EPA Public Access

Author manuscript

Chem Eng J. Author manuscript; available in PMC 2018 September 19.

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Published in final edited form as:

Chem Eng J. 2017 September 1; 323: 270–277. doi:10.1016/j.cej.2017.04.045.

Mechanisms and effectivity of sulfate reducing bioreactors using a chitinous substrate in treating mining influenced water

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Abstract

Mining-influenced water (MIW) is one of the main environmental challenges associated with the mining industry. Passive MIW remediation can be achieved through microbial activity in sulfate-reducing bioreactors (SRBRs), but their actual removal rates depend on different factors, one of which is the substrate composition. Chitinous materials have demonstrated high metal removal rates, particularly for the two recalcitrant MIW contaminants Zn and Mn, but their removal mechanisms need further study. We studied Cd, Fe, Zn, and Mn removal in bioactive and abiotic SRBRs to elucidate the metal removal mechanisms and the differences in metal and sulfate removal rates using a chitinous material as substrate. We found that sulfate-reducing bacteria are effective in increasing metal and sulfate removal rates and the duration of operation in SRBRs, and that the main mechanism involved was metal precipitation as sulfides. The solid residues provided evidence of the presence of sulfides in the bioactive column, more specifically ZnS, according to XPS analysis. The feasibility of passive treatments with a chitinous substrate could be an important option for MIW remediation.

Graphical abstract

Keywords

Passive remediation; Sulfide; Acid mine drainage; Anaerobic bioreactors

1. Introduction

Acidic, metal-laden mining-influenced water (MIW) is formed when iron sulfides are oxidized to sulfates, and is one of the main environmental challenges associated with the mining industry [1], [2], [3]. MIW remediation typically involves acidity neutralization, sulfate removal, and the removal of dissolved and particulate contaminants (metals and metalloids) [3], [4]. Metal removal by anaerobic treatment in passive systems using sulfate-reducing bacteria (SRB) may cover these three processes and is an interesting alternative to the traditional remediation involving oxidation and pH adjustment [5]. The Sulfate Reducing Bioreactors (SRBRs) need alkalinity and carbon sources to neutralize acidity and promote biomass growth, except for a few reported cases in which metal removal was achieved without the inclusion of an additional alkalinity source [6], [7]. Under anaerobic conditions, SRB reduce sulfate to hydrogen sulfide, which reacts with divalent metal cations to precipitate as stable metal sulfides [1], [8], [9], [10], [11]:

Where $\text{M}^{2+} = \text{Zn}^{2+}, \text{Fe}^{2+}, \text{Ni}^{2+}, \text{Cu}^{2+}, \text{Pb}^{2+}$ and CH_2O represents the substrate.

Other metallic cations precipitate as hydroxides (e.g., $\text{Fe}^{3+}, \text{Cr}^{3+}, \text{Al}^{3+}$), (bi-) carbonates (e.g. $\text{Fe}^{2+}, \text{Mn}^{2+}$), or co-precipitate with the generated sulfides; however, sulfide precipitation tends to be dominant in SRBRs [12]. The organic substrate composition, neutral pH (ideally 5 to 8), reducing conditions (−100 to −200 mV [12]), appropriate temperature (SRB tolerate −5 to 75 °C), and the influent chemistry (SO_4^{2-} concentration) are important to have efficient sulfate and metal removal. SRB need simple and soluble molecules (e.g., lactate, acetate, glucose, etc.) as carbon sources because they do not efficiently process complex organic carbon sources (e.g. sawdust, hay, compost, etc.) [1]. Therefore, SRB synergy with cellulolytic or fermentative bacteria is necessary to break down these complex molecules into simple electron donors [13]. Several mixtures of easily biodegradable (e.g. manure) and more recalcitrant carbon sources (e.g. alfalfa, wood chips, hay, etc.) have been reported to yield high sulfate reduction rates [1], [10], [14]. The organic substrate also serves as a porous support for microbial attachment, metal precipitation, and in many cases, as filtration media in SRBRs. Even though, as seen in Eq. (1), the substrate generates alkalinity, many carbon sources do not provide enough buffering capacity by themselves and, for the treatment of acidic MIW, lime or other alkaline material (e.g. steel slag [15], paper mill waste [1], mussel shells [16], etc.) is typically added to increase pH prior to the anaerobic treatment or as a part of the substrate mixture [1]. Chitinous materials, obtained from crustacean shells (e.g. crab, shrimp), mollusks, insects, and others, contain chitin, the second most abundant natural polymer on earth, and have been tested as a

biosorbents [2], [17] and as a SRBR substrates [3], [14], [18], [19]. The composition of the used chitinous material (made out of crab shells) Chitorem SC-20® (JRW Bioremediation, Lenexa, KS) which is 40% (w/w) CaCO_3 , 30% protein, 20% chitin polymer, 7% moisture, and 3% ash, has all the necessary ingredients to serve as an efficient SRBR substrate. The CaCO_3 acts as the alkalinity source, the protein as the electron donor, and the chitin polymer as substrate and after degradation as an electron donor. The gradual degradation of SC-20 continuously occurring in the bioreactor replenishes these three elements in the system. One additional SC-20 advantage is its 6.9 carbon: nitrogen ratio, which is suitable for an effective substrate because low nitrogen availability limits SRB growth [1], [18].

