Chemolithotrophic Denitrification of Nitrate Contaminated Groundwater Using Sulfur-Bearing Minerals

John Sutton
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Chemolithotrophic Denitrification of Nitrate Contaminated Groundwater Using Sulfur-Bearing Minerals

by

John Sutton

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering
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DEDICATION

For Beatriz Dunoyer de Segonzac; without her love, motivation and patience, this would not have been possible.
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ABSTRACT

Nitrate contamination in groundwater resources poses a serious threat, not only to the health of those who would drink it, but also as a potential nutrient for eutrophication. Current methods of nitrate removal can efficiently remove nitrate below 10 mg/L as nitrogen, but may require resources unavailable to small community water systems (SCWS). Sulfur-oxidizing biological denitrification is a promising alternative that may be more suitable for SCWS. Sulfur-oxidizing denitrification has previously been shown to have a high nitrate removal efficiency; however, it produces water high in sulfate. Utilization of sulfide-bearing minerals, rather than elemental sulfur as an electron donor, may yield similar nitrate removal efficiencies without the production of unwanted byproducts.

In this study, five minerals were examined through batch and column experiments to investigate suitable electron donors for autotrophic denitrification: sphalerite ((Zn,Fe)S), pyrite (FeS$_2$), pyrrhotite (Fe$_{1-x}$S$_2$), iron (II) sulfide, and molybdenite (MoS$_2$). Throughout the batch experiments, MoS$_2$ performed poorly, removing less total inorganic nitrogen (TIN) than the non-inoculated control and was thus not considered for future tests. Fe$_{1-x}$S$_2$ tracked with the positive S$^0$ pastille control during the first trial, though underperformed during the second trial and was therefore eliminated due to inconsistent performance. Iron(II) sulfide underwent dissimilatory nitrate reduction to ammonium (DNRA), effectively removing no TIN, and was thus not considered for the column experiments. Pyrite and sphalerite both had similar nitrogen species profiles throughout all trials and were therefore considered for the column experiment.

Two side-by-side upflow packed-bed reactors were operated over the course of 312 days, one with pyrite and pumice, and the other with sphalerite and pumice. Phases 1-3 of the experiment (Days 0-70) were operated as an acclimation phase where the procedure for the columns was altered to obtain consistent results. Phases 4-5 were operated with an approximate HRT of 24 hours and target influent
concentrations of 100 mg/L NO$_3$-N, 10 mg/L NO$_2$-N, 10 mg/L PO$_4^{3-}$-P, and 300 mg/L alkalinity as HCO$_3^-$. Throughout phase four (days 71-221), the pyrite and sphalerite column product waters had average nitrate concentrations of 48 mg/L and 43 mg/L respectively (as N). Though no significant (P>0.05) sulfate production was observed in the product water, 24 and 23 mg/L of sulfur oxyanions, i.e. sulfur oxidation intermediates, were produced in the pyrite and sphalerite columns, respectively. Additionally, the pyrite and sphalerite column product waters had average nitrite-nitrogen concentrations of 5.8 and 5.3 mg/L respectively, which greatly exceeds the 1 mg/L NO$_2$-N primary drinking water standard. Phase 5 (days 222-312) of the column study included 4.1 mg/L of yeast extract in the feed stock in order to account for organic carbon required for denitrifiers for biosynthesis. During this phase, the average nitrate-nitrogen concentrations in the influent, pyrite product water, and sphalerite product water were 90 mg/L, 69 mg/L and 68 mg/L respectively. Furthermore, the average sulfate concentrations in the influent, pyrite product water, and sphalerite product water were 116 mg/L, 118 mg/L and 122 mg/L respectively. Additionally, the pyrite and sphalerite columns produced about 21 and 27 mg/L, respectively, of intermediate sulfur oxyanions. The low sulfate formation during phases four and five of the column experiment suggest that chemolithotrophic denitrification via sulfur oxidation was not the sole pathway for denitrification. Ultimately, pyrite and sphalerite did not achieve safe levels of nitrate or nitrite under the given reactor configuration, though improving surface reactivity may yield a greater removal efficiency.
CHAPTER 1: INTRODUCTION

Nitrate (NO$_3^-$) contamination in groundwater sources of drinking water can, upon ingestion, convert to nitrite (NO$_2^-$) and cause adverse health effects such as methemoglobinemia in infants (Wright et al. 1999). Nitrate can enter a groundwater system through infiltration from surface waters which have been contaminated by anthropogenic sources such as agricultural runoff, industrial runoff, or improperly treated wastewaters (Ghafari et al., 2009). In an attempt to limit the risk to vulnerable individuals, the United States Environmental Protection Agency (USEPA) enforces maximum contaminant levels (MCLs) for NO$_3^-$ and NO$_2^-$ in public drinking water at 10 mg/L (as N) and 1 mg/L (as N) respectively (USEPA, 2016).

Reverse osmosis, electro-dialysis, and ion exchange are common technologies used by municipalities to treat nitrified waters which all provide high quality treatment of NO$_3^-$ contaminated waters. However, these techniques either require a large amount of electricity or necessitate the disposal of concentrated waste brines (Elmidaoui et al., 2003; Schoeman and Steyn, 2003; Ghafari et al., 2009). Compounding upon the issue of brine disposal, these methods require regular maintenance to prevent membrane scaling/fouling while risking potential contamination of the treated water with counter ions (Greenlee et al., 2009; USEPA, 2016).

One popular method used to achieve a low-nitrate product water without the aforementioned disadvantages is heterotrophic denitrification. Though this method achieves nearly complete removal of nitrate and nitrite, it requires the addition of a potentially costly organic carbon source which must be constantly monitored to prevent excess dissolved organic carbon (DOC) within the product water (Moon et al., 2004; Ergas and Rheinheimer, 2004; Zhang et al., 2012). Furthermore, heterotrophic denitrification produces high amounts of biomass, which could breakthrough into the product water, thus contaminating
the product water with additional DOC and increasing the risk of forming disinfection by-products (Sengupta et al., 2007). DOC within drinking water has created non-compliance issues for small community water systems (SCWS), with regards to total coliform and disinfection by-product (DPB) guidelines (Rubin, 2013; Oxenford and Barrett, 2016). Therefore, SCWS may not be able to utilize heterotrophic denitrification techniques to treat contaminated drinking water sources due to limited financial, technological, and managerial resources.

