroom compost used in these tests. Two anonymous reviewers are thanked for their helpful comments to improve the manuscript.
3.8. References


Fig. 3.1. Comparison of the changes in pH (A and B), alkalinity (C and D), and acidity (E and F, note different scales for y-axis) over time in microcosms containing MIW, sediment, and three different substrates. Closed symbols correspond to active microcosms and open symbols to killed controls. Data points represent duplicate average measurements; error bars represent 1 standard deviation.
Fig. 3.2. Changes in sulfate (A), ammonium (B), and dissolved organic carbon (C) over time in active microcosms containing MIW, sediment, and three different substrates. Theoretical initial sulfate concentration was calculated based on charge balance; the range (defined by horizontal dotted lines) represents average ± 1 standard deviation. Data points represent duplicate average measurements; error bars represent 1 standard deviation.
Fig. 3.3. Changes in sodium (A and B), potassium (C and D), calcium (E and F), and magnesium (G and H) over time in microcosms containing MIW, sediment, and three different substrates. Closed symbols correspond to active microcosms and open symbols to killed controls. Data points represent duplicate average measurements; error bars represent 1 standard deviation.
Fig. 3.4. Changes in aluminum (A and B), iron (C and D), and manganese (E and F) over time in microcosms containing MIW, sediment, and three different substrates. Closed symbols correspond to active microcosms and open symbols to killed controls. Data points represent duplicate average measurements; error bars represent 1 standard deviation.
Fig. 3.5. Saturation indices for calcite (CaCO$_3$), rhodochrosite (MnCO$_3$), gibbsite (Al(OH)$_3$), alunite (KAl$_3$(SO$_4$)$_2$(OH)$_6$), goethite (FeOOH), and pyrite (FeS$_2$) in active microcosms containing MIW, sediment, and (A-CC) chitin complex, (B-LAC) sodium lactate, and (C-SMC) spent mushroom compost.
Table 3.1. Initial characteristics of MIW, solid substrates, and sediment used in the microcosm tests.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MIW water$^1$</th>
<th>Sediment$^2$</th>
<th>SC-20 Chitin$^2$</th>
<th>Mushroom compost$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2.95</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Alkalinity, as CaCO$_3$</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Acidity, as CaCO$_3$</td>
<td>153</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Sulfate</td>
<td>480 - 650$^3$</td>
<td>618</td>
<td>684</td>
<td>15,814</td>
</tr>
<tr>
<td>Chloride</td>
<td>6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Aluminum</td>
<td>10</td>
<td>361</td>
<td>2.8</td>
<td>10.1</td>
</tr>
<tr>
<td>Iron</td>
<td>10</td>
<td>587.3</td>
<td>10.4</td>
<td>156.5</td>
</tr>
<tr>
<td>Manganese</td>
<td>15</td>
<td>169.7</td>
<td>36.6</td>
<td>96.9</td>
</tr>
<tr>
<td>Calcium</td>
<td>72</td>
<td>1.184</td>
<td>28,890</td>
<td>16,237</td>
</tr>
<tr>
<td>Magnesium</td>
<td>61</td>
<td>314</td>
<td>1,715</td>
<td>2,514</td>
</tr>
<tr>
<td>Sodium</td>
<td>2.6</td>
<td>232.6</td>
<td>27,320</td>
<td>1,875</td>
</tr>
<tr>
<td>Potassium</td>
<td>2.3</td>
<td>89</td>
<td>1,503</td>
<td>10,998</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.63</td>
<td>4.6</td>
<td>17</td>
<td>47</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>NA</td>
<td>3.0</td>
<td>1,730</td>
<td>1,211</td>
</tr>
<tr>
<td>Copper</td>
<td>&lt; 0.02</td>
<td>0.8</td>
<td>4.0</td>
<td>9.2</td>
</tr>
<tr>
<td>Ammonium</td>
<td>&lt; 0.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Dissolved organic carbon</td>
<td>0.83</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Total Carbon, %</td>
<td>NA</td>
<td>4.16</td>
<td>18.3</td>
<td>27.2</td>
</tr>
<tr>
<td>Total Nitrogen, %</td>
<td>NA</td>
<td>0.17</td>
<td>2.85</td>
<td>1.51</td>
</tr>
<tr>
<td>Moisture, %</td>
<td>NA</td>
<td>31.9</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^1$Units are mg/L.

$^2$Values are for extractable elements and units are mg/kg on a dry basis, unless otherwise specified.

$^3$Estimated from charge balance calculations.

NA: Not analyzed.
Table 3.2. Rates of alkalinity production, acidity removal, and sulfate reduction in active microcosms containing chitin complex (CC), sodium lactate, and spent mushroom compost (SMC).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CC</th>
<th>Sodium Lactate</th>
<th>SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkalinity production</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag period, $t_{\text{initial}}$ (d)</td>
<td>1</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Rate (mg CaCO$_3$/L-d)</td>
<td>26.5</td>
<td>30.3</td>
<td>0</td>
</tr>
<tr>
<td><strong>Acidity removal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag period, $t_{\text{initial}}$ (d)</td>
<td>1</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Rate (mg CaCO$_3$/L-d)</td>
<td>-25.2</td>
<td>-12.7</td>
<td>-10.1*</td>
</tr>
<tr>
<td><strong>Sulfate reduction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag period, $t_{\text{initial}}$ (d)</td>
<td>9</td>
<td>27</td>
<td>50</td>
</tr>
<tr>
<td>Rate (mg SO$_4^{2-}$/L-d)</td>
<td>-17.8</td>
<td>-24.8</td>
<td>0</td>
</tr>
</tbody>
</table>

*Acidity removal rate was calculated for the first 12 days of incubation.
CHAPTER 4
Manganese Removal from Mine Impacted Water Using Crab-Shell Chitin: I. Role of Associated Minerals in Precipitation

4.1. Abstract

The performance of crab-shell chitin as a substrate for the abiotic and anoxic treatment of mine impacted water (MIW) was assessed to isolate its chemical and physical treatment mechanisms. Alkalinity generation and metal (Mn, Fe, Al) removal with crab-shell chitin were evaluated and compared to those obtained using limestone in closed-system and kinetic tests. Raw (R-SC20) and deproteinized (DP-SC20) crab-shell chitin were tested and compared to evaluate the effect of chitin-associated minerals and proteins. Systems for all tests were prepared and operated inside an anaerobic chamber by mixing crab-shell chitin or limestone with anoxic, synthetic MIW (SMIW) at predetermined ratios.

In closed systems, 5 g/L of R- or DP-SC20 completely removed (≥95%) both Mn and Fe from single-metal SMIW (initial concentration = 10 mg/L). After 72 h, pH increased from 3 to 9.2-10.2, while 83-187 mg CaCO$_3$/L of alkalinity was generated. In contrast, 5-125 g-limestone/L only raised the pH to 7.8-8.3, leading to lower alkalinity levels (56-63 mg CaCO$_3$/L) and poor metal removal efficiencies (≤85%). In kinetic tests with 5 g-DP-SC20/L, removal of ≥95% of the initial metal load was achieved after 0.5, 6, and 48 h for Al, Fe, and Mn, respectively. Geochemical calculations (PHREEQC) indicate that limestone-treated systems were close to equilibrium with calcite, while octacalcium phosphate (Ca$_4$H(PO$_4$)$_3$) appears to be a controlling phase in systems treated with R- and DP-SC20. Precipitation of aluminum hydroxides and rhodochrosite (MnCO$_3$) and/or MnHPO$_4$ are the probable mechanisms for Al and Mn removal. In the case of iron, geochemical calculations point to the precipitation of iron hydroxides; however, visual observations suggest the formation of green rust, a precursor of other, more stable phases like goethite or lepidocrocite. Several factors could be attributed to the faster changes observed with R- and DP-SC20 compared to limestone: a significantly larger surface area, the presence of phosphates, and the release of organic compounds. These results are the first to verify and quantify the capacity of crab-shell chitin to treat MIW abiotically.
4.2. Introduction

In the search for more efficient ways to passively remediate the very recurrent issue of mine impacted waters (MIW), a great variety of organic substrates have been evaluated. There are numerous reports of promising results using waste materials like animal manure, vegetable compost, sawdust, and leaf mulch (Johnson and Hallberg, 2005; Gibert et al., 2002). Crab-shell chitin has also proven to be a promising substrate for the passive treatment of MIW. Rapid pH and alkalinity increases, biological sulfate reduction, and metal removal have been observed under laboratory conditions (Daubert and Brennan, 2007; Korte et al., 2008; Robinson-Lora and Brennan, 2008; Robinson-Lora and Brennan, 2009b). Of special interest is the outstanding efficiency of crab-shell chitin to remove Mn compared to other substrates in both laboratory and field studies. In laboratory microcosms tests using spent mushroom compost, sodium lactate, and crab-shell chitin, the latter was the only substrate capable of promoting significant Mn removal in both live and killed systems (>73%, Robinson-Lora and Brennan, 2008). In field studies, enhanced Mn removal (86%) was observed with crab-shell chitin, compared to other substrates (50% for ethanol, wood chips/hay, wood chips/corn stover; Venot et al., 2008).

The rapid changes in pH and alkalinity and a portion of the metal removal have been attributed to the dissolution of chitin-associated carbonates. These carbonates (especially CaCO$_3$ in the form of calcite or aragonite) are naturally present in the shell of crabs and other crustaceans to provide structural strength (Percot et al, 2003) and had been previously identified as responsible for the removal of metals. Lee and colleagues (1997, 2004) used crab (Protunus trituberculatus) shell particles containing approximately 17% chitin, 10% protein, and 58% calcium carbonate to remove Pb and Co from metal-bearing wastewater. Maximum lead and cobalt uptakes of 1300 mg Pb/g and 510 mg Co/g were obtained, and SEM-EDS and XRD analyses indicated the formation of Pb- and Co-carbonate precipitates and their sorption onto the crab shell surface. While promising for the treatment of heavy metals, their tests were conducted under conditions different from those typically found in acidic MIW. In particular, the low pH, presence of high concentrations of sulfate, and variety of metals (mainly Fe and Al) in MIW could affect
both carbonate dissolution and manganese removal, and therefore requires further evaluation.

The aim of this study was to evaluate the role of chitin-associated minerals in MIW treatment under abiotic and anoxic conditions and to develop a better understanding of the mechanisms that drive the observed changes in systems where metal oxidation is limited. The performance of two different purities of crab shell (raw and deproteinized) was assessed and compared to limestone. In particular, we focused on the removal of Mn, since this metal has shown to be unusually difficult to remove in most conventional treatment systems.

4.3. Methods

4.3.1. Chemicals

All chemicals used in this study were reagent grade or better. Ultra High Purity argon gas (UHPAG) was purchased from MG Industries (Malvern, PA). ChitoRem® SC-20 (minimally processed crab shell), derived from Dungeness crab (JRW Bioremediation, LLC, Lenexa, KS), was used as an example of chitinous material. Raw SC-20 was rinsed with deionized water to remove readily soluble salts and dried overnight at 50°C. The obtained material (R-SC20) was sieved (No. 7 and 140) to remove big particles (>2.80 mm) and fines (< 0.106 mm). Another fraction was deproteinized according to protocols described in previous studies (Percot et al., 2003) using 1 N NaOH; the absorbance of the supernatant was measured at 280 nm, following the completion of the reaction indicated by the attainment of a maximum plateau. Particles were then washed with deionized water until neutrality of the rinsate, and dried and sieved as above to produce DP-SC20. Limestone (LS) was obtained from a local quarry (Martin Limestone, PA), pulverized using a mortar and pestle, and then sieved (No. 20 and 140) to obtain two particle size fractions: 0.85 – 0.106 mm (particulate) and < 0.106 mm (fine).

