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

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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.

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1. 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. (1)):

$$4FeS_{2(s)} + 15O_{2(aq)} + 14H_2O_{(l)} \rightarrow 4Fe(OH)_{3(s)} \downarrow + 8SO_{4(aq)}^{2-} + 16H_{(aq)}^{+} \quad (1)$$

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, Eqs. (2) and (3)):

$$CH_2O_{(s)} + SO_{4(aq)}^{2-} + 6H_{(aq)}^{+} \rightarrow H_2S_{(aq)} + CO_{2(aq)} + 3H_2O \quad (2)$$

$$Me_{(aq)}^{2+} + H_2S_{(aq)} \rightarrow MeS_{(s)} \downarrow + 2H_{(aq)}^{+} \quad (3)$$

where CH_2O 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

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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_8H_{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. Methods

2.1. 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® 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 ($C_8H_{13}NO_5$), ~12% protein ($C_{16}H_{24}O_5N_4$), 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).

2.2. 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 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 $\leqslant$ 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 1).

2.3. 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

Table 1
Sampling locations and initial characteristics of AMD and sediments used in the microcosm test.

Site name:	Beech Creek (BC)	North Fork (NF)	Cherry Run (CR)	Kittanning Run
Approximate sampling location (GPS):	41.08598°, −77.86725°	41.051015°, −77.96397°	41.05428°, −77.95562°	40.49781°, −78.47633°
Water				
ORP (mV)	286	438	437	ND
pH	3.51	3.50	3.25	2.95
EC (μS)	788	382	483	ND
Alkalinity (mg/L $CaCO_3$)	0	0	0	0
Acidity (mg/L $CaCO_3$)	68	30	60	153
Sulfate (mg/L)	393	181	293	570[a]
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	51.0	39.0	31.9[b]

MCL: National Secondary Standard for drinking water. ND: Not determined.
[a] Estimated value based charge balance calculations (PHREEQC).
[b] Approximate sampling location for sediment: 40.49660°, −78.46230°.

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 0.2 mL/L conc. HNO_3 for dissolved metals, and −10 °C for VFAs).

2.4. 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 titrations 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 0.2 mL/L conc. HNO_3 for dissolved metals, and −10 °C for VFAs). Periodically, independent samples for sulfide were taken from the effluent port and immediately analyzed.

2.5. 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 H_2SO_4 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 Electronic Annex 1 in the online version of this article). 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.

3. Results

3.1. Microcosm test

A very rapid pH increase was observed in all chitin-amended bottles (actives, Fig. 1A). Circumneutral pH was achieved after only 2 days of treatment, and continued to increase until it reached a plateau around pH 7.5 after 7 days. No significant differences in pH were observed among the three treated AMD sets ($\alpha = 0.05$). 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. 1B and C). No significant differences were found in these parameters among the three AMD sets at each sacrificial event ($\alpha = 0.05$). Average rates for alkalinity production and acidity removal corresponded to 37.9 and −27.5 mg $CaCO_3$/L day, respectively. Parallel to alkalinity, a steady increase in calcium concentration at a rate of 34.5 mg/L day was observed during the first 9 days of treatment, when a plateau of 217 mg/L was reached (see Electronic Annex 2). Electrical conductivity also steadily increased in active microcosms, reaching a maximum of 1632 ± 115 μS/cm by the end of the test (see Electronic Annex 2). No significant differences were found in the final levels of these four parameters among the three AMD sets ($\alpha = 0.05$).

Based on triplicate MPN analysis, average estimates of the initial acetate-utilizing SRB population in the sediment used as a microbial source was 14×10^3, 12×10^3, and 18×10^4 cells per gram (on a dry basis) for sites BC, NF, and CR, respectively. Changes

