THE EFFECTS OF SUBSTRATE SELECTION AND INOCULATION ON THE PERFORMANCE OF LABORATORY-SCALE SULFATE-REDUCING BIOREACTOR COLUMNS

by

Jeffrey Andreas Ladderud

A thesis submitted to the Faculty and the Board of Trustees of the Colorado School of Mines in partial fulfillment of the requirements for the degree of Master of Science (Hydrology).

Golden, Colorado

Date _____

Signed: _____

Jeffrey Andreas Ladderud

Signed: _____

Dr. Jonathan O. Sharp
Thesis Advisor

Golden, Colorado

Date _____

Signed: _____

Dr. Terri S. Hogue
Hydrologic Science and Engineering
Program Head

Abstract

This thesis focuses on the geochemistry of two sets of laboratory-scale sulfate-reducing bioreactors (SRBRs) that examine the effects of substrate selection and inoculation on SRBR performance as it relates to zinc immobilization from actual mine-influenced waters (MIWs). Eight, 20 L down-flow columns that contained substrate permutations of alfalfa, woodchips, sawdust, and walnut shells were operated for more than 1.5 years. Analysis of the results demonstrated that alfalfa hay is an important, relatively-recalcitrant carbon source for the microorganisms within an SRBR. Metal precipitates occurring on the organic substrate were analyzed by energy-dispersive x-ray spectroscopy (EDX) using a random grid and running average. Water samples collected from ports along the length of the columns were analyzed with inductively coupled plasma atomic emission spectroscopy (ICP-AES). This combination of analytical techniques offers unique insights into metal removal and precipitation patterns in an SRBR. EDX determined zinc, sulfur, and calcium elemental abundance can be effectively organized on a ternary diagram to reveal spatial, temporal, and operational trends in these systems. In the active SRBR systems, this was manifested as a shift away from gypsum-like ($CaSO_4 \bullet 2H_2O$) toward sphalerite-like (ZnS) precipitates in the columns over an approximately 1/2 year time period. Images acquired through scanning electron microscopy (SEM) revealed crystal habits suggestive of the assigned minerals. In a subsequent set of experiments, results from eight up-flow, bench-scale columns all containing alfalfa hay and woodchips but seeded with various inoculation permutations (MIW, substrate, and anaerobic digester granules (ADGs)) and operated for 93 days highlight the importance of a viable inoculation source as a means of promoting rapid sulfate reduction and subsequent zinc removal as a zinc sulfide precipitate. Only columns inoculated with active ADGs demonstrated significant sulfide generation accompanied by the removal of Zn, Ni, and Co. Observations of Zn, Ni, and Co removal in columns not demonstrating significant sulfide generation reinforce the importance of sorption as

a secondary metal removal mechanism whose importance compared to metal sulfide generation dwindles over time, as evidenced by diminishing metal removal rates in these columns with time (compared to steady and complete removal of Zn, Ni, and Co in the sulfide-generating columns). The dissolution of iron sulfides in the ADGs and subsequent precipitation of comparatively insoluble zinc sulfides and cadmium sulfides is another potential mechanism of metal removal in the columns. The geochemical data acquisition from these experiments is designed to accompany concurrent microecological studies to collectively enhance our understanding of the biogeochemistry of these systems and advance the field of SRBR technology. Independently, the data show that appropriate substrate and inoculation considerations are needed for rapid SRBR establishment.

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Acknowledgements

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CHAPTER 1: INTRODUCTION

Mines have been part of the North American landscape since before the establishment of the United States. Hopi Indians, residing in present-day Arizona, used coal to bake pottery made from clay (Turner and Lofgren, 1966). These pre-colonial coal and clay mines were but the advent of an impressive era of western mining activity. Gold rushes, coal demand and the search for uranium (among other resources) have peppered the pastoral American West with head shafts and tailings piles, ghost towns and abandoned adits, the legacy of a time in which environmental concerns were far from the forefront of a miner's mind. More concerning than the potential aesthetic displeasures resulting from orphaned infrastructure is the environmental threat of mine waste pollution. The extraction of mineral resources inevitably disturbs the landscape. Crushed, excavated waste rock can interact with the atmosphere releasing heavy metals into the surrounding environment as well as producing acid mine drainage (AMD) (Cohen, 2006). Ore processing can also contribute pollutants such as cyanide, which is leached through tailings piles to extract gold.

Although active mining operations come and go, the environmental impacts of mining are not ephemeral. Current legislation requires mining operations to reclaim the mine site as part of its closure (Gorton III, 2009). This legal and ethical obligation alone is enough to provide work to environmental engineers for decades to come. Add to this the mitigation of pollution from abandoned mines that pre-date environmental legislation such as CERCLA, and it becomes clear that long-term, economic mine remediation solutions are of paramount importance. Presently, mineral holdings are being concentrated among fewer and fewer large mining corporations who are in turn becoming increasingly responsible for numerous and

diverse mine pollution sites. By partnering with such corporations, public research institutions such as the Colorado School of Mines (CSM) can contribute to the effort of working toward responsible and sustainable solutions for reclaiming mining-impacted environments.

AMD refers to mine-influenced waters (MIWs) characterized by low pH values (sometimes less than 1) (Tsukamoto et al., 2004). This acidity is often accompanied by high concentrations of dissolved heavy metals such as iron, copper, zinc and nickel as well as high sulfate concentrations (Neculita et al., 2008b). The production of AMD is a naturally-occurring process that is often exacerbated by mining activity and accelerated by certain bacteria (Akcil and Koldas, 2006). More specifically, the physical and biological oxidation of metal sulfides (e.g. pyrite or marcasite) generates hydrogen ions, which by definition, lower the pH of the water. Perhaps the most prevalent of these reactions is:

$$FeS_2(s) + \frac{7}{2} O_2(g) + H_2O(l) \rightarrow Fe^{2+}(aq) + 2\ SO_4^{2-}(aq) + 2\ H^+(aq)$$ (Kalin et al., 2006)

Here, sulfide is oxidized to sulfate while oxygen is reduced. Additionally, ferric iron oxidation, ferric iron hydrolysis, and enhanced oxidation of ferric sulfide ions are also physical means of producing AMD (Kalin et al., 2006). The oxidation of metal sulfides as described in the equation above is often the result of mining activity. As metal ore is fractured and exhumed from the ground (an anoxic environment), it becomes exposed to oxygen in the air and rainwater and subsequently oxidizes.

Once oxygen has been introduced by physical processes, AMD production is often accelerated by microorganisms such as *Acidithiobacillus ferrooxidans* (Brown et al., 2002; Mielke et al., 2003). This acidophilic chemolithotroph oxidizes ferrous iron to ferric iron that then creates sulfuric acid via the reaction:

$$FeS_2(s) + 14\ Fe^{3+}(aq) + 8\ H_2O(l) \rightarrow 15\ Fe^{2+}(aq) + 2\ SO_4^{2-}(aq) + 16\ H^+(aq)$$

Beyond catalyzed acid production, *A. ferrooxidans* is also responsible for creating conditions that favor the precipitation of complex, yellow iron(III) sulfate called jarosite [$HFe_3(SO_4)_2(OH)_6$] (Madigan et al., 2009). Jarosite (sometimes called "yellow boy" by coal miners) is an aesthetic pollutant in AMD. Another iron oxidizing bacterium, *Leptospirillum ferrooxidans*, lives in the same habitats as *A. ferrooxidans* and is more prevalent at extreme temperatures and pH values (30-50 °C and pH~0.5) (Gould and Kapoor, 2003).

Especially in closed mines with no pumping systems, oxygenated groundwater can interact with exposed minerals for perpetuity, thereby leaching toxic levels of acid, sulfates, metals and metalloids into the surrounding environment creating a legacy pollution problem. Furthermore, AMD can also form in tailings piles, waste rock, and soil heaps, where higher oxygen concentrations and increased surface area can lead to more rapid AMD generation than that which occurs in the mine itself (Johnson and Hallberg, 2005). It has been estimated that spending in the US on AMD prevention and abatement via chemical means is on the order of $1 million per day (Kleinmann and Hedin, 1993).

The ubiquitous presence of AMD presents a global environmental health challenge. A multi-factor pollutant, AMD affects ecosystems through acidity, salinization, metal toxicity, and sedimentation processes (Gray, 1997). These pollution pathways place chemical, physical, biological, and ecological pressures on ecosystems, leading to ecological instability (Gray, 1997). Further complicating the issue is the fact that the sources and influences of AMD vary from site to site, rendering blanket remediation strategies ineffective (Santamaria et al., 2014).

Over the past several decades, scientists and engineers have been developing strategies to remediate environments affected by mine-influenced water (MIW). As a result of the surge of advances in microbiological techniques beginning in the 1990s, biological remediation strategies have become an increasingly more viable strategy for MIW treatment.

One possible mechanism for passive biological treatment with fundamental interest as well as industry-relevant applications involves sulfate-reducing bioreactors (SRBRs) as a means of treating mine-influenced water (MIW, often called AMD).

These bioreactors capitalize on the metabolic process of sulfate reducing bacteria, which reduce sulfate ions in the MIW to sulfide ions (Sheoran and Sheoran, 2006). The sulfide ions then react with divalent metal cations such as Pb^{2+}, Cd^{2+}, Cu^{2+}, and Zn^{2+}, some of which are acutely toxic, to form metal sulfide precipitates (Azabou et al., 2007). This contrasts with the traditional, chemically and infrastructure-intensive, hydroxide precipitation method for metal removal from MIW, which features the formation of metal hydroxide precipitates as solution pH increases. The potential advantages of an SRBR process over traditional hydroxide precipitation include lower precipitate solubility products, chemical requirements and sludge volumes (Whang et al., 1982; Peters et al., 1985). Furthermore, selective precipitation and the fact that many metal-refining operations are designed for sulfide ore processing mean that metal recovery and reuse are more feasible under a metal sulfide precipitation scheme (Esposito et al., 2006). This may be useful in recovering some of the capital and maintenance costs of a passive treatment system, which are already estimated at less than 1/2 and 1/20 the cost of conventional treatment costs, respectively (Eger and Lapakko, 1988).

The development of a successful SRBR involves optimizing substrate composition, residence time, and influent geochemistry (Tsukamoto et al., 2004). With each treatment scenario presenting a unique set of conditions, this process might involve several iterations of laboratory- and pilot-scale bioreactors before a full-scale reactor is implemented (Gusek, 2001; Sánchez-Andrea et al., 2014). However, once successfully employed, an SRBR can be used to precipitate heavy metals such as zinc, nickel, and cobalt out of MIW. These can then be recycled (economics permitting) or properly disposed when reactor substrate is retired.

Ongoing research aims to diminish the limitations that currently hinder SRBR deployment. This remediation strategy can potentially operate nearly maintenance free on a simple set of locally-derived materials which labels it an especially promising option for MIW treatment in rural, inaccessible settings (Sheoran et al., 2010). A series of bench-scale laboratory experiments are useful to optimize the parameters involved in SRBR design and operation as well as better understand treatment in these systems. On a more fundamental level, developing a set of diagnostic tools and procedures to evaluate the performance of SRBRs in a timely manner may prove to be a valuable resource to the mine reclamation industry. Making connections between the geochemistry of an SRBR and its microbiology is one strategy that can be used towards this goal. Microbial metabolism creates reactants, such as sulfide, and the appropriate metal oxidation states to indirectly facilitate metal precipitation and removal as sulfide, carbonate, and/or oxyhydroxide solids (Lewis, 2010). Metal sulfides, so long as they remain unoxidized, are less soluble and bio-available than other metal species (Wildeman and Updegraff, 1997; Lewis and van Hille, 2006) Therefore, the identity of precipitates forming in SRBRs needs to be understood to minimize the potential for the dissolution of metal precipitates and thus aqueous metal remobilization. To these ends, a larger collective research project has focused on coupling next-generation phylogenetic sequencing with geochemical data from inductively coupled plasma atomic emission spectroscopy (ICP-AES), scanning electron microscopy (SEM), and energy-dispersive x-ray spectroscopy (EDX) to explore the relationship between microbes and geochemistry in an SRBR. This approach was conducted primarily through two laboratory-scale column experiments. The first centered on manipulating permutations in column substrate material while the second investigated the role of microbial inoculation and seeding conditions on operational establishment and performance. This thesis presents the design, operational and geochemical components of these experiments and will be coupled to microbial investigations for dissemination in scientific peer-reviewed journals.

CHAPTER 2: EFFECTS OF SUBSTRATE ON ZINC PRECIPITATION

SRBRs are poised for colonization by sulfate reducing bacteria (SRB) as sulfate (the electron acceptor) and organic carbon (the electron donor) are in excess. AMD typically contains only low concentrations of soluble organic carbon, and thus an electron donor must be amended in conjunction with AMD to establish a thriving sulfate reducing system (Kolmert and Johnson, 2001). Although small carbon molecules including alcohols (e.g. ethanol), organic acids (e.g. lactate and acetate), and sugars (e.g. sucrose) may be directly dosed into an SRBR (Kolmert and Johnson, 2001; Sierra-Alvarez et al., 2006; Bayrakdar et al., 2009; Sheoran et al., 2010), these reagents are relatively expensive and short-lived. Furthermore, these simple molecules may be utilized by a wide variety of microorganisms which may out-compete the SRB in the reactor (Koschorreck et al., 2010). Instead, research is often focused on complex carbon sources introduced as a solid reactor matrix such as corn stover, hay, straw, pine wood, sawdust and mushroom compost (Johnson and Hallberg, 2003; Song et al., 2012). These compounds provide electron donor molecules which are more slowly released within the SRBR, generally through the assistance of cellulose-degrading microorganisms (Gibert et al., 2004; Logan et al., 2005). The slow release of organic acids from the complex substrate sustains the SRB population for a longer period of time when contrasted with the more chemically-defined, soluble substrates and may increase the life of the reactor before substrate replacement is necessary (Sheoran et al., 2010). Furthermore, many of these compounds are locally available and typically less expensive (Gibert et al., 2004). Empirical evidence suggests that a mixture of readily biodegradable substrates (e.g. manures and organic sludges, which can also provide

relevant microbes as expanded in the next chapter) and more recalcitrant ones (e.g. woodchips and alfalfa) promote more optimal community establishment and sulfate reduction (Waybrant et al., 1998; Cocos et al., 2002; Waybrant et al., 2002; Zagury et al., 2006; Neculita et al., 2011).

2.1 Objectives/Questions

Researchers have used a variety of different organic substrates when designing and building SRBRs (Neculita et al., 2007; Sánchez-Andrea et al., 2014). However, it appears that the selection of a specific substrate is often biased by local availability and what has worked well in previous experiments (Chang et al., 2000; Neculita et al., 2007; Sánchez-Andrea et al., 2014; Kim et al., 2014; Santamaria et al., 2014). Research has been conducted toward characterizing some of the natural organic carbon materials used in SRBRs, such as various woodchips, sawdust, grass, composts, and manures, for parameters including carbon, nitrogen, and lignin content (e.g. Gibert et al., 2004; Coetser et al., 2006; Zagury et al., 2006), and more recent studies have explicitly linked substrate composition to microbial community development (Lindsay et al., 2011; Hiibel et al., 2011). However, attempts to describe relationships between substrate parameters, such as organic carbon content, and the ability of said substrate to support sulfate reduction and metal removal have been unsuccessful due to weak correlation between substrate parameters and performance metrics (Zagury et al., 2006; Neculita et al., 2011). We are aware of little research at present that specifically examines the relationships between organic substrate composition, microbial community development and SRBR geochemical performance using cutting-edge technologies such as next-generation sequencing and synchrotron analysis. The following experiment is designed to help fill in this gap in the literature by establishing laboratory systems than enable this form of exploration.

Specifically, an array of eight pilot scale (20 L) down-flow columns were used to investigate the relationship between organic substrate and zinc removal while investigating the efficacy of emerging applications of analytical tools such as next-generation phylogenetic

sequencing, energy-dispersive x-ray spectroscopy (EDX), and synchrotron elemental and mineralogical analyses. This thesis was designed to develop an SEM-EDX-based semi-quantitative approach, in addition to ICP-AES, to assess mineralogical differences between samples collected from the eight columns, and to compare and contrast the merits of SEM-EDX against those of ICP. Geochemical precipitation data as discerned by electron microscopy and EDX will be presented in this manuscript while sequencing and synchrotron data can be found in forthcoming publications.

The experiment was designed with the following questions in mind:

- Can the presence of certain substrates or a mix of substrates be linked to enhanced zinc removal?
- Can analysis of precipitates using EDX agree with aqueous mass removal as quantified by ICP, and can this be used to enhance understanding of metal removal over space and time?
- Can a synthesis of EDX spectra acquired at a microscopic scale using a running average approach provide a reliable portrayal of the mineralogy formed on the column substrate at a large scale?
- Does this form of geochemical analysis further understanding of stability and/or immobilization of metals in the columns?

2.2 Materials and Methods

Eight down-flow PVC column pilot-scale bioreactors were constructed and operated in southeastern Arizona in collaboration with an industry sponsor. The columns were operated for 498 days.

2.2.1 Column Design and Operation

The columns were 52” in height and had an inner diameter of 6” for a total volume of 24 L when empty. Five liquid sample ports were installed along the column length, in addition to an effluent port, and three solid sample ports (labeled ports 1 (top), 3, and 5), which consisted of a slotted plastic tube placed perpendicular to the major axis of the column. The ports were located as indicated in Figure 2.1. The bottom 4 inches of the columns were filled with glass marbles. The SRBR columns were filled with either single, regionally-sourced organic substrates (ponderosa pine woodchips, sawdust, alfalfa hay, and walnut shell) or mixtures thereof (Table 2.1) to a volume of 18 L. These recalcitrant, solid substrates were selected based on precedent for prior bioreactor performance (e.g. Sheoran et al., 2010). In addition, limestone was mixed in with the organic substrates at a fixed weight percent basis. The limestone adds alkalinity and also serves as a bulking agent (Amos and Younger, 2003). Four pre-packed grab bags were placed into each of the solid sampling ports to facilitate substrate sampling during column operation. The substrate in these bags was of identical permutation to, and in full communication with the surrounding column environment. Each of the eight columns (and the sample bags therein) had a different substrate permutation, thus, they contain different organic and limestone masses (Table 2.2).