4.3.2. Characterization of particles

The chemical composition of all solids was determined in duplicate by lithium metaborate fusion, followed by ICP-AES analyses (Perkin-Elmer Optima 5300) at the Materials Characterization Laboratory at The Pennsylvania State University. Rock
standards were used to calibrate the results. The total carbon and nitrogen of the obtained solids were analyzed by combustion, using a Fisons NA 1500 Elemental Analyzer, at the Agricultural Analytical Services Laboratory at the Pennsylvania State University. Mineralogical composition of the solids was determined by powder XRD (Scintag, Inc., Cupertino, CA). The surface area of the solids was measured in duplicate by physical adsorption of N\textsubscript{2} and calculated using the BET (Brunauer, Emmett and Teller) method with a Micrometrics Instrument Corporation ASAP 2020. The morphology and composition of the solids were observed and measured using a FEI Quanta 200 environmental scanning electron microscope (ESEM) equipped with an Oxford Inca 200 EDS.

4.3.3. Synthetic MIW (SMIW) solutions

Tests were conducted using synthetic MIW (SMIW) to limit the affect of other metals (likely present in natural MIW) on the results. SMIW solutions were prepared using Na\textsubscript{2}SO\textsubscript{4} (1.4 g/L or 10 mM) as the background salt. Appropriate amounts of FeCl\textsubscript{2}, Mn(NO\textsubscript{3})\textsubscript{2}, and/or Al(NO\textsubscript{3})\textsubscript{3} were added to generate single or multiple metal (Fe, Mn, and/or Al) solutions at the desired initial concentrations. The initial pH was adjusted to 3.0 (unless otherwise specified), by adding appropriate amounts of 1 N H\textsubscript{2}SO\textsubscript{4}. Prior to use, solutions were deaerated with UHPAG for 2 – 3 h and equilibrated in an anaerobic chamber (Coy Laboratory Products, Inc., Grass lakes, MI) for at least 24 h.

4.3.4. Experimental setup for closed systems to evaluate total Mn removal under different solid loadings and Mn concentrations

Closed systems were used to evaluate the Mn-removal capacities of R-SC20, DP-SC20, and LS under different conditions. Systems were in prepared in glass centrifuge tubes, inside an anaerobic chamber. Filtered (0.2 µm), anoxic SMIW (40 ml) was mixed with predetermined amounts of R-SC20, DP-SC20, or limestone. To evaluate the effect of solid load, SMIW with an initial Mn concentration of 10 mg/L was treated with solid loads ranging from 2 to 25 g/L for DP-SC20, 2 to 12.5 g/L for R-SC20, and 5 to 125 g/L for limestone. To evaluate the effect of the initial manganese concentration, a fixed solid load of 5 g/L (R-SC20 or DP-SC20 only), was used to treat SMIW with initial Mn concentrations.
ranging from 10 to 250 mg/L. Systems were prepared in triplicate, with duplicate blanks (without solid addition). Tubes were sealed and continuously stirred on an orbital shaker for 72 h. The 72-h contact time was chosen based on preliminary tests conducted in our laboratory to ensure that all chemical transformations in the systems were completed. After the contact time was elapsed, the tubes were opened and the pH of the solution was measured inside the anaerobic chamber. In addition, two subsamples were taken and filtered (0.2 µm). One of the subsamples was promptly (<6 h) analyzed for alkalinity and the other was preserved with 1.5 ml/L conc. HNO₃ for analyses of dissolved metals, S, and P.

4.3.5. Experimental setup kinetic tests to evaluate the rate of Mn removal under varying metals, pH, and dissolved oxygen conditions

Kinetic tests were conducted to evaluate manganese removal and alkalinity generation rates using R-SC20 or DP-SC20 under different conditions (Table 4.1). Reactors (2 L) were operated inside an anaerobic chamber with continuous stirring at 300 rpm. A fixed solid load of 5 g/L was used, which was added at t = 0. The pH was continuously monitored and duplicate samples (10 ml) were taken at predetermined time intervals of t = 0, 10, 20, and 30 min, and at 1, 2, 6, 12, 24, and 72 h. Samples were filtered (0.2 µm) and promptly (<6 h) analyzed for alkalinity. A subsample was diluted (1:10) and preserved in acidified-anoxic deionized water (2 ml/L conc. HNO₃) for metal, S, and P analyses.

4.3.6. Analytical methods

Electrodes were used to measure ORP (platinum electrode, ORION 9778 BN), pH (Accumet® BASIC, AB15 connected to a Thermo-ORION pH probe), and ammonium concentrations (ISE ORION 9512). Alkalinity was measured by titrations with 0.02 N H₂SO₄ according to the procedure described in Standard Methods (titration end point pH 4.5, APHA, 2005). Dissolved metal, sulfur, and phosphorus concentrations were measured by inductively coupled plasma emission spectrometry (ICP, Leeman Labs PS3000UV) at the Materials Characterization Laboratory at The Pennsylvania State University. Volatile fatty acids (VFAs) were determined by high performance liquid
chromatography (HPLC, Waters 2695) as described in Robinson-Lora and Brennan (2009a). The ascorbic acid spectrophotometric method (APHA, 2005) was used to measure the concentration of orthophosphate.

Statistical analyses of the collected data were performed using MINITAB® statistical software (Minitab Inc., State College, PA). The geochemical computer program PHREEQC (Parkhurst and Appelo, 1999) was used to estimate the saturation indexes (SI) of several aluminum, iron, and manganese phases. Equilibrium constants used in the calculation were taken from the MINTEQA2, version 4.0, database (USEPA, 1999). The concentrations of chloride and nitrate released from the added metal salts were estimated based on the initial, measured concentration of their associated metals (Fe for Cl\textsuperscript{−}, and Mn + Al for NO\textsubscript{3}−).

4.4. Results

4.4.1. Characterization of solids

While very limited porosity was detected in the LS samples (Table 4.2), a moderate porosity was measured in the chitinous materials, with DP-SC20 having a higher porosity than R-SC20. Results from the chemical characterization (Table 4.2) revealed that calcium is the main component of LS (56.5% as CaO), followed by carbon (8.6%) and traces of silicon, aluminum, iron, magnesium, and potassium (<1%). In R-SC20 and DP-SC20, calcium was also the main component, but its levels were almost half of those found in LS (29 – 38% as CaO). In addition, R- and DP-SC20 also contained similar levels of carbon (19 – 23 %) and moderate levels of magnesium (1.8 – 2.5% as MgO), phosphorus (3.7 – 5.0% as P\textsubscript{2}O\textsubscript{5}), and nitrogen (1.4 – 3.9 %). Other elements were present at trace levels (< 1%). Calcite was detected as the major component of all three solids, with small quartz impurities in LS (Fig. 4.1). The source of phosphorus in the chitinous materials, determined by XRD after calcination of a sub-sample of R-SC20 (550 °C for 2 h), appears to correspond to hydroxyapatite (data not shown). SEM images reveal the greater porosity of the chitinous materials over limestone, which is coincident with BET results (Fig. 4.1).
4.4.2. Closed systems to evaluate total Mn removal under different solid loadings and Mn concentrations

In systems with initial Mn concentrations of 10 mg/L, about 89% of Mn was removed with a solid load of 2 g-DP-SC20/L, while >95% removal was achieved with a solid load of 5 g/L. Higher Mn removal was observed when higher loads of DP-SC20 were used, achieving 99.7% removal with 25 g-DP-SC20/L (Fig. 4.2A). Similar results were obtained when R-SC20 was used, with Mn removals of 85 – 97% for solid loads of 2 – 12.5 g/L. In contrast, in systems with a limestone load of 5 g/L, removals of <8% were observed. Even at much higher loads (125 g/L), the addition of limestone (particulate or fine) promoted only 76 and 85% removal of Mn, respectively. In general, higher removals were observed with fine limestone than with the particulate material. Although Mn removal values were significantly different (p-value=0.000) between fine and particulate limestone solids, the differences did not exceed 10%. A direct relationship between the initial and the final manganese in the systems seems to exist. As the initial manganese concentration in the systems increased, its removal by both DP-SC20 and R-SC20 decreased (Fig 2B). For both solids, manganese removal decreased to ~60% when Mn = 250 mg/L.

After 72 h, the pH of systems amended with DP-SC20 increased from 3 to 9.4 – 10.2 and 67 – 150 mg CaCO₃/L of alkalinity was generated (Fig. 4.2C and E). Slightly lower pH values were measured in systems treated with R-SC20 (pH 9.2 – 9.6), yet almost double alkalinity concentrations were reached by the end of the contact time (115 – 223 mg CaCO₃/L). In contrast, limestone (particulate or fine) addition only raised the pH to 7.8 – 8.3, and generated much lower alkalinity levels (56 – 63 mg CaCO₃/L). For a fixed solid load of 5 g/L DP-SC20 or R-SC20, the increase in the initial manganese concentration resulted in smaller increases in pH values (Fig. 4.2D). For both solids, the observed final pH decreased to 7.7 – 7.9 when Mn = 250 mg/L. On the other hand, increases in Mn appear to have opposite effects on the final alkalinity, depending on the type of solid used (Fig. 4.2F). While alkalinity drastically increased with increases of Mn in R-SC20-treated systems (from 176 to 525 mg CaCO₃/L), a minor decrease was observed in DP-SC20-treated systems (from 83 to mg 55 CaCO₃/L).
Along with the increase in alkalinity, calcite dissolution from all the evaluated materials resulted in the occurrence of important concentrations of calcium by the end of the test. Final Ca concentrations increased with the solid load for R-SC20 and limestone, as well as with the increase in the initial Mn concentration (Fig. 4.2G and H). Calcium concentrations were always much higher with R-SC20 than with the other solids (71 – 234 mg/L), while in limestone-treated systems, Ca levels had very little variation (49 – 66 mg/L). Systems treated with DP-SC20 exhibited the lowest levels of calcium (23 – 41 mg/L). In addition, an inverse relationship was observed between the final Ca concentration and the solid load in the DP-SC20 systems. The cause of this distinct observation is not clear, but it may be attributed to the occurrence of Ca/Mn co-precipitation.

Phosphorus release from DP-SC20 and R-SC20 was also observed (Fig. 4.2I and J). Concentrations did not appear to be affected by either the solid load or the initial concentration of Mn, but they seem to be dependent on the type of solid used. Higher concentrations were always measured in R-SC20-treated systems (0.8 – 1.7 mg/L P) than in those treated with DP-SC20 (≤ 0.2 mg/L P). Measurements using the ascorbic acid spectrophotometric method confirmed that the speciation of the released phosphorus corresponds mainly to orthophosphates (data not shown).