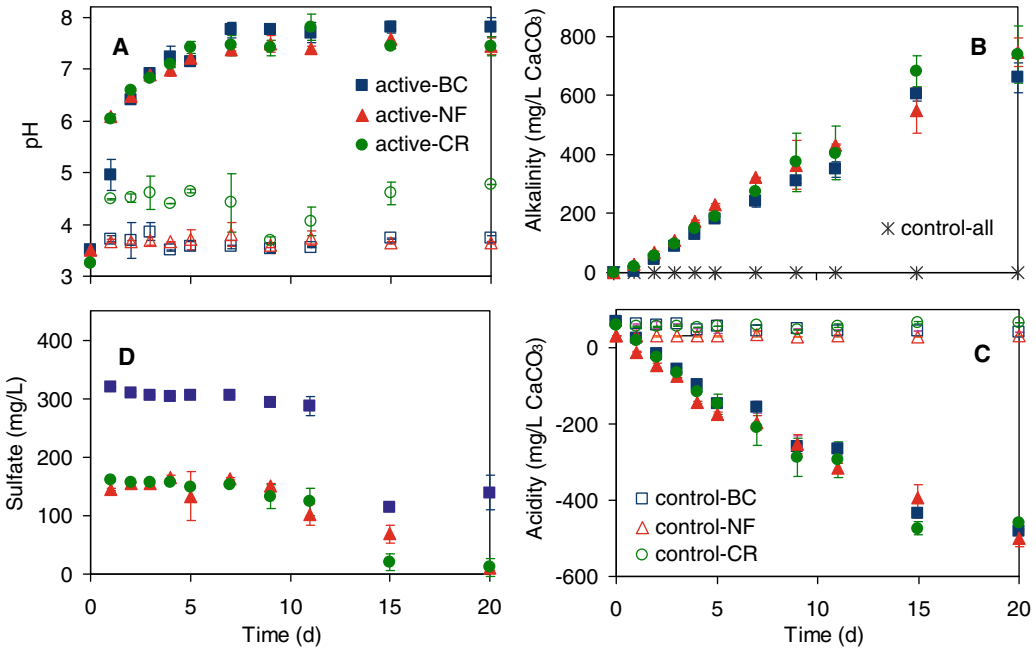

Fig. 1. Changes in pH (A), alkalinity (B), acidity (C), and sulfate (D), over time in active (closed symbols, with crab-shell chitin) and control (open symbols, no chitin) microcosms containing AMD and sediment from three different locations in central Pennsylvania (BC = Beech Creek; NF = North Fork; CR = Cherry Run). Data points represent duplicate average measurements; error bars represent 1 standard deviation.

in sulfate concentration were insignificant during the first 7 days of treatment (coefficient of variance <10%). The activity of the SRB started to be evident after this period (Fig. 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 day ($p \leqslant 0.01$). Based on the mass of substrate, these sulfate reduction rates were −23.7 to −31.1 mg SO_4^{2-}/L day g chitin. Similarly, sulfide accumulation started to be evident after 7 days of treatment. In addition to the characteristic rotten-egg smell of hydrogen 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 (see Electronic Annex 2). 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 5 days of treatment, ORP rapidly decreased from 387 ± 87 mV to −148 ± 33 mV (see Electronic Annex 2). Later changes were slower, reaching a minimum of −271 ± 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 (see Electronic Annex 2). 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 1 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, and 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.

3.2. Column test

During the initial stagnant incubation period, a rapid increase in pH was observed in all columns. After 4 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). 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). 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, 2000 mg C/L from VFAs, and 4610 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), 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