Table 2.1 Column substrate composition by weight percent

		Column							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Substrate	**Limestone**	30%	30%	30%	30%	30%	30%	30%	30%
	Woodchips	50%	-	35%	35%	-	-	70%	-
	Sawdust	10%	35%	-	35%	-	70%	-	-
	Alfalfa Hay	10%	35%	35%	-	70%	-	-	-
	Walnut Shells	-	-	-	-	-	-	-	70%

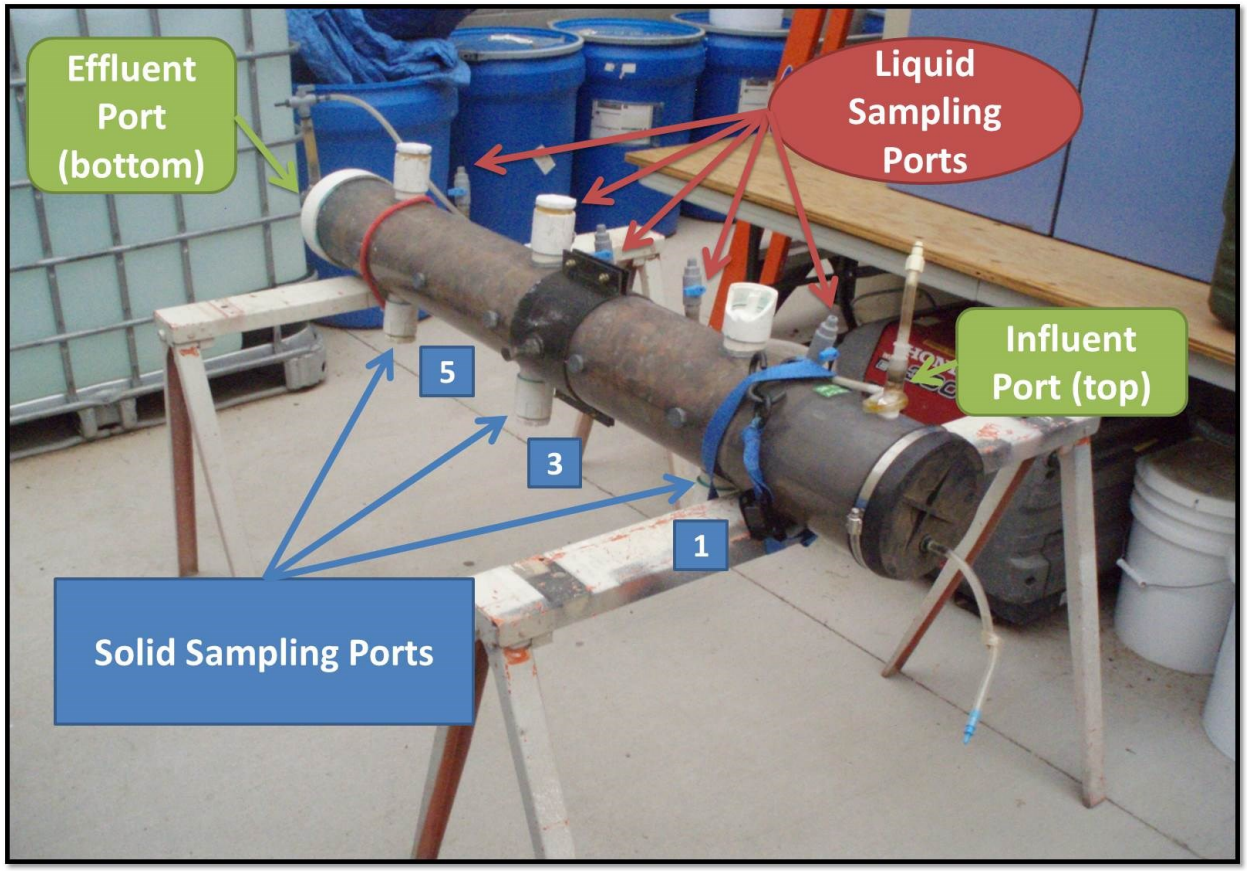

Figure 2.1 Column design

Table 2.2 Column substrate composition by mass

		Column							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Substrate	**Limestone (g)**	1152	1080	1074	1591	1065	1873	1152	4756
	Woodchips (g)	1947		1282	1841	-	-	2789	
	Sawdust (g)	392	1317	-	1787	-	4369	-	-
	Alfalfa Hay (g)	403	1332	1276	-	2449	-	-	-
	Walnut Shells (g)	-	-	-	-	-	-	-	9617
	Total (g)	3894	3729	3632	5219	3514	6242	3941	14373

The columns were fed mining influenced water (MIW) collected in May 2012 from a nearby field site in Arizona and stored for the duration of the experiment in a 2500-gallon plastic tank at the experimental site. The columns were packed on May 23, 2012 and filled with MIW that was re-circulated at a rate of 15 L/day. On June 15, 2012, 520 mL of a mixed-culture

sulfate-reducing laboratory enrichment was added to the columns for inoculation purposes. Beginning on July 30, 2012 the columns were fed a continuous flow of MIW at a rate of 400 mL/day. The MIW had a pH ~ 6.5, near zero alkalinity, and time-averaged major ion constituents as listed in Table 2.3. Notably, the water was poor in Al and Fe. Influent water chemistry was monitored via weekly ICP and ion chromatography (IC) samples, revealing that the concentrations of zinc, calcium, and sulfate fed into the columns decreased to 80, 87, and 86 percent of the original values, respectively, over the course of the experiment. Geochemical modeling (Visual MINTEQ, Gustafsson) indicates that the influent MIW is slightly over-saturated with respect to gypsum ($CaSO_{4(s)}$) at 25°C. The decrease in influent calcium concentration over the course of the experiment suggests gypsum precipitation, though no precipitate was visually observed due to the opacity of the holding tank. Following the September 2012 sampling event, columns 1, 3, 4, and 7 were re-inoculated on October 29, 2012 using 1 L of effluent from Column 2. Column 6 was re-inoculated using 0.5 L of Column 2 effluent. Following the May 2013 sampling event, columns 1, 4, 6, and 7 were again re-inoculated using 389, 522, 624, and 394 grams composted cow manure, respectively on June 12, 2013. The manure was added to the top of these SRBRs. Flow rates for the MIW were manipulated between 0 mL/day and 800 mL/day in an attempt to optimize metal removal rates, with well-performing columns (those removing relatively high amounts of Zn) receiving higher flow rates.

Table 2.3 Average influent water chemistry: major ions

Ion	Sulfate	Chloride	Calcium	Magnesium	Manganese	Sodium	Zinc
Conc. (mg/L)	4940	30	560	770	4	180	170

2.2.2 Column Sampling

Pre-packed substrate bags from each of the solid sampling ports were collected under a stream of N_2 gas for DNA extraction (not discussed in this paper) and SEM-EDX analysis after

349 and 498 days of flow through operation (May 2013 and October 2013). Liquid samples for geochemical analysis were collected from liquid sampling ports 1, 3, and 5 on each of the eight columns after 110, 349 and 498 days of flow through operation. Additionally, water samples were collected on a weekly basis from the influent MIW tank as well as from the effluent ports of each of the eight columns. These samples were analyzed for metals using ICP, anions using IC, and sulfate using colorimetric spectroscopy assays (CSAs). The SEM-EDX samples were stored in ambient laboratory conditions for several months prior to analysis. Water samples were collected from the top, middle and bottom ports of each column under a stream of N_2 gas on days 110, 349, and 498 for ICP analysis. These samples were collected from the top down and collected before retrieval of solid samples to avoid imparting a sampling bias that could be introduced from downward water migration or gas introduction. All ICP samples were acidified with concentrated nitric acid on site, and filtered with a 0.45 µm membrane within 48 hours of collection. The ICP samples were analyzed for elemental constituents using inductively coupled plasma atomic emission spectroscopy (ICP-AES). The difference in zinc concentrations between influent and effluent ICP samples was used to calculate the zinc removal rate (normalized to the variable column flow rates) in each column, which in turn was used as a performance metric. Sulfate readings measured using IC were generally approximately 10% larger than those measured using the CSA. As with zinc, the difference in sulfate concentrations between influent and effluent CSA samples (normalized to the variable column flow rates) was used to calculate the sulfate reduction rate in each column, which in turn was used as a performance metric.

2.2.3 Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray Spectroscopy (EDX)

Metal precipitates (e.g. zinc-sulfur-type precipitates) associated with dried, archived substrate samples were visualized with electron microscopy and further characterized for

elemental analysis using a Hitachi TM 1000 SEM (Hitachi Ltd., Tokyo, Japan) system equipped with a Quantax 50 EDX system. Multiple pieces of sawdust/woodchip/alfalfa/walnut shell were spread in a single layer onto a 15 mm by 15 mm square of adhesive carbon tape and EDX spectra were acquired at randomly selected positions following standard procedures for biovolume fraction quantification using digital image analysis (e.g. Daims and Wagner, 2007; Almstrand et al., 2013). Running averages were calculated for the molar percent of sulfur, zinc, and calcium. Thirty spectra were acquired for each sample. By n=30 spectra, the running average and associated 95% confidence interval for the molar percent of zinc, calcium, and sulfur had leveled out in all samples, indicating that sufficient spectra had been acquired (Figure 2.2).

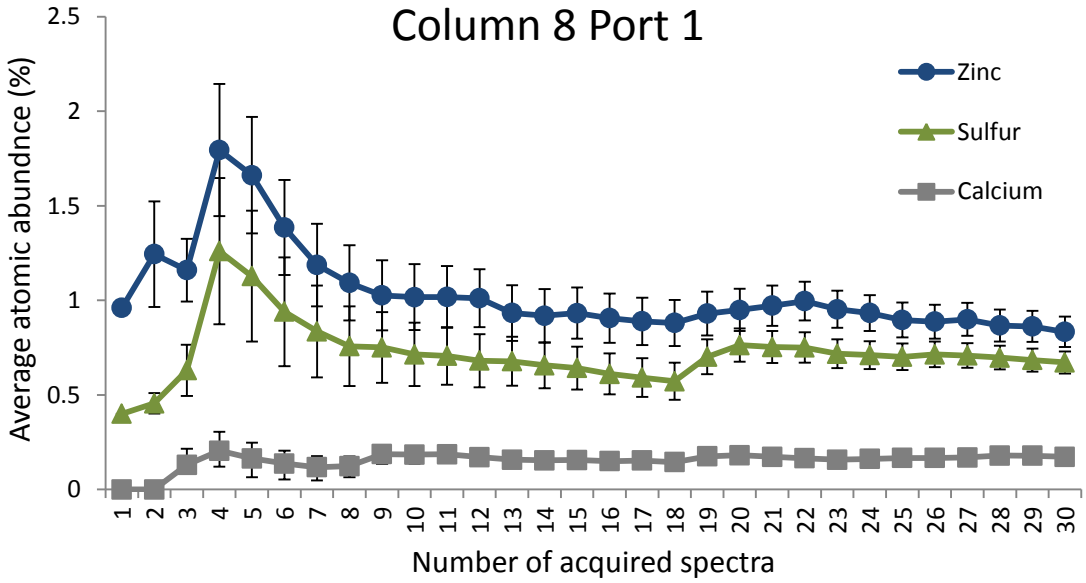

Figure 2.2 Representative plot showing running averages of molar percent Ca, Zn, and S taken from 30 randomly collected SEM-EDS spectra. Error bars = 95% confidence interval.

2.3 Results and Discussion

Typically, analysis of the aqueous phase using inductively coupled plasma atomic emission spectroscopy (ICP-AES) is the default means of assessing metal removal rates in an

SRBR (e.g. Jong and Parry, 2006; Santamaria et al., 2014). Metal concentrations are measured in both the influent and effluent water, and the difference is recorded as the amount of metals retained by (or released from) the SRBR. Although this method is straightforward and generally accurate, complimentary analytical methods can better illuminate metal removal mechanisms within the SRBRs as well as the relationship between metal precipitation and substrate.

2.3.1 SEM-EDX and Ternary Diagrams

An SEM-EDX-based semi-quantitative approach was developed and employed in concert with ICP-AES, to assess mineralogical differences between samples. The method, which represents a major contribution of this thesis to the larger scope of work, was adapted from the acquisition methods used for quantitative fluorescence microscopy and digital image analysis of microbial biofilms in domestic wastewater treatment (e.g. Almstrand et al., 2013). Rather than images, as were collected in the precedent, spectra were acquired in a strictly randomized manner. This allows for reliable quantification of heterogeneous samples given that data from enough fields of view (FOVs) are utilized (Daims and Wagner, 2007). The running average for molar percent and the associated 95% confidence intervals revealed that 30 spectra per sample port were sufficient sampling depth for obtaining a representative average molar abundance of elements in the sample (Figure 2.2). In comparison, Kaksonen et al. (2003) used a total of 18 randomly selected spots for EDS analysis of metal precipitate composition in an up-flow anaerobic sludge blanket reactor. Few of the running averages in the present experiment were markedly different between n=18 and n=30 spectra (data not shown) suggesting that using 18 rather than 30 spectra may be an acceptable method of optimizing resources when using SEM-EDX analyses in future research.

Analysis via SEM-EDX is complimentary to ICP-AES analysis but limited in applicability to contaminant mass balance (Jong and Parry, 2005; Neculita et al., 2008b). Whereas ICP data

report absolute aqueous concentrations for a homogeneous effluent, EDX analyses report relative mass ratios for the atomic constituents of solid substrate and precipitate within a sample of a complex, heterogeneous system. The SEM provides a visual image of the sample while the EDX can offer insights as to the possible mineralogies present in the image. This SEM image can be valuable both in identifying precipitated minerals based on visible crystal structure (Figure 2.3), and in observing the proximity of said minerals to specific substrates (e.g. gypsum crystals observed on woodchips) and to microbes (Figure 2.4). The SEM-EDX analysis of any given sample offers a unique snapshot of the substrate. It is, however, important that this snapshot is treated as such, and that it is not necessarily extrapolated to be representative of an entire zone within a column, though this can be overcome by taking a sufficient number of different samples. Furthermore, while EDX provides molar and weight percent analyses of precipitates, it is not able to ascertain chemical formulas for the precipitated mineral as x-ray diffraction (XRD) or electron diffraction patterns from a transmission electron microscope (TEM) are able to do. The use of the SEM-EDX approach must, therefore, be carefully evaluated prior to undertaking as the process of randomly acquiring spectra by hand is time and labor intensive (2 hours per sample for 48 samples plus data entry and analysis—approximately 150 hours for this project). Though not true for this experiment (where SEM-EDX data provided a valuable, convergent line of evidence supporting inferences about zinc removal mechanisms), in many cases, the bulk geochemistry from ICP-AES analysis and/or total metal deposition analysis via acid digestion of the substrate is sufficient when metal removal data from an SRBR is required.

In order to better quantify and spatially organize the EDX data, the average zinc, sulfur, and calcium molar percentages from 30 spectra were normalized to 100% and plotted on a ternary diagram.

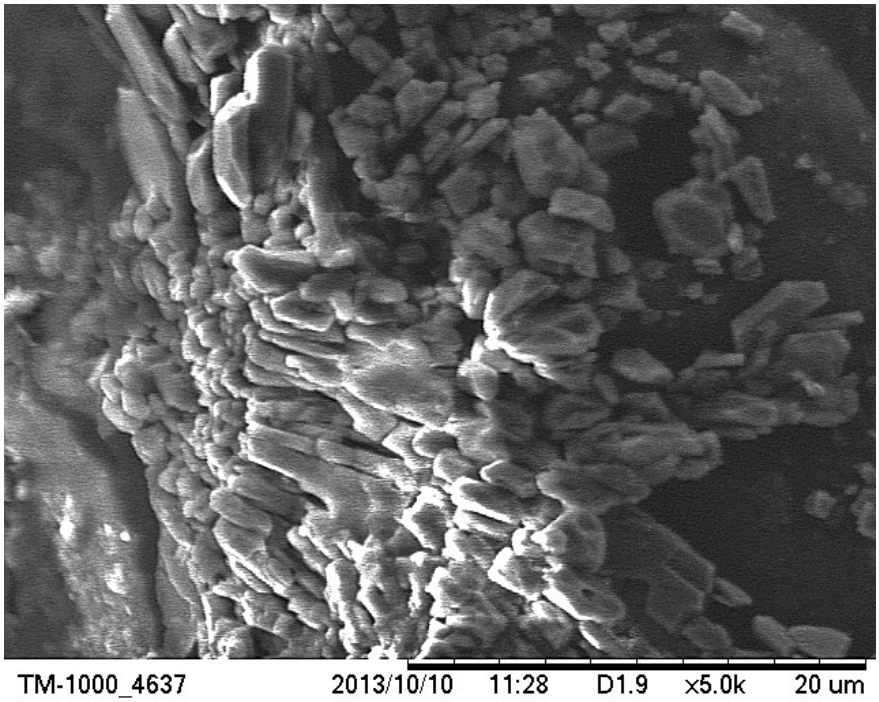

Figure 2.3 The crystal habit in this SEM image of woodchip substrate from Column 7 is suggestive of gypsum ($CaSO_4 \cdot 2H_2O$), as has been observed by Amos and Younger (2003) in another biological treatment system for AMD.