Chemolithotrophic denitrification is an alternative method of removing nitrate and nitrite from contaminated groundwater sources. This method relies on inorganic electron donors such as zero-valent sulfur (S\(^0\)), hydrogen gas (H\(_2\)), reduced metals (Fe(II) and Mn(II)), and sulfur-bearing minerals to reduce nitrate and nitrite to nitrogen gas. This method also uses inorganic carbon as a source of carbon for biosynthesis, negating the need for chemical addition of an organic carbon source and limiting the amount of DOC in the treated water. Also, due to the slow growth kinetics of the chemolithotrophs that participate in autotrophic denitrification, there is much less sludge produced when compared to heterotrophic denitrification (Krayzelova et al., 2014). This could create a niche application for autotrophic denitrification in drinking-water sources. However, chemolithotrophic denitrification is not without its disadvantages. For instance, utilization of zero-valent sulfur (S\(^0\)) as an electron donor for denitrification results in elevated sulfate concentrations within the product water, high consumption of alkalinity, and the potential production of sulfur by-products such as thiosulfate (S\(_2\)O\(_3^{2-}\)), sulfite (SO\(_3^{2-}\)), and sulfide (S\(^2-\)) (Oh et al., 2001; Park and Yoo, 2009). Elevated levels of sulfate or sulfate precursors above 250 mg/L would violate the sulfate National Secondary Drinking Water Regulation (NSDWR) and would likely impact the aesthetic qualities of the drinking water source.

Sulfur-bearing minerals such as pyrite (FeS\(_2\)), iron sulfide (FeS), and pyrrhotite (Fe\(_{1-x}\)S) have been studied for their abilities to act as electron donors for autotrophic denitrification in both natural and engineered systems (Torrento et al., 2010; Li et al., 2013; Li et al., 2016; Tong et al., 2017). Many have found that utilizing sulfur-bearing minerals results in less sulfate production than using zero-valent sulfur or sodium thiosulfate, thereby reducing one of the negative consequences. However, this method requires
a longer hydraulic residence time than zero-valent sulfur or sodium thiosulfate to achieve equivalent denitrification efficiencies and thus leads to a quality-over-quantity trade-off given the same reactor volume. Tong et al. (2017) proposed the following stoichiometric equation for pyrite autotrophic denitrification.

\[
0.364\text{FeS}_2 + 0.116\text{CO}_2 + \text{NO}_3^- + 0.821\text{H}_2\text{O} + 0.023\text{NH}_4^+ \rightarrow 0.364 \text{Fe(OH)}_3 + 0.023\text{C}_5\text{H}_7\text{O}_2\text{N} + 0.50\text{N}_2 + 0.729\text{SO}_4^{2-} + 0.480\text{H}^+
\] (1)

While the majority of previous studies have examined the capacity of iron-sulfur minerals to promote denitrification in wastewater or in-situ ground water systems, these minerals have not been previously studied for denitrification applications in small community drinking water systems, let alone in a side-by-side study. This application could potentially benefit from the higher-quality product derived from utilizing iron-sulfur minerals rather than S\(^0\) or S\(_2\)O\(_3\)\(^-\), especially as drinking water has very stringent limits on chemical and biological constituents. Additionally, sulfur-bearing minerals such as sphalerite (ZnS) and molybdenite (MoS\(_2\)) have not been examined for their capabilities to act as electron donors for autotrophic denitrification, and thus their removal efficiencies are unknown.

Therefore, the overall objective of this research is to determine the capacity of easily obtainable sulfur-bearing minerals to support chemolithotrophic denitrification in nitrate-contaminated drinking water sources. This overall objective will be achieved through the following goals:

1) Screen for candidates that best achieve removal of nitrate and nitrite through batch reactor studies of sulfur-bearing minerals.

2) Observe the long-term removal efficiencies of the best-performing sulfur-bearing minerals from goal two through a side-by-side up-flow packed-bed reactor study.
CHAPTER 2: LITERATURE REVIEW AND MOTIVATION

2.1 Nitrate Pollution in Small Community Drinking Water Systems

2.1.1 National Primary Drinking Water Regulations (NPDWR)

Following the nationwide adoption of Title XIV of the Public Health Service Act, also known as the Safe Drinking Water Act (SDWA), the United States Environmental Protection Agency (USEPA) created national regulations for harmful contaminants that could impact human health once ingested. National Primary Drinking Water Regulations (NPDWR) only apply to public drinking water systems, as private wells fall under the responsibility of the owner. Contaminants that are included within the NPDWR must fall below a maximum contaminant level (MCL) which is primarily determined by a tolerable risk, regarding public health, and secondly, by best available treatment technology. Essentially, public drinking water stewards should strive to provide safe drinking water in the most technologically and economically feasible way.

Specifically, a NPDWR is enacted if public health can be impacted, if the contaminant exists or is likely to exist within the public drinking water system at harmful levels, and if the regulation of the contaminant will benefit those served by the public drinking water system. With regard to this study, the NPDWR contaminants that are most likely to be impacted by treatment of groundwater with sulfur-bearing minerals are those listed in Table 2.1. These were determined by cross-referencing the NPDWR contaminants with the major and minor elements of each mineral considered in this study. In total, pyrite, sphalerite, molybdenite, and pyrrhotite may contain the following major and trace elements: Fe, S, As, Au, Cd, Hg, In, TI, Ga, Ge, Sb, Sn, Pb, Ag, Mn, Mo, Ni, Co, Cu, Se, V, and Zn. (Mindat, 2019). This list contains contaminants in the NPDWR (Table 2.1), the National Secondary Drinking Water Regulation (NSDWR) (Table 2.2), and others not regulated by the EPA for drinking water.
Table 2.1: National Primary Drinking Water Regulations for contaminants of concern in this study.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>MCL (mg/L; TT&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbidity</td>
<td>TT&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Antimony</td>
<td>0.006</td>
</tr>
<tr>
<td>Arsenic</td>
<td>0.010</td>
</tr>
<tr>
<td>Barium</td>
<td>2</td>
</tr>
<tr>
<td>Beryllium</td>
<td>0.004</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.005</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.1</td>
</tr>
<tr>
<td>Copper</td>
<td>TT&lt;sup&gt;a&lt;/sup&gt;; Action Level&lt;sup&gt;b&lt;/sup&gt; = 1.3</td>
</tr>
<tr>
<td>Lead</td>
<td>TT&lt;sup&gt;a&lt;/sup&gt;; Action Level&lt;sup&gt;b&lt;/sup&gt; = 0.015</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.002</td>
</tr>
<tr>
<td>Nitrate (as Nitrogen)</td>
<td>10</td>
</tr>
<tr>
<td>Nitrite (as Nitrogen)</td>
<td>1</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.05</td>
</tr>
<tr>
<td>Thallium</td>
<td>0.002</td>
</tr>
</tbody>
</table>

a. TT refers to treatment technique. That is to say that the product water must be treated using an approved treatment technique.

b. Action Level refers to the contaminant concentration that is exceeded in 10% of samples and thus requires further treatment.