4.4.3. Kinetic tests to evaluate the rate of Mn removal under varying metals, pH, and dissolved oxygen conditions

In kinetic tests, removal of ≥95% of the initial Mn load was achieved after 24 h in most systems (Fig. 4.3A and B). Similar results were observed in single- and 2-metal systems, as well as under aerobic conditions, while the presence of the three metals simultaneously or the use of R-SC20 resulted in slightly lower removal (93% at t = 24 h), although similar removal was achieved by the end of the test. Even when Mn was increased to 100 mg/L, the addition of 5 g-DP-SC20/L promoted 81.9% removal in 72 h. The initial pH of the SMIW also affected Mn removal: faster removal was achieved with pH₀ = 4 (>95% after 12 h), while pH₀ = 2 resulted in a significantly reduced removal (only 87.5% by the end of the test).
Rapid increases in pH were observed during the first 2 h of the experiment, and then slowed to reach a steady plateau by ~10 h. Systems with single or multiple metals, at initial concentrations of 10 mg/L, reached final pH values of 8.9 – 9.9 (Fig. 4.3C). Similar changes were observed when the reactor was operated under aerobic conditions or when R-SC20 was used (Fig. 4.3D). The presence of aluminum and higher Mn concentrations appeared to result in lower pH values, likely due to the formation of Al-hydroxides. Different initial pH values resulted in different rates of pH change (Fig. 4.3D): the system with pH\textsubscript{o} = 4 reached a pH of 9.7 after only 1 h, while the system with pH\textsubscript{o} = 2 only reached a final pH of 8.4 by the end of the test (72 h).

Rapid increases in alkalinity were also observed in all systems (Fig. 4.3E and F). Changes in most systems were relatively similar, with final concentrations ranging between 64 and 106 mg/L as CaCO\textsubscript{3}. Lower alkalinity was observed in the system with higher Mn concentrations, while higher alkalinity was generated in the systems with aluminum or lower initial pH. However, in the system with DP-SC20 at pH\textsubscript{o} = 2, alkalinity levels started to decrease after 24 h, reaching 118 mg/L as CaCO\textsubscript{3} by the end of the test. The use of R-SC20 promoted sustained increases in alkalinity and significantly higher (at least two-fold) final alkalinity levels than in the other systems, presumably due to the hydrolysis of chitin-associated proteins.

The dissolution of the chitin-associated carbonates also resulted in important changes in Ca, (Fig. 4.3G and H). Concentrations rapidly increased during the first 2 h of operation, reaching a plateau by 6 h in most cases. Similar Ca concentrations were reached by the end of the tests for Mn and Mn/Fe systems, as well as under aerobic conditions (32 – 36 mg/L). Lower concentrations were observed when pH\textsubscript{o} = 4 (20 mg/L). In contrast, higher concentrations were observed in the rest of the systems: when 10 mg/L of Al were present, Ca reached 57 – 63 mg/L, while 100 mg/L of Mn and the use of R-SC20 promoted the release of 89 – 100 mg/L Ca. Tremendously higher Ca concentrations (312 mg/l) were observed when pH\textsubscript{o} = 2, due to enhanced calcium carbonate dissolution at low pH. In addition, changes in Mg and Sr concentrations were also observed, following very similar trends to those observed with calcium (data not shown). This is not unexpected since Mg and Sr are structurally associated with Ca in
the crab shell. However, final Mg and Sr concentrations were much lower than Ca (9 – 40 and 0.6 – 4.8 mg/L, respectively).

Rapid phosphorus release was observed in most tests (Fig. 4.3I and J), except when pH$_o$ = 4 for which P was below the detection limit (0.05 mg/L) throughout most of the test. The maximum P concentrations were reached within 30 min, without exceeding 2.3 mg/L in most cases. The use of R-SC20 or an initial pH of 2.0 promoted higher P concentrations, reaching 4.75 and 27.6 mg/L, respectively. After this initial peak, P concentrations rapidly decreased, leveling around 0.3 mg/L on average (0.9 mg/L with R-SC20 and pH$_o$ = 2) by the end of the test. Measurements using the ascorbic acid spectrophotometric method confirmed that the speciation of the released phosphorus corresponds mainly to orthophosphates (data not shown).

In most systems, 100% removal of Al and Fe were achieved after 0.5 and 12 h, respectively (Fig. 4.4). When the initial Mn concentration was increased to 100 mg/L, the total removal of iron was delayed to 48 h. As the pH of the systems increased, aluminum was released back to the solution after 6 h, resulting in a final Al removal of 58 – 98%. The removal of iron and aluminum was also accompanied by the formation of green (in systems with Fe) and white (in systems with Al) precipitates.

The almost immediate release of small amounts of formate was observed only when R-SC20 was used. The concentration of formate remained relatively constant during the first 24 h of treatment at around 0.12 mM (4 mg/L). Afterwards, its concentration slowly increased, reaching 0.4 mM (19 mg/L) by the end of the test. Other VFAs (acetate, propionate) were detected only after 48 h of treatment, at concentrations of 0.3 – 0.5 mM (data not shown).

4.5. Discussion

4.5.1. Mineral dissolution and alkalinity

Based on the chemical composition of the solids used, limestone particles have about 60% more calcite than crab-shell chitin. However, this higher provision of alkalinity source did not result in greater pH and alkalinity changes in systems containing limestone. Under aerated conditions with calcite addition, the presence of atmospheric CO$_2$ limits the maximum pH achievable to pH ~8.3. However, in this study, tests were conducted in
a CO₂-depleted environment for which thermodynamic calculations show that calcite dissolution can result in pH > 9. Several arguments could explain the lower changes observed in systems treated with limestone, compared to those treated with raw and deproteinized crab-shells. BET analyses and SEM visualization reveal a larger surface area of the R- and DP-SC20 in comparison to that of limestone. Indeed, limestone particles appeared much more compact and less porous. According to Stumm and Morgan (1996), the limited surface area of limestone should result in slower dissolution rates, especially once the system has reached near-neutral pH. However, researchers have argued that BET surface area is not the best parameter to correlate dissolution rates (Cubillas et al., 2005). Authors suggest that not all the measured BET surface area is really available for dissolution, and that the use of geometric surface area is a better proxy to report and compare reactive surface areas for CaCO₃ dissolution rates. Nonetheless, when using geometric surface area, the normalized dissolution rates of calcite and aragonite were found to be comparable for both abiogenic (pure minerals) and biogenic (bivalves; Cubillas et al., 2005) sources. If this is the case, and since the particle size ranges of the materials used in the present study are similar, the greater BET area and porosity of the crab-shell chitin cannot explain the faster dissolution of its associated carbonates. This suggests that the biogenic character of the chitin-associated carbonates may give them a higher reactivity, making them dissolve more readily. It has been suggested that the reactivity of different faces of a crystal is different, and that biogenic carbonates may exhibit preferential crystal orientation (Cubillas et al., 2005). This preferential orientation may be responsible for the enhanced dissolution observed here.

Another major difference between the materials used is the presence of other minerals (mainly phosphates) as well as organic compounds (mainly proteins) associated with chitin in the crab shells. It is also possible that not only proteins but also some readily soluble minerals were removed during the preparation of DP-SC20, causing some of the differences observed between it and the raw material. The much higher calcium concentrations observed in the R-SC20 system indicate that the extent of CaCO₃ dissolution was greater than in the systems treated with DP-SC20. In addition, a rapid release of formate and other fatty acids from R-SC20 was observed. These organic compounds are likely the product of the hydrolysis of residual proteins present in this
minimally processed material, since tests assessing microbial activity indicated that it was negligible. Such molecules could have partially contributed to the higher alkalinity measured with R-SC20, as well as the faster dissolution of its associated carbonates.

In most cases, after 72 h of contact time in the closed tests, less than 2.5% of the initial calcium (based on the solid load) was released from the evaluated solids. However, while calcium and alkalinity concentrations varied with the load of R- or DP-SC20, final values in limestone-treated systems were very similar, regardless of the loading. Indeed, thermodynamic calculations indicate that limestone systems were close to equilibrium with respect to calcite while systems treated with either DP-SC20 or R-SC20 were supersaturated with respect to this mineral (Fig. 4.5A). It is suspected that the presence of VFAs and other organic compounds, released from the untreated material, promotes higher supersaturation (Stumm and Morgan, 1996). It is also possible that the presence of another more caustic, fast dissolving mineral (like Ca(OH)$_2$) in the crab shells is the responsible for driving the pH to values close to pH 10. Such types of minerals were not detected during the characterization of the solids, suggesting that they are present in trace quantities, if at all. It appears from geochemical modeling that calcium concentrations in systems treated with either DP-SC20 or R-SC20 are controlled by the precipitation of octacalcium phosphate (Ca$_4$H(PO$_4$)$_3$) (Fig. 4.5A). This phase is known as a precursor for the formation of apatite (Stumm and Morgan, 1996). This suggests that the phosphates released from the chitinous materials play an important role in the observed changes.

4.5.2. Manganese removal

The removal of Mn observed in this study can be attributed to sorption and/or (co)precipitation. Sorption onto organic substrates has been already reported to occur in passive MIW treatment (Webb et al, 1998; Willow and Cohen, 2003) and chitin has been identified as promising sorbent for metals like zinc, copper, cadmium, lead, and iron (Benguella and Benaissa, 2002; Rae and Gibb, 2003; Karthikeyan, et al., 2005). Results from the tests conducted to quantify Mn showed that sorption onto chitin or its associated proteins is occurring (Chapter 5). However, the values of the estimated sorption capacity indicate that this mechanism cannot account for the observed removal under slightly acidic conditions. Mn can also be sorbed onto the calcite surface, as has been previously
reported (Zachara et al., 1991). Co-precipitation of calcium and manganese could also occur: Sibrell et al., (2007) reported significant removal of manganese via this mechanism in pulsed limestone beds, at pH values below 8.3.

The most common strategy for Mn removal from aqueous systems is oxidation, which requires high pH since abiotic and biological rates are slow for pH < 8.0. Previous researchers have also reported poor Mn removal in the presence of Fe (Johnson and Younger, 2005). In spite of the high pH values reached at early times in the present study, Mn oxidation can be ruled out since the experiments were performed in an anaerobic chamber, and the removal of Mn and Fe were observed to occur simultaneously. Furthermore, there is a great similarity between the results obtained for the aerobic test and those obtained under anaerobic conditions. This indicates the absence of Mn oxidation, and that the Mn removal mechanisms are likely the same as under anaerobic conditions. In addition, Mn oxidation is usually characterized by the formation of black precipitates, which were not observed in the present study.

Geochemical calculations indicate the possible precipitation of several minerals (Fig. 4.5B). After a 72-h contact time, all systems appear to be supersaturated with respect to rhodochrosite (MnCO$_3$) and manganese hydrogen phosphate (MnHPO$_4$). The formation of manganese carbonates such as MnCO$_3$ and kutnahorite (MnCa(CO$_3$)$_2$) has been previously reported under reductive conditions (Waybrant et al., 1998), as well as in aerated systems (Bamforth et al., 2006). In this case, it is plausible to suspect that the poor Mn removal observed in the limestone systems is not due to insufficient alkalinity/carbonate provision, but rather to slow kinetics. Indeed, high levels of supersaturation are often found in natural anaerobic environments, where precipitation of rhodochrosite is expected to limit the concentration of Mn in solution (Jensen et al., 2002). Lebron and Suarez, (1999) showed that precipitation of manganese carbonates is kinetically regulated and that rhodochrosite-supersaturated solutions (SI = 2 – 5) can remain metastable for at least 72 h; a time frame that is comparable with the residence time of many types of passive treatment systems (Ziemkiewicz et al., 2003). In contrast, it is possible that the significantly higher concentrations of calcium and carbonates released by the dissolution of the chitin-associated calcite in R- and DP-SC20 treated systems thanks to their much higher levels of rhodochrosite-supersaturation. Another
A plausible explanation is the co-precipitation of Ca and Mn, which has been observed in previous studies (Sibrell et al., 2007).