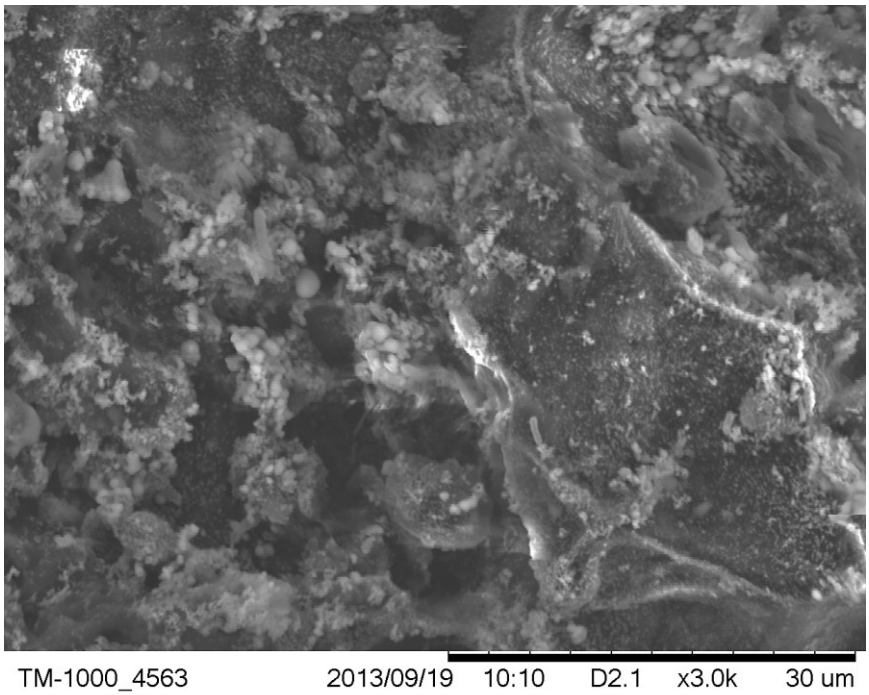

Figure 2.4 Spherical zinc-sulfur type precipitate (likely sphalerite (e.g. Labrenz et al., 2000)) on walnut shells from Column 8. Note the proximity of the microbe and the precipitate in the upper left quadrant.

Although other elements present in the column influent and carbon-based substrate, notably carbon and oxygen, were detected by the EDX (Figure 2.5), additional components were filtered as the ternary relationship between zinc, sulfur, and calcium was of interest to this study. This is because these other elements either didn't form insoluble precipitates with sulfur (e.g. Mg) or were present at very low concentrations in the influent water relative to Zn and Ca (e.g. Ni, Cu). The relatively simple chemistry of this MIW (compared to other MIWs) was convenient in that the absence of Fe and Al made for less clogging in the columns. Zinc is a favorable contaminant to study as it behaves similarly to other more-toxic metals (e.g. Cd) but is less toxic and therefore safer to work with in an experimental setting.

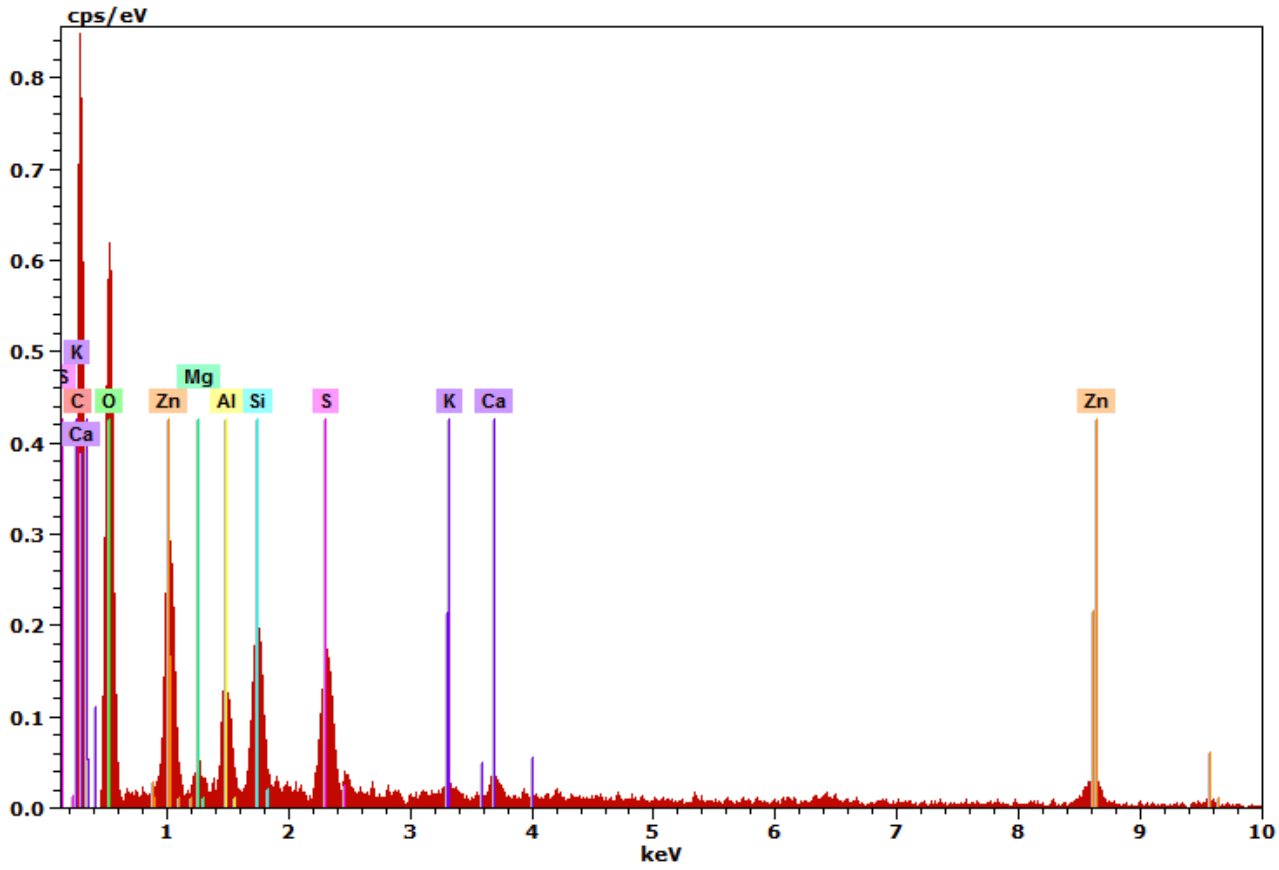

Figure 2.5 An example EDX spectrum from Column 2, Port 1, October, 2013. Though other elements present in the influent water or the carbon-based substrate were detected by the EDX, only S, Ca, and Zn were considered in this study.

Spatial and temporal trends within and between substrates were graphed using ternary diagrams portraying the relative abundance of these three end-member species in a triangular space (whereas typical x-y coordinate plots can only compare two variables). In this triangular space, any given data point has a value for each of three axes, the sum of which is 100%. The vertices of the triangle represent 100% relative abundance of that end member while the edge opposite that vertex represents 0% of that end member. Ternary plots provide an alternative means to bar graphs, which are often used to present multivariate data. A ternary plot is good for more densely juxtaposing large amounts of data than is possible in a bar graph. However, the aggregation of data in a ternary plot runs the risk of drowning out individual data points. Thus, ternary plots are recommended for observing large-scale trends and groupings as was useful in addressing our research objectives. Bar graphs in turn are often more suitable for zooming in on smaller sub-sets of data.

Plotting EDX data on a ternary diagram allows for a visual analysis of the relative abundances of zinc, sulfur and calcium over space and time in our system (Figure 2.6). An underlying assumption of our analysis is that varying relative abundances of zinc and calcium are likely controlled by the speciation of sulfur. If sulfide is the dominant species, then zinc will precipitate as ZnS whereas a high concentration of sulfate will result in the precipitation of gypsum ($CaSO_4 \cdot 2H_2O$) (Elliott et al., 1998; Amos and Younger, 2003; Johnson and Hallberg, 2005; Zagury et al., 2006; Neculita et al., 2008a). Both zinc-sulfur and calcium-sulfur precipitates were observed using an SEM (Figure 2.3, Figure 2.4). Precipitation of zinc-sulfur in column 8 was as spherical aggregates (likely sphalerite), similar to ZnS precipitates observed in other mine water environments (Labrenz et al., 2000; Gammons and Frandsen, 2001; Labrenz and Banfield, 2004; Church et al., 2007) (Figure 2.7). The speciation of sulfur is largely controlled by the activity of sulfate-reducing bacteria, which are able to reduce soluble S(VI) to S(-II) via enzymatic reduction associated with their metabolism (Widdel, 1988). Influent water

was rich in sulfate but poor in sulfide. Thus, the reduction of sulfate to sulfide by SRBs such as *Desulfosporosinus* and *Desulfurispora* is a prerequisite for zinc removal as zinc sulfide in this system (Kaksonen et al., 2008; Alazard et al., 2010).

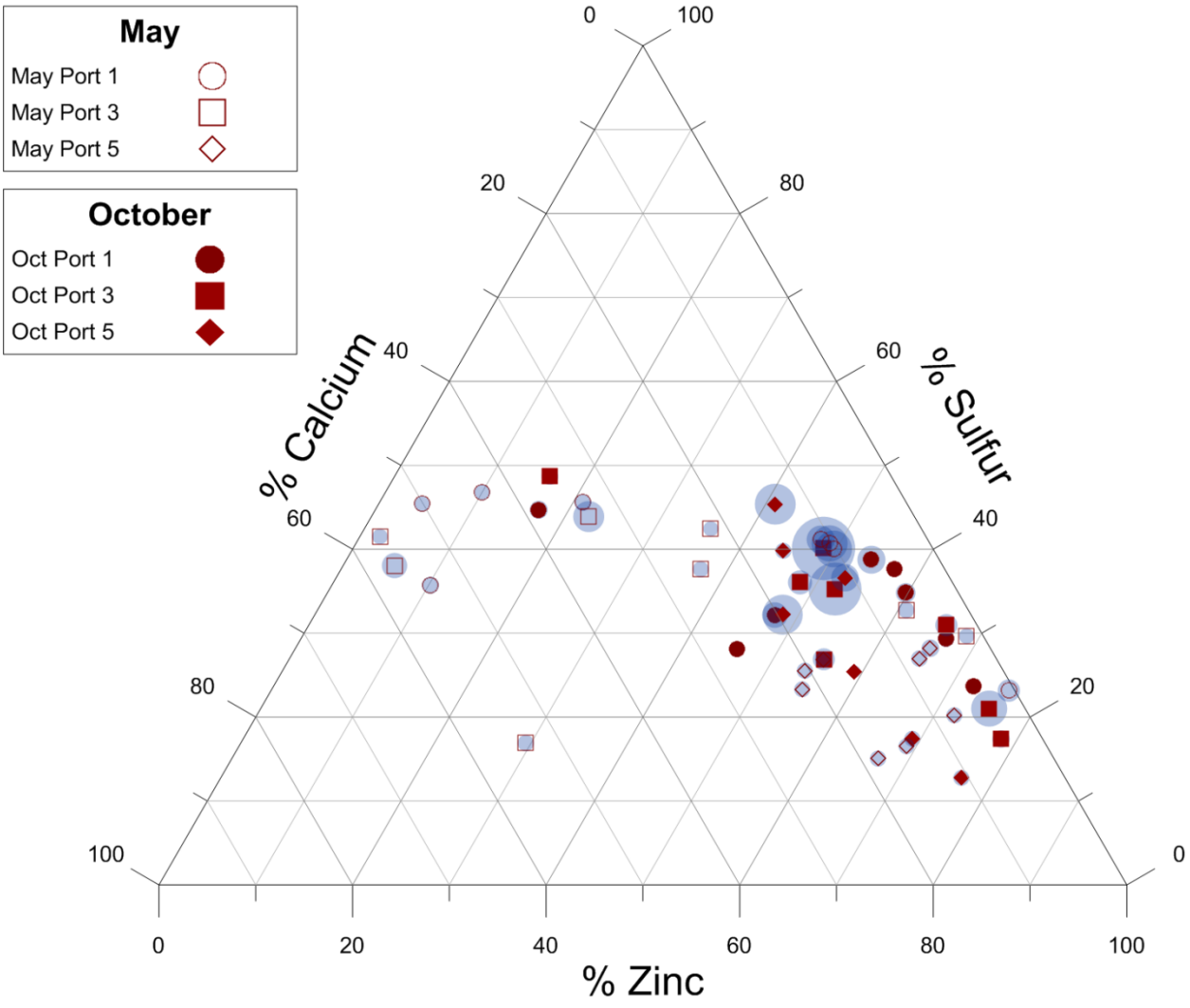

Figure 2.6 A ternary diagram of the relative molar fractions of Zn, S, and Ca, as analyzed using EDX, from the May and October 2013 sampling events of all eight columns. Each data point is the average of 30 randomly-acquired fields. The blue shaded halos around the data represent the relative mass of zinc removal per day at that point in time and space, as measured by ICP-AES, for the column segment ending at the port represented by the EDX data point (Table 2.4).

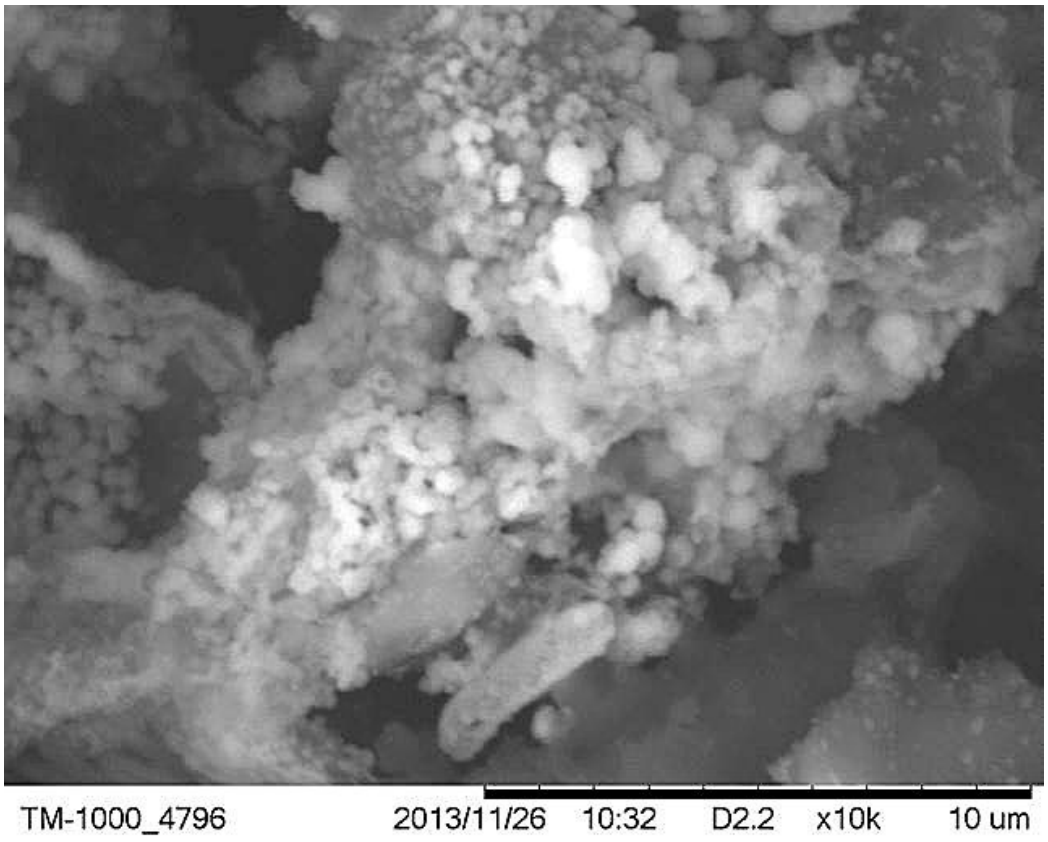

Figure 2.7 Zinc-sulfur-type precipitates in column 8 exhibit similar morphologies to the one-micron diameter spherical aggregate ZnS precipitates collected from biofilms in a flooded Pb-Zn mine in Wisconsin and described by Labrenz et al. (2000) in the journal Science.

Overall, the relative molar fractions of zinc were higher in October than in May (Figure 2.8). With the exception of column 4 port 3 and column 6 port 1, the normalized data for October cluster between approximately 40% and 80% zinc, 15% to 45% sulfur, and 5% to 25% calcium. This contrasts with the May data, which have a wider spread along the zinc and calcium axes. Thus, over a time span of 149 days, the columns have generally evolved from systems with significant proportions of calcium sulfate toward zinc-sulfur-precipitate-dominated systems. As a bias of analysis, ternary plots are limited to three elements and hence only show the relative abundances of the chosen elements: zinc, sulfur, and calcium. Thus, this temporal evolution may not be the same for absolute quantities of zinc-sulfur and calcium-sulfur precipitates, which could instead be discerned through ICP data and putative geochemical

modeling. However, coupling EDX and ICP data suggest that the temporal evolutions of these relative and absolute quantities track each other fairly well, with the highest zinc removal rates from the ICP data set generally coinciding with a Zn:S molar ratio of approximately 1:1 in the EDX data set (Figure 2.8).