Aside from the potential introduction of non-native metals to the source water, this study focuses on the removal of nitrate through denitrification, including all consequential intermediate products, such as nitrite, nitric oxide, and nitrous oxide. Nitrate earned a NPDWR when scientific evidence linked it to methemoglobinemia in infants (Wright et al., 1999). When ingested, nitrate is reduced to nitrite, which hinders the ability of blood to deliver oxygen to vital organs. This is of particular concern to infants and fetuses as their gut microbiology is particularly vulnerable due to a limited ability to produce nitrite reductase. Aside from this, several studies have shown that imbibition of nitrate-contaminated drinking
water could lead to an elevated risk of bladder cancer (Chiu et al., 2007; Jones et al., 2016), thyroid cancer (Ward et al., 2010; Kilfoy et al., 2011), colon cancer (De Roos et al., 2003; Espejo-Herrera et al., 2016), birth defects (Holtby et al., 2014; Brender et al., 2013), low birthweight (Migeot et al., 2013), and preterm birth (Bukowski et al., 2001; Stayner et al., 2017). However, it is crucial to note that the Center for Disease Control and Prevention (CDC) has developed conflicting research with regard to nitrate acting as a direct carcinogen (Agency for Toxic Substances and Disease Registry, 2012). Nevertheless, limiting nitrate and nitrite levels in drinking water has been determined to reduce the risk to public health and thus will be the primary focus of this research.

2.1.2 National Secondary Drinking Water Regulations (NSDWRs)

In addition to the NPDWR set forth by the USEPA, certain aesthetic qualities should also be maintained in public drinking water. Qualities such as odor, taste, and appearance may be negatively impacted by contaminants such as sulfur, iron, manganese, etc. without necessarily posing a direct risk to public health. These contaminants are designated with NSDWR. For this study, the major elements and common impurities of candidate minerals were cross-referenced with the NSDWR in order to determine the consequences of the chosen drinking water treatment system (Table 2.2).

Chemolithotrophic denitrification using pyrite and other sulfur-based electron donors commonly produces excess sulfate (Tong et al., 2017). Excess sulfate has been shown to impart the water with a bitter taste while leading to a higher risk of gastrointestinal discomfort. This must be controlled to produce a product water that adheres to the NSDWR. Furthermore, mineral impurities may result in the release of copper and zinc. Thus, the product water will require monitoring to prevent any aesthetic degradation.
Table 2.2: National Secondary Drinking Water Regulations for contaminants of concern in this study.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Secondary Standard (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum</td>
<td>0.05-0.2</td>
</tr>
<tr>
<td>Chloride</td>
<td>250</td>
</tr>
<tr>
<td>Copper</td>
<td>1.0</td>
</tr>
<tr>
<td>Iron</td>
<td>0.3</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.05</td>
</tr>
<tr>
<td>pH</td>
<td>6.5-8.5</td>
</tr>
<tr>
<td>Sulfate</td>
<td>250</td>
</tr>
<tr>
<td>Zinc</td>
<td>5</td>
</tr>
</tbody>
</table>

2.2 Small Community Water Systems and Method Suitability

According to the SDWA, a public water system refers to a distribution system capable of providing water for public consumption (Safe Drinking Water Act, 1986). Water quality in a public water system ultimately falls under the jurisdiction of the USEPA and is thus subject to the NPDWR and NSDWR detailed in sections 2.1.1 and 2.1.2.

The SDWA separates community water systems (CWS) into three distinct groups: those that serve a population between 25-500 people, those that serve a population between 500-3,300, and those that serve a population between 3,300-10,000 (Safe Drinking Water Act, 1974). Others have placed distinctive names to these groups, labeling CWS that serve fewer than 3,300 people as “very small” community water systems (VSCWS), and those that serve between 3,300 and 10,000 people as “small” community water systems (SCWS) (Hunter, 2008).

The drinking water resources of SCWS, especially rural ones, are particularly vulnerable to nitrate contamination due to nearby agricultural activities that rely on fertilizers or animal manures (Hunter, 2008). NO₃-N levels above 2-3 mg/L typically indicate anthropogenic nitrate contamination.
(Foster et al., 1982; Kross et al., 1993; Mueller et al., 1995). Some communities in rural areas in the US have developed such severe nitrate contamination issues that they abandon their wells and seek alternative sources (Schuff 1992; Spalding and Exner, 1993; Lasserre et al., 1999). According to Schaider et al. (2016), out of 41,781 observed CWS, systems that service a population between 25-500 are more likely to exceed 5 mg/L of NO₃-N than small or medium sized systems. However, systems that serve between 3,300-10,000 people were observed to have higher than average nitrate concentrations (Schaider et al., 2016). Thus, developing novel treatment technologies targeted towards SCWS would prove advantageous, and help alleviate water stress.

Now that the problem has been identified, existing solutions can be scrutinized. Reverse osmosis (RO) treatment of nitrate-contaminated groundwater creates a very high-quality product water. However, the product water requires remineralization to become potable. Calcium concentrations above 180 mg/L will cause the RO membrane to clog frequently, increasing operational costs (Harries et al., 1991; Kunz, 1997). Furthermore, disposal of the waste brine created during the RO process has been shown to account for roughly 60% of the total operational costs, which compound upon an already high capital cost (Green and Shelef, 1994). Additionally, this process does not solve the nitrate-contamination issue at large, instead, it merely moves the nitrate to a brine, which requires its own treatment. This essentially passes the chain of nitrate custody from the owner/operator to a third party. Utilizing groundwater that has high hardness will only serve to increase the waste brine disposal frequency, and therefore the operational cost. Thus, for SCWS that treat groundwater for drinking water use, RO would be a poor option.