Precipitation is a common removal mechanism for MIW metals: Cu, Pb, Zn, Cd, and As have been reported to form insoluble sulfide compounds when in contact with H_2S [20]. Mn is the most difficult metal to be removed [20], and typically precipitates as carbonate or sulfide [21], [22]. Cohen [22] found that sorption strength of metals in humic materials was $\text{Fe} = \text{Cu} \gg \text{Zn} \gg \text{Mn}$, that metal sorption proceeded until all sites were saturated, and that sorption could continue for days after SRB activity ended. Venot et al. [14] studied SO_4^{2-} , Mn, and Zn removal in bioreactors using a chitinous product, ethanol, and other mixed substrates, reporting higher Mn removal rate with the chitinous substrate than with the other substrates. Sulfate and Zn removal rates were higher with ethanol and woodchips and hay. Robinson-Lora and Brennan [3] compared SC-20 with lactate and with spent mushroom compost as substrates in sacrificial batch microcosms. The microcosms with SC-20 had higher alkalinity, but similar sulfate removal rates as lactate. Aluminium and iron formed hydroxides and pyrite with all the substrates; Mn was only removed (>73%) by SC-20, likely as rhodochrosite. Hedrich and Johnson [23] used a two-module system to remove metals from mine water. The first module consisted of a pH controlled aerobic bioreactor that aimed for iron removal as schwertmannite followed by the precipitation of CuS and CdS by reaction with H_2S coming from the second module; this second module was an anaerobic SRBR in which SRB was used to precipitate ZnS. The whole unit was more complex than a typical SRBR and required NaOH addition, but achieved high purity in the schwertmannite and ZnS precipitates, with Zn effluent concentration as low as <1 mg/L (not consistent due to flowrate variations). Rötting, Cama, Ayora, Cortina and De Pablo [24] removed Cd, Ni, and Co from metallic solution using caustic magnesia to increase pH in passive systems. This method was effective, providing very low Cd concentration in the effluent (0.005–0.020 mg/L), but these authors did not evaluate Zn or Mn with this method.

Still, we found unanswered questions about chitinous substrate in SRBRs: What is the expected longevity of the chitinous substrate for Zn removal? What is the actual influence of the SRB activity in sulfate reduction? What are the mechanisms of metal removal under microbial activity and under abiotic conditions? This study aims to answer these questions and to elucidate the SRBRs metal removal mechanisms by studying Cd, Fe, Mn, and Zn removal using a chitinous substrate; to find differences in metal removal mechanisms in bioactive and abiotic reactors with a chitinous substrate; and to find evidence of sulfur and metals precipitation in the experimental solid residues.

2. Experimental section

2.1. Mine water samples

The Formosa Mine (Douglas Co, OR) was a Cu and Zn mine that currently holds 15,300 m³ of low-grade ore stored in the mine workings. Water discharges from the mine site are considered a threat for nearby ecosystems and drinking water sources due to Cr, Cu, Pb, and Zn contamination [25]. The Formosa adit mine water (AMW) was collected using a Mini Monsoon sampling pump (Proactive Environmental Products, Bradenton, FL) into four 208 L (55-gal) drums, followed by pressurization with nitrogen to keep a low dissolved oxygen(D.O.) concentration in the water and to prevent metal oxidation during transportation and storage.

2.2. Column fill materials origin and characterization

Chitorem SC-20® was obtained from JRW Bioremediation LLC, quartz sand was purchased from Global Drilling Suppliers, Inc. (Cincinnati, OH). The 1:3 SC-20:sand mixture was prepared to add porosity in the substrate and to avoid clogging. Solid substrates characterization included: moisture by ASTM Method D2216–10; particle size distribution(PSD) by ASTM Method D6913–04, using mesh sizes of 2, 0.422, 0.251, 0.178, and 0.075 mm; elemental composition by acid digestion (U.S. EPA Method 3051) followed by Inductively Coupled Plasma-Atomic Emission Spectrometric (ICP-AES) analysis (EPA Method 6010B) with an IRIS Intrepid (Thermo Scientific, MA) instrument.