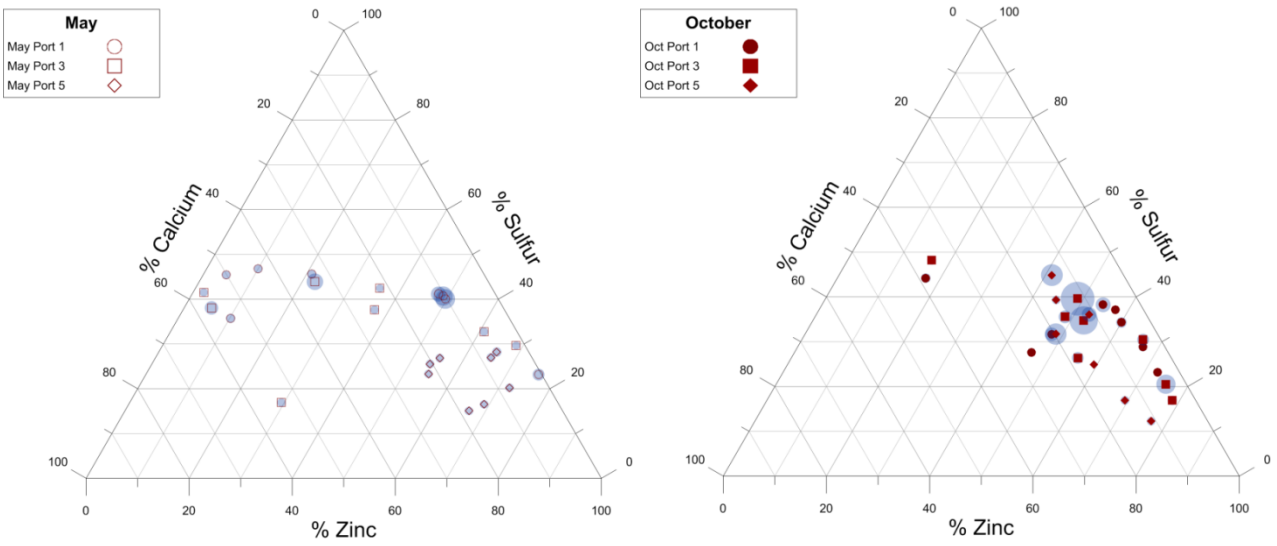

Figure 2.8 Relative molar abundances of Zn, Ca, and S for all eight columns in May of 2013 (left) were more variable than those analyzed in October 2013 (right). The shaded halos around the data represent the relative mass of zinc removal per day at that point in time and space, as measured by ICP-AES (Table 2.4).

2.3.2 ICP and Sulfate Data

This study explored zinc precipitation using SEM-EDX as an alternative, supplementary means of investigating SRBR performance, though it relied on ICP data from aqueous samples as the ultimate measure of performance. A running total of the milligrams of zinc removed per gram of substrate is presented in Figure 2.9. From this plot it is evident that, in terms of this metric, Columns 2, 3, and 5 are far outperforming the other columns. These three columns share a commonality in that they all contain at least 35% alfalfa (Table 2.1). Column 5, which has an organic substrate entirely of alfalfa, is especially interesting in that the ternary data from

all three ports converges temporally from May to October (Figure 2.10). Column 1, which contained 10% alfalfa, had higher zinc removal rates by day 498 than did columns 4, 6, and 7 which contained only limestone and sawdust and/or woodchips, but a lower zinc removal rate than columns 2, 3, and 5 had. Thus, it may be surmised that alfalfa is a valuable constituent to the substrate composition. Though Column 8 does not perform as well as columns 2, 3, and 5 given the metric presented in Figure 2.9, which normalizes zinc removal to substrate mass, it is important to note that Column 8, which had a much larger substrate mass than did columns 2, 3, and 5, still removed nearly 100% of the influent zinc throughout the duration of the experiment.

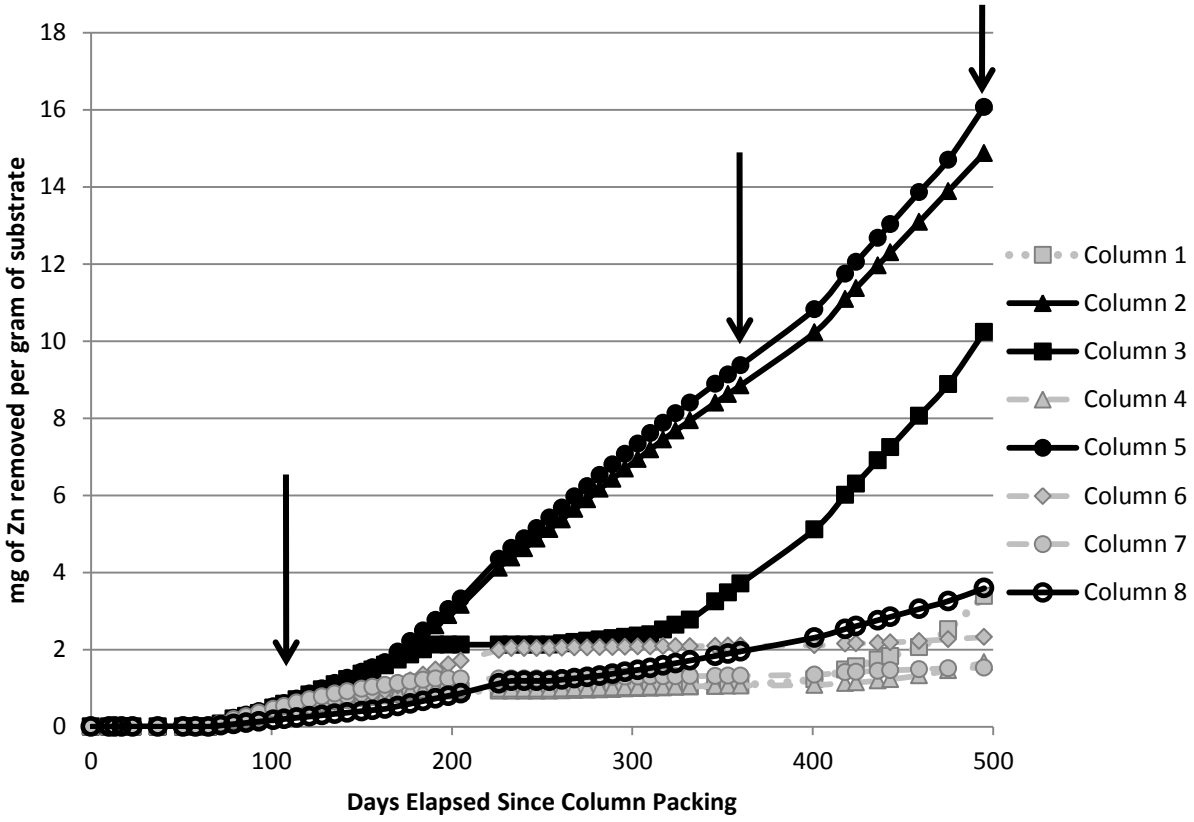

Figure 2.9 A flow-adjusted history of zinc removal in the eight columns, normalized to total substrate mass. Arrows denote the substrate sampling events at 110, 349, and 498 days elapsed time from the packing of the columns. After 498 days column 5 had the highest zinc removal rate (when normalized to the total mass of organic substrate present) followed by columns 2, 3, 8, 1, 6, 7, and 4.

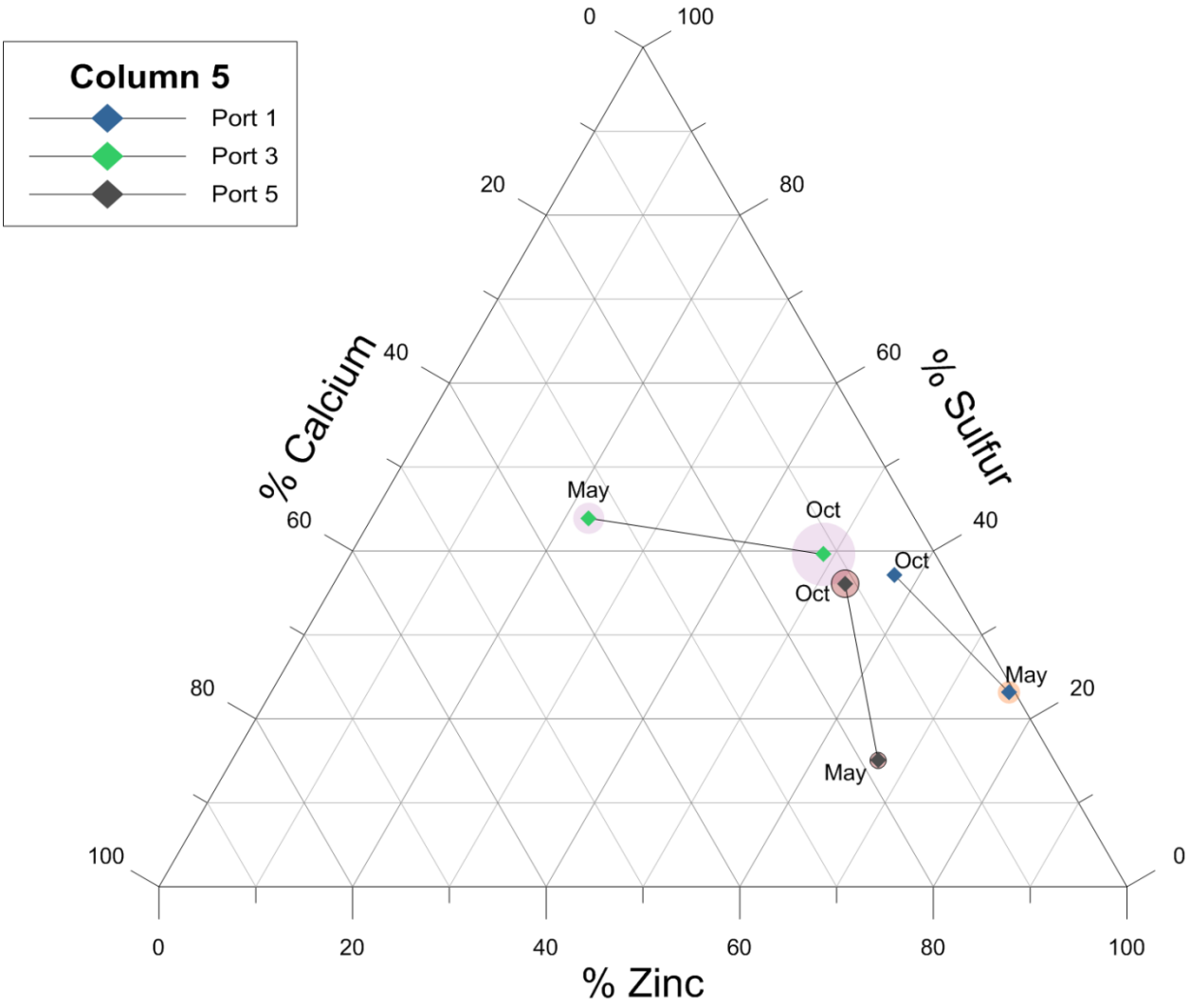

Figure 2.10 The temporal and spatial evolution of Column 5 (70% alfalfa). This column is somewhat unique among its peers in that the three disparate data points from May converge 149 days later to a tighter cluster of October data points. The shaded halos around the data represent the relative mass of zinc removal per day at that point in time and space, as measured by ICP-AES (Table 2.4).

The ICP data presented in Table 2.4 highlight spatial trends in the columns. In September, 2012, 110 days after the columns were packed, most of the columns are removing approximately 35-50 mg Zn/day between ports 1 and 3, though Column 1 is removing only 22 mg Zn/day and Column 7 has the majority of zinc removal occurring between the influent port and port 1. In May, 2013, 349 days after the columns were packed, the zone of highest zinc removal rates has generally moved upward spatially in columns 2, 3, 5, and 8, while zinc

removal rates in columns 1, 4, 6, and 7 is nearly non-existent. This marks a differentiation between the columns containing ≥35% alfalfa or walnut shells, and the columns containing little or neither of these substrates. By October, 2013, 498 days after the columns were packed, the zone of highest zinc removal rates had migrated downward in columns 2, 3, 5, and 8, and zinc removal had once again picked up in columns 1, 4, and 6. Furthermore, Column 1, which contained 10% alfalfa, had a higher net zinc removal rate than columns 4 and 6, which contained no alfalfa. Column 7 remained inactive with respect to zinc removal. The rejuvenation of columns 1, 4, and 6 between the May and October sampling events was likely due to the fact that these columns were re-inoculated with dried cow manure on June 12, 2013. Similar performance-boosting effects from re-inoculation have been documented in other SRBR experiments (Gusek, 2001). Another revelation from Table 2.4 is that, while there was limited, localized re-mobilization of zinc in some columns (as indicated by a negative zinc removal rate), the columns all had a net effect of zinc immobilization. Furthermore, Figure 2.9 illustrates that the zinc removal rate in each column improved over time. However, some of these rates may be too low to reach target treatment goals or to justify SRBR capital costs. It is important to note that column flow rates were adjusted based on column performance, with well-performing columns receiving higher flow rates and thus more influent zinc. As a result, the difference in zinc removal rates between well- and poorly-performing columns as reported for Day 349 and Day 498 in Table 2.4 is greater than it would be had all columns operated under an identical influent flow rate.

If sulfate removal rates are used as a performance metric, then Columns 2, 3, 5, and 8 loosely outperform Columns 1, 4, 6, and 7 (Figure 2.11). However, Column 1 exhibits a noticeable increase in sulfate reduction rate beginning at Day 400. One explanation for this is that the re-inoculation event on Day 385 may have, in conjunction with the 10% alfalfa in the substrate, stimulated sulfate reducing activity in the column.

Table 2.4 Temporal and spatial zinc removal after 110 days (A), 349 days (B), and 498 days (C) of operation.

Zinc removal rate in mg/d by segment								
	C1	C2	C3	C4	C5	C6	C7	C8
Alfalfa Content	10%	35%	35%	-	70%	-	-	(Walnut Shells)
9/10/2012--Day 110								
In to 1	3	-2	-8	-5	-3	0	36	1
1 to 3	22	46	34	35	46	52	2	51
3 to 5	3	3	20	13	-5	0	0	0
Net	28	47	46	42	38	51	38	51
5/7/2013--Day 349								
In to 1	7	125	70	1	52	5	2	125
1 to 3	-1	0	55	3	73	-4	1	0
3 to 5	0	0	0	1	0	2	0	0
Net	6	125	125	5	125	3	3	125
10/3/2013--Day 498								
In to 1	118	-3	49	63	16	10	4	9
1 to 3	70	42	31	0	170	39	-1	242
3 to 5	0	149	165	0	65	-22	1	0
Net	188	188	244	63	251	26	4	251

These data corroborate well with the zinc removal data presented in Figure 2.9, in which Columns 2, 3, 5, and 8 again demonstrate the best performance, with Column 1 showing improved zinc removal after Day 400. It is interesting to note that there are some data points in which more sulfate is leaving the columns than is entering. This might imply the dissolution of previously precipitated gypsum. The negative values may also be a result of the hydraulic residence time of the columns since the influent and effluent sulfate concentrations were measured at the same time even though a parcel of water theoretically takes on the order of one month to travel through the column (depending on the flow rate and substrate porosity) and influent sulfate concentrations varied with time (between approximately 4290 mg/L and approximately 5580 mg/L as measured using a CSA). Though the sulfate concentration of the MIW varied with time, the system was never sulfate limited (Figure 2.12).

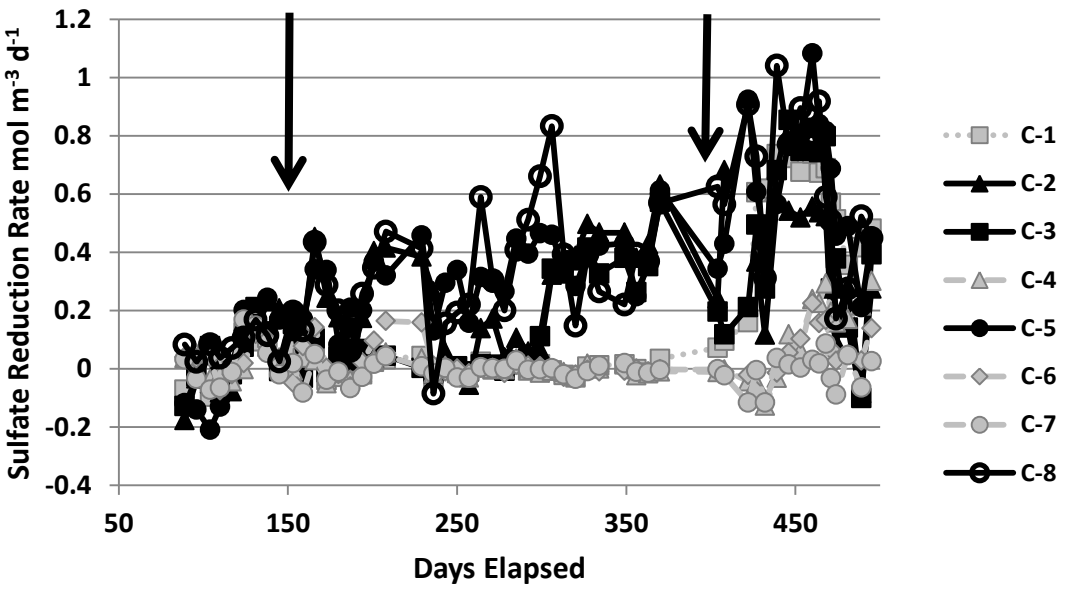

Figure 2.11 Sulfate removal rates from each column (influent minus effluent concentration normalized to flow rate, as measured using colorimetric spectroscopy assays). A negative value signifies more sulfate was released from the column than was present in the influent water. Generally, Columns 2, 3, 5, and 8 had higher sulfate removal rates than did Columns 1, 4, 6, and 7. This relationship correlates well with zinc removal data which shows Columns 2, 3, 5, and 8 as removing the most zinc. Vertical arrows represent re-inoclulation events at 159 and 385 days of elapsed time.