Electrodialysis is another popular option that has been utilized for drinking-water treatment. This process involves an electric current that forces ions through a pair of semipermeable membranes. Like RO, electrodialysis requires brine disposal, which may not be feasible for SCWS. Furthermore, the feed water needs to have a turbidity less than 2 NTU, otherwise, the efficiency of the process decreases and may even cease entirely if the turbidity is extreme (Hunter, 2008).

Aside from the previously described methods, ion exchange (IX) is a common method used to remove ionic contaminants from water using charged beads or resins. Typically, positively charged beads
are used to remove negatively charged ions, such as nitrate and nitrite, while negatively charged beads remove positively charged ions such as calcium and magnesium or iron. While effective, IX produces the most cumbersome brine due to added counter ions that are used to regenerate the resins (Ceval et al., 1995).

2.3 Chemolithotrophic Denitrification

2.3.1 Denitrification Overview

Biological denitrification is an efficient process for removing nitrate from most sources of contaminated water if conditions are carefully controlled. Heterotrophic denitrification occurs when microbes utilize organic carbon for both cell synthesis and microbial respiration via nitrate reduction. Autotrophic denitrification differs from heterotrophic denitrification in that an inorganic carbon source, such as bicarbonate (HCO$_3^-$) or carbon dioxide (CO$_2$), is required for cell synthesis. Autotrophic denitrification, in this study, proceeds using an inorganic electron donor, such as hydrogen gas or sulfur, and can therefore be considered chemolithotrophic denitrification. Often, this microbial respiratory process occurs in natural environments when conditions are appropriate for ubiquitous, facultative, anaerobic microorganisms to thrive. However, should oxygen be present, the facultative microorganisms will prefer oxygen as an electron acceptor over nitrate. Thus, controlling environmental conditions through engineered systems can help ensure that biological denitrification will proceed.

Biological denitrification usually operates via four enzymatic reduction processes: NO$_3^-$ to NO$_2^-$ via nitrate reductase, NO$_2^-$ to NO via nitrite reductase, NO to N$_2$O via nitric oxide reductase, and finally N$_2$O to N$_2$ via nitrous oxide reductase. Anoxic conditions are generally required for denitrification as nitrite reductase and nitrous oxide reductase are sensitive to elevated dissolved oxygen concentrations (Oh et al., 1999; Sabba et al., 2016). Should the activity of these enzymes become inhibited, intermediates may accumulate, which may pose a greater threat to human health than nitrate. This issue may be further exacerbated by limited microbial access to phosphate (Kim et al., 2002; Hunter, 2003).
When utilizing biological denitrification in ex-situ reactors, media that facilitate biomass growth are often necessary to maintain consistent performance. Furthermore, treating drinking water using biological denitrification requires secondary treatment, such as filtration, aeration, and disinfection, to elevate the quality of the product water to drinking water standards (Roennefahrt, 1986; Dahab and Sirigina, 1994; Green and Shelef, 1994; Hunter and Follett, 1997; Silverstein and Carlson, 1999). One of the major issues with this method is possible contamination of the product water with bacteria and bacterial by-products, which is common in systems using heterotrophic denitrification. Furthermore, bacteria and other organic sources of carbon can form harmful disinfection by-products (DBP) during disinfection via chlorination. Utilizing chemolithotrophic denitrification can limit the concentration of bacteria post denitrification due to low biomass yields (Lampe and Zhang, 1997; Flere and Zhang, 1999).

Chemolithotrophic denitrification generally requires controlling several key factors to achieve sufficient denitrification rates. First and foremost, limiting dissolved oxygen (DO) within the system is crucial for nitrate to be used as the terminal electron acceptor. The general oxidation-reduction potential for this process is around -100 to 100 mV, though siderite (FeCO$_3$) has been found to promote denitrification at redox potentials of 200 mV (Mo et al., 2005; Di Capua et al., 2017). Limiting the oxygen within reactors can be achieved by purging the solution with nitrogen gas (He et al., 2018). Second, providing sufficient electron donor, inorganic carbon, and other nutrients for chemolithotrophs to thrive is necessary. Chemolithotrophic denitrifiers, such as *Thiobacillus*, tend to have higher observed growth rates when ammonium is used as a nitrogen source for biosynthesis, though inoculum derived from activated sludge has been shown to be more resistant to nutritional stress (Baalsrud and Baalsrud, 1954; Li et al., 2016; Di Capua, 2016). Furthermore, the addition of phosphorus has been shown to improve the resistance of denitrifying organisms to hydraulic loading shock and help stifle nitrite accumulation by relieving nutritional stress on bacteria (Hunter, 2003; Wang et al., 2018). Third, should conditions within the reactor become acidic (<pH 5), the production of enzymes may become inhibited, leading to the accumulation of harmful intermediates such as nitrite and nitric oxide (Baeseman et al., 2006). Fourth, Dries et al. (1988) found that hard water (317-375 mg CaCO$_3$/L) caused precipitation
within a few weeks of packed-bed reactor (PBR) operation, which ultimately reduced treatment efficiency due to surface fouling and pore clogging. The precipitation of mineral solids might limit the mass transfer and decrease biomass activity (Lee and Rittman, 2003; Arvin and Kristensen, 1982; Flora et al., 1993). Thus, controlling water hardness through the addition of bicarbonate, rather than limestone, is a viable solution that may limit the amount of scale formation and improve the rate of chemolithotrophic denitrification (Sahinkaya and Dursun, 2012; Di Capua et al., 2015).

2.3.2 Viable Electron Donors and Reaction Stoichiometry

Chemolithotrophic denitrification is a broad category of denitrification processes that can include the utilization of hydrogen gas, arsenic, iron, intermediate sulfur oxyanions, and sulfur-bearing minerals, among others, as electron donors. Generally, chemolithotrophic denitrification benefits from a low biomass yield and not requiring any added organic carbon. These factors result in lower operational costs, as opposed to heterotrophic denitrification, for many of the above electron donors with the added benefit of reducing the amount of post-treatment chlorine demand or DBP precursors (Pu et al., 2014). Aside from the general benefits of chemolithotrophic versus heterotrophic denitrification, each of the previously stated electron donors distinguishes itself with benefits and drawbacks.