2.3. Column bioreactor design, set-up and operation

Three Plexiglass columns (volume 1.15 L, length 101 cm, inner diameter 3.81 cm) were operated in up flow configuration (Fig. S1). The Bioactive and the Abiotic columns were filled with a pre-homogenized SC-20: sand (1:3) mixture (140 g of SC-20 and 460 g of quartz sand). The Control Column was filled with sand only (460 g). A layer of 5 mm autoclaved glass beads was placed below and above the column fillings in all cases. Two influent reservoirs (glass, 11 L) were kept in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) to avoid iron oxidation and precipitation under continuous gentle stirring (PC-410 D Corning stirring plates). The Bioactive Column was fed from reservoir 1 via an Ismatec IP (Wertheim, Germany) 78023–02 multichannel peristaltic pump, with AMW. The Abiotic and Control columns were fed with the AMW with 0.13 g/L of sodium azide (Thermo Fisher Scientific, Waltham, MA), added to assure abiotic condition, via an Ismatec IP 12383–00018 pump. The Bioactive Column was inoculated with 20 mL of SRB-containing biomass obtained from a SRBR operating in Colorado (see details in the SI). The columns were flooded, and then allowed to stagnate for nine weeks to foster biomass growth in the Bioactive Column. After the 9-week period, the columns operation started with a flow rate of 80 mL/day to obtain a 100 h Hydraulic Retention Time (HRT), which was determined by a previous Li tracer study (the porous volume was measured as 0.345 L in the Bioactive Column). Oxidation reduction potential (ORP) was continuously monitored in all columns using ORP probes (Cole-Parmer, Vernon Hills, IL) connected to a Prober-pH 8 interface and recorded in a computer. The effluent was collected in 3-L Tedlar bags. The aqueous influent and effluent of the columns were analyzed weekly for pH (EPA Method 9040B), alkalinity(EPA Method 310.1), dissolved elements (filtered in 0.45 µm PVDF

media) by EPA Method 6010B in an IRIS Intrepid (Thermo Scientific, MA) Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES), anions (including sulfate, also filtered in 0.45 μm PVDF media) by EPA Method 300.0 in a Dionex ICS-2000 Ion Chromatographer (Thermo Scientific, MA), volatile fatty acids by EPA Method 5560B using an Agilent 6890 N Gas Chromatographer – Flame Ionization Detector (Agilent Technologies, Inc., Santa Clara, CA), sulfide by HACH Method 8131 using a HACH DR890 Colorimeter (Hach, Loveland, CO). At the 119th day of operations, the flow rate was doubled to reduce HRT to 50 h. The operational periods were determined according to the Zn breakthrough and were: 472 days for the Bioactive Column and 421 days for the Abiotic and the Control columns. Effluent samples were collected in Tedlar bags to avoid ambient air exposure. The gaseous phase was also collected in tedlar bag and analyzed weekly for composition using an Agilent 6890 GC-TCD. Gas volume was measured using a graduated syringe. The H_2S concentration in the collected gases was measured using a Jerome 631-X H_2S analyzer (Arizona Instruments, AZ).

2.4. Biomass analyses

A Census DNA [26] for SRB was performed by Microbial Insights Inc. (Rockford, TN) in selected samples. The biofilm samples were extracted with a syringe from the center of the columns and frozen for transportation. The ribosomal RNA (rRNA) of the 407th day columns effluent was quantified using the qPCR methods described in Pitkänen, Ryu, Elk, Hokajärvi, Siponen, Vepsäläinen, Räsänen and Santo Domingo [27] in duplicate.

2.5. Solid residue analyses

Post reaction column solids were characterized for the content and oxidation state of sulfur. The columns solid residues were collected at the end of the experiments in three portions for each column: top, middle, and bottom. These residues were dried at 110 °C overnight under oxic conditions. X-ray Diffraction (XRD), X-ray Photoelectron Spectroscopy (XPS) and X-ray Absorption Near Edge Structure (XANES) were performed to investigate sulfur speciation and possible associations in the solid residues. The solids elemental composition was performed by acid digestion (U.S. EPA Method 3051) followed by Inductively Coupled Plasma-Atomic Emission Spectrometric (ICP-AES) analysis (EPA Method 6010B) with an IRIS Intrepid (Thermo Scientific, MA) instrument. X-ray Diffraction (XRD) was performed in an X'Pert-MPD PW3040/00 Diffractometer (PANalytical, Westborough, MA) using $\text{CuK}\alpha$ radiation at 0.02°/min ranging from 10° to 90° and HighScore Plus® software was used for the data analysis. X-ray Photoelectron Spectroscopy (XPS) was done on a Kratos Axis 165 Auger/XPS using a mono-Al beam. Samples were made into a pellet (~1 mm) and mounted on carbon tape. XPS analysis was performed in 2 modes: surface survey in the range of 0–1200 eV to evaluate surface elemental composition and 155–175 eV range of S 2p binding energy (BE) region. Oxidation state of sulfur was evaluated based on the peak fitting using the Igor Peak fitting module. Fitting of the XPS spectra was performed based on the available data on the binding energy peak position within different sulfur compounds, with sulfur at varying oxidation states. The detailed information on the BE for elements can be found on the NIST database (srdata.nist.gov/xps). All peaks were assigned based on the 2 characteristic BE peaks for sulfur: $2p_{1/2}$ and $2p_{3/2}$. The fitting procedure was performed so

as to maintain the shift between the $2p_{1/2}$ and $2p_{3/2}$ peaks in the range of 1–1.2 eV, if possible, and intensity ratio of 1:2.