The alternative sink for manganese in the systems treated with either DP-SC20 or R-SC20 is precipitation as a phosphate mineral (MnHPO$_4$). This phase is highly insoluble and has been reported to occur when hydroxyapatite is used for the control of acid drainage (Evangelou, 1995). Removal of Mn aided by the presence of phosphates has also been reported in anaerobic digestors via precipitation (Carliell-Marquet and Wheatley, 2002). In addition, the negative charge of the phosphate group can also induce metal chemisorption. In recent studies, phosphate pretreatment significantly increased the adsorption capacity of rice husk as a sorbent for lead, copper, zinc, and manganese removal (Mohan and Sreelakshmi, 2008). However, as indicated by the geochemical calculations (Fig. 4.5B), all systems were significantly oversaturated with respect to MnHPO$_4$, despite the high Mn removal. It is possible that phosphorus had preferentially precipitated as Ca$_4$H(PO$_4$)$_3$ limiting the formation of MnHPO$_4$. For most systems, P concentrations were clearly limited by the precipitation of either of the considered phases (Fig. 4.5C). In addition, the release of VFAs and other organics could also be the cause of the slightly lower Mn removal in systems treated with R-SC20, since organic compounds can act as chelating agents, increasing the solubility of metals (Ahumada et al., 2001; Carliell-Marquet and Wheatley, 2002).

### 4.5.3. Effect of coexisting metals and initial pH

The presence of aluminum had some effects on the final pH and alkalinity of the systems, but did not negatively affect Mn removal. In this case, the precipitation of Al hydroxides consumed some hydroxyl ions, leading to lower pH values. Aluminum hydroxides have been shown to act as a sorbent or partner for co-precipitation (Gibert et al., 2005). Therefore, it is likely that Al precipitation partly contributed to the removal of the other metals (Mn and Fe), leading to higher alkalinity values due to the excess carbonate ions that were not used for Mn/Fe precipitation. Aluminum solubility is pH dependant, having a narrow range of pH within which its hydroxides are insoluble (pH 5 – 8). As the pH in the systems continued increasing with time, Al speciation shifted from Al(OH)$_3$ to Al(OH)$_4^-$, leading to partial redissolution (Fig. 4.6). It appears that the
presence of other metals inhibited the extent of this redissolution, as the lowest amount of Al released back in the system was observed with 100 mg/L of Mn. The redissolution of aluminum is not desirable but it can be easily limited by controlling the residence time under continuous-flow conditions. In previous column studies using raw crab-shell chitin with a 12-h contact time, we have reported complete and sustained Al removal (Robinson-Lora and Brennan, 2009b).

The presence of iron did not negatively influence the removal of Mn. Instead of the typical orange ferric hydroxides, green-colored precipitates were observed in the Fe-containing systems. These precipitates likely correspond to green rust and are an indication that iron oxidation was limited. Calculated SI values indicate the possible formation of iron hydroxides (ferrihydrite, goethite, lepidocrocite), for which (carbonate or sulfate) green rust has been identified as a precursor (Abdelmoula et al., 1996).

Initial pH of the treated solution had a clear impact on the performance of the systems. The faster changes in pH that occurred when pH_0 = 4, promoted the fastest Mn removal of all tests. In contrast, the harsher conditions dictated by a lower initial pH (pH_0 = 2.0) promoted greater dissolution of the chitin-associated carbonates, revealed by the much higher Ca and alkalinity concentrations. These conditions were not enough to promote Mn removal, however. The delay in metal removal may be associated with the lower pH of the system. The decrease in Ca at later times could be due to calcite re-crystallization, suggesting the occurrence of Mn/Ca co-precipitation, or the precipitation of octacalcium phosphate (Ca₄H(PO₄)₃).

4.6. Conclusions

Results from the present study demonstrate the important role of chitin-associated minerals (carbonates and phosphates) and proteins in the passive remediation of MIW using crab-shell chitin. The intricate and complex structure formed by these three components results in a relatively high surface area compared to other common treatment materials. This characteristic, combined with an apparently greater reactivity of its chitin-associated minerals and aided by the release of organic compounds and phosphates, makes crab-shell chitin an attractive alternative material for fast alkalinity generation and the treatment of historically difficult Mn-bearing MIW. More research is needed to
evaluate the performance and longevity of this material under continuous-abiotic conditions.

4.7. Acknowledgements

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4.8. References


Gibert, O., de Pablo, J., Cortina, J. L., Ayora, C. 2002. Treatment of acid mine drainage by sulfate-reducing bacteria using permeable reactive barriers: A review from
laboratory to full-scale experiments. Reviews in Environmental Science and Biotechnology, 1:327–33.


Fig. 4.1. SEM micrographs (left panel), EDS spectra (central panel), and XRD patterns (right panel) of R-SC20, DP-SC20, and limestone. (▼) calcite (CaCO₃), (○) quartz (SiO₂).
Fig. 4.2. Final manganese concentration, pH, alkalinity (as CaCO$_3$), calcium, and phosphorus concentrations in SMIW systems treated with DP-SC20, R-SC20, particulate limestone (Part-LS), and fine limestone (Fine-LS). Left panel: Effect of solid load when Mn$_o$ = 10 mg/L. Right panel: Effect of initial manganese concentration with a solid load of 5 g/L. Initial pH = 3.0. Values represent triplicate averages; error bars represent one standard deviation.
Fig. 4.3. Manganese, pH, alkalinity, calcium, and phosphorus changes in reactors containing SMIW and 5 g/L of DP-SC20 or R-SC20 (right panel). Initial metal concentrations were 10 mg/L each, except for Mn100/Fe/Al (left panel), where Mn₀ = 100 mg/L. Initial pH was 3.0, and tests were conducted anaerobically, unless otherwise specified (right panel). See detailed experimental conditions in Table 4.2. Values represent duplicate averages; error bars represent one standard deviation. Note secondary axis for Ca and P for selected data series.
Fig. 4.4. Iron (A) and aluminum (B) changes in reactors containing SMIW and 5 g/L of DP-SC20. Initial metals concentrations were 10 mg/L each, except for Mn100/Fe/Al (panel B), where Mn$_0$ = 100 mg/L, and initial pH was 3.0. Detailed experimental conditions for each test are provided in Table 4.2. Values represent duplicate averages; error bars represent one standard deviation.
Fig. 4.5. Relationship between final pH and final concentration of Ca (A), Mn (B), and P (C) in all the evaluated systems in this study (72 h contact time). Lines represent the concentration of the element in equilibrium with: calcite (CC), hydroxylapatite (HAP), octacalcium phosphate (OCP), rhodochrosite (MnC), and MnHPO$_4$ (MnP). Calculations for calcite and rhodochrosite assumed a total C concentration of 1 mM.
Fig. 4.6. Relationship between pH and Al concentration in kinetic tests containing aluminum. Lines represent the concentration of the element in equilibrium with amorphous Al(OH)$_3$ (AOA) and gibbsite (GIB). Detailed experimental conditions for each test are provided in Table 4.2. Values represent duplicate averages.
Table 4.1. Operating conditions for kinetic tests.

<table>
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<tr>
<th>System</th>
<th>Fe</th>
<th>Mn</th>
<th>Mn/Fe</th>
<th>Mn/Al</th>
<th>Mn/Fe/Al</th>
<th>Mn100/Fe/Al</th>
<th>Mn pH=2</th>
<th>Mn pH=4</th>
<th>Mn aerobic</th>
<th>Mn RSC20</th>
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<td>pH_0</td>
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<td>2.91</td>
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<td>3.12</td>
<td>2.91</td>
<td>3.07</td>
<td>2.00</td>
<td>4.13</td>
<td>3.03</td>
<td>3.02</td>
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<tr>
<td>Mn_a</td>
<td>-</td>
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<td>9.1</td>
<td>10.0</td>
<td>9.9</td>
<td>101.2</td>
<td>9.6</td>
<td>10.1</td>
<td>10.0</td>
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<tr>
<td>Fe_a</td>
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<td>-</td>
<td>9.1</td>
<td>-</td>
<td>9.6</td>
<td>9.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Al_a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9.2</td>
<td>9.5</td>
<td>9.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Solid: DP-SC20, R-SC20

*aConcentration in mg/L.

Table 4.2. Chemical composition and surface area of the solid materials.

<table>
<thead>
<tr>
<th>Sample</th>
<th>R-SC20a</th>
<th>DP-SC20a</th>
<th>Limestonea</th>
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<tr>
<td>CaO (%)</td>
<td>29.0 (1.7)</td>
<td>38.4 (4.8)</td>
<td>56.5 (0.7)</td>
</tr>
<tr>
<td>MgO (%)</td>
<td>1.68 (0.12)</td>
<td>2.41 (0.13)</td>
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</tr>
<tr>
<td>SrO (%)</td>
<td>0.30 (0.02)</td>
<td>0.37 (0.05)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SiO_2 (%)</td>
<td>&lt;0.05</td>
<td>0.34 (0.37)</td>
<td>0.98 (0.71)</td>
</tr>
<tr>
<td>P_2O_5 (%)</td>
<td>3.58 (0.21)</td>
<td>4.63 (0.51)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Na_2O (%)</td>
<td>0.60 (0.16)</td>
<td>0.48 (0.08)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Al_2O_3 (%)</td>
<td>0.20 (0.19)</td>
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<td>0.45 (0.06)</td>
</tr>
<tr>
<td>Fe_2O_3 (%)</td>
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<td>0.04 (0.04)</td>
<td>0.16 (0.04)</td>
</tr>
<tr>
<td>MnO (%)</td>
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<td>0.06 (0.01)</td>
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<tr>
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<td>&lt;0.05</td>
<td>0.38 (0.38)</td>
</tr>
<tr>
<td>C (%)</td>
<td>22.83</td>
<td>18.88</td>
<td>8.58</td>
</tr>
<tr>
<td>N (%)</td>
<td>3.91</td>
<td>1.38</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Surface area (m²/g) 14 (0.1) 35 (7.0) < 1

aData correspond to average of duplicate measurements, except for C and N; values in parentheses correspond to one standard deviation.
CHAPTER 5
Manganese Removal from Mine Impacted Water Using Crab-Shell Chitin: II. Role of Chitin and Associated Proteins in Biosorption

5.1. Abstract
The manganese removal capacity of two purities of crab-shell chitin was evaluated under different pH conditions by means of kinetic tests and sorption isotherms. Demineralized (DM-SC20) and demineralized/deproteinized (DMP-SC20) crab-shell chitin were tested and compared to evaluate the contribution of chitin and its associated proteins to biosorption. The kinetics of manganese adsorption onto both types of solids was well described by the pseudo-second order model. The adsorption rates depended on the pH of system and the type of solid, with faster changes occurring under alkaline conditions and with DMP-SC20 \( k_2 = 0.411 – 0.535 \) g/mg min) than with DM-SC20 \( k_2 = 0.125 – 0.197 \) g/mg min). The adsorption equilibrium isotherms were best fit by the Langmuir, rather than Freundlich, model. The maximum sorption capacity \( q_m \) was found to depend greatly on the pH of the solution, with minimal or no sorption observed at pH < 5. At higher pH regimes, \( q_m \) values ranged from 0.165 (at pH 5.4) to 0.981 (at pH 8.7) for “pure” chitin (DMP-SC20) and increased from 0.878 (at pH 5.2) to 5.437 (at pH 8.6) when both chitin and protein were present (DM-SC20). Results clearly suggest that the chitin-associated proteins offer additional sorption sites for manganese.