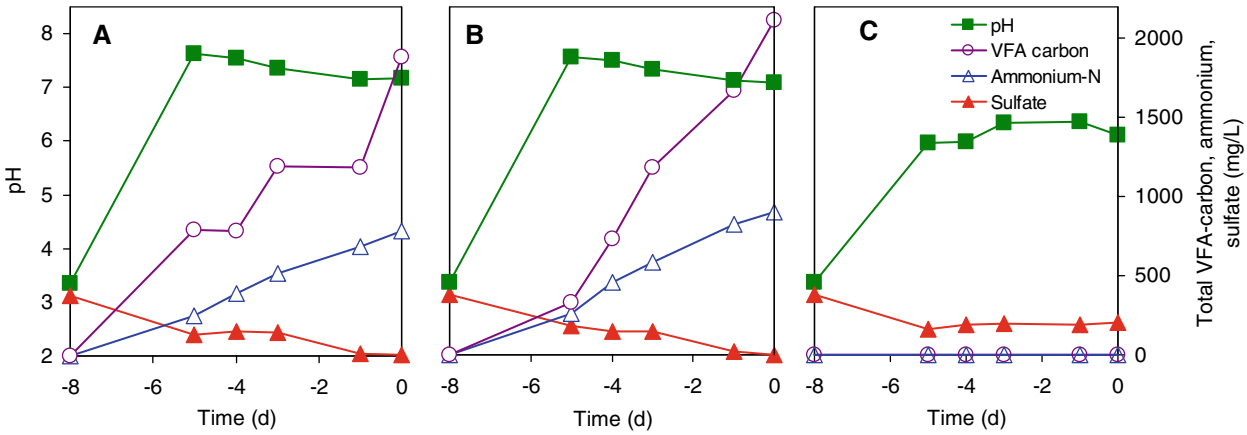

Fig. 2. Changes in sulfate, ammonium, total carbon from VFAs, and pH for active columns 1 (A) and 2 (B), and the control column (C), during the pre-run incubation period. Negative values on the *x*-axis correspond to the number of days prior to the beginning of continuous flow.

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 and 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. 3A).

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 4610 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 to day 102. At t = 102 days, 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. 3B). By the end of the experiment (t = 125 days, 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. 3C). 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% and 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 days (174 PV), followed by iron breakthrough at t = 109 days (234 PV), and aluminum breakthrough was never detected (Fig. 3D–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 days. 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).

4. Discussion

4.1. Microcosm test

When converted to mequiv./L day, the generation rates for alkalinity and calcium are extremely similar (0.82 and 0.86 mequiv./L day, 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 and 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