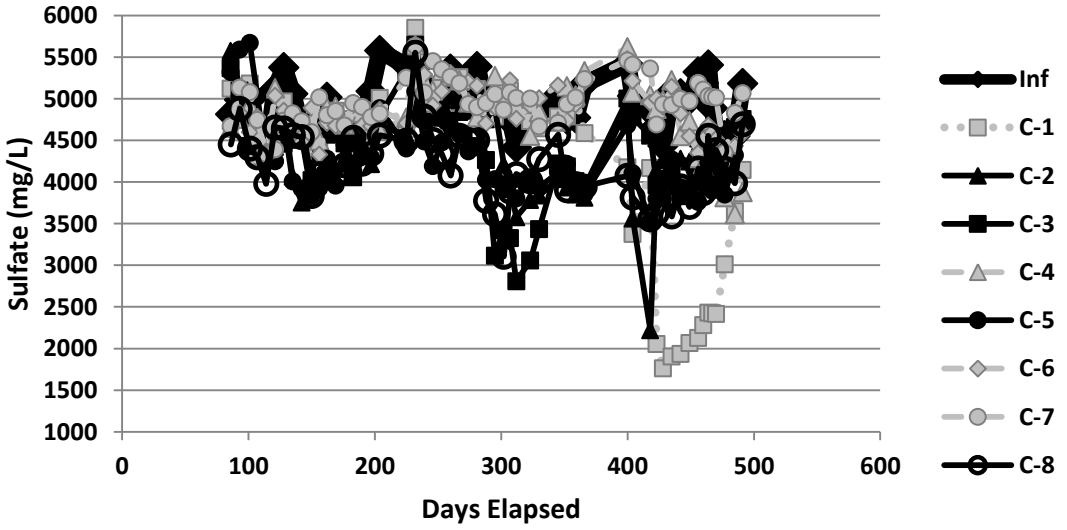

Figure 2.12 Sulfate concentrations in the influent (MIW) and effluent of the eight columns as measured using CSAs. With influent sulfate concentrations consistently above 4000 mg/L, the system was never sulfate limited.

2.3.3 Discussion and Summary

Cohen and Straub (1992) included hay in their passive treatment system, which removed metals with 98-100% efficiency. The hay served as a bulking agent to promote hydraulic conductivity in the system, and also to enhance SRB activity. In the columns of the present investigation, crushed limestone was added to the substrate mixture to act as a bulking agent and to add alkalinity to the system. McCauley et al. (2009) found that mussel shells were better suited than limestone for this role, though local availability may dictate the practicality of using this substrate (as was the case for these inland columns). Hiibel et al. (2011) found that there were no significant differences in performance between ethanol, hay/woodchip, and corn stover/woodchip reactors over a one year period, though metal and sulfate loading rates in their columns were much lower than the present study. The columns of Hiibel et al. (2006) were able, therefore, to fully treat the zinc. Similar to the present study, Hiibel et al. (2006) found sulfate removal to be highly variable. Schmidtova and Baldwin (2011) found a mixture of molasses, alfalfa, and orchid grass to be among the highest performing substrates in terms of sulfate reduction rate among the five substrate permutations tested.

Using shaded halos as a means of weighting the data points on the ternary diagrams can be illustrative in tying together EDX and ICP-AES data. In Figure 2.6, the shaded halos were created from ICP data collected from aqueous samples from each of the substrate sampling ports found on all columns. This allows for a finer spatial discretization regarding zinc removal when compared to a simple influent minus effluent concentration difference. Specifically, the shaded halos represent zinc removal rates, in mg/d, for each segment of the columns (Table 2.4). Notably, the largest shaded halos (and therefore the highest zinc removal rates) occur in October in the region where Zn:S equals approximately 5:4. This is nearly a 1:1 ratio, and though not conclusive, it further supports ZnS mineralogy. Similarly, Azabou et al.,

(2007), Kaksonen et al. (2003), and Neculita et al. (2008b) all observed Zn:S molar ratios of nearly, but not exactly, 1:1 when using EDX analysis of metal precipitates in bioreactors.

One possible explanation for the excess of zinc relative to sulfur is the precipitation of zinc carbonate. Geochemical modeling (Visual MINTEQ) suggests that the increased concentration of carbonate ions present in the columns (relative to the influent water) due to the dissolution of limestone may lead to the over-saturation and/or precipitation of zinc carbonate. Since this process, when compared to that of zinc sulfide precipitation, is relatively independent of microbial sulfate reduction, and since all columns receive an identical influent water and contain an identical weight percent of limestone, it stands to reason that zinc carbonate precipitation should occur equally among all eight columns. This is supported by the fact that zinc removal rates in all eight columns were very similar after 110 days of column operation (Table 2.4). Presumably, the effects of zinc removal through zinc carbonate precipitation were less important relative to those of zinc removal through zinc sulfide precipitation after 349 days of column operation, the sampling point by which the well-performing columns had differentiated themselves from the poorly-performing columns.

It was not possible to identify zinc carbonate precipitates using an EDX-SEM approach. This is because EDX analysis is unable to differentiate between carbon in the organic substrate and carbon in a zinc carbonate precipitate. In contrast, the sulfur signature from a zinc sulfide precipitate is detectable using EDX. Employing x-ray diffraction (XRD) or examining electron diffraction patterns from a transmission electron microscope (TEM) are other methods that could be utilized to better discern the identities of the precipitates observed in this study, provided that the precipitates have a crystalline structure.

This experiment, which focused on manipulating substrate permutations in eight 20 L pilot-scale reactors, demonstrated that using 35% alfalfa as a relatively-recalcitrant (compared

to labile substrates such as manure or compost) organic substrate is an effective means of removing zinc as zinc-sulfur-type precipitate from an Al- and Fe-poor MIW in Arizona. Literature states that the C/N ratio of alfalfa is approximately one order of magnitude less than that of woodchips, suggesting that perhaps the relatively high nitrogen in the alfalfa is what makes this substrate more effective than woodchips for use in an SRBR (Bainbridge, 2007; USDA NRCS, 2011). The experiment explored the use of an SEM-EDX semi-quantitative analysis as a supplement to traditional analytical tools such as ICP-AES. The SEM-EDX combination is a powerful tool that can offer unique insights as to the precipitative processes occurring within an SRBR. However, the SEM-EDX approach should be carefully evaluated before use as it is time and labor intensive and its results are confined to the spatial zones analyzed. Nonetheless, data from EDX analyses can be effectively represented on a ternary diagram, especially to observe large-scale spatial and temporal trends. Analysis via SEM, EDX, and ICP provided data sets that were in agreement with one another with regards to zinc-sulfur-type precipitation in alfalfa-rich columns. Adding data from aqueous digests of the precipitate-laden substrate samples would be a means of contributing a parallel line of evidence that would presumably further strengthen these results.

CHAPTER 3: EFFECTS OF MICROBIAL INOCULATION ON BIOREACTOR ESTABLISHMENT

Whereas the 20 L, Arizona columns in the last chapter investigated the effects of manipulating the organic substrate used in the reactors, this experiment was designed to test the effects of various seeding and inoculation conditions on the geochemistry and microbiology of the system. This research is important because inoculation is a common component of many passive remediation systems (e.g. García et al., 2001; Martins et al., 2009; Sahinkaya et al., 2009; Cruz Viggi et al., 2010; Montoya et al., 2013), and comparisons with negative controls have demonstrated that inoculating an SRBR reduces start up times and enhances sulfate reduction and metal removal rates (Chang et al., 2000; Jong and Parry, 2003). However, to our knowledge, only one research group has published work explicitly examining the effects of inoculation on bioreactor performance (Pereyra et al., 2005; Pruden et al., 2007; Pereyra et al., 2012). Our approach of robust geochemical analysis coupled to next-generation phylogenetic analysis has promise to enhance understanding of the effects of inoculum on those properties and increase our collective understanding of these fascinating systems.

3.1 Objectives/Questions

This study explores the geochemistry of eight up-flow, bench-scale SRBRs (~500 mL) packed with solid organic material (1:1 weight ratio of weathered sawdust and hay). Substrate selection was informed by the prior experiments to enable a representative but rapid potential for SRBR establishment. To this end, the columns were fed with a blend of actual MIWs and subject to four inoculum permutations in duplicate (A: filter sterilized MIW, B: MIW, C: MIW plus an additional microbial-active inoculum (anaerobic digester granules, ADG), D: MIW plus

sterilized ADG inoculum). The laboratory-bench-scale columns went online on December 15, 2013 and were taken offline after operating for 93 days on March 18, 2014. This enabled differentiation between the duplicate variables and destructive sampling for geochemical and microbial analyses. Weekly samples of the SRBR influent and effluent were collected and analyzed for sulfate and sulfide via colorimetric spectroscopy assays, and for dissolved organic carbon (DOC) using a Shimadzu TOC-L CSH Total Carbon Analyzer. ICP analysis for metals was conducted at monthly intervals. This suite of geochemical data was used to address the objective of this thesis chapter, namely, identifying temporal trends in each of the lines of data within the suite and analyzing how well these trends converge to support a unified interpretation of the geochemical processes occurring in the columns over time. As separate aspects of the study, once processed, periodic genetic sequencing of the SRBR substrate will detail the evolution of the microbial community and synchrotron analysis of the SRBR substrate will offer insights as to metal speciation and spatial distribution. Together, these geochemical and microbiological data sets paint a more holistic picture as to the effects of influent water and inoculum on the development of an SRBR.

The experiment is designed with the following questions in mind:

- What is the effect of inoculation of native microbes hosted in MIW on SRBR column sulfate reduction and metal removal rates?
- What is the effect of self-inoculation by microbes associated with the organic substrate on SRBR column sulfate reduction and metal removal rates?
- What is the effect of inoculation using anaerobic digester granules with an active microbial community on SRBR column sulfate reduction and metal removal rates?

3.2 Materials and Methods

Eight up-flow column bench-scale bioreactors were constructed and operated at the Colorado School of Mines (Golden, CO). The columns were operated for 93 days.

3.2.1 Column Design

The mining-influenced water (MIW) collected in the Colorado Rockies proximal to the I-70 corridor (hereafter referred to as VC water) was pre-treated with 671 ± 16.6 g of sterilized limestone housed in two Chromaflex™ upflow (480 mL/day) chromatography glass columns (L:30 cm, ID:4,8 cm, V: 543 mL Kimble-Chase, Rockwood, TN, USA) in order to raise pH from 4.5 to 7.2. This water was then fed into eight up-flow (43.2 mL/day, hydraulic residence time 5.5 days) Chromaflex™ columns (L:15 cm, ID:4.8 cm, V:271 mL). Each of the eight columns was packed with 15 g weathered weight of organic substrate (7.5 g and 7.5 g weathered weight of barley hay and ground pine woodchips respectively). Both the hay and the woodchips were weathered for one year in atmospheric conditions near Golden, CO. Twelve mesh bags (2.5 cm by 2.5 cm in size) each containing 0.30 g of ground and sieved substrate (1:1 weathered weight hay and woodchips) were placed in the top of each column to allow for periodic substrate sampling with minimal disruption of the SRBRs. The organic material was soaked in synthetic MIW (SMIW) similar to the composition of the VC water used in this experiment (November 2013, 79.4 mg/L Zn, 71.7 mg/L Mn, 107 mg/L Mg, 800 mg/L SO_4^{2-}) for 48 hours before packing. The A columns were inoculated with the organic substrate only. Unlike columns B-D, they controlled for the inoculation event with SMIW that had been sterilized via sterile filtration (0.2 µm) and hence, initial colonization was limited to the hay/woodchip substrate. The B columns were inoculated with the organic substrate and received non-sterile MIW to enable exposure to microbes derived from both MIW and organic substrate. The C columns received an additional inoculum of 8 g (wet-weight) of homogenized active anaerobic digester granules (MillerCoors

anaerobic wastewater digester in Golden, CO) pre-soaked together with the organic substrate. The D columns received the same inoculum as the C columns; however these granules were sterilized via three cycles in the autoclave prior to inoculation. The columns were topped off with SMIW after packing. All columns were protected against artificial and natural light in order to prevent photosynthetic oxygen generation in the columns.

3.2.2 Column Influent

MIW was collected every ~20 days from the VC subsurface capture system outlet (VC, pH 3.4) (Idaho Springs, CO, USA) and from a tributary to Clear Creek (CC, pH 7.2) derived from the raw water collection system at the Golden water treatment facility in Golden, CO. During an initial acclimatization phase, the two MIWs were pretreated in the limestone columns and mixed at a ratio of 1:1 (Blend 1) before being fed into the SRBRs at a pH of approximately 7.2. After approximately four SRBR pore volumes (24 days of operation) the ratio was changed to 1:2 (CC:VC) (Blend 2) for the duration of the experiment in order to avoid SO_4^{2-} limiting conditions in the SRBRs. Average SRBR column influent constituent concentrations of selected elements with concentrations above the ICP-AES detection limit are presented in Table 3.1.

Table 3.1 Column influent constituent concentrations (mg/L)

Ion	Sulfate	Calcium	Magnesium	Manganese	Nickel	Zinc
Blend 1	966	269	65	34	0.3	30
Blend 2	1167	355	92	52	0.4	48
VC	1840*	251	107	72	0.6	79

*Not measured but extrapolated from Blend 1 and Blend 2 assuming all sulfate comes from VC

3.2.3 Column Sampling

Once a month (January 8, February 4, and March 18, 2014), a single mesh bag was removed from the top of each column under a stream of N_2 gas. The extracted bag was

replaced with a new mesh bag of identical substrate composition. Columns B-D were destructively sampled at the end of the experimental period in an anaerobic chamber under an N_2:H_2 (95%:5%) atmosphere. Substrate was sampled from the bottom and middle sections of each column. Approximately 2 g of material was allowed to dry in the anaerobic chamber for at least 2 days prior to collection for subsequent geochemical analyses.

Column influent and effluent water was sampled on a weekly basis. Samples were filtered (0.45 µm pore size), acidified (pH < 2) with nitric or phosphoric acid and kept at 4°C until SO_4^{2-}, ICP and DOC analyses were performed. Additional weekly aqueous samples for S^{2-} were collected under near anaerobic conditions and analyzed immediately.

3.2.4 Geochemical Analyses

SO_4^{2-} and S^{2-} were analyzed on a Hach DR5000 spectrophotometer (Hach Company, Loveland, CO, USA) utilizing the USEPA SulfaVer 4 (measurement range: 2 to 70 mg/L SO_4^{2-}) and Methylene Blue (detection limit: 5 to 800 µg/L S^{2-}) Methods, respectively. Sulfate samples were collected on a weekly basis. Effluent water was collected from the columns until approximately 12 mL of effluent was obtained (a sampling time of approximately 7 hours). Samples were passed through a 0.45µm syringe filter. They were then acidified with 3 drops of 70% Ultrex ultrapure nitric acid and refrigerated at 4°C until the time of analysis. The samples were diluted 20x immediately prior to analysis so that readings fell within the measurement range.

It is important to note that all weekly sulfate tests conducted on or after January 15, 2014 (Day 31) included the analysis of a 20x dilution of a 1000 mg/L sulfate standard solution. The average of these standard analyses was 807 mg/L and the standard deviation was 23.5 mg/L (n=9). Running other 1000 mg/L and 100 mg/L sulfate standards yielded a similar trend of analyses yielding measured values that were 80% of the labeled standards. This is a strong

indication that there is error in the column effluent sulfate measurements due to an improperly calibrated spectrophotometer. Accordingly, reported sulfate values have been corrected in Excel by dividing all values by a factor of 0.8.

Sulfide samples were collected weekly from the effluent tube of each column using a Leur-Lock syringe that had been purged three times with nitrogen gas. Beginning with the February 19 sampling event, the effluent tube lines were drained into a separate Leur-Lock syringe and allowed to re-fill prior to the sample collection in order to better avoid particulate matter in the sample (which could interfere with the spectrophotometry reading). Sample volumes were generally 1 mL, unless the effluent tubing contained less that amount, in which case the maximum available volume of effluent was collected. The syringe tips were sealed with Parafilm and the samples were analyzed immediately in order to reduce sulfide volatilization and oxidation. Dilution of at least 10x was necessary due to the low sample volumes. Column C effluent was diluted 20x immediately prior to analysis so that readings fell within the measurement range.

DOC was analyzed in the laboratories at the Colorado School of Mines using a Shimadzu TOC-L CSH Total Carbon Analyzer with an effective detection limit of 0.9 ppm (Shimadzu Corporation, Kyoto, Japan).

3.3 Results and Discussion

Geochemical analyses of these columns included weekly sampling of influent and effluent waters for sulfide, sulfate, DOC, and dissolved metals.

3.3.1 Sulfide, Sulfate, and Dissolved Organic Carbon (DOC)

Weekly sulfate measurements ranged from 300 mg/L to 1375 mg/L (Figure 3.1). Generally, sulfate levels from the column effluents paralleled the influent sulfate concentration indicating that it was not a limiting electron acceptor in these systems. Sulfate levels in columns

A, and B were nearly the same as that of the influent water (an average concentration of approximately 965 mg/L by the end of the experiment). Sulfate levels in the D columns were slightly lower than that of the influent water (an average concentration of approximately 935 mg/L by the end of the experiment) while that of the C columns was markedly lower than that of the influent (an average concentration of 735 mg/L in column C1 and 820 mg/L in column C2). This difference is more visibly evident when only data from the influent and columns C and D are presented (Figure 3.2). The difference in influent and Column D effluent sulfate prior to day 40 may be the result of gypsum precipitation, though no gypsum-like precipitates were visually observed in this column. Sulfide level readings ranged from 20 to 8900 µg/L (Figure 3.3). Columns A, B, and D had similar sulfide levels which were approximately one order of magnitude smaller than those of the C columns demonstrating more robust sulfate reduction in the C columns.