5.2. Introduction
Manganese is a common contaminant found in mine impacted waters (MIW) derived from coal and metal mining (Hallberg and Johnson, 2005). In untreated MIW, manganese concentrations can vary considerably, from < 1 mg/L to hundreds of mg/L, with an average of 10 to 25 mg/L (USEPA, 2008). When ingested or inhaled at high concentrations (>10 mg/d), this metal has been observed to cause neurological disorders in humans (USEPA, 1999). Although it is typically found at lower concentrations in MIW and has a lower toxicity than most of its other metal co-contaminants, Mn still affects the appearance, taste, and odor of water. For these reasons, discharges from mining activities to surface water in the USA must comply with National Pollution
Discharge Elimination System (NPDES) of the Clean Water Act, which limits the monthly average dissolved Mn concentration in the effluent to 2 mg/L.

Over the last three decades many treatment technologies have been developed to address and correct the deleterious effects of mining on the quality of natural streams. However, among the possible contaminants found in MIW, Mn has been found notoriously difficult to remove (Johnson and Younger, 2005; Sibrell et al., 2007). The most common approach for the removal of Mn is its oxidation followed by its precipitation as manganese oxide ($\text{MnO}_2$). However, this requires high pH since abiotic and biological oxidation rates are slow for pH $< 8.0$, and can also be inhibited by the presence of Fe (Johnson and Younger, 2005), the most prevalent metal contaminant in coal mining discharges. In addition, under reducing conditions, partial manganese removal has been reported and attributed to its precipitation as rhodochrosite ($\text{MnCO}_3$, Waybrant et al., 1998).

In recent studies, crab-shell chitin has shown relatively high efficiency to remove Mn under reducing conditions in both laboratory and field studies (Daubert and Brennan, 2007; Korte et al., 2008; Robinson-Lora and Brennan, 2008; Venot et al., 2008). This efficient acidity and metal removal may be attributed to the dissolution of chitin-associated carbonates, which are naturally present in the shells of crabs and other crustaceans to provide structural strength (Percot et al, 2003). The role of this chitin-associated mineral was previously evaluated, and results point to an enhanced calcite dissolution, followed by a fast precipitation of manganese carbonates and/or phosphates (Chapter 4). On the other hand, adsorption has been identified as an important method of metal sequestration, especially under moderately acidic conditions. At initial stages, sorption onto the organic substrate or onto Al-Fe-(oxy)hydroxides has been observed (Webb et al, 1998; Willow and Cohen, 2003; Gibert et al, 2005; Neculita et al, 2007). Although most authors agree on the fact that this biosorption could be a transient phenomenon, results show a great sorption capacity for a variety of substrates, and some authors have recently suggested that it be used as the sole mechanism for remediation of MIW. Inexpensive sorbents like fly ash, red mud, pine bark, bentonite, zeolites (Zoumis et al, 2000), palm fruit bunch, maize cob (Nassar et al, 2004), vegetable compost (Gibert et al, 2005), lignite (Mohan and Chander, 2006), and rice husk (Chockalingam and
Subramanian, 2006) have been tested with promising results. Utgikar et al (2000) also suggested applying metal adsorption as a preliminary step to decrease metal concentrations prior to biological sulfate reduction to prevent microbial inhibition due to metal toxicity.

The aim of this study was to evaluate the role of chitin and its associated proteins in the removal of manganese from MIW, due to sorption processes, under abiotic and anoxic conditions. The performance of two different purities of crab-shell chitin (demineralized and demineralized/deproteinized) was assessed and compared under different pH regimes. This study was conducted in an effort to develop a better understanding of the mechanisms that drive the observed changes in systems treated with a complex material, such as crab-shell chitin, where oxidation of this metal is limited.

5.3. Methods

5.3.1. Chemicals

All chemicals used in this study were reagent grade or better. Ultra High Purity argon gas (UHPAG) was purchased from MG Industries (Malvern, PA). ChitoRem® SC-20 (minimally processed crab shell), derived from Dungeness crab (JRW Bioremediation, LLC, Lenexa, KS), was used as an example of chitinous material, and was purified further as follows. ChitoRem® SC-20 from the manufacturer was rinsed with deionized water to remove readily soluble salts and then demineralized using 1 N HCl, according to protocols described in previous studies (Percot et al., 2003). The completion of the reaction was followed by measuring the pH of the supernatant until the pH remained acidic and stable after a new addition of the hydrochloric acid solution. Particles were then washed with deionized water until neutrality of the rinsate, and dried overnight at 50°C. The obtained demineralized material (DM-SC20) was sieved using sieves No. 7 and 140 to remove big particles (>2.80 mm) and fines (< 0.106 mm). A fraction of the obtained DM-SC20 was deproteinized using 1 N NaOH (Percot et al., 2003) while measuring the absorbance of the supernatant at 280 nm until it reached a maximum plateau, indicating the completion of the reaction. Particles were then washed with deionized water until neutrality of the rinsate, and dried and sieved as above to produce the demineralized and deproteinized product, DMP-SC20.
5.3.2. Characterization of particles

The chemical composition of all solids was determined by lithium metaborate fusion followed by ICP-AES analyses (Perkin-Elmer Optima 5300) at the Materials Characterization Laboratory at The Pennsylvania State University. Rock standards were used to calibrate the results. The total carbon and nitrogen of the obtained solids were analyzed in duplicate by combustion, using a Fisons NA 1500 Elemental Analyzer, at the Agricultural Analytical Services Laboratory at the Pennsylvania State University. The surface area of the solids was measured in duplicate by physical adsorption of N\textsubscript{2} and calculated using the BET (Brunauer, Emmett and Teller) method with a Micrometrics Instrument Corporation ASAP 2020. Sample morphology was observed using a FEI Quanta 200 environmental scanning electron microscope (ESEM).

Acid-base discontinuous titrations were performed at a constant ionic strength (0.1 M NaNO\textsubscript{3}), using a separate portion of the solids (2 g/L DM- or DMP-SC20) for each point of the curve. A known amount of HCl was added to set the initial pH < 3 and then a standardized NaOH solution was used as titrant. The final pH was measured after a contact time of three hours. This rather long contact time was found to be necessary to obtain a stable pH reading. The choice for the down-up titration and the use of one sample per point was based on results from preliminary attempts to characterize these solids in our laboratory that suggested their instability at high pH. Indeed, deproteination can occur with alkaline treatment (Percot et al., 2003). Therefore, this experimental setup was found to be adequate to minimize changes in the solids during the characterization.

5.3.3. Synthetic MIW (SMIW) solutions

Manganese removal tests were conducted using synthetic MIW (SMIW) to limit the affect of other metals (likely present in natural MIW) on the results. To better imitate the conditions found in MIW, solutions were prepared using Na\textsubscript{2}SO\textsubscript{4} (1.4 g/L or 10 mM) as the background salt. The initial pH was adjusted to the desired value by adding appropriate amounts of 1 N H\textsubscript{2}SO\textsubscript{4} or 1N NaOH. Solutions were deaerated with UHPAG for 2 – 3 h, and then appropriate amounts of Mn(NO\textsubscript{3})\textsubscript{2} were added to adjust the initial Mn concentration to the desired value. Prior to use, solutions were equilibrated in an anaerobic chamber (Coy Laboratory Products, Inc., Grass lakes, MI) for at least 24 h.
5.3.4. Kinetic tests

Kinetic tests were conducted to evaluate manganese removal rates under different pH regimes (acidic, pH ~5, and alkaline, pH ~9), with an initial Mn concentration of 10 mg/L. Reactors (400 ml) were operated inside an anaerobic chamber with continuous stirring at 500 rpm to ensure complete suspension of the solids. A fixed solid load of 5 g/L (DM- or DMP-SC20) was used, which was added at t = 0. The pH was continuously monitored and duplicate samples (10 ml) were taken at predetermined time intervals of t = 0, 10, 20, and 30 min, and at 1, 2, 6, 12, 24, 48, and 72 h. Samples were filtered (0.2 µm), diluted (1:10), and preserved in acidified-anoxic deionized water (2 ml/L conc. HNO₃) for Mn analyses.

5.3.5. Batch sorption tests

Manganese sorption onto DM- and DMP-SC20 was evaluated in closed systems, as a function of pH (pH 5 to pH 9) and Mn concentration (0.5 to 250 mg/L). Systems were prepared in duplicate inside an anaerobic chamber, by mixing 20 ml of filtered (0.2 µm), anoxic SMIW with 0.1 g of DM- or DMP-SC20 in acid-washed glass vials. Blank systems (without the addition of solids) were prepared in singlet. Vials were sealed and continuously stirred on an orbital shaker for 72 h. The 72-h contact time was chosen based on preliminary tests conducted in our laboratory to ensure that all chemical transformations in the systems were completed. After the contact time was elapsed, the vials were opened and the pH of the solution immediately measured inside the anaerobic chamber. Samples were filtered (0.2 µm), diluted (1:10), and preserved in acidified-anoxic deionized water (2 ml/L conc. HNO₃) for Mn analyses. The concentration of manganese sorbed onto the solid (qₑ, mg/g) was calculated using Eq. 5.1:

\[ qₑ = \frac{(C₀ - Cᶠ)}{a} \]  

where \( C₀ \) and \( Cᶠ \) are the initial and final concentration of manganese in solution (mg/L), respectively, and \( a \) is solid load used (g/L).

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectrometry (Bruker Optics IFS 66/S, Germany) was used to identify the functional groups involved in the Mn adsorption. For each type of solid, three conditions were selected for analysis: 1) equilibrated at pH 5, 2) equilibrated at pH 9, and 3) after Mn adsorption at pH 9. The
selected solid samples were pressed into a self-supporting disk using a 7mm stainless steel dye and hand press. These disks were then pressed onto a ZnSe ATR crystal for analysis at an angle of incidence of 45 degrees. The spectra were obtained from 800 to 4000 cm\(^{-1}\) at a resolution of 6 cm\(^{-1}\) with an accumulation of 400 scans.

5.3.6. Analytical methods

The pH was measured using a hand-held VWR® sympHony® pH meter provided with a Gel 3-in-1 electrode. Daily calibrations were carried out using buffers of pH 4, 7, and 10 and slopes were consistently greater or equal to 98.5% of the Nerst value. Dissolved metal concentrations were measured by inductively coupled plasma emission spectrometry (ICP, Leeman Labs PS3000UV) at the Materials Characterization Laboratory at The Pennsylvania State University.

Statistical analyses of the collected data were performed using MINITAB® statistical software (Minitab Inc., State College, PA). The geochemical computer program PHREEQC (Parkhurst and Appelo, 1999) was used to estimate the speciation of manganese in solution. The concentrations of nitrate released from the added metal salts were estimated based on the initial, measured concentration of their associated metals.

5.4. Results

5.4.1. Characterization of solids

Results from the chemical analyses indicate that both solids are mainly composed of carbon and nitrogen, with almost negligible residues of chitin-associated minerals (Table 5.1). The measured carbon and nitrogen contents in DMP-SC20 are very close to the theoretical values for pure chitin (C\(_8\)H\(_{15}\)O\(_5\)N). The percentage of protein in DM-SC20 can be estimated based on its nitrogen content using Eq. 5.2 (Percot et al., 2003):

\[
\%P = 6.25(\%N - 6.9) \quad (5.2)
\]

where \(\%P\) is the percentage of proteins, \(\%N\) is the percentage of nitrogen, and 6.25 and 6.9 correspond to the theoretical percentage of nitrogen in protein and chitin, respectively. Therefore, the percentage of proteins in DM-SC20 corresponds to ~23%. SEM micrographs (Fig. 5.1) reveal a somewhat similar structure of both materials, consisting of a series of stacked layers with limited visible porosity. Low porosity was indeed
measured by BET analysis, with no significant difference between the two solids (Table 5.1).