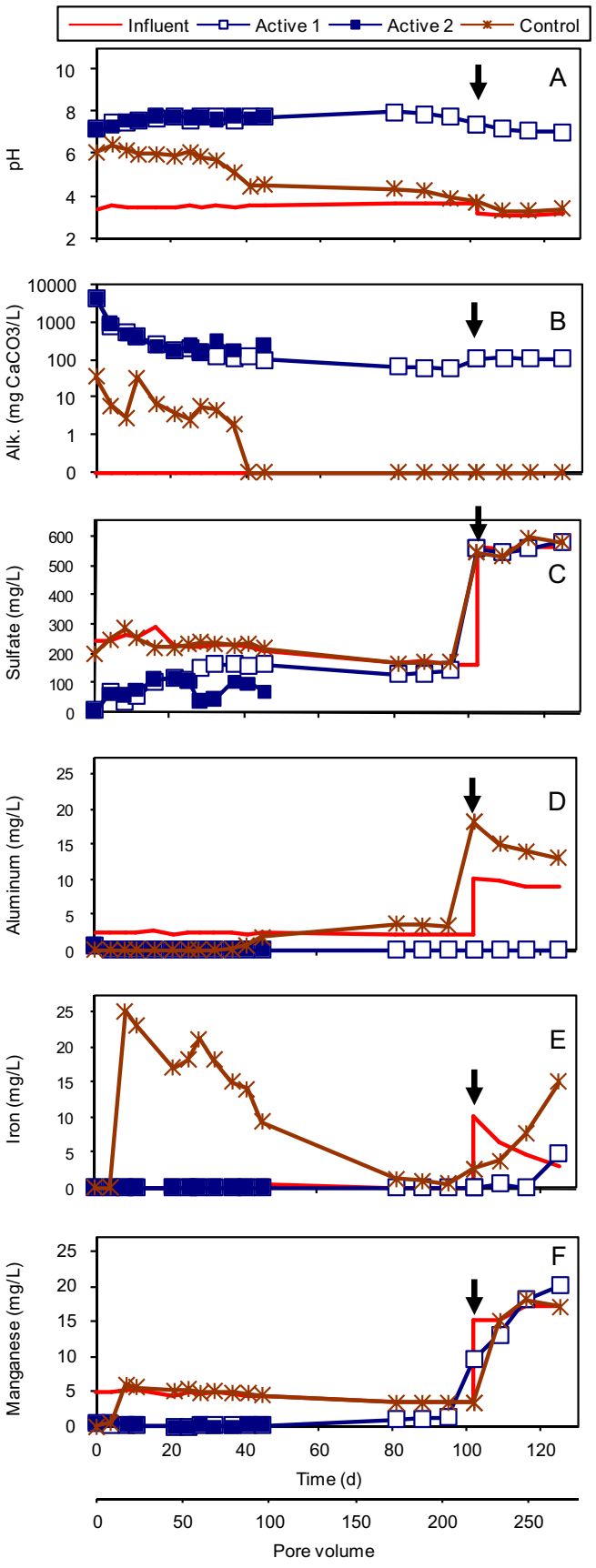

Fig. 3. Changes in pH (A), alkalinity (B), sulfate (C), aluminum (D), iron (E), and manganese (F) in active (with chitin) and control (no chitin) columns during continuous-flow operation. The arrow indicates the point at which the source of influent water was changed.

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 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 an observed plateau of calcium ion concentration after 9 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 $CaCO_3$. SRB 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 ~1/4 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 ± 2.6 mg/L day). In previous batch studies by others, sulfate removal rates ranged between 3 and 109 mg/L day, 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 day 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).

4.2. 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 h) 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. 4). 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. Contrastingly, since pH in the active column was maintained near neutral, aluminum was always retained. Calculated saturation indexes (SI > 0, Fig. 4) indicate that the removal of aluminum in the active columns was due to precipitation of hydroxide phases (gibbsite, diaspore, and/or 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 sulfides (FeS, mackinawite, and pyrite) and ferric (hydr)oxides (ferrihydrite, hematite, and 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. 4). 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. 4) indicate that the column system was highly supersaturated with respect to calcium carbonate minerals and suggest rhodochrosite 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 been previously 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, and 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 $CaCO_3$) giving a carbonate-derived neutralization capacity of more than 15,600 mg as $CaCO_3$. 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 $CaCO_3$ in each column. Based on the measured acidity of the influent, the total acidity input over the course of the column test was 2750 mg, whereas the net alkalinity released (based on effluent titrations) corresponded to 10,300 mg as $CaCO_3$, with a contribution of 1930 mg from biological sulfate reduction (1853 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 atom is converted into carbonate alkalinity; in reality, some incomplete oxidation of the complex molecules derived from chitin can be expected. Also, a fraction of the available (organic) carbon will be used for biomass synthesis, and the rate of biological alkalinity

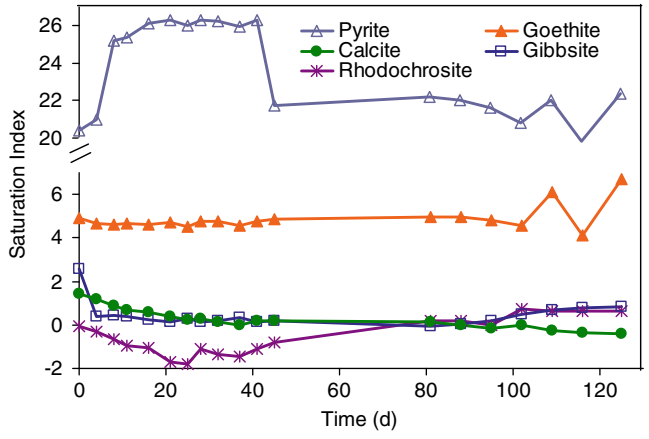

Fig. 4. Calculated saturation indices for representative mineral phases of aluminum (gibbsite, $Al(OH)_3$), iron (pyrite, FeS_2, and goethite, FeOOH), manganese (rhodochrosite, $MnCO_3$), and calcium (calcite, $CaCO_3$) in active (with crab-shell chitin) Column 1 during continuous-flow operation.

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 10 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 US 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.

5. 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 3 days.
- Rapidly stimulated the activity of SRB, sustaining removal rates of -13.6 ± 2.6 mg SO_4^{2-}/L day.
- 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).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2008.11.063.

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