Hydrogen gas ($H_2$) has previously been shown to be an excellent electron donor for chemolithrophic denitrifiers for several key reasons, though its drawbacks may make its operational costs too high for SCWS. The stoichiometric reaction for hydrogenotrophic denitrification is:

$$H_2 + 0.355NO_3^- + 0.049CO_2 + 0.355H^+ \rightarrow 0.010C_5H_7O_2N + 0.172N_2 + 1.143H_2O$$  \hspace{1cm} (2)

Hydrogen gas boasts a high diffusivity into biofilms, which improves the overall denitrification rate by increasing microbial access to an electron donor (Kurt et al., 1987). However, as a flammable gas, hydrogen requires extra care when handling to maintain safe and stable operating conditions. Furthermore, the oxidation-reduction potential (ORP) in the reactor must be kept above -50 mV and the pH between 7.6 and 8.6 to promote denitrification, which requires careful monitoring of reactor conditions (Lee and Rittman, 2003; Rezania et al., 2005a; Rezania et al., 2005b; Ghafari et al., 2009;
Ghafari et al., 2010; Di Capua et al., 2016). Rezania et al. (2005a) determined that pH values below pH 7.6 can result in the availability of inorganic carbon to become limited while values above 8.6 will decrease the overall hydrogenotrophic activity. Additionally, the temperature of the reactor must remain between 25-35 °C to obtain maximum treatment efficiency (Zhou et al., 2007). Ergas and Reuss (2001) encountered issues with hydrogenotrophic denitrification in terms of its low solubility.

Utilization of ferrous iron as an electron donor for chemolithotrophic denitrification has been shown to have lower denitrification rates than other electron donors due to the low redox potential between the donor and acceptor (+230 mV) (Devlin et al., 2000; Hedrich et al., 2011). Nonetheless, an equation proposed by Straub et al. (1996) for denitrification using ferrous iron as an electron donor is:

$$Fe^{2+} + 0.2NO_3^- + 2.4H_2O \rightarrow Fe(OH)_3 + 0.1N_2 + 1.8H^+$$

Here, alkalinity is consumed and ferric hydroxides are produced. These may precipitate in the reactor and can add a metallic taste to the product water which, should be avoided if possible to provide desirable drinking water. Overall, ferrous iron is not recommended for use as an electron donor for SCWS.

Arsenic has been investigated for its ability to act as an electron source for chemolithotrophic denitrification but should not be used for drinking water treatment due to its severe toxicity. Nonetheless, arsenic is a novel electron donor that can have alternative applications, especially in natural waters already contaminated with As$^{+3}$. Rhine et al. (2006) proposed the following stoichiometric equation for denitrification using arsenic:

$$H_3AsO_3 + 0.4NO_3^- \rightarrow 1.6H^+ + HAsO_4^{2-} + 0.2N_2 + 0.2H_2O$$

Arsenic can often be found in the +3 and +5 oxidation states, with the +3 state (arsenite) being much more toxic, mobile, and bioavailable than the +5 oxidation state (Neff, 1997). Therefore, careful handling of As$^{+3}$ addition to the denitrification reactor must be adhered to limit risks to human and environmental health. Though the denitrification process converts As$^{+3}$ to As$^{+5}$, complete removal of arsenic from the system requires the addition of clay, an adsorbent with a high concentration of aluminum. This would require reactor maintenance as well as proper disposal of arsenic-laden clay. However, chemolithotrophic denitrification using As$^{+3}$ has been shown to produce stable results over long observation periods (Sun et
al., 2009; Sun et al., 2010b). Careful application of arsenic for denitrification purposes is required as As$^{+3}$ concentrations above 3.5 mM will result in full inhibition of denitrification (Sun et al., 2008). Furthermore, this application should be limited to waters with natural arsenic contamination and should not be applied to drinking water treatment.

Aside from hydrogen gas, iron, and arsenic-based chemolithotrophic denitrification, a group of intermediate sulfur oxyanions have shown promising results. One of the main concerns with treating drinking water with chemolithotrophic bacteria is the elevated sulfate levels in the product water. Thiosulfate ($S_2O_3^{2-}$) has been shown to be an excellent electron donor for culturing microbes tolerant to hydraulic loading shocks and elevated concentrations of toxic metals that can provide high rates of denitrification (Pethkar et al., 2003; Di Capua et al., 2017; Khanongnuch et al., 2018). Di Capua et al., 2017 proposed an overall denitrification reaction for thiosulfate:

$$S_2O_3^{2-} + 1.24NO_3^- + 0.45HCO_3^- + 0.09NH_4^+ + 0.11H_2O \rightarrow 0.09C_5H_7O_2N + 0.40H^+ + 0.62N_2 + 2SO_4^{2-} \quad (4)$$

In this equation, the nitrate/sulfate ratio is approximately 0.62. Hydrogen sulfide (H$_2$S) can often be found in anoxic waters contaminated by anthropogenic waste (Garcia De Lomas et al., 2006; Jiang et al., 2009; Shao et al., 2009). A proposed denitrification reaction using hydrogen sulfide is:

$$HS^- + 1.23NO_3^- + 0.573H^+ + 0.438HCO_3^- + 0.027CO_2 + 0.093NH_4^+ \rightarrow 0.093C_5H_7N_2O + 0.866H_2O + 0.614N_2 + SO_4^{2-} \quad (5)$$

Here, the molar nitrate/sulfate ratio is 1.23, which is much higher than that of thiosulfate, indicating less production of sulfate for the same removal of nitrate. While controlling the sulfate concentrations in the product water is important for adhering to NSDWRs, maintaining an appropriate initial NO$_3^-$-N to sulfur ratio is crucial to controlling the overall chemolithotrophic denitrification efficiency. Should this ratio be higher than the stoichiometric value, then chemolithotrophic denitrification using hydrogen sulfide will proceed completely, and the dominant sulfur product will be sulfate. However, if the initial ratio is at or lower than the stoichiometric value, i.e. there is not enough electron acceptor for all the electron donor,
then zero-valent sulfur (ZVS) will precipitate even though full oxidation is more thermodynamically favorable (Cardoso et al., 2006; Moraes et al., 2012). Though the utilization of hydrogen sulfide requires the careful balance of initial sulfide concentrations to maintain a healthy biofilm, it is the only intermediate sulfur oxyanion to produce alkalinity, thus helping maintain an appropriate environment for chemolithotrophic microorganisms (Di Capua et al., 2019). However, the volatile nature of hydrogen sulfide can result in unreliable rates of denitrification unless the application is carefully administered (Cardoso et al., 2006; Sabba et al., 2018). Furthermore, H₂S can be highly toxic and quite odorous, which can present a safety hazard to workers while also impairing the aesthetic quality of the product water.