Titration results for both DMP- and DM-SC20 indicate the presence of at least two inflection points (Fig. 5.2A). The end points of the titrations were estimated based on the graphic method proposed by Gran (Stumm and Morgan, 1996), and the total active sites corresponded to 0.3 and 0.8 mmol/g for DMP- and DM-SC20, respectively. The net surface charge ($Q_H$, mol/g) as a function of the pH of the suspension was calculated using Eq. 5.3 (Stumm and Morgan, 1996):

$$Q_H = \frac{(C_A - C_B + [OH^-] - [H^+]})/a}$$

where $C_A$ and $C_B$ are the molar concentrations of the acid and the base, respectively, in the suspension after each titrant addition, and $a$ is solid load used (g/L). Net negative charges were observed on the solids when pH > 5.6 for DMP-SC20 and pH > 5.1 for DM-SC20 (Fig 5.2B). For a given pH value, surface charges were higher on DM-SC20.

Experimental data can be described assuming the solid surface consists of a discrete number of weakly monoprotic acidic sites ($\equiv S_i$) that dissociate according to Eq. 5.4 and 5.5:

$$\equiv S_i H \leftrightarrow \equiv S_i^- + H^+$$

$$K_{app,i} = \frac{[\equiv S_i^-][H^+]}{[\equiv S_i H]}$$

where $\equiv S_i H$ and $\equiv S_i^-$ are protonated and diprotonated species of the $i^{th}$ type of acidic site, and $K_{app,i}$ is the apparent deprotonation constant (Cox et al., 1999; Pagnanelli et al., 2004). Combining the mass balance for the $i^{th}$ acidic site with Eq. 5.5 it is possible to relate $[\equiv S_i^-]$ to the pH of the system and the constant parameters $K_{app,i}$ and $[\equiv S_i]_T$:

$$[\equiv S_i^-]_T = [\equiv S_i^-](1 + [H^+] / K_{app,i})$$

Therefore, the net negative charge concentration on the solid can be expressed as:

$$Q_H = \sum_i [\equiv S_i^-] = \sum_i \frac{[\equiv S_i^-]_T}{1 + [H^+] / K_{app,i}}$$

For the present study, three different acidic sites were assumed to be present on the evaluated solid. A non-linear regression method was performed to fit the data obtained from the alkalimetric titrations and estimate the six adjustable parameters ($[\equiv S_1]_T$, $[\equiv S_2]_T$, $[\equiv S_3]_T$, $K_{app,1}$, $K_{app,2}$, $K_{app,3}$, Table 5.2). High correlation coefficients and low standard
errors were obtained for both types of solids, indicating that the three-site model (represented by the dotted lines in Fig. 5.2B) describes the experimental data well. The estimated concentration of the acidic sites is always higher for DM- than for DMP-SC20.

5.4.2. Kinetic tests

Kinetic tests were conducted to evaluate the metal removal rates by the solids under both acidic and alkaline pH regimes, with an initial manganese concentration of 10 mg/L. Qualitatively, a similar behavior was observed in all the evaluated systems. The majority of the Mn removal occurred during the first six hours (Fig. 5.3). However, slight changes were observed to occur throughout the duration of the tests, especially in those systems treated with DM-SC20. Nevertheless, those changes did not lead to significantly different removals (p-value = 0.4) than the calculated average for t ≥ 6 h. Under acidic conditions (pH ~5), Mn uptake by both types of solids was observed to be very low (Fig. 5.3A), as expected due to the limited amount of negative sites at low pH. After a contact time of 72 h, only 3 and 10% of the initial manganese in solution (Mn₀ = 10 mg/L) was removed by DMP- and DM-SC20, respectively. In contrast, higher amounts of manganese were removed by both types of solids under alkaline conditions (pH ~9, Fig. 5.3B). After 72 h, 35 and 83% Mn was removed by DMP- and DM-SC20, respectively.

Along with manganese removal, the addition of the evaluated solids also caused changes in the pH of the solution. These changes were faster, yet smaller, under acidic conditions (from pH 4.8 to pH 5.1 in less than 30 min), than under alkaline conditions (from pH 9.2 to pH 8.2 by the end of the tests).

5.4.3. Manganese adsorption isotherms

Sorption data were interpreted in terms of the Langmuir (Eq. 5.8) and the Freundlich (Eq. 5.9) sorption models:

\[ q_e = q_m \frac{K_L C_e}{1 + K_L C_e}, \]  

\[ q_e = K_F C_e^{1/n}, \]

where \( q_e \) and \( C_e \) are the equilibrium concentration of the adsorbate on the solid (mg/g) and in the liquid phases (mg/L), respectively; \( q_m \) is the maximum adsorption capacity.
(mg/g) according to the Langmuir model; \( K_L \), \( K_F \), and \( n \) are constants. Based on the correlation coefficients (Table 5.3), the data show a good agreement with the two models. However, the obtained fitted curves for the Freundlich model have higher standard errors (\( s_e \)) and over-predict the sorption capacity of the solids at high metal concentrations. For this reason, the Langmuir model (represented by solid lines in Fig. 5.4) was chosen as the best fit to describe the data.

For both types of solids, the extent of manganese removal strongly depended on the pH of the system (Fig. 5.4). However, as solutions became more alkaline, this pH-dependency decreased. Under slightly acidic conditions, a 30-fold decrease in the hydronium ion concentration (i.e. from pH 5.2 to pH 6.7) resulted in a 3-fold increase of the maximum adsorption capacity (\( q_m \)). Under slightly alkaline conditions, a ten-fold decrease in the hydronium ion concentration (i.e. from pH 7.6 to pH 8.6) resulted in only a 10% increase of \( q_m \). In addition, for the same pH regime, the adsorption capacities of DM-SC20 were 5.3 – 7.3 times higher than those calculated for DMP-SC20.

Similar ATR-FTIR spectra were obtained from both solids at all the evaluated conditions (Fig. 5.5). Bands in all spectra are in close agreement to those previously reported for \( \alpha \)-chitin extracted from crustacean shells (Cardenas et al., 2004; Dolphen et al., 2007). This is especially true for DMP-SC20 samples. However, certain differences were found in DM-SC20 with respect to the pure material (compare Fig. 5.5A-C to Fig. 5.5D-F). A shift in the band intensity ratio was observed between the two types of solids within the region 2930 – 2880 cm\(^{-1}\); in DMP-SC20 samples, the band at 2880 cm\(^{-1}\) had a higher intensity than the band around 2930 cm\(^{-1}\), while the opposite was true for DM-SC20 samples. The double band in the region 1655 – 1620 cm\(^{-1}\) in DMP-SC20 spectra disappeared in DM-SC20 and was replaced by a wider single band at 1623 cm\(^{-1}\) with a shoulder at around 1655 cm\(^{-1}\). The band at 1522 cm\(^{-1}\) in DMP-SC20 samples shifted to 1537 - 1549 cm\(^{-1}\) in DM-SC20; additional bands also seemed to appear at lower wavenumbers, resulting in a shoulder at around 1500 cm\(^{-1}\). A series of unresolved bands also appeared in the 1450 – 1430 cm\(^{-1}\) region in DM-SC20 spectra while only a single band was present in DMP-SC20 samples, at 1416 cm\(^{-1}\). Finally, while only a double band was observed in DMP-SC20 samples at 1204 and 1260 cm\(^{-1}\), spectra for DM-SC20 samples contained additional bands within this region. Although relatively minor,
spectra from samples of the same type of solid also exhibited some differences between each other as a result of the different treatment conditions. A small shoulder at around 1730 cm\(^{-1}\) appeared only in solids equilibrated at pH 5 (Fig. 5.5A and D). For DM-SC20 samples, slight shifts occurred in the band around 1540 cm\(^{-1}\), as well as within the 1204 – 1256 cm\(^{-1}\) region (Fig. 5.5D – F).

5.5. Discussion

5.5.1. Potentiometric titration of solid

Recent studies have evaluated the potential of chitin and chitosan (its deacetylated derivative) as sorption agents. Chitin and chitosan have both been shown to be excellent metal ligands for heavy metal complexation. However, biosorption is a complex process that has not been very well understood since it can involve several mechanisms such as complexation, chelation, ion exchange, and physical adsorption. It has been suggested that the metal forms a coordination complex with the nitrogen or oxygen in chitin (Volesky and Holan, 1995). Numerous studies have shown the ability of chitinous materials to remove both cationic and anionic contaminants including not only metals but also sulfate (Moret and Rubio, 2003; Niu and Volesky, 2003; Zhou et al., 2005; de Oliveira Franco et al, 2004; Karthikeyan et al, 2005). In most studies on metal removal using chitinous materials, researchers have used relatively pure chitin polymers, extracted from fungi or crustaceans. However, a few groups have recently evaluated the use of raw chitinous materials for the removal of metals.

In the present study, the sorption capacity of a rather complex material was studied. Crab shells are composed by chitin fibrils, arranged with a variety of proteins and clusters of minerals (Raabe et al., 2005). Several studies have reported the composition of chitin-associated proteins in crustaceans, by means of their amino acid residues (Shahidi and Synowiecki, 1991; Percot et al., 2003; Iijima, 2005). With differences in their concentrations, aspartatic (pK 2.09, 3.86, and 9.82) and glutamatic acids (pK 2.19, 4.25, and 9.67), histidine (pK 1.82, 6.04, and 9.17), alanine (pK 2.35 and 9.69), lysine (pK 2.18, 8.95, and 10.53), tyrosine (pK 2.20, 9.11, and 10.07), and arginine (pK 2.17, 9.04, and 12.48) are commonly found in the evaluated organisms. However, the exact structure of chitin-associated proteins remains somewhat unknown. After demineralization, the
obtained DM-SC20 particles consist of a mixture of chitin and its associated proteins. After the deproteinization process, it is believed that DMP-SC20 consists mainly of chitin: Percot et al. (2003) reported protein residues < 0.6%. However, due to the intricate relationship between this biopolymer and the proteins, it is likely that some minor protein residues were still present in DMP-SC20. In addition, crustacean shells also contain small amounts of lipids and carotenoids, which were not removed during demineralization or deproteinization. Nevertheless, results from carbon and nitrogen analyses reveal that almost pure chitin was indeed obtained.

The acid-base characteristics of sorption sites are important when determining the potential performance of a sorbent. Solid surface charge changes as a function of pH indicate the likelihood of cation adsorption. In the present study, the net-negative charges that were observed only for pH > 5.1 or 5.6 indicate that cation adsorption will be electrostatically inhibited under the typically acidic conditions of MIW. On the other hand, the estimation of the $pK_{app}$ of the potential sorption sites gives an indication of the functionality of those sites. In the case of chitinous materials, two main functional groups are expected to be present and responsible for adsorption. Each chitin monomer (N-acetylglucosamine) contains one carbonyl and one amine group. These two functional groups are also present in the chitin-associated proteins. In general, pKa values for carboxylic acids range between 2 and 6, while for amines values are usually >8 (Stumm and Morgan, 1996). Therefore, for DMP-SC20, the first site detected ($\equiv S_1$) is attributed to carboxylic groups, while the second and third ($\equiv S_2$ and $\equiv S_3$) are attributed to amine groups. Similarly, for DM-SC20, $\equiv S_1$ and $\equiv S_3$ correspond to carbonyl and amine groups, respectively. Although somewhat higher than the typical value, $pK_{app2}$ for DM-SC20 likely corresponds to a carboxylic group. The estimated concentration of sorption sites, based on the regression calculations, in DMP-SC20 (0.37 mmol/g) is greatly exceeded by DM-SC20 (1.10 mmol/g). These values are somewhat higher than those estimated from the graphic method (0.8 and 0.3 mmol/g for DM- and DMP-SC20, respectively). Given that the surface areas of the solids were not significantly different, the difference in the concentration of sites between DMP- and DM-SC20 is an indication that the chitin-associated proteins remaining in DM-SC20 play an important role in its overall sorption capacity.
5.5.2. Kinetic tests

These tests were conducted with two main purposes: (1) to evaluate manganese uptake rates and (2) to define an appropriate contact time to ensure that equilibrium had been reached for adsorption isotherms.