X-ray Absorption Near Edge Structure (XANES) was performed in fluorescence mode on the Low Energy X-ray Absorption Spectroscopy Beamline at the Center for Advanced Microstructures and Devices (CAMD) in Baton Rouge, LA. Samples were crushed into a fine powder, mounted on kapton tape and sealed with mylar plastic wrap. Sample preparation and experimental details are described by Hormes [28]. The beam energy was calibrated using zinc sulfate, setting it to the S K-edge peak at 2481.4 eV. Sulfur spectra were taken under vacuum at a pressure of 46 Torr and scanned with step widths of 1 eV for the pre-edge region between 2449 and 2469 eV; 0.2 eV between 2469 and 2499 eV, the edge region, and 0.5 between 2499 and 2520 eV. Sulfur reference compounds were used to perform linear combination fitting (LCF) to get composition data using ATHENA. All data was normalized and analyzed in ATHENA by IFEFFIT [29].

2.6. Visual Minteq simulations

The chemical equilibrium software Visual Minteq Version 3.0/3.1 [30] (VMinteq) was used to estimate the elemental concentration and speciation at the experimental conditions described above to help elucidate the metal removal mechanisms involved using the Davies method for activity correction. The reported $\text{Log } K_{\text{sp}}$ was taken from the Visual Minteq database.

3. Results and discussion

3.1. Characterization of Column fill materials

The sand size was 92.4% 0.425–2 mm, and the SC-20 had a greater portion of fines (Table S1). 140 g of SC-20 was used to obtain a total of 32 g of carbon in the columns (carbon content in SC-20 was 22.8%, Table S2). The SC-20 elemental composition was dominated by Ca, P, Mg, S, and Sr, along with smaller concentrations of Al, Fe, Mn, and Zn (Table S3), which may be released into the water during the first few days of operations.

3.2. Mine water characterization and possible SRB inhibition

The collected AMW contained high concentrations of Fe, Ca, Zn, Mg, Cu, sulfates, and its pH was ~3 (Table 1). Some studies have shown that elevated influent metal concentrations inhibit sulfate reduction. Azabou et al. [31] reported that 5 mg/L of Cu, 41 mg/L of Fe, or 71 mg/L of Zn inflicted 50% of maximal growth rate decay in SRB (IC_{50}), and 10 mg/L of Cu, 60 mg/L of Fe, or 125 mg/L of Zn were established as maximum tolerated concentrations (MTC) in an isolated *Desulfomicrobium* strain SA2. In our study, Cu (16.8 mg/L) and Fe (106 mg/L) were found in concentrations higher than the MTC and Zn (73.9 mg/L) higher than the IC_{50} which suggested that the SRB performance could have been affected.

3.3. Columns operation and performance

After the 9-week stagnant period, the columns operation started with a HRT of 100 h (or 1.65 pore volume (PV)/week) aiming to reach a stable anaerobic environment and effective Zn removal with effluent concentrations <0.1 mg/L. This low Zn concentration in the

effluent was observed by the 14th day in the Bioactive Column and on the 49th day in the Abiotic Column. After 119 days of successful Zn removal, the flow rate was doubled to reduce HRT to 50 h (3.29 PV/week). The Bioactive and Abiotic Columns reached steady metal removal at this new HRT. The Bioactive Column reached Zn breakthrough with an effluent concentration of 4.68 mg/L by day 458 with slowly decreasing pH from neutral down to 3.86 (Fig. 1), and it was discontinued after 472 days. The Abiotic Column reached Zn breakthrough with a concentration of 1.66 mg/L by day 166, when pH was 6.64. Biomass activity and biosorption could have played a significant role in extending Zn removal for additional 306 days in the Bioactive Column. The inert control column had high Ca and Na concentrations released in the first week, and after that the effluent and influent had similar concentrations. The dissolution of precipitated compounds when pH decreased towards the end of the experiment resulted in metal breakthrough, and an increase in metal concentrations in the bioreactors effluent. The rRNA quantification of the total microbial population (active cells) in the columns effluent (Fig. S2) had a higher count in the Bioactive Column (1.51×10^6 copy/ml of sample) than the Abiotic Column (3.51×10^3 copy/ml of sample), which means that the added sodium azide produced a 99.8% inhibition. This was also reflected in the low SRB detected ($<2.00 \times 10^3$ cells/mL) in the Abiotic Column (Fig. S3). A discussion of the important variables that were monitored during this experiment follows.