ZVS is an intermediate sulfur product capable of acting as an electron donor for chemolithotrophic denitrification. On the one hand, it is inexpensive, easy to handle, and functions simultaneously as a biofilm growth media and as an electron donor (Di Capua et al., 2015). On the other hand, ZVS consumes alkalinity, thus requiring an added buffer. This can be seen in the following equation proposed by Mora et al. (2014):

\[
S^0 + 0.876NO_3^- + 0.343H_2O + 0.379HCO_3^- + 0.023CO_2 + 0.080NH_4^+ \rightarrow 0.080C_2H_7O_2N + 0.824H^+ + 0.44N_2 + SO_4^{2-}
\]

(6)

Limestone and bicarbonate are both common additives as they can act as a buffer and carbon source. However, limestone may increase hardness in the reactor and may lead to phosphorus precipitation (Di Capua et al., 2015). One alternative to using limestone is oyster shells. Sengupta et al., 2007 observed several benefits to utilizing oyster shells, namely a better rate of buffer dissolution, an increased ability to host microorganisms, a better product water turbidity, and an economic benefit. ZVS has a nitrate/sulfate ratio of 0.876 which is better than thiosulfate, but worse than hydrogen sulfide.

Though several inorganic sulfur- and iron-bearing substances have been studied for their capacity to promote chemolithotrophic denitrification, pyrite (FeS₂) is by far the most studied. Pyrite is an alternative electron donor for chemolithotrophic denitrification that has been observed in natural groundwater systems and has been applied to the treatment of wastewater with low organic carbon
concentrations (Jorgensen et al., Tong et al., 2017). Tong et al. (2017) proposed the following reaction for pyrite-mediated denitrification:

\[
0.364 \text{FeS}_2 + 0.116 \text{CO}_2 + \text{NO}_3^- + 0.821\text{H}_2\text{O} + 0.023\text{NH}_4^+ \rightarrow 0.364\text{Fe(OH)}_3 + 0.023\text{C}_5\text{H}_7\text{O}_2\text{N} + 0.50\text{N}_2 + 0.729\text{SO}_4^{2-} + 0.480\text{H}^+ \tag{7}
\]

Utilizing pyrite as an electron donor provides a nitrate/sulfate ratio of approximately 1.372, which is higher than all of the intermediate sulfur oxyanions, which indicates that for every mole of nitrate reduced to \( \text{N}_2 \), pyrite will produce less sulfate than the intermediate sulfur oxyanions and ZVS. Furthermore, some studies have observed that in nitrified aquifers with pyrite present, sulfate has often been below the stoichiometric amount, though this could be attributed to a low nitrate/sulfate concentration that could result in the accumulation of intermediate sulfur oxidation products (Miotlinski, 2008; Zhang et al., 2009). When comparing pyrite-based denitrification to intermediate sulfur oxyanion-based denitrification in continuous flow systems, pyrite has been shown to reduce alkalinity consumption and sulfate by-product production (Tong et al., 2017).

### 2.4 Nitrate Remediation using Sulfur-Bearing Minerals

Sulfur-bearing minerals can be divided into two groups: monosulfides and disulfides. Monosulfides include greigite (magnetic \( \text{Fe}_3\text{S}_4 \)), Mackinawite (\( \text{Fe}_{1.11}\text{S} \)), galena (\( \text{PbS} \)), sphalerite (\( \text{ZnS} \)), and pyrrhotite (magnetic \( \text{Fe}_{0.9}\text{S} \)), among others. Generally, these minerals tend to be acid-volatile and should not come into contact with acid for long periods of time. The disulfide group includes marcasite (orthorhombic \( \text{FeS}_2 \)) and pyrite (cubic \( \text{FeS}_2 \)). Of these two categories, pyrite and pyrrhotite are the only minerals that have been previously shown to act as electron donors for chemolithotrophic denitrification.

Chemolithotrophic denitrification rates using sulfur-bearing minerals can be improved by several methods. In general, adding electron donor in excess of the electron acceptor can improve the rate of denitrification by increasing the available surface area of the donor (Sierra-Alvarez, 2007). Effectively, this serves to decrease the initial \( \text{NO}_3^- : \text{N/Sulfur} \) ratio, thus promoting complete denitrification at the cost of potential introduction of intermediate sulfur oxyanions. Pu et al. (2014) found that pretreatment of the
pyrite surface with hydrochloric acid improves the rate of denitrification, likely due to the removal of surface impurities such as iron hydroxides or oxidized sulfur species. Additionally, this pretreatment has been shown to roughen the surface of the mineral, improving its capacity to carry biofilms (Pu et al., 2014). Others have shown that acid pretreatment of pyrite brings more ZVS to the mineral surface which can act as a readily available electron donor (Mirzoyan et al., 2014). However, Tong et al. (2018) determined that acid pre-treatment of pyrite had little effect on long-term nitrate removal efficiencies. However, pretreatment of monosulfide minerals using HCl should occur for a short amount of time to prevent excess HS\(^{(g)}\) volatilization. The disulfides are more tolerant than the monosulfides to acidic conditions and can therefore be exposed to acid pretreatment for longer periods (Santos et al., 2016).

Further process improvement may be achieved by promoting a mixotrophic metabolism (Tong et al., 2018). A mixotrophic denitrification metabolism occurs in a system when both chemolithotrophs and heterotrophs are present and contributing to the overall observed denitrification rate. However, this process still requires the addition of an external organic carbon source, which, unless carefully monitored, may result in DOC breakthrough.