Several models have been tested to describe the kinetics of metal adsorption onto chitinous materials (Ngah et al., 2005; Vijayaraghavan, 2006). Among these models, the pseudo-first order (Eq. 5.10), pseudo-second order (Eq. 5.11), and intraparticle diffusion (Eq. 5.12) models were selected and tested with the results obtained in this study:

\[
\log(q_e - q_t) = \log q_e - \frac{k_1}{2.303}t
\]  
(5.10)

\[
\frac{dq}{dt} = k(q_e - q_t)^2
\]  
(5.11)

\[
q_t = k_i t^{0.5}
\]  
(5.12)

where \(q_e\) and \(q_t\) are the adsorbate concentration in the solid (mg/g) at equilibrium and at time \(t\) (min), respectively, and \(k_1\) (min\(^{-1}\)), \(k_2\) (g/mg min), and \(k_i\) (mg/g min\(^{0.5}\)) are rate constants. To facilitate the evaluation, Eq. 5.10 was integrated and transformed into a linear form (Eq. 5.13)

\[
\frac{t}{q_t} = \frac{1}{k_2q_e^2} + \frac{t}{q_e}
\]  
(5.13)

To minimize errors due to the changes observed at later times in the tests, the kinetic models were evaluated over the range of \(t \leq 6\) h. Results of the linearized forms of the three evaluated models clearly show that the pseudo-second order model (Eq. 5.11) is the best at describing the observed Mn adsorption. While very poor or no correlation was found for the pseudo-first order and the intraparticle model, correlation coefficients for the pseudo-second order model were close to one at pH 9 (Table 5.4). However, the reduced sorption observed at pH ~5 did not fit the expected behavior, decreasing the correlation. The higher values of the rate constant obtained for DMP-SC20 suggest that manganese uptake is faster onto chitin than onto the chitin-protein mixture (present in DM-SC20). However, the higher \(q_e\) values obtained with DM-SC20, corroborates the idea that the chitin-associated proteins offer additional sites for manganese adsorption.
The extended duration of the kinetic experiments (72 h) was selected based on previous studies using chitinous materials where minimal or no changes were observed after 24 – 48 h. Although some changes were observed at later times in the tests presented here, statistical analysis indicates that the values for \( t \geq 24 \text{ h} \) are not significantly different. Therefore, the 72-h contact time can be considered appropriate to ensure that equilibrium was reached.

5.5.3. Manganese adsorption isotherms

The process of manganese adsorption onto DMP- and DM-SC20 is well described by the Langmuir model. The essential characteristics of the Langmuir model can be expressed in terms of the separation factor, \( R_L \), a dimensionless parameter defined by Eq. 5.14 (Webber and Chakravorti, 1974):

\[
R_L = \frac{1}{1 + K_L C_0} \quad (5.14)
\]

where \( C_0 \) is the initial concentration. \( R_L \) indicates the characteristics of the adsorption: unfavorable for \( R_L > 1 \), linear for \( R_L = 1 \), favorable for \( 0 < R_L < 1 \), and irreversible for \( R_L = 0 \). For this study, \( R_L \) values calculated for initial Mn concentrations between 0.5 and 250 mg/L were all between 0.01 and 0.99. This indicates a favorable adsorption of manganese onto both types of solids.

On the other hand, the sorption capacity of DMP-SC20 is greatly exceeded by that calculated for DM-SC20. As was mentioned before, this is an indication that the chitin-associated proteins present in DM-SC20 are greatly responsible for the observed removal of manganese. In this case, a change from \(~100\% \) chitin (in DMP-SC20) to \(~1:3 \) protein-to-chitin content in DM-SC20 leads to an increase of sorption capacity of 5.3 – 7.3 times. This additional sorption capacity related to the chitin-associated proteins is due to the functional groups present in their constituent amino acids. Among the five most common amino acids found in the crustacean shells, two are acidic in character (aspartic and glutamic acids), each providing an extra carboxylic group, while other three are basic (histidine, lysine, and arginine), each providing an extra amine group. Under the conditions at which the tests were conducted (pH 5 – 9), it is expected that the carboxylic groups were deprotonized, while the amine groups were positively charged. Under slightly acidic conditions, it is likely that the hydronium ions out-competed the
manganese in solution, limiting its binding to the amine groups and leaving the carboxylic groups primarily responsible for the observed adsorption. As the activity of the hydronium ions decreased under alkaline conditions, manganese was able to be sorbed onto the available amine groups.

FTIR spectroscopy is a useful material characterization tool to get conformational information. With the use of ATR accessories, information is collected from the surface of the analyzed sample as the depth of penetration of the evanescent wave is usually only few microns. Therefore, the collected ATR-FTIR spectra (Fig. 5.5) can provide insights into the functional groups that were present and active during Mn adsorption. The close agreement found between the FTIR spectra of DMP-SC20 samples and those reported in literature corroborates once more the fact that this material was indeed pure chitin. Samples of the same material appeared not to be affected by the different treatment conditions. This is especially true for DMP-SC20. It is possible that the little Mn removal obtained with this solid make changes in FTIR hard to detect. The only noticeable difference between the spectra of DMP-SC20 samples is the occurrence of a shoulder at ~1730 cm\(^{-1}\) for the sample equilibrated at pH 5. This feature (also noticeable for the DM-SC20 at pH 5) could be associated with vibration of carbonyl groups (Pretsch et al., 2009) and might have been enhanced by the slightly acidic conditions. Two of the main differences between DMP- and DM-SC20 spectra are associated with the amide I (C=O stretching (\(\nu_{C=O}\)) at 1660 – 1620 cm\(^{-1}\)) and amide II (N-H bending (\(\delta_{NH}\)) at 1550 – 1520 cm\(^{-1}\)) vibrations (Cardenas et al., 2004; Focher et al., 1992). Since the peptide bond in proteins is in essence an amide, it is not surprising to find additional vibration bands associated to the presence of proteins in DM-SC20. The position of protein-amide bands is affected by the secondary structure (Byler and Susi, 1986). Therefore, the observed shifts and unresolved shape of these bands are likely due to conformational changes associated with changes of H-bonds as well as the effect Mn bonding. This constitutes additional evidence for the role of proteins in the removal of Mn. An additional difference between DMP- and DM-SC20 is the changes in intensity ration in the 2930 – 2880 cm\(^{-1}\) region, associated with CH, CH\(_2\), and CH\(_3\) stretching vibrations (\(\nu_{CH}\), Byler and Susi, 1986). Such variations are therefore likely to the different alkane sidechains of the chitin-associated proteins. The remarkable similarities between all spectra within the
signature or fingerprint region (wavenumber < 1500 cm\(^{-1}\)) indicate that the chitin structure is the main component responsible for the shape and location of the observed bands. It is possible that the relatively low content of each type protein in DM-SC20 resulted is low signals, barely detected by the instrument and masked by the stronger signals of chitin structure. However, some variations were detected in the regions of 1450 – 1430 and 1260 – 1204 cm\(^{-1}\). The first of these regions corresponds to bending vibrations of the C-H bonds (\(\delta_{\text{CH}}\)), while the latter corresponds to N-H bending (Cardenas et al., 2004). These additional, unresolved bands observed in the DM-SC20 spectra are likely due to particular features of the chitin-associated proteins and their secondary structure. Furthermore, the changes in the intensity ratio of the N-H bending bands may also indicate conformational changes due to the pH conditions and the sorption of Mn.

Previous studies have evaluated the manganese removal capacity of several natural and synthetic sorbents. In general, reported maximum sorption capacities ranged between 2 and 30 mg/g (Nassar et al., 2004; Vaghetti et al., 2009; Vijayaraghavan et al., 2009), with exceptionally high values observed for pecan nutshell (98 mg/g) and Arthrobacter sp. biomass (406 mg/g, Vaghetti et al., 2009). Since all tests were conducted under different conditions, a direct comparison with the results obtained in this study cannot be made. However, qualitatively speaking, the maximum Mn adsorption capacities of DMP- and DM-SC20 appear to be within the lower end of the reported range. The very high sorption values reported with pecan nutshell and Arthrobacter sp. biomass are likely due to the abundance of negatively charged functional groups such as carboxylic acids and phenol in comparison to those found in chitin and its associated proteins. The relatively poor Mn sorption observed with pure chitin also contradicts previous studies where metals like zinc, copper, cadmium, and lead were efficiently removed (Benguella and Benaiissa, 2002; Rae and Gibb, 2003). According to the Irving – Williams order, the complex stability of Mn\(^{2+}\) ions is well below those of the formerly mentioned transition metals (Stumm and Morgan, 1996). The stability of Mn could explain the relatively low sorption observed here. In fact, the same behavior was also noticed with Arthrobacter sp. biomass: Mn uptake was the lowest in comparison to Pb, Cu, and Zn (Vaghetti et al., 2009). Another factor that could have negatively influenced the Mn sorption onto the evaluated solids was the presence of sulfate in the evaluated
systems. The addition of sulfate to the SMIW was intended to better imitate the typical conditions found in affected streams. However, high levels of sulfate can lead to the formation of sulfate-manganese complexes. Thermodynamic calculations indicate that with 1000 mg/L of sulfate in solution, manganese speciation consists of ~73% as Mn\textsuperscript{2+}(aq) and ~27% as MnSO\textsubscript{4}(aq). Therefore, the occurrence of manganese complexation could have partially inhibited its sorption onto the evaluated chitinous materials.

The main objective of the present study was to evaluate the individual contributions of chitin and its associated proteins to manganese removal when using a complex substrate such as crab shell particles. The results indicate that both chitin and protein are able to partially adsorb manganese. However, the presence of proteins significantly increases the sorption capacity of the material. For field applications, untreated crab-shell particles are the most cost-effective alternative. In this raw material, the proportion of protein-to-chitin has been reported as high as 1.2:1 (Robinson-Lora and Brennan, 2009), which greatly exceeds the one reported here (~0.3:1). Consequently, higher manganese removal due to sorption onto the protein sorption sites could be expected. On the other hand, the acidic conditions commonly found in MIW might inhibit this sorption process. However, chitin-associated carbonates have proven to be an excellent source of buffering capacity, rapidly increasing the solution pH. As a result, it can be expected that the chitin-associated proteins will contribute to the removal of manganese, once the pH of the solution is increased due to the dissolution of chitin-associated carbonates.