3.3.1. pH and alkalinity—The chitinous substrate was able to add alkalinity and effectively neutralize the influent pH (Fig. 1 and Fig. S4). The high alkalinity concentrations (up to 22,800 mg/L as CaCO_3) continually decreased, but were always higher in the Bioactive Column than in the Abiotic Column. Interestingly, both columns had similar Ca concentrations in the effluent throughout the experimental period (Fig. S6); hence, this alkalinity difference could be, at least in part, due to microbial activity [10].

3.3.2. Oxidation reduction potential (E_h)—Initial E_h values for the Abiotic Column were around -200 mV; however, by the 56th day it experienced a rapid increase, reaching positive values since then (Fig. 1). The Bioactive Column was steadily operating in the reducing region and reached the neutral region on the 458th day of the process. It seemed that the biomass was again responsible for holding the system under reducing conditions, and therefore, allowing a longer period of metal removal activity.

3.3.3. Metal removal performance and mechanisms—Different metals have different removal mechanisms in SRBRs and those mechanisms and their kinetics could be different at different stages of the process [1]. Each one of the studied metals had several processes influencing their behavior.

3.3.3.1. Cadmium: Cadmium present in the influent MIW at a level of 0.267 mg/L was effectively removed, becoming undetectable (<0.007 mg/L) in the effluent of the chitinous substrate columns (Fig. 1). The Abiotic Column experienced Cd breakthrough starting on the 218th day of operation (47.8 PV), while the Bioactive Column did not experience Cd breakthrough. Cadmium mostly exists as Cd^{2+} and can be removed in a sulfate-reducing bioreactor by a combination of biosorption onto the substrate and biomass surface, and by

precipitation. Pagnanelli et al. [32] observed that bioprecipitation accounted for 23%, while biosorption accounted for 77% of total Cd removal in microcosms inoculated with SRB. It is apparent that microbial activity was responsible for the added Cd removal capacity, mainly increasing available S^{2-} for precipitation and also by increasing the available adsorption surface through biomass growth and chitin polymer degradation. VMinteq simulation estimated that Cd would almost entirely precipitate as greenockite ($\text{Log } K_{sp} = -14.02$). However, the precipitated CdS concentration was too low to be observed in the solid residues using XPS, XANES or XRD. Total cadmium in the solid residues was found to be the highest at 96.3 mg/kg in the Bioactive Column middle and 23.2 mg/kg in the Abiotic Column top (Table S4).

3.3.3.2. Iron and aluminum: The initial concentration of iron in AMW was 106 mg/L, but decreased to an average of 30 mg/L during the storage period. Since Fe^{2+} readily reacts with ambient oxygen to become Fe^{3+} , precipitating as $\text{Fe}(\text{OH})_3$ at $\text{pH} > 3$, an anaerobic chamber was used to avoid further Fe oxidation. Nevertheless, the AMW stored in drums for 8 weeks prior to the stagnant period and during the operational process, in spite of having positive nitrogen pressure, decreased the Fe concentration to 11 mg/L. Overall, Fe average concentration in the influent was 28.4 ± 5.89 mg/L. Iron was effectively removed in the Bioactive Column (< 0.105 mg/L), but suffered a breakthrough that started on the 218th day of operation with 77.4 PV (Fig. 1). Iron removal was achieved by precipitation as FeS ($\text{Log } K_{sp} = -18.5$), according to the VMinteq simulation (at $\text{pH } 6.5$, 100% of the iron precipitated). As pH decreased, Fe precipitation rate also decreased and Fe breakthrough was observed at $\text{pH} < 6.5$. Interestingly, Fe did not suffer breakthrough in the Abiotic Column, most probably due to Fe^{2+} precipitation as FeCO_3 and as $\text{Fe}(\text{OH})_2$ (a green color was observed in the column during the experiment) or as $\text{Fe}(\text{OH})_3$ which happens at $E_h > -120$ mV with iron being oxidized [33]. The release of precipitated iron was observed in the Bioactive Column, as the effluent concentration after the 380th day was increasingly higher than the 30 mg/L AMW concentration, indicating that part of the precipitated Fe was solubilized, which happens at $\text{pH} < 5$ [34]. This was also corroborated by the Fe concentrations found in the solid residues with higher levels in the Abiotic Column (1010–4790 mg/kg) than in the Bioactive Column (206–420 mg/kg). Some $\text{Fe}(\text{OH})_3$ precipitation likely also happened in the Control Column because the solid residues showed Fe levels in the range 748–1300 mg/kg. Aluminum is, along with iron, responsible for the acidity of the mine water and had high concentration in the influent (17.0 mg/L) and its removal was also effective (Fig. S5) by precipitation as diaspore ($\text{Log } K_{sp} 6.87$) and hercynite ($\text{Log } K_{sp} 13.85$) according to Visual Minteq.