### 2.4.1 Utilization of Sulfur-Bearing Minerals in Wastewater Treatment

Nitrified wastewater treatment using sulfur-bearing minerals has been performed using pyrrhotite and pyrite. Zhang et al. (2019) found that natural pyrrhotite yielded slower denitrification rates than synthetic pyrrhotite, which they attributed to the greater specific surface area of synthetic pyrrhotite over natural pyrrhotite (Vaclavkova et al., 2014). Furthermore, Zhang et al. (2019) observed that both synthetic and natural pyrrhotite were capable of supporting a active biofilm. Li et al. (2016a) utilized pyrrhotite in an upflow packed-bed reactor study consisting of three reactors: 1) limestone only, 2) pyrrhotite only, and 3) pyrrhotite and limestone. Each was fed synthetically nitrified wastewater four times per day from days 1-218 days of the study followed by real wastewater from days 218-249. Even though pyrrhotite belongs to the monosulfide group that are acid-volatile, they pre-treated the pyrrhotite with 10% HCl for 2 hours followed by a series of DI rinses until a pH of 7 was achieved in the rinse water. They operated their
columns at an HRT of 12 hours, which has previously been shown to be the optimal HRT for conventional biological nutrient removal activated sludge systems (Brown et al., 2011; Xu et al., 2014). Li et al. (2016) observed that biomass in the column was concentrated towards the bottom of the column and decreased by mass along the height of the column. Furthermore, they observed secondary minerals on the surface of the pyrrhotite which were determined to be FePO$_4$ and CaHPO$_4$. Precipitation of ZVS, P$_2$O$_5$ and Fe$_3$O$_5$ has also been observed when siderite (FeCO$_3$) is used, likely linked to ease of access to the metal ion in the mineral matrix (Wang et al., 2019). This system achieved a removal efficiency of 75.8 ± 1.2% at an HRT of 12 hours while the system developed by Zhang et al. (2019) obtained a nitrate removal efficiency of 96.2±7.1% at an HRT of 12 hours. They further increased the system HRT to 48 hours and saw nitrate removal efficiencies increase 99.7±0.9%.

Pyrrhotite-based denitrification has been shown to require certain reactor conditions to achieve sufficient nitrate removal rates. The temperature range for utilizing pyrrhotite as the sole electron donor is between 4-40 °C (Trouve et al., 1998; Zhang et al., 2019). Another limitation observed by Zhang et al. (2019) was nitrite accumulation and surface adsorption of phosphate beyond influent total organic nitrogen concentrations of 38 mg/L and phosphate concentrations of 12 mg/L.

Treating nitrified wastewater at the lab scale using pyrite has been well studied, especially when comparing its efficiency as an electron donor to ZVS. Kong et al. (2016) found that pyrite produces less sulfate while consuming less alkalinity for the same amount of nitrate reduced compared to ZVS. Tong et al. (2018) found that the highest denitrification rates using pyrite in upflow packed-bed reactors can be achieved with 1250 mg volatile suspended solids (VSS)/L, pyrite particles sizes of 0.82-1.02 mm, and a pyrite dose of 125 g/L for an influent containing 100 mg/L NO$_3^-$-N. The small particle size and large dose is necessary as the biomass is unable to access sulfur beyond the mineral surface (Bosch et al., 2012). Due to the fact that attached biofilms must occur on the mineral surface, Fe$^{3+}$ precipitation may encrust microorganisms, preventing access to nitrate and other nutrients (Klueglein et al., 2014; Nordhoff et al., 2017).
2.4.2 Utilization of Sulfur-Bearing Minerals in Groundwater Treatment

In-situ treatment of nitrified groundwater by pyrite has previously been associated to natural remediation processes. Pauwels et al. (2000) observed complete denitrification within an aquifer with lower observed sulfate than expected which they attributed to observed amorphous iron-sulfate precipitates. They hypothesized that these were likely crystallized jarosite and natroalunite that precipitated to control elevated sulfate concentrations. However, jarosite and natroalunite typically precipitate under acidic conditions that were not observed within the aquifer. Pauwels et al. (2000) confirmed the thermodynamic possibility of jarosite precipitation in relatively neutral waters using EQ3 code (Wolery et al., 1990; Baron and Palmer, 1996). Furthermore, they searched for evidence of heterotrophic denitrification, but determined that there was insufficient dissolved organic carbon for the observed nitrate reduction. Ultimately, Pauwels et al. (2000) attributed the lack of sulfate production to jarosite precipitation driven by an absence of carbon minerals.

2.4.3 Utilization of Chemolithotrophic Denitrification in Drinking Water Treatment

Though sulfur-bearing minerals have not been utilized as electron donors for chemolithotrophic denitrification for strict drinking water treatment, ZVS has been commonly used both as the sole electron donor and as a mixotrophic constituent. Sahinkaya et al. (2011) found that mixotrophic denitrification had the following advantages over ZVS-limestone autotrophic denitrification: 1) increased nitrate removal efficiency, 2) decreased effluent sulfate concentration, 3) decreased alkalinity demand, and 4) decreased effluent hardness. Sahinkaya et al. (2015) used synthetic contaminated tap water with 50-75 mg/L NO₃⁻-N and 575 mg/L CaCO₃ as NaHCO₃ as a buffer was utilized in a membrane biofilm reactor (MBfR) along with ZVS during the autotrophic phases of their experiment. They later added 75-150 mg/L methanol (28 & 56 mg/L DOC respectively) to simulate mixotrophic conditions. They found that the mixotrophic denitrification rates were higher than those observed during the autotrophic phase. Furthermore, they found that sulfate concentrations in the effluent could be controlled via the addition of DOC and even reduced the concentration of intermediate sulfur oxyanions in the reactor effluent. They observed
complete denitrification of 75 mg/L NO$_3^-$-N with an effluent sulfate concentration of 225 mg/L when the feed methanol/NO$_3^-$ ratio was 1.67 g/g.

Strictly speaking, no study has attempted to create a system capable of denitrifying contaminated drinking water using sulfur-bearing minerals as the sole electron donor. That being said, denitrification of groundwater and wastewater could easily apply to drinking water treatment given the appropriate scope. Huang et al. (2011) operated a heterotrophic up-flow packed bed reactor to remove >85% of NO$_3^-$-N and then supplemented the remainder with ferrous sulfide to limit excess organic carbon and sulfate in the effluent. As evidenced from the above discussion of electron donors and target waters, the majority of studies either focus on sulfur-bearing minerals to treat wastewaters and ground waters while drinking water treatment has often been limited to hydrogenotrophic denitrification or ZVS.