5.6. Conclusions

Results from the present study demonstrate the role of chitin-associated proteins in the removal of manganese from MIW using crab-shell chitin. Results indicate that both types of biomolecules are capable of sorbing manganese. However, the removal capacity of the chitinous materials significantly increases when chitin-associated proteins are present. The available sorption sites are likely associated with both carboxylic and amine functional groups. The adsorption equilibrium isotherms, well described by the Langmuir model, indicate that the maximum sorption capacity greatly depends on the pH of the solution. While essentially no sorption was observed at pH <5, a 6-fold increase in the sorption capacity of both types of materials was observed when the conditions
changed from acidic (pH 5) to alkaline (pH 9). More research is needed to evaluate the persistence and reversibility of this mechanism under continuous-abiotic conditions.

5.7. Acknowledgements
This material is based upon work supported by the National Science Foundation under Grant No. CBET-0644983.
5.8. References


Fig. 5.1. SEM micrographs showing the similar physical structure of DMP-SC20 (chitin) and DM-SC20 (chitin + proteins).

Fig. 5.2. Potentiometric characterization of DMP- and DM-SC20: (A) Alkalimetric titration of solids and (B) Net-negative surface charges calculated from titration curves. Dotted lines correspond to three-site model prediction.
Fig. 5.3. Manganese uptake onto DMP- and DM-SC20 under (A) acidic and (B) alkaline conditions. Data points represent averages of duplicate samples; error bars are smaller than the symbol size.
Fig. 5.4. Manganese adsorption onto 5 g/L of (A) DMP-SC20 and (B) DM-SC20 at different pH values. Data points represent duplicate averages; error bars represent one standard deviation. Solid lines correspond to Langmuir fitting model.
Fig. 5.5. ATR-FTIR spectra of DMP-SC20 equilibrated at pH 5 (A) and before (B) and after (C) Mn sorption at pH 9, and DM-SC20 equilibrated at pH 5 (D) and before (E) and after (F) Mn sorption at pH 9. Shaded areas correspond to wavenumber regions associated with stretching (ν) and bending (δ) vibrations of the indicated bonds.
Table 5.1. Chemical composition and surface area of the solid materials.

<table>
<thead>
<tr>
<th>Solid</th>
<th>DMP-SC20</th>
<th>DM-SC20</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (%)</td>
<td>44.3 (1.5)(^a)</td>
<td>46.2 (0.8)(^a)</td>
</tr>
<tr>
<td>N (%)</td>
<td>6.9 (0.1)(^a)</td>
<td>10.5 (0.1)(^a)</td>
</tr>
<tr>
<td>CaO (%)</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>MgO (%)</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>P(_2)O(_5) (%)</td>
<td>0.06</td>
<td>0.39</td>
</tr>
<tr>
<td>Na(_2)O (%)</td>
<td>0.13</td>
<td>0.02</td>
</tr>
<tr>
<td>Al(_2)O(_3) (%)</td>
<td>0.09</td>
<td>0.13</td>
</tr>
<tr>
<td>SrO (%)</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>BaO (%)</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Fe(_2)O(_3) (%)</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>K(_2)O (%)</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>MnO (%)</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Surface area (m(^2)/g)</td>
<td>3.0 (0.9)(^a)</td>
<td>2.0 (1.1)(^a)</td>
</tr>
</tbody>
</table>

\(^a\)Data correspond to average of duplicate measurements; values in parentheses correspond to one standard deviation.

Table 5.2. Adjustable parameters for three-site model from non-linear regression of titration data.

<table>
<thead>
<tr>
<th>Solid</th>
<th>([= S_1])(_T) (mmol/g)</th>
<th>([= S_2])(_T) (mmol/g)</th>
<th>([= S_3])(_T) (mmol/g)</th>
<th>pK(_{app,1})</th>
<th>pK(_{app,2})</th>
<th>pK(_{app,3})</th>
<th>(r^2)</th>
<th>(s_e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMP-SC20</td>
<td>0.07</td>
<td>0.17</td>
<td>0.14</td>
<td>4.46</td>
<td>7.71</td>
<td>9.68</td>
<td>0.999</td>
<td>0.015</td>
</tr>
<tr>
<td>DM-SC20</td>
<td>0.28</td>
<td>0.31</td>
<td>0.51</td>
<td>3.96</td>
<td>6.45</td>
<td>10.11</td>
<td>0.998</td>
<td>0.015</td>
</tr>
</tbody>
</table>
Table 5.3. Results obtained from the fitting of the isotherms to Langmuir and Freundlich adsorption models.

<table>
<thead>
<tr>
<th>Solid</th>
<th>pH</th>
<th>( q_m )</th>
<th>( K_L )</th>
<th>( r^2 )</th>
<th>( s_e )</th>
<th>( K_F )</th>
<th>( n )</th>
<th>( r^2 )</th>
<th>( s_e )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMP-SC20</td>
<td>5.4</td>
<td>0.165</td>
<td>0.085</td>
<td>0.995</td>
<td>0.011</td>
<td>0.008</td>
<td>1.268</td>
<td>0.849</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>0.446</td>
<td>0.120</td>
<td>0.998</td>
<td>0.023</td>
<td>0.058</td>
<td>2.133</td>
<td>0.891</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>0.900</td>
<td>0.307</td>
<td>0.857</td>
<td>0.054</td>
<td>0.198</td>
<td>2.631</td>
<td>0.871</td>
<td>0.201</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>0.981</td>
<td>0.440</td>
<td>0.876</td>
<td>0.058</td>
<td>0.252</td>
<td>2.877</td>
<td>0.873</td>
<td>0.279</td>
</tr>
<tr>
<td>DM-SC20</td>
<td>5.2</td>
<td>0.878</td>
<td>0.016</td>
<td>0.946</td>
<td>0.151</td>
<td>0.022</td>
<td>1.370</td>
<td>0.950</td>
<td>0.340</td>
</tr>
<tr>
<td></td>
<td>6.7</td>
<td>3.271</td>
<td>0.133</td>
<td>0.980</td>
<td>0.395</td>
<td>0.353</td>
<td>1.920</td>
<td>0.913</td>
<td>1.046</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>4.972</td>
<td>0.340</td>
<td>0.970</td>
<td>0.333</td>
<td>0.863</td>
<td>2.282</td>
<td>0.942</td>
<td>1.719</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
<td>5.437</td>
<td>0.439</td>
<td>0.963</td>
<td>0.298</td>
<td>1.279</td>
<td>2.697</td>
<td>0.908</td>
<td>1.473</td>
</tr>
</tbody>
</table>

Table 5.4. Kinetic parameters for pseudo-second order adsorption model.

<table>
<thead>
<tr>
<th>Solid</th>
<th>pH 5.04 ± 0.07</th>
<th>pH 8.62 ± 0.14</th>
<th>pH 5.05 ± 0.02</th>
<th>pH 9.01 ± 0.14</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMP-SC20</td>
<td>0.063</td>
<td>0.643</td>
<td>0.179</td>
<td>1.875</td>
</tr>
<tr>
<td>DM-SC20</td>
<td>0.411</td>
<td>0.535</td>
<td>0.125</td>
<td>0.197</td>
</tr>
<tr>
<td>( q_e ) (mg/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( k_2 ) (g/mg min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.871</td>
<td>0.999</td>
<td>0.991</td>
<td>1.000</td>
</tr>
</tbody>
</table>
CONCLUSIONS

This study evaluated chitinous materials (crab shells, SC-20) as a multifunctional substrate for the passive treatment of mine impacted waters. Results demonstrated that the complexity of SC-20, composed by chitin, proteins, and minerals (mainly carbonates and phosphates), resulted in a variety of changes where each of the SC-20 components played an important role for the overall treatment. Under biologically active conditions, the organic components of SC-20 (chitin and proteins) served as electron donor sources, sustaining the activity of sulfate reducing bacteria. Abiotic tests showed that these two components can also provide sorption sites for the removal of manganese. Simultaneously, the chitin-associated minerals rapidly dissolved due to their great reactivity, increasing the pH and changing the systems from net acid to net alkaline. Such rapid changes in pH and alkalinity were beneficial for both biologically active and abiotic treatments as they 1) created more suitable conditions for the prompt onset of SRB and 2) induced to the supersaturation of the system with respect to a variety of metal minerals (carbonates and/or phosphates), promoting their precipitation.

Steady treatment was also achieved under continuous-flow, biologically active conditions. In columns packed with SC-20 alone as substrate, alkalinity was constantly generated and acidity and metals (Fe, Al, and Mn) were removed. Metals removal was likely due to the combined effect of precipitation and sorption. The probable mineral phases formed are aluminum hydroxides, manganese carbonates (i.e. rhodochrosite) and/or phosphates, and iron hydroxides and/or sulfides.

In comparison to other commonly used substrates, the addition of SC-20 alone resulted in faster changes and more efficient treatment. This was especially true for manganese removal. Under biologically active conditions, SC-20 addition promoted partial removal of this metal, while no removal was observed with spent mushroom compost or sodium lactate. Under identical abiotic conditions, manganese removal achieved with SC-20 was more than 10 times larger than those obtained with limestone.

The results summarized above show the alternative material SC-20 as a promising option for both biologically active and abiotic treatment of mine impacted waters. This represents a unique characteristic of this material that, to our knowledge, has not been
reported before. Crab shells could be used therefore alone or in combination with other organic substrates to support biological treatment systems, such as constructed wetlands or permeable reactive barriers. Alternatively, this material could replace or amend limestone in abiotic systems (like limestone drains), enhancing the removal of manganese. Crab shells could also be used for a rapid polishing of streams where manganese is a concern. Although SC-20 is more expensive than other waste materials commonly used, its addition results in a more rapid removal. This implies the need for shorter retention times and, therefore, less space for the required level of treatment, compensating the initial investment on the material.

More research is needed to assess the performance and longevity of this material under continuous-flow, abiotic conditions. Continuous-flow tests should also attempt to identify the accumulated phases and corroborate the finding of this study. Of special interest is the verification of the role of phosphate minerals and the consequences of their exhaustion for the removal efficiency. For field applications, it will be also beneficial exploring the use of bigger particles to reduce head losses. In this case, it will be necessary to evaluate the effect of the particle size on the removal rates, reactivity (passivation), and longevity.

Finally, we consider this study as a further step in the understanding of how complex materials like crab shells could be advantageously used for remediation purposes. Based on the promising results that were obtained, it would also be worth to expand the scope to new materials and pollutants. As it was observed with Al, Fe, and Mn, the removal of other metals present in MIW may be possible by the addition of SC-20. This possibility should be also tested. On the other hand, it will be valuable to evaluate the potential of other materials with similar characteristics to those found in crab shells (i.e. complex structure and composition) for its use as substrates.
APPENDIX

Method for the estimation of SRB population in the sediment inoculum used for microcosms tests.

Sediment samples (0.5 – 1 g) were transferred using aseptic techniques to 50-ml sterile centrifuge tubes, containing 10 ml of sterile-anoxic MgSO₄ solution (1 mM). Tubes were put in an orbital shaker for 2 hours to gently homogenize the mixtures. Serial tenfold dilutions were then prepared, using sterile-anoxic MgSO₄ solution (1 mM). Freshly prepared Baar’s medium (4.5 ml) was dispensed into culture tubes while it was still warm to minimize oxygen inclusion. The MPN media tube was inoculated with 0.5 ml of the appropriate dilution. Serial dilutions were made to obtain a $10^2 – 10^7$ dilution of the initial sediment – MgSO₄ solution mixture, with three tubes at each dilution. Tubes were incubated at 20°C for 30 days and observed for the formation of a black precipitate (ferrous sulfide) as the indication of SRB activity.
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EDUCATION
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