3.3.3.3. Manganese: Manganese concentration in the influent was uniform at 1.53 mg/L during the experimental period, and was effectively removed in the Bioactive Column up to the 161st day of operations (47.8 PV) and in the Abiotic Column up to the 126th day of operations (31.3 PV; Fig. 1). Manganese is usually present as Mn^{+2} in mine water and its removal is usually problematic because it is soluble in the pH range of 4.5 to 8 in which the bioreactors usually operate, and because its oxidation is kinetically slow, contrary to iron oxidation [21], [35]. Manganese removal in bioreactors depends on pH and E_h , and its precipitation as rhodochrosite (MnCO_3), kutnahorite ($\text{CaMn}(\text{CO}_3)_2$) and as alabandite

(MnS) has been reported [21]. Precipitation as these minerals is further favored by microbial activity, but the specific mechanisms still need further study. Hallberg and Johnson [35] reported that Mn does not readily form MnS, and has not significantly been removed in SRBRs, though it may precipitate as rhodochrosite. Medírcio, Leão and Teixeira [36] reported a maximum of 90% Mn precipitation in the presence of Cd in batch tests with SRB, and observed that the presence of Cd increased Mn removal because it coprecipitated with Cd. Robinson-Lora and Brennan [18] used crab-shell chitin to remove Mn, Al, and Fe from mine water in SRB columns and reached 171 PV in operation with Mn removal, reporting that Mn removal was driven by precipitation of carbonate phases and adsorption. In our study, E_h had values in the range -217 to 120 mV in the Bioactive Column at pH 7.96, decreasing with time to 2.99, which according to Fig. 1 in Bamforth et al. [21] allowed alabandite precipitation. Later, with lower pH and higher E_h , soluble $MnSO_4$ was formed. The same Mn behavior was possible in the Abiotic Column, with the difference that experienced an earlier breakthrough. Hence, microbial activity allowed an increase in Mn removal capacity. However, as in the case of iron, a large excess of Mn was released after 380 days of operation of the Bioactive Column. In both cases, the breakthrough occurred when the system reached a pH ~ 6 , suggesting that decreasing pH allowed the dissolution of the precipitated manganese. In both columns, the most probable removal mechanism for Mn could have been adsorption and precipitation as alabandite, as previously explained. Nevertheless, Visual Minteq did not estimate Mn precipitation in the columns simulations, but reported the association of Mn with HS^- to form $MnHS^+$. The highest reported manganese concentrations in the solid residues were: 96.3 mg/kg in the Bioactive Column middle and 23.2 mg/kg in the Abiotic Column Top, but Mn was not detected by XANES or XRD.

3.3.3.4. Zinc: The Zn level in the influent was ~ 72.9 mg/L, and was undetectable in the effluent for a week (<0.005 mg/L) and was removed (to <0.4 mg/L) in the Bioactive Column for 458 days. Zinc breakthrough (concentrations >0.5 mg/L) started on day 458 in the Bioactive Column with 186 PV and on day 161 with 47.8 PV in the Abiotic Column (Fig. 1). Since the treatment's main goal was Zn removal, the biomass allowed for an extended operational capacity of 138 PV. Zinc is another toxic metal that is difficult to remove from mine water because its hydroxides precipitate at pH >8 [37], but is effectively removed in SRBRs because it tends to precipitate as sphalerite (ZnS) [31]. VMinteq simulation estimated that all Zn present in the system would precipitate as ZnS (Log $K_{sp} -10.8$). The zinc concentrations found in the solid residues in the Bioactive Column (maximum of 7500 mg/kg) and in the Abiotic Column (maximum of 3070 mg/kg) were high enough to be detected and identified, most likely as ZnS by XPS (see Sulfur discussion), proving that ZnS precipitation was an important Zn removal mechanism.

3.3.4. Sulfate reduction—Sulfate concentration in the AMW during the experimental time was 1990 mg/L, which is higher than 630 mg/L, the stoichiometrically required amount to precipitate all cations as sulfides (calculations not shown). Sulfate was removed to an average of 361 mg/L during the experimental period with HRT of 100 h, then increased to an average of 759 mg/L for HRT of 50 h. Nevertheless, sulfate concentrations continuously increased in the Bioactive Column effluent since the flowrate was increased (Fig. 2a).

Sulfate concentration in the Abiotic Column effluent also increased with the increase in the flowrate, from an average of 811 mg/L for 100 h HRT to an average of 893 mg/L for 50 h HRT. Sulfate concentrations in the Control Column effluent were similar to the influent, with the exception of the first two weeks of operation, probably because of some reduction occurring during the stagnant period. In the Bioactive Column, Zn and SO_4^{2-} breakthroughs were observed simultaneously, suggesting that an important mechanism for Zn removal was ZnS precipitation. In the Abiotic Column, Zn breakthrough occurred earlier than SO_4^{2-} breakthrough, suggesting that Zn precipitation as ZnS was less important in this column, and suggesting that biomass activity made a difference in Zn removal. Sulfate removal rate increased with the flowrate increase in both columns. The average sulfate removal rate in the Bioactive Column was 2.49 mol/(m³*day) for 100 h HRT, while it reached an average of 4.32 mol/(m³*day) for 50 h HRT, reaching a maximum of 6.11 mol/(m³*day) in week 61, prior to the breakthrough (Fig. 2b). The average sulfate removal rates in the Abiotic Column were 2.03 and 3.97 mol/(m³*day) for 100 h and 50 HRT, respectively, and reached a maximum of 5.80 mol/(m³*day) in week 60, the last week this column was in operation. Total sulfur in the solid residues was found to be at 1760 to 5300 mg/kg in the Bioactive Column, 432 to 658 mg/kg in the Abiotic Column, and lower concentrations (maximum 67.1 mg/kg) in the Control Column (Table S4).