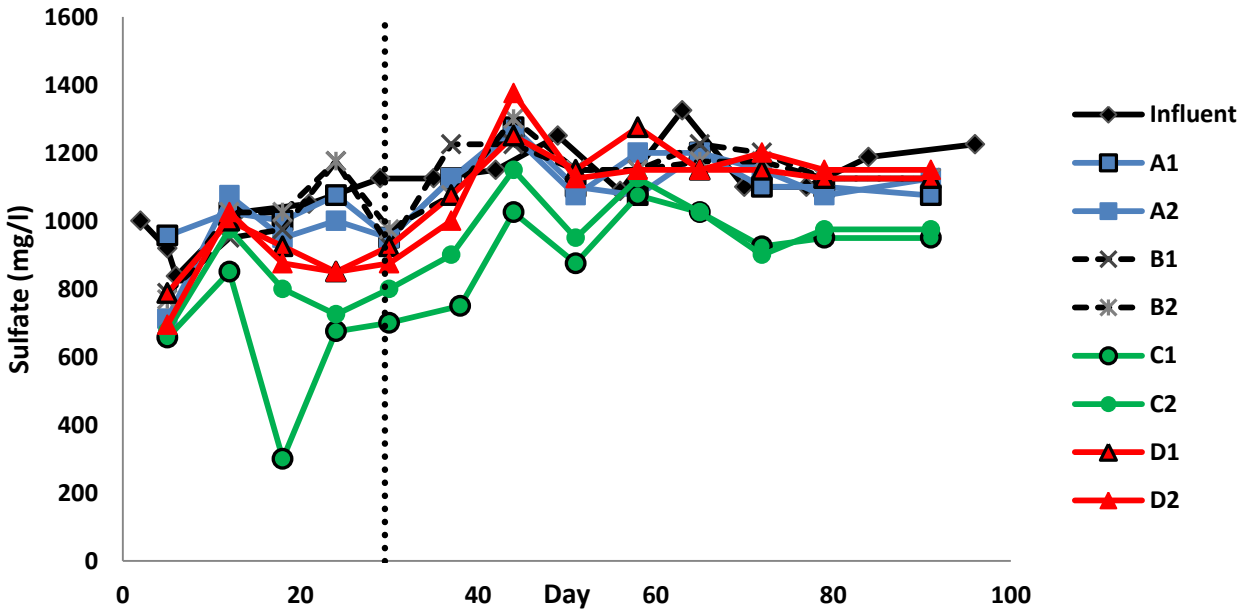

Figure 3.1 Effluent sulfate data for replicate columns plus influent as measured using the HACH SulfaVer 4 protocol. The dashed vertical line represents the shift from a 1:1 to 1:2 CC:VC influent ratio after 24 days of operation plus an additional 5 days to account for the column residence time in order to facilitate a better direct comparison between influent and effluent concentrations. As noted in the sulfate analysis protocols section of the report, due to uncertainty this figure should primarily be interpreted qualitatively to indicate more pronounced sulfate reduction in the C replicates when contrasted with the other systems.

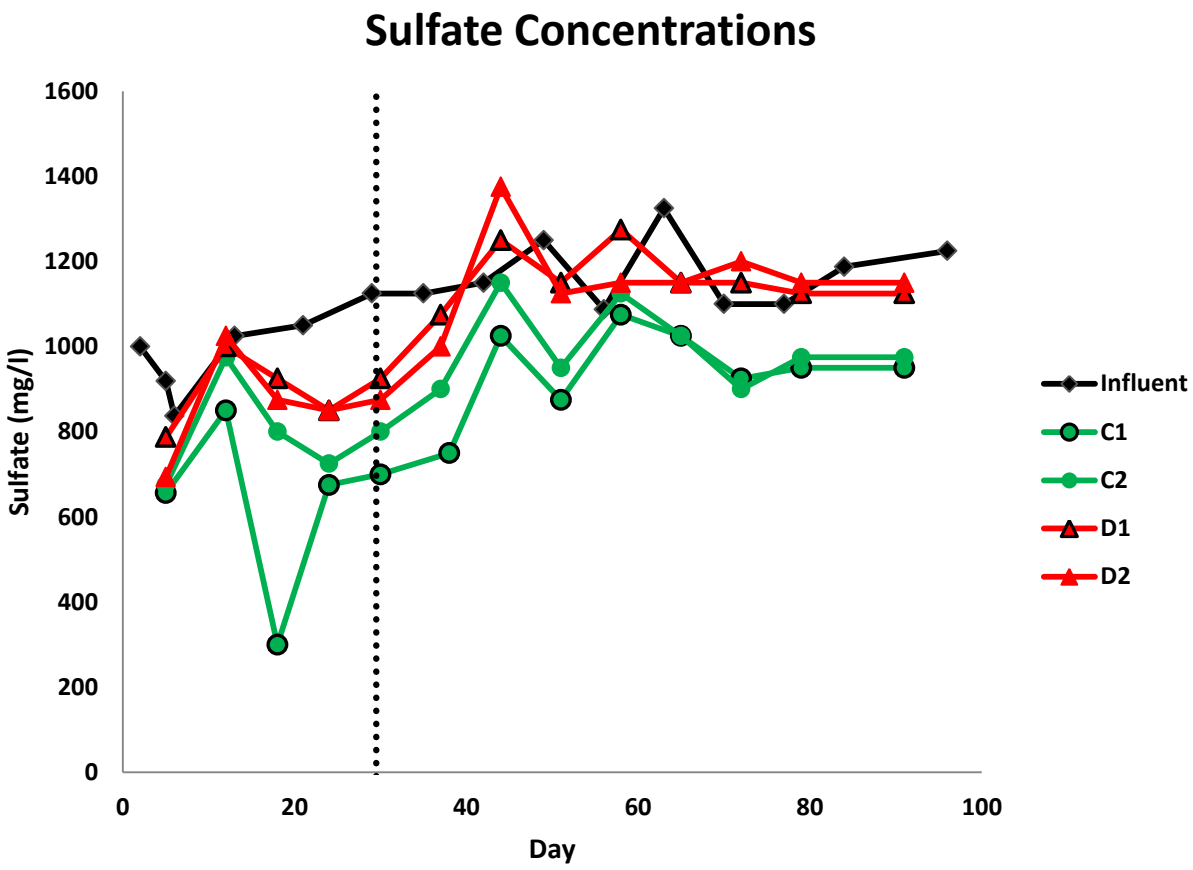

Figure 3.2 Effluent sulfate data for column C and D replicates plus the column influent as measured using the HACH SulfaVer 4 protocol. The dashed vertical line represents the shift from a 1:1 to 1:2 CC:VC influent ratio after 24 days of operation plus an additional 5 days to account for the column residence time in order to facilitate a better direct comparison between influent and effluent concentrations.

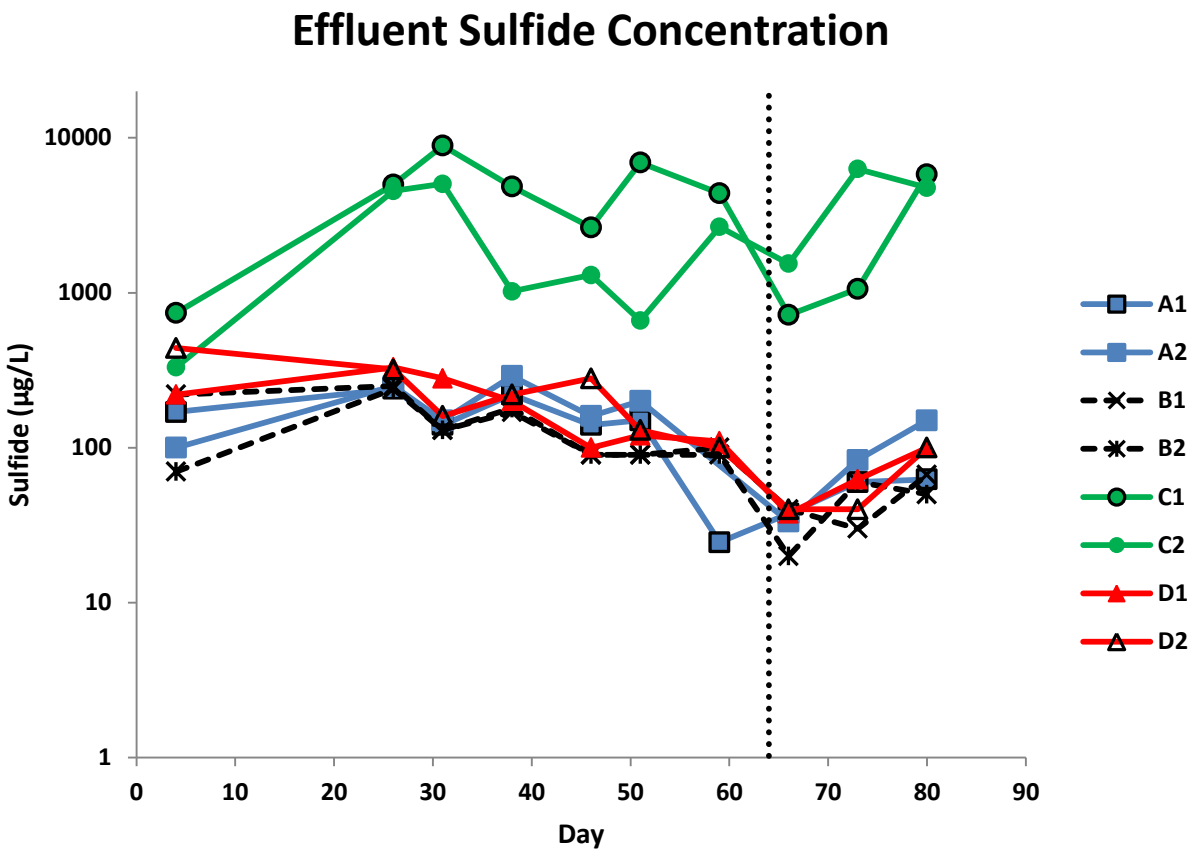

Figure 3.3 Effluent sulfide concentrations for all 8 columns as measured using the HACH Methylene Blue protocol. The C column effluent contains approximately one order of magnitude more sulfide than that of the A, B, and D columns. This suggests that only the C columns were actively reducing sulfate. Data points to the left of the dotted line were collected using an improper spectrophotometer blanking technique and are thus susceptible to being artificially high values.

DOC levels ranged from 24 to 172 mg/L (Figure 3.4). Overall, the data depict a downward trend in DOC consistent with leaching from the solid substrate. The DOC readings for the C columns were consistently lower than those of columns A, B, and D suggesting more consumption of the soluble organics, though the difference is not statistically significant (unpaired t-test, $p>0.1$). The lower effluent DOC readings in the C columns suggests a higher level of microbial activity in those columns relative to the A, B, and D columns because heterotrophic microbial metabolism and growth require the uptake of organic carbon.

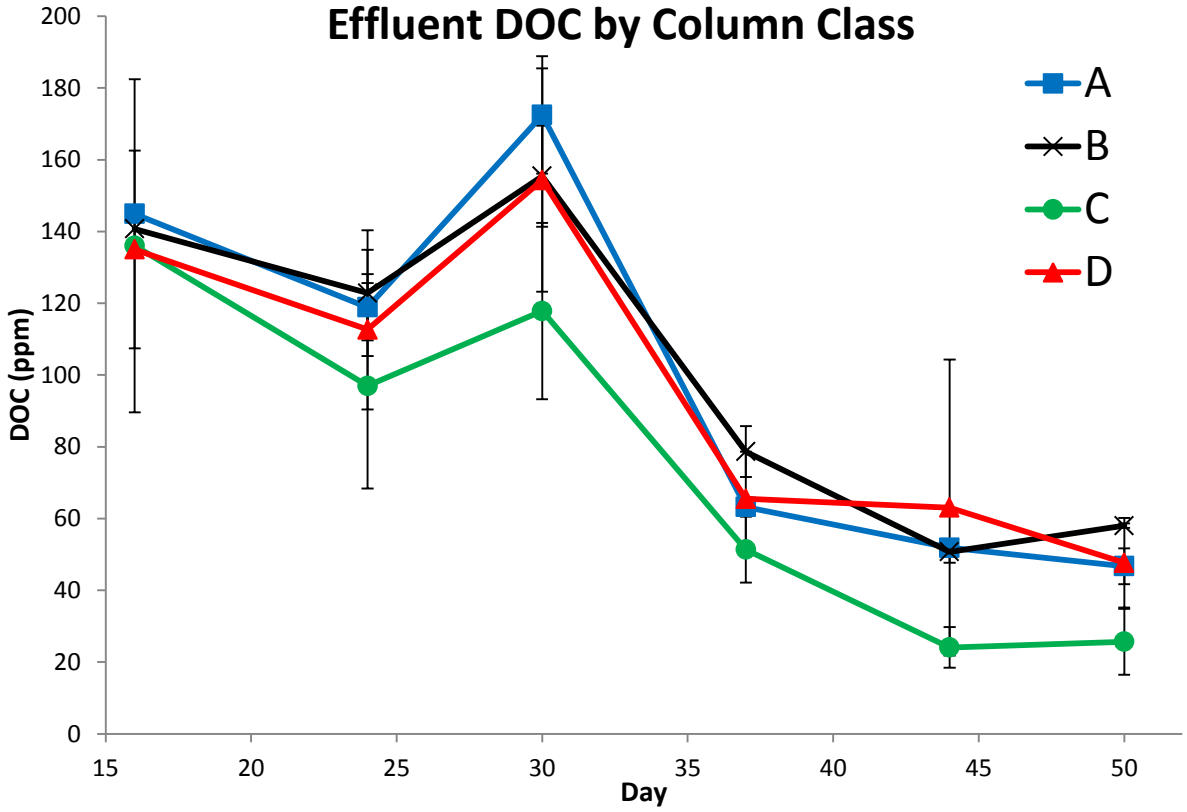

Figure 3.4 There is a trend toward slightly less DOC in the effluent of the C columns that becomes evident when the replicate columns of each class are averaged. This suggests a higher level of microbial activity in the C columns because heterotrophic microbial metabolism and growth require the uptake of organic carbon. Error bars represent one standard deviation.

By collectively interpreting the sulfate, sulfide, and DOC data, it appears that the A, B, and D columns all had established microbial communities capable of sulfate reduction; but that the sulfate-reducing activity in these columns paled in comparison to that of the C columns. This suggests that the active ADGs served as a more effective inoculum than did the substrate, MIW, or sterilized granules. Comparing the sulfide and sulfate data between the A and B columns isolates MIW as a manipulated variable and reveals that the non-filtered MIW had little additional effect on sulfate reduction rates. All columns contained non-sterilized substrate; yet columns A, B, and D had minimal sulfate reduction, suggesting that inoculation with sulfate alone is not an effective means or boosting sulfate reduction. Comparing the sulfide and sulfate

data between the C and D columns isolates the ADGs as a manipulated variable and reveals that inoculation via active ADGs significantly improves sulfate reduction rates in a column.

While all column effluents had decreasing amounts of DOC with time (a likely indicator of microbial growth, though also potentially just a result of a slow washing of the labile carbon in the substrate), and all column effluents had measurable amounts of sulfide and sulfate, the C column effluents consistently contained approximately 15% less sulfate and 10 times more sulfide than the A, B, and D columns, where sulfide concentrations in the diluted effluent samples of the A, B and D columns were consistently near the 5 µg/L lower detection limit for the analytical method used. The sulfate measurements in all columns were well within the measurement range for the analytical method. Sulfate concentrations in the A, B, and D column effluents were generally very similar to the influent sulfate concentrations for those columns. The higher sulfide concentrations and lower sulfate concentrations in the C columns, especially when compared with the influent sulfate concentrations, provide evidence that sulfate reduction is occurring in the C columns much more than in the A, B, and D columns, and reinforces the usefulness of an inoculum to accelerate the establishment of a sulfate reducing community in an SRBR. This argument is supported by the smell of H_2S gas emanating from the C columns as well as the formation of black precipitates in the C columns. No odor was noticed from the A, B, or D columns, nor was black precipitate observed in these columns. Given that the odor threshold for H_2S gas is as low as 10 µg/L (OSHA, 2014), the measured sulfide concentrations of approximately 100 µg/L should be interpreted cautiously. Visual and olfactory observations have been used by others as evidence of sulfate reduction (Gammons and Frandsen, 2001; Pereyra et al., 2005; Lewis, 2010).

It is important to note that prior to the February 19, 2014 sampling campaign (Day 66) the spectrophotometer was improperly blanked for sulfide measurements using DI water without the HACH sulfide reagents. The adoption of the proper blanking procedure (using DI water plus

the reagents) on February 19 potentially explains the lower sulfide concentrations observed on and after that date. It is also worth noting that the correction in blanking procedure yielded sulfide readings in diluted samples of the effluent of columns A, B, and D that were near or below the detection limit of 5 µg/L. Turbidity can also introduce error in the form of artificially high readings. The physical and chemical properties of sulfide and potential for complexation within the column, along with dilution requirements coupled to spectrophotometric limitations, limit the absolute quantitative nature of these measurements. We therefore estimate the effective sulfide detection limit in this experiment to be approximately 100 µg/L. Hence measurements should be taken with a qualitative perspective indicating a clear generation of sulfide in the C columns with an uncertain stoichiometric release, and possible sulfide generation in the A, B, and D columns.

3.3.2 Soluble Metals

ICP data from the column effluents offer additional insights into plausible metal removal mechanisms of the columns. Note that the A columns are not included in this analysis because, unlike sulfate readings, where the filtered A influent MIW exhibited similar sulfate concentrations to the unfiltered B-D influent MIW, zinc concentrations in the A column influent were approximately 20% lower than zinc concentrations in the B-D column influents, possibly due to the filtration of colloidal zinc carbonate that may have formed during limestone pre-treatment of the influent water. ICP results from the A columns were thus not directly comparable to ICP results from columns B-D.