2.5 Application of Sulfur-Bearing Minerals in Bioreactors

2.5.1 Packed-Bed Reactors (PBR)

Typically, packed-bed reactors (PBR) function on the principles that microbes will form biofilms around a carrier material and that the flow of the system, either up-flow or down-flow, resembles a plug flow system. A PBR will require a biofilm carrier material and regular maintenance. Karanasios et al. (2010) described the ideal carrier material as having a high specific surface area while maintaining a sufficient size and porosity to prevent pore clogging. Excess porosity/small grain sizes can cause nitrogen gas bubbles to become trapped by the biofilm carrier material, increasing pressure and causing fluctuations in bed porosity (Di Capua et al., 2015). ZVS is well studied as an electron donor for chemolithotrophic denitrification, as it is a superb electron donor and facilitates biofilm growth (Di Capua et al., 2015). However, others have seen that the surface of ZVS may be too toxic for biomass growth (Boles et al., 2012). Too much biofilm growth can result in pore clogging and channeling which can reduce denitrification efficiency. Furthermore, the majority of studies mentioned in section 2.4 utilize synthetic water to prevent clogging. Flere et al. (1999) found that real groundwater can foul the column.
faster than synthetic groundwater. However, utilizing actual groundwater can yield insights into the practicality of treating actual contaminated waters for drinking water use.

The efficiency of sulfur PBRs depends primarily on the hydraulic retention time (HRT) of the reactor. Generally, HRTs between 8-20 h can result in almost complete denitrification, though increasing the HRT may be necessary for elevated nitrate loading rates by allowing more time for reactions to proceed (Zhao et al., 2011; Di Capua et al., 2015; Tong et al., 2018). Should the HRT be too low, then incomplete denitrification may occur, or at worst, washout of biomass.

Overall, PBRs are simple to handle and are generally effective in terms of capital and operational costs. The choice between utilizing either natural or polymeric biofilm carriers depends on the available finances of the SCWS. Additionally, PBRs boast high nitrate removal efficiencies when coupled with low nitrate loading rates and can be further improved with organic supplementation. This reactor configuration would be ideal for groundwater due to its low total suspended solids (TSS) and natural nitrate loading rates (Di Capua et al., 2015).

2.5.2 Fluidized-Bed Reactor (FBR)

Fluidized-bed reactors (FBR) have several advantages over PBRs: 1) they have a more efficient contact between the biomass and substrate, 2) they are capable of supporting high biomass concentrations, 3) they can have a high treatment capacity which leads to 4) allowing for a smaller reactor volume to be used for a given nitrate concentration and thus a 5) shorter required HRT. However, this method requires a higher power input and more frequent maintenance than the PBRs and is unable to use solid electron donors due to high fluidization rates. Therefore, FBRs would not be recommended for SCWS using a solid electron donor such as sulfur-bearing minerals (Di Capua et al., 2015).

2.5.3 Membrane Biofilm Reactor (MBfR)

A membrane biofilm reactor (MBfR) akin to that used by Sahinkaya et al (2015) to treat nitrified drinking water comes with its own set of advantages and disadvantages. For one, a MBfR has excellent
nitrate removal capabilities and a compact volume. However, it frequently fouls, which can require maintenance and/or spare membranes, increasing the operational costs. Furthermore, this type of reactor is not recommended for waters with high alkalinity or high concentrations of heavy metals, which limits the target water (Di Capua et al., 2015). This method would therefore be unsuitable for treatment of natural Floridan Aquifer groundwater due to high alkalinity.
CHAPTER 3: MATERIALS AND METHODS

3.1 Mineral Acquisition and Processing

Six sulfur-based electron donors were utilized throughout the course of this study: zero-valent sulfur, pyrite, sphalerite, pyrrhotite, FeS, and molybdenite (Appendix 1). The pyrite was sourced from Dinosaurs Rock Inc. (Stafford, Texas) for all microcosm trials and column experiments. Their pyrite was sourced from Spain (personal communication with Dinosaurs Rock Inc.). Sphalerite was obtained as 0.5 kg massives from a private seller located in Nazareth, PA. Pyrrhotite was obtained as 1 kg massives from an eBay seller located in Ukraine. Zero-valent sulfur was obtained as a 90% pure elemental sulfur fertilizer in the form of small pastilles from Martin Midstream Partners (Seneca, IL). FeS was obtained as 1 kg synthetic fused sticks from VWR (Radnor, PA). Molybdenite was obtained from Kidz-Rocks (Newbury Park, California). Pyrite and sphalerite were analyzed via XRD to confirm their purity. The zero-valent sulfur and synthetic FeS purities were provided from their respective sellers.

Pyrite, sphalerite, pyrrhotite, FeS, and molybdenite were all treated using an HCl-pre-treatment method to remove oxidation products on the surface as well as to provide a better surface for biofilm growth (Li et al., 2016; Tong et al., 2017). This procedure has previously been shown to improve chemolithotrophic denitrification rates (Torrento et al., 2010; Pu et al., 2014; Tong et al., 2017). First, the minerals were manually crushed and sieved in U.S. standard sieves to achieve a particle size of 1-2 mm. This diameter had previously been found to be ideal for column studies using pyrite (Tong et al., 2017), and was therefore applied to all minerals in this study. The sieves were shaken manually for approximately 10 minutes to ensure that the particles had separated.

Afterwards, 200 g of each mineral was soaked in 1 L of 10% (v/v) HCl (Fisher Scientific, 99% purity, Pittsburgh, PA) for two hours in a fume hood. During acid washing, the pyrrhotite and FeS rinses
began to produce gas, believed to be hydrogen sulfide based on odor; this phenomenon has also been observed (Li et al., 2016). Therefore, I recommend that pre-treatment of these minerals should be avoided in the future. The acid/mineral wastewater was deposited into a hazardous waste container in a fume hood. The solid mineral portion was then rinsed a minimum of 10 times with deionized water until a stable rinse water pH between 6.5-7.5 was achieved. The pH of the rinse water was measured in triplicate using a calibrated Oakton probe on an Orion 5 Star benchtop system. The rinsed minerals were then placed in Pyrex beakers and dried at 103 °C for four hours to remove moisture and then stored in bags purged with nitrogen gas to avoid oxidation.

### 3.2 Microcosm Construction and Operation

Three microcosm trials were performed to satisfy the first objective of this research. Each trial was performed using different concentrations of biomass and slightly different microcosm configurations (Table 3.1). I performed these experiments with my fellow student, Erica Dasi, under the guidance of Dr Laura Rodriguez-Gonzalez. The first microcosm trial followed a similar set-up to that of He et al. (2018) and consisted of 500 mL glass bottles containing solid electron donor, biomass, and groundwater spiked with nitrate. Bottles for the first