Sulfides (hydrogen sulfide and metal sulfides) in the effluent were detected in the Bioactive Column in concentrations higher than 1 mg/L in the first 140 days of operation, and then decreased to stabilize in concentrations from 0.01 to 0.04 mg/L, suggesting that SRB activity was higher during those initial 140 days (Fig. S7). The Abiotic Column had decreasing sulfide concentrations from 2.5 to 1 mg/L detected up to day 35, and then decreasing further, maintaining <0.04 mg/L concentrations starting from day 161. The sulfide and H₂S detected in the effluent and gas phase of the Abiotic and Control columns could be due to the reducing effect of sodium azide reacting with the influent sulfate.

In spite of not maintaining anoxic conditions for the solid residues during desiccation, storage, and analyses, the sulfur speciation provided evidence of sulfide generation in the columns. The XPS and XANES spectra indicated (Fig. 3, Fig. 4 and Table S5) changing oxidation state of the deposited sulfur within the Bioactive Column. The characteristic peaks in XPS at ~160 eV corresponded to sulfide ions, and were identified (Fig. S8) with high probability as different ZnS species [38]. The appearance of the broadening peak is a result of multi-peak overlap within the typical region of more than one sulfur species. The small peak located ~167 eV is an indication of sulfite presence, with a good agreement for ZnSO₃[39]. Similarly, peaks at ~170 eV found in the Bioactive Column middle and in the Abiotic Column residues originated from sulfate species, most likely ZnSO₄[40]. It was interesting to find the peak in the Bioactive Column middle at 163.5 eV (Fig. 5), a characteristic binding energy of the thiol compounds [41] without the presence of inorganic S²⁻, indicating that the possible reduction mechanism was sulfate reduction to organic sulfur followed by mineralization. The increasing fraction of S²⁻ compounds towards the top of the bioreactor could be due to the sulfur solubilization which started with the sulfate breakthrough. In the middle of the column, less than 50% of the deposited sulfur was found as sulfide, indicating the progressive nature of the sulfur solubilization. As suggested by XPS data, Zn concentration was found to be highest among potential counter ions (Fig. S8)

and XANES spectra fitted the best to zinc sulfur compounds (Fig. S9). It is possible that the ZnS that precipitated in the Bioactive Column and resisted oxidation during desiccation was present as sphalerite. Steger and Desjardins [42] showed that ca. 0.6×10^{-3} moles/mole of sphalerite were oxidized at 52 °C in 840 h experiments. The XRD did not detect any sulfide minerals, probably due to low concentrations in the solid residues (XRD data not shown).

The results obtained in the Abiotic Column were in stark contrast with the Bioactive column because no sulfide was found in the solid residues, but sulfate and sulfite were detected in the XPS spectrum (data not shown). The sulfate removal mechanisms in the Abiotic Column could have been adsorption (as observed by Moret and Rubio [43]) and precipitation as sulfide, sulfite, and sulfate as corroborated by Visual Minteq.

3.3.5. Acetic acid—Acetic acid started with high concentrations in the experimental period in the Bioactive and Abiotic columns, and decreased until reaching Below Reporting Limit (BRL) concentrations (<0.48 mg/L) starting at day 281 (Fig. S10). The highest acetic acid concentration was measured on day 7 for the Bioactive Column (20600 mg/L), reflecting the high microbial activity occurring during the stagnant period, but decreasing during operation. The presence of acetic acid in the reactor implies that some type of bacteria, other than SRB, were degrading the protein or the chitin polymer, providing the SRB with a suitable carbon source. The Abiotic Column had a peak at day 14 with 9070 mg/L, implying that some portion of the proteins present in the chitin suffered degradation in spite of the lower microbial activity in this column.