In the case of zinc, the C columns removed nearly all zinc from the influent water (dropping the concentration from 30-50 mg/L down to, in most cases, less than 0.2 mg/L) (Figure 3.5). Initially, the D columns also removed some zinc, though much less than the C columns (dropping the concentration from 30-50 mg/L down to roughly 10-15 mg/L). However,

by Day 58 the D and B column effluent zinc concentrations were roughly equivalent to the zinc concentrations in the influent water. This suggests that zinc was precipitating out of solution as a zinc sulfide in the C columns through reaction with sulfide. It is possible that the visible precipitate observed at the influent end of the C columns was zinc sulfide. Zinc removal in the B columns was possibly the result of sorption to the organic substrate. The slight removal of zinc in the D columns may be the result of minor sulfide production in the early stages of D column operation, or more likely it's due to iron sulfide, which was present on the ADGs, dissolving and leading to the precipitation of the much less soluble zinc sulfide. After 72 days of operation the Zn concentrations in the D column effluents equaled that of the influent, indicating all of the iron sulfide initially present had been replaced by zinc sulfide. Thus it can be said that inoculation with ADGs is an effective means of improving zinc removal in an SRBR.

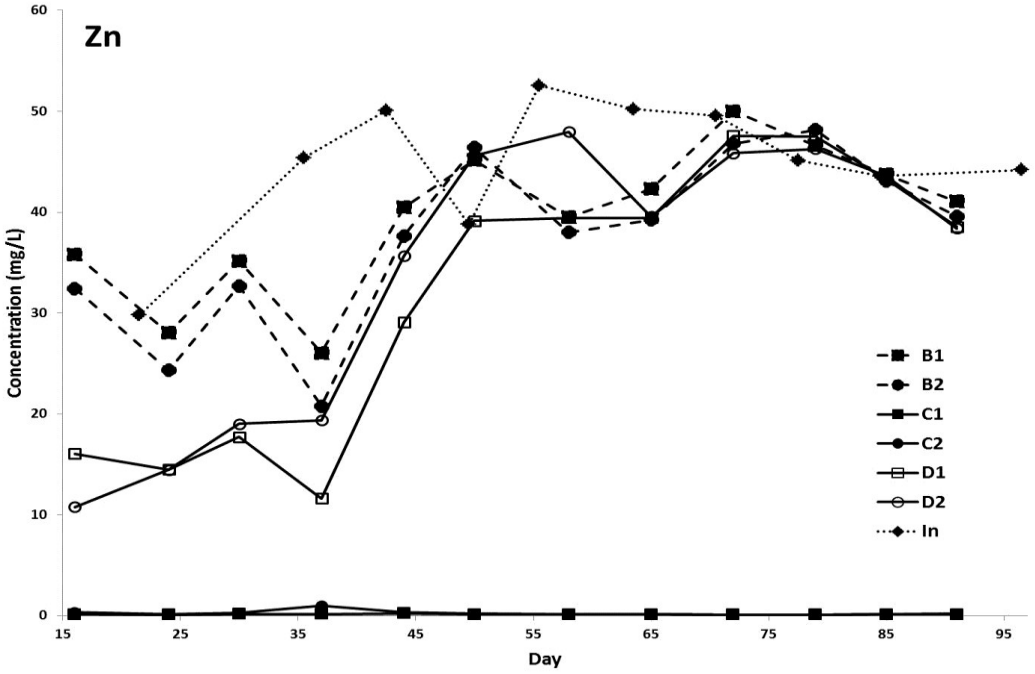

Figure 3.5 Concentration of zinc in the column influent and effluent. The D columns had some initial zinc removal while the C columns consistently removed all of the influent zinc. This, coupled with the sulfide/sulfate data, suggests that precipitation with sulfide is the primary removal mechanism for zinc in the C columns. Note that influent samples were measured concurrently with effluent samples but have been shifted 5.5 days later to account for the hydraulic residence time of the columns and to allow for a more direct comparison between influent and effluent ICP data.

Cobalt ICP analyses produced data with trends similar to that of zinc (Figure 3.6). The C columns removed nearly all of the cobalt while the B and D column effluent cobalt levels were similar to the column influent concentrations. As for zinc, this suggests that sulfide precipitation is likely the primary cobalt removal mechanism in the C columns. Sorption appears to play only a minor role in cobalt removal as effluent concentrations in the B and D columns were only slightly lower than the influent Co concentration for the first 44 days of column operation, after which all three concentration data sets overlapped. Thus it can be said that inoculation with ADGs is an effective and important means of promoting cobalt removal in an SRBR.

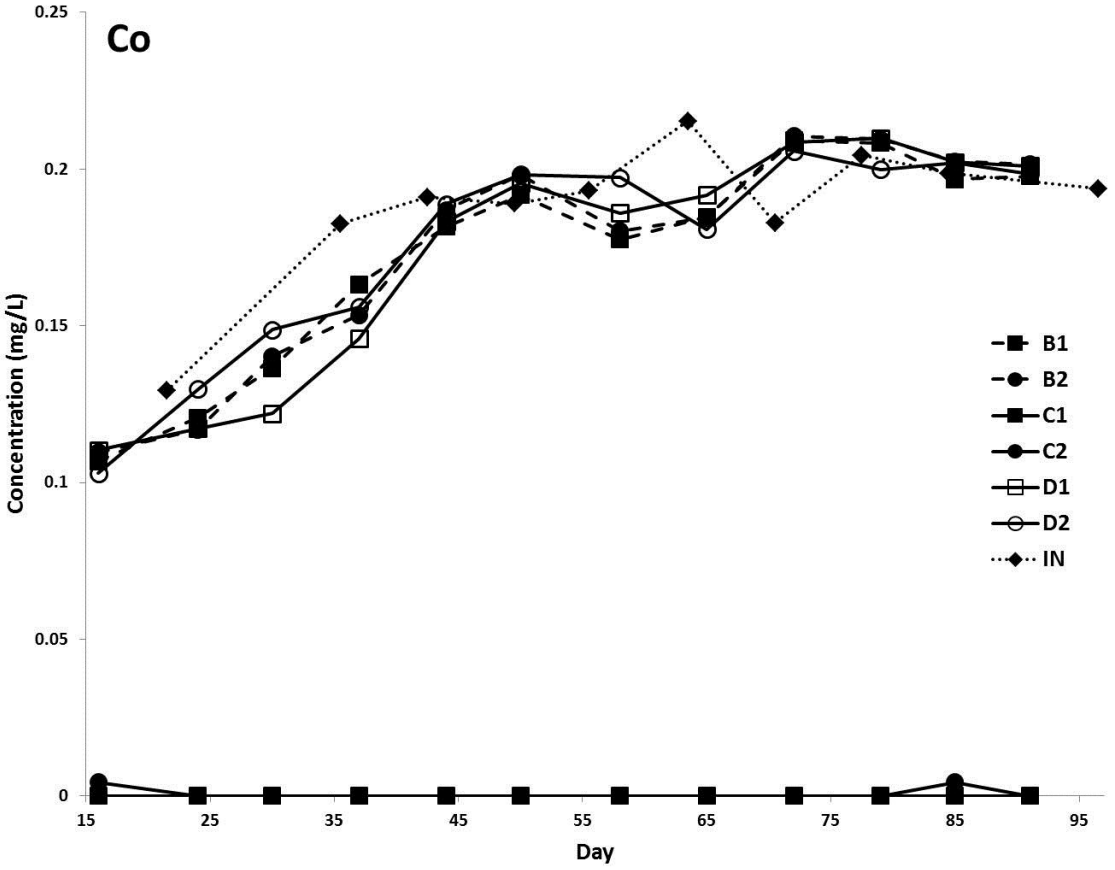

Figure 3.6 Concentration of cobalt in the column influent and effluent. The B and D columns had some initial cobalt removal while the C columns consistently removed all of the influent cobalt. This, coupled with the sulfide/sulfate data, suggests that precipitation with sulfide is the primary removal mechanism for cobalt.

Nickel data is slightly different from that of zinc and cobalt in that, while the C columns once again remove nearly all of the nickel from solution, the B and D columns also remove a pronounced amount of nickel, especially in the earlier stages of the experiment (Figure 3.7). One explanation for this is that there are multiple metal removal mechanisms. It is likely that in addition to precipitation with sulfides (as probably occurs in the C columns), sorption to the organic substrate is also occurring. After Day 44 (as was also the case with cobalt), nickel removal in the B and D columns is much less pronounced suggesting sorption sites had reached saturation after that time. The fact that the C columns, which were distinct in their comparatively high degree of sulfate reduction, continuously removed nearly all of the nickel while the B and D columns did not suggests that inoculation with ADGs is an effective means of improving nickel removal in an SRBR.

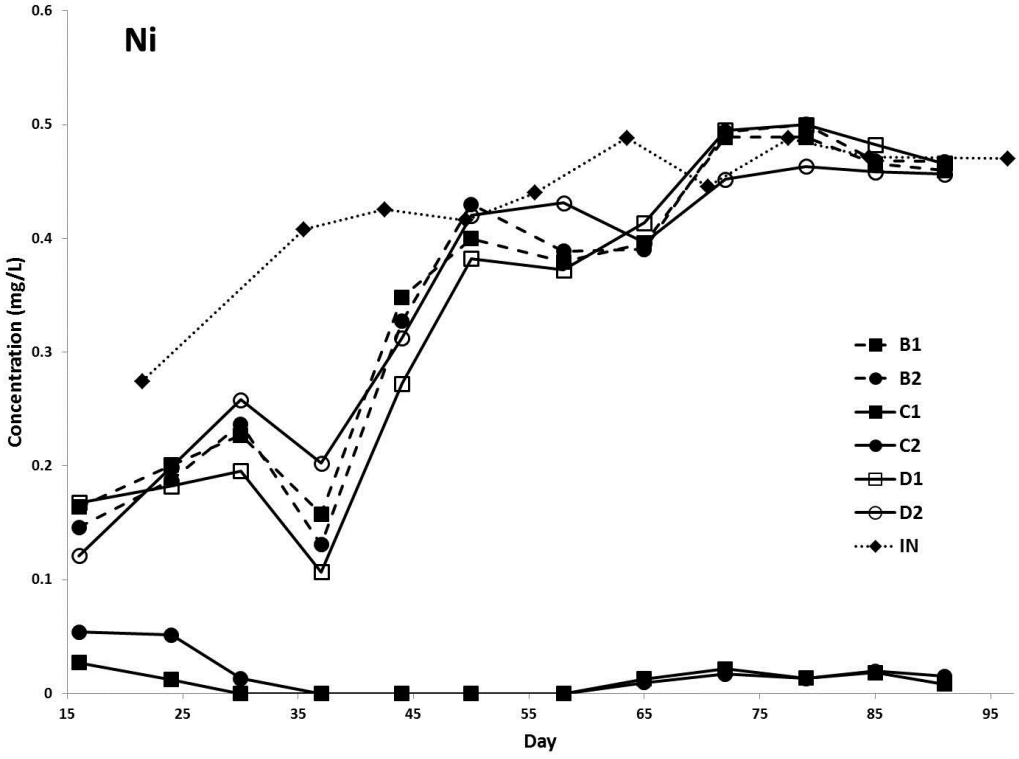

Figure 3.7 Concentration of nickel in the column influent and effluent. The B and D columns initially removed some nickel while the C columns removed most nickel throughout the experiment. This, coupled with the sulfide/sulfate data, suggests that precipitation with sulfide is the primary removal mechanism for nickel.

The C and D columns remove nearly all cadmium from the influent water while the B columns remove only some cadmium (Figure 3.8). This suggests the dissolution of iron sulfide present in the anaerobic digester granules and subsequent precipitation of much less soluble cadmium sulfide. Beginning on Day 72, some cadmium was observed in the effluent of the D columns, suggesting that there was insufficient iron sulfide remaining in the ADGs to dissolve and provide a sulfide source for the continued precipitation of cadmium sulfide. The sustained total removal of cadmium in the C columns after Day 72 can be explained in that the C columns still had available sulfide through the process of microbial sulfate reduction. Removal of cadmium in the B columns may have been due to sorption of cadmium to the organic substrate.

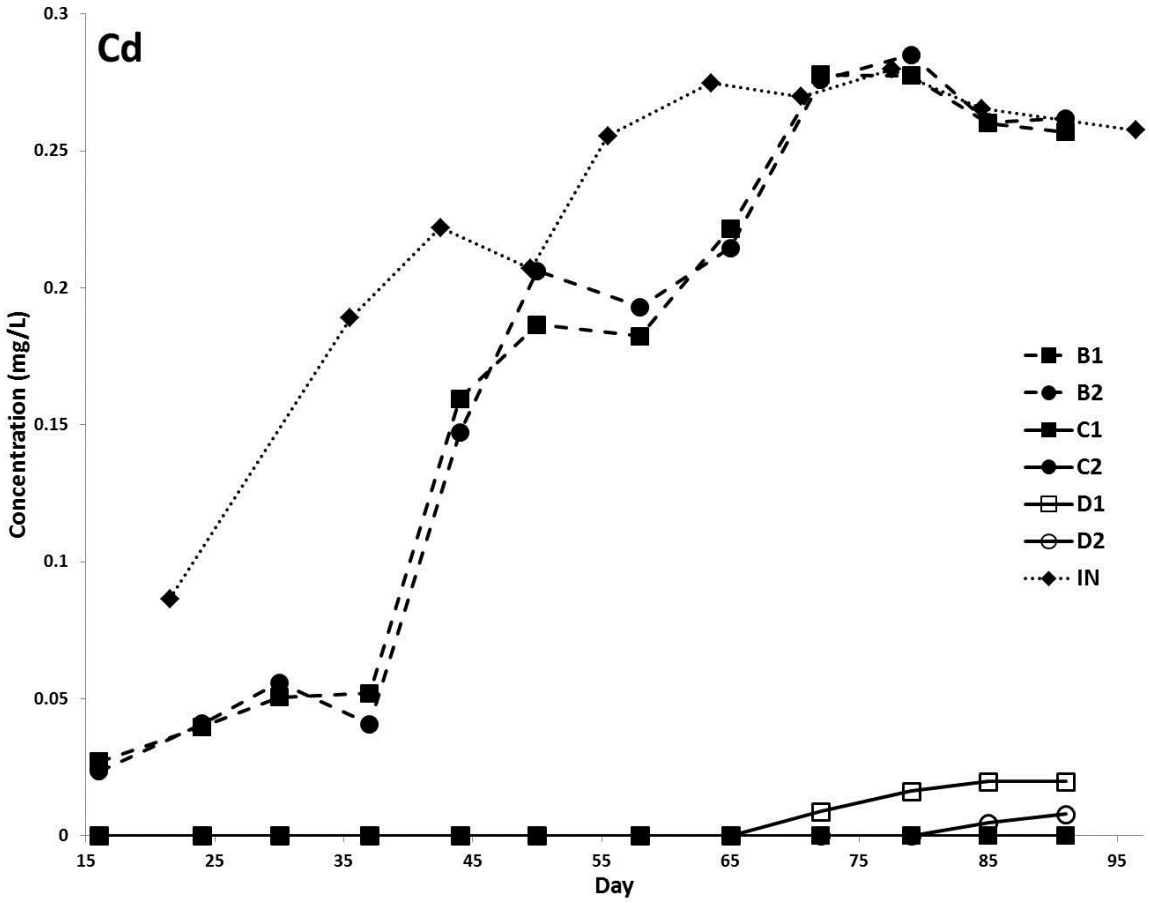

Figure 3.8 Concentration of cadmium in the column influent and effluent. Both the C and D columns removed much more cadmium from the influent water than did the B columns. This suggests that sorption to the anaerobic digester granules may have been the removal mechanism for cadmium.

There was typically no iron in the influent solution (detection limit=0.003 mg/L), nor was there ever more than 0.36 mg/L iron in the B column effluent (Figure 3.9). It can therefore be concluded that the iron measured in the C and D column effluent is released from the anaerobic digester granules as the associated iron sulfide dissolved and that the dissolved iron did not originate from the hay/woodchip substrate mixture consistent to all columns. The D columns released more iron than the C columns and also released iron for longer than the C columns, likely because the extra sulfide production in the C columns (via microbial sulfate reduction) prevented the iron sulfide from dissolving by keeping the aqueous iron and sulfide levels above the solubility limit of the iron sulfide. By Day 72, no more soluble iron was being released by any of the systems. Also of note is the fact that there was no visible yellow/orange ferric oxide precipitate in the iron-eluting columns, which, as expected, suggests reducing conditions inside of the columns.

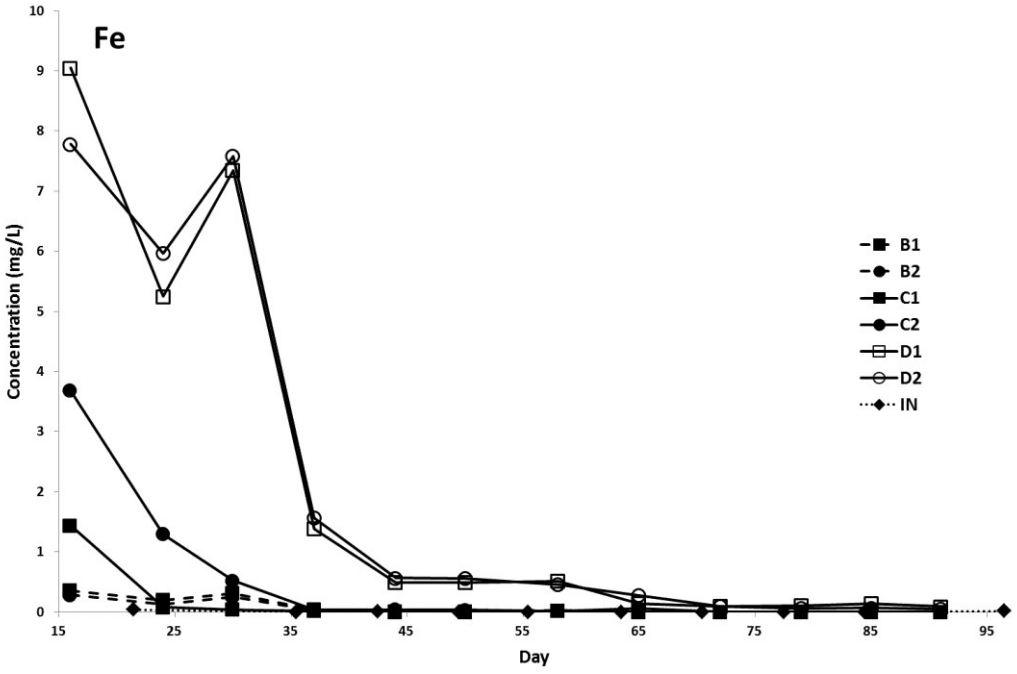

Figure 3.9 Concentration of iron in the column influent and effluent. Note that there is no iron in the influent water or in the B column effluent. These data suggest a release of iron from the anaerobic digester granules. The D columns released iron in greater quantities and for a longer period of time because of extra sulfide generation in the C columns via microbial sulfate reduction.

Silica effluent concentrations consistently exceeded the influent concentration (Figure 3.10). This suggests that silica was being released from the weathered substrate in the columns. The columns did not remove either magnesium or manganese from solution (Figure 3.11).

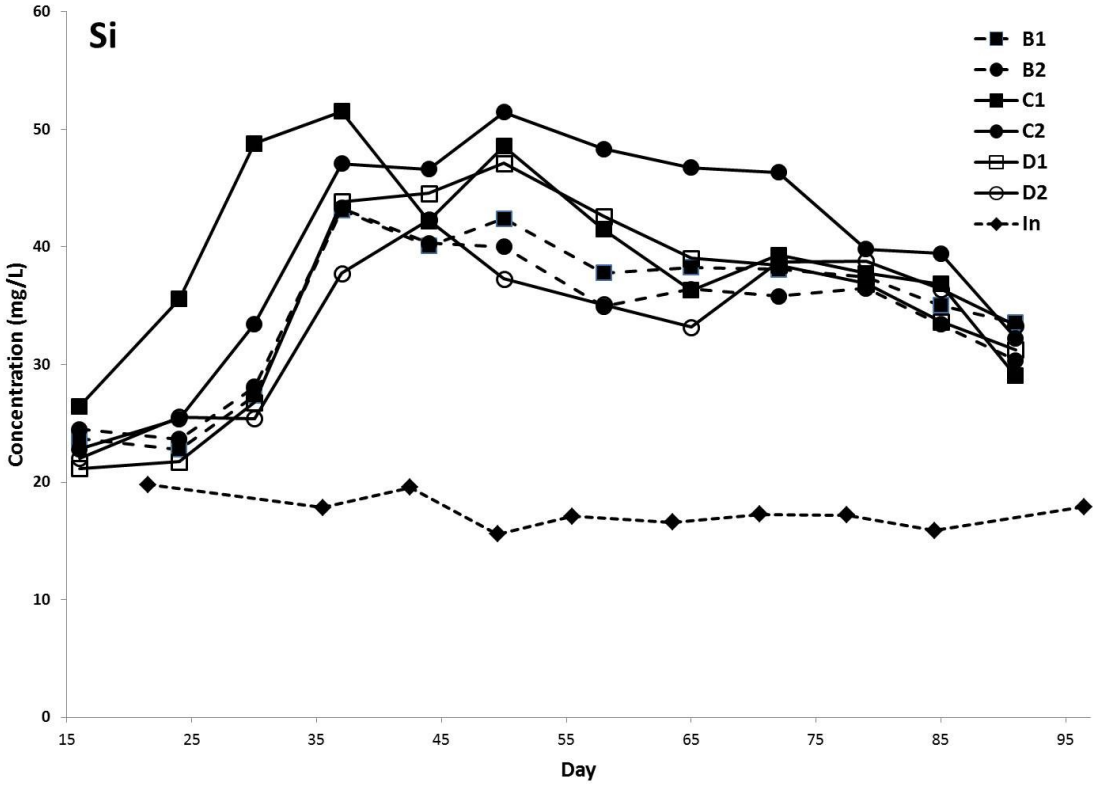

Figure 3.10 Concentration of silica in the column influent and effluent. Higher concentrations of silica in the column effluents than in the influent water is indicative of a release of silica from the weathered substrate.

3.3.3 Discussion and Summary

In summary, the C columns, which represented the only system that received a viable inoculation from an anaerobic digester, had increased sulfate removal as reduction when contrasted with the other columns. This inoculation strategy is therefore an important means of improving reactor performance and warrants the potential additional costs of the inoculum.

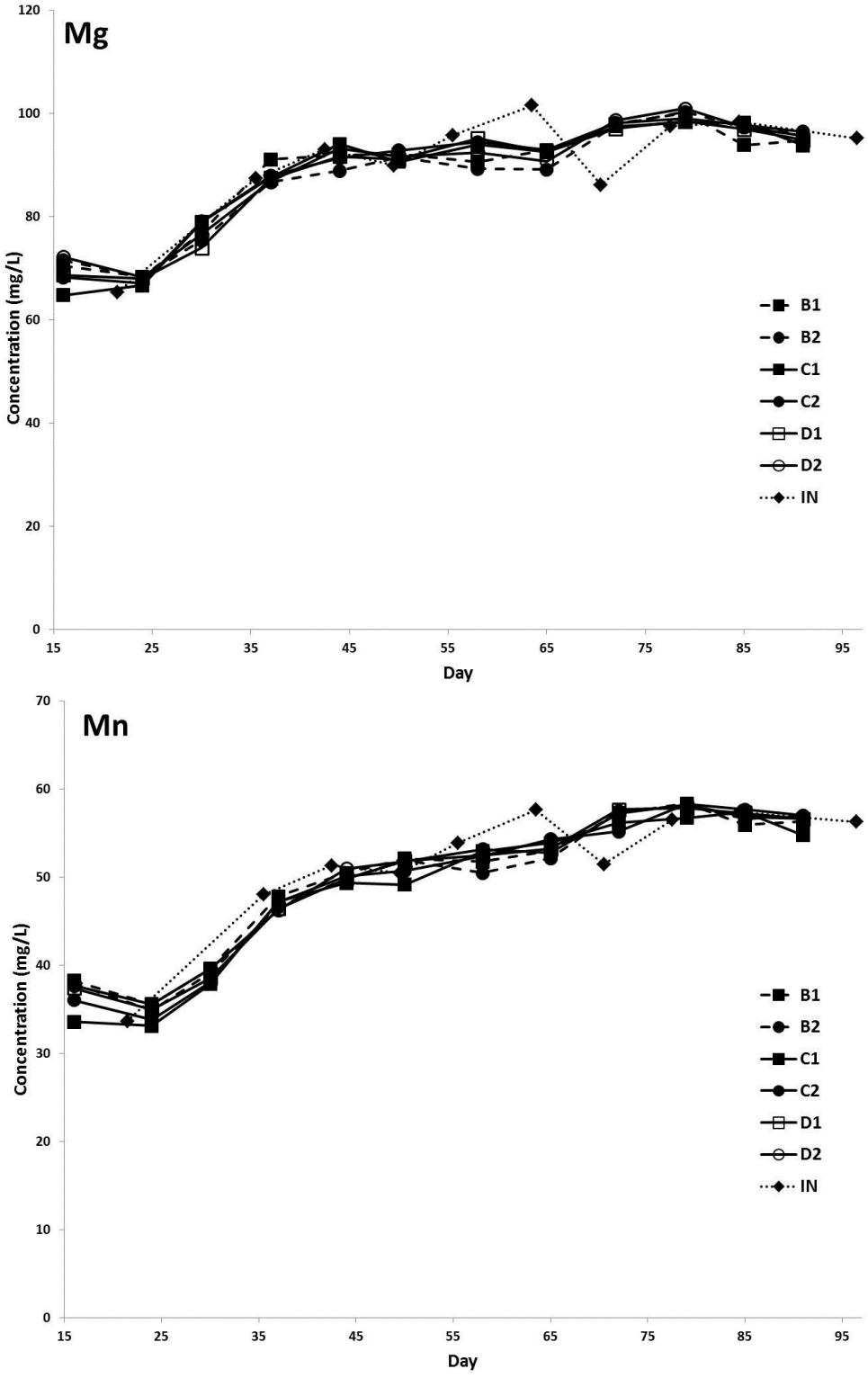

Figure 3.11 Concentration of magnesium (top) and manganese (bottom) in the column influent and effluent. These data suggest that none of the columns were removing magnesium or manganese from the influent water.

Metal mobility in the columns was influenced by: sorption to the organic substrate, release from the organic substrate, sorption to the anaerobic digester granules, release from the anaerobic digester granules, and precipitation as metal sulfides, with sulfide being supplied both by microbial sulfate reduction, and by the dissolution of relatively soluble iron sulfide present in the anaerobic digester granules. Sorption and sulfide precipitation are among the primary means of metal removal in the reducing environments of sulfidogenic bioreactors (Wildeman and Laudon, 1989; Sheoran and Sheoran, 2006). Inoculation with non-sterilized ADGs contributed to metal removal via both of these mechanisms in this experiment. Field evidence suggests that the importance of sorption relative to metal sulfide precipitation dwindles as substrate sorption sites saturate with time (Machemer and Wildeman, 1992; Gammons and Frandsen, 2001). In the long-term, metal sulfide precipitation is a more desirable removal mechanism because sorbed metals have the potential to remobilize if oxidizing conditions degrade the organic substrate (Tessier et al., 1979). Furthermore, the precipitation of metal sulfides potentially generates additional, less-reversible sorption sites for additional metal removal (Jong and Parry, 2004). Both removal mechanisms have demonstrated success in laboratory-scale experiments (e.g. Sahinkaya et al., 2009; Pinto et al., 2011). Many divalent metal cations are liable to precipitate as metal sulfides (e.g. Co^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}) (Sheoran et al., 2010). This list supports the hypothesis that zinc, nickel and cobalt were all removed via metal sulfide precipitation in this experiment.

Manganese is notoriously hard to remove from MIW due to its high solubility (Silva et al., 2012). It can cause health effects at levels as low as 0.5 mg/L (Zhu et al., 2009). It is not surprising that there was minimal Mn removal in this experiment as most experiments designed to treat Mn have done so either under aerobic conditions (e.g. Rose et al., 2003) or using specific sorption strategies under both aerobic (e.g. Taffarel and Rubio, 2010) and anaerobic conditions (e.g. Robinson-Lora and Brennan, 2010). Though it only occurred in modest

concentrations compared to many MIWs, it is interesting that iron would be mobilized from the anaerobic digester granules given the relative propensity for iron to sorb to substrate materials (Machemer and Wildeman, 1992; Gibert et al., 2005). This release was much less in the C columns than in the D columns, suggesting that some of the iron released from the ADGs can be re-stabilized as an iron sulfide precipitate. The benefits of using ADGs as an inoculum well outweigh the minor negative side effect of transient iron release. Given that magnesium and silicon are not linked to adverse health effects, there is a paucity of information in the literature regarding the removal of these two ions from MIW though this isn't too concerning as they are not all that relevant to performance variables.

CHAPTER 4: CONCLUSIONS, IMPLICATIONS AND FUTURE DIRECTIONS

The data collected in these experiments play an integral and synergistic role with ongoing microbiological research to tell a holistic story regarding the fundamental processes taking place inside of sulfate-reducing bioreactors and its link to community evolution that accompanies those processes.

Eight, 20 L reactors with varying substrate permutations were operated over the course of 497 days. There are several insights that can be learned from these columns:

1. Using energy-dispersive x-ray spectroscopy (EDX) to examine metal precipitation in the columns can effectively provide additional information beyond that given by ICP, though the process is much more time and labor intensive than ICP alone (2 hours per sample for 48 samples plus data entry and analysis—approximately 150 hours for this data set). The extra time considerations stem from the fact that an EDX approach requires many more data points per sample than does ICP because EDX samples are by nature more heterogeneous than aqueous collections for ICP. The benefit of a heterogeneous sample is that it is possible, given an adequate number of data points, to examine spatial relationships between substrate, precipitate, and potentially microorganism. This is assisted by the use of an SEM in conjunction with EDX.
2. Ternary diagrams, which are commonly used in geologic sciences, can be a useful tool in analyzing EDX data. Ternary plots are recommended for observing large scale trends and groupings while bar graphs are often more suitable for examining smaller sub-sets

of data. Ternary plots employed in this experiment revealed a temporal trend of increased zinc-sulfur signatures from May to October of 2013. ICP data superimposed on the ternary diagram affirmed that the highest zinc removal rates generally coincided with Zn:S ratios of approximately 1:1, and that zinc removal rates were higher in October 2013 than in May 2013. Spatial trends in the data were more easily discernible from a color-coded data table. The zone of highest zinc removal rate was between port 1 and port 3 after 110 days, receded to the region between the influent port and port one after 349 days, and migrated downward to the zone between port 1 and port 5 after 498 days.

3. In terms of zinc removal, columns containing at least 35% alfalfa demonstrated the best performance. Column 1, which contained only 10% alfalfa, had a near-zero net zinc removal rate in May 2013 (Day 349), on par with the columns containing no alfalfa. However, by October 2013 (Day 498) Column 1 had an improved zinc removal rate in between those of the columns containing 35% alfalfa and those of the columns containing no alfalfa. Collectively this suggests that alfalfa is a better recalcitrant substrate than either sawdust or woodchips in this experiment. In addition to its use as a carbon source, the alfalfa may also serve as a bulking agent to facilitate flow of MIW through the reactor. Literature suggests that mixing a recalcitrant substrate with a more labile one (such as manure) provides the best reactor performance and startup. The labile substrates may also serve as a significant microbial inoculum (as examined in the second set of experiments).

Previous studies have had mixed success in linking substrate characteristics to sulfate reduction rates. Zagury et al. (2006) found that they could not predict sulfate reduction based on characterizing the dissolved organic carbon, easily available substances, and the C/N ratio of the substrate. In contrast, Neculita and Zagury (2008) found that higher C/N, COD/SO_4^{2-}, and DOC/SO_4^{2-} ratios in the substrate were all associated with better sulfate-reducing conditions.

Similarly, Schmidtova and Baldwin (2011) found a positive correlation between initial C/N ratios of the substrate and the sulfate reduction rate. Coetser et al. (2006) found that a high carbohydrate and crude fat content, and a low crude fiber content helped to drive sulfate reduction. A characterization of the C/N, COD/SO_4^{2-}, and DOC/SO_4^{2-} ratios of the substrates used in these columns would thus be an important next step in this study.

Eight laboratory-bench-scale columns were constructed to examine the influence of various inoculation conditions on sulfate reduction and metal removal rates within the columns. The results of these inoculation experiments provide a geochemical foundation that becomes increasingly novel and elucidating when coupled to larger project efforts to bridge geochemical insights with molecular biology. Important results include:

1. The fact that the C columns displayed much more evidence of sulfate reduction than any of the other columns highlights the usefulness of an active anaerobic, organic rich inoculum in facilitating sulfate reduction. The fact that there was little difference in sulfide and sulfate data between the A and B columns suggests that the microorganisms present in the MIW are insufficient to act as an inoculum in the timespan of these column experiments (~3 months). The marked difference in sulfide and sulfate data between the C and D columns suggests that the addition of active microorganisms associated with anaerobic digester granules was responsible for accelerating the evolution of an SRBR capable of metal sulfide precipitation.
2. ICP data suggest, and literature corroborates, that sorption is an important metal removal mechanism in an SRBR, especially in during the initial phases of operation. This is evidenced by B and D column data for Co, Ni, and Zn, and B column data for Cd which all show more pronounced metal removal earlier in the experiment. Literature suggests that, while sorption-bound metals are relatively stable, they are more easily mobilized than metal sulfide precipitates as pH decreases.

3. Low sulfide concentrations in the D columns may have led to the dissolution of iron sulfide initially present in the anaerobic digester granules, thereby providing an alternative source of aqueous sulfide (microbial sulfate reduction being the primary source of aqueous sulfide in standard SRBRs). The precipitation of cadmium and zinc with this aqueous sulfide is another potentially significant removal mechanism for these two metals in the D columns. After Day 72 it is hypothesized that all iron sulfide in the D column ADGs had dissolved, leading to decreased removal efficiencies of cadmium and zinc in the D columns.

It is important to remember that substrate and inocula are but two of several parameters that potentially affect SRBR performance. Numerous studies have investigated other such parameters such as: pH (Elliott et al., 1998; Jong and Parry, 2006; Jiménez-Rodríguez et al., 2009), redox potential (Lefèvre et al., 2013), temperature (Moosa et al., 2005), SRB growth surfaces (Glombitza, 2001), hydraulic retention time (Gibert et al., 2004), hydraulic conductivity (Benner et al., 2001), and metal loading rates (Utgikar et al., 2001; Utgikar et al., 2002; Utgikar et al., 2003). Also important are non-scientific factors such as cost, terrain considerations, and the challenges associated with scaling up a reactor design (Gusek, 2004). Additionally, other AMD remediation strategies such as sorption to various substrates without sulfide precipitation, phytoextraction, and dosing with alkali may be considered as alternatives to SRBRs (Johnson and Hallberg, 2005). However, these alternative strategies run a higher risk of metal remobilization than does precipitating metals as metal sulfides in an SRBR (Tessier et al., 1979). Similar to SRBRs, they can also be hindered by a lack of widespread applicability (e.g. Ernst, 2005; Mendez and Maier, 2008).

Sulfate-reducing bioreactors have been labeled a promising technology; however much is still not known about their fundamental processes. Seeing into the “black box” could better inform both design and operation leading to more reliable and effective implementation at

orphaned and abandoned mines. The operational design and geochemical data gained from these two experiments is being used to support larger efforts in both of these systems. Forthcoming genetic sequencing, substrate digests, TEM electron diffraction patterns, and synchrotron analyses will all contribute to filling in the understanding gaps associated with the fascinating, complex systems that evolve within SRBRs.

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