3.3.6. Gas volume and composition—The Bioactive Column started with high gas volumes collected, reaching a peak of 610 mL at day 28, and then decreased (Fig. S11). Nitrogen was the main constituent of the gas in this column, and in all columns (Fig. S12). The high nitrogen content in the gas could be due to the high content of nitrogen in the anaerobic chamber holding the influent reservoirs. In fact, the Abiotic Column and the Control Column had more gas volumes collected than the Bioactive Column, although these were dominated by nitrogen. Carbon dioxide had high concentrations in the first days of the operational period in the Bioactive Column, but was even higher in the Abiotic Column for three weeks, then decreased in both (Fig. S13). After that, CO₂ was present at lower concentrations and stabilized at 6–8% in the Bioactive Column and $<1\%$ in the Abiotic Column after 180 days of operations. The presence of CO₂ in the Abiotic Column is the result of the high carbonate content of the SC-20, which was solubilized and then a portion of it was converted to CO₂ and dragged by the gases (mainly nitrogen) moving upflow and collected in the Tedlar bag. The fact of having a consistently high amount of CO₂ in the Bioactive Column could be related to the higher organics degradation rate due to biomass activity. At first, the microbial population degraded protein and the chitin polymer onto acetate. Then methanogens produced CH₄ and CO₂ from acetate. A portion of the generated CO₂ could have been used by methanogens to generate more methane. The lower CO₂ concentrations and the period (around 180 days of operation) support this argument because these coincide with the period in which the Bioactive Column reported methane generation (Fig. S14). Hydrosulfuric acid was reported in the Bioactive Column in higher concentrations in the initial period, but with intermittencies, then occasionally after 180 days

of operation (Fig. S15). H₂S was also observed in the Abiotic (5 times) and Control columns (4 times), which could be the consequence of sodium azide reducing sulfates.

4. Conclusions

The comprehensive study of aqueous phase, gas phase, biomass, and solid residue analyses provided evidence that microbial activity enhanced Cd, Fe, Mn, and Zn removal in SRBRs, while chemical precipitation and adsorption also occurred. Evidence of sulfide formation, probably ZnS according to XANES, was found in the SRBR residues, proving that this could be an important mechanism for Zn removal. In fact, the chitinous substrate was particularly effective in obtaining high removal rates for Mn and Zn. Sulfate removal rates were higher than those reported in the literature [1], [10], [14] for chitinous substrate and others, suggesting that its use could increase the remediation efficiency of acidic mine waters. In spite of the lower mobility of sulfide precipitates, as compared to hydroxides or carbonates, to make it truly a passive system, field systems would probably require the design of other systems to prevent metal leaching from the generated sludge, which was observed at low pH. Further study of sulfate reduction mechanisms is warranted to increase efficiency in field applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded by the Engineering Technical Support Center and performed at the National Risk Management Research Laboratory of the U.S. EPA, Cincinnati, Ohio. The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Mention of trade names and commercial products does not constitute endorsement or recommendation for use. The authors want to thank Mr. Christopher Cora, a project manager at the U.S. EPA Region 10 office for his technical and logistics assistant at the Formosa site. We also acknowledge JRW Bioremediation LLC, particularly Michael Sieczkowski, for providing the Chitorem SC-20 and constructive suggestions used in this study. We also thank Dr. Raghuraman Venkatapathy for his comments on the manuscript, Dr. Jorge Santo Domingo for his help with the molecular microbiology analyses, and Mr. Christopher Holder for laboratory work.

Abbreviations

SRB	Sulfate-Reducing Bacteria
AMW	Adit Mine Water
AMWSA	Adit Mine Water with Sodium Azide
BRL	Below Reporting Limit
MTC	Maximum Tolerated Concentration
PV	Pore Volume
XRD	X-ray Diffraction
XPS	X-ray Photoelectron Spectroscopy

XANES	X-ray Adsorption Near Edge Structure
BE	Binding Energy
MTC	Maximum Tolerated Concentrations

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Fig. 1.

pH, ORP, Cadmium, Iron, Manganese, and Zinc content measured during the experimental time. ORP was measured with a probe inside the column, while all others were measured in the aqueous influent and effluent. ORP data was adjusted and reported as E_h .

Fig. 2. Sulfate content measured in the columns influent and effluent (a) and calculated sulfate reduction rate (b) during the experimental time. All columns had a 100 h hydraulic retention time (HRT) for the first 119 days of operation, after that HRT was reduced to 50 h.

Fig. 3.
Example of XPS spectral fitting – Bioactive Column Top.

Fig. 4.
XANES spectra of solid residue samples.

Fig. 5.
XPS analysis of sulfur range in solid residue samples.

Table 1.

pH, elemental composition, and sulfate content in the Bioactive Column Influent.

Sample Name	Bioactive Column Influent
pH	2.48 ± 0.06
Al (mg/L)	17.0 ± 1.1
As (mg/L)	<0.036
Ca (mg/L)	81.7 ± 5.2
Cd (mg/L)	0.296 ± 0.014
Cu (mg/L)	17.1 ± 0.8
Fe (mg/L)	24.0 ± 6.13
Mg (mg/L)	20.7 ± 1.3
Mn (mg/L)	1.67 ± 0.15
Ni (mg/L)	0.060 ± 0.009
Pb (mg/L)	0.069 ± 0.022
Zn (mg/L)	72.9 ± 4.6
Sulfates (mg/L)	1986 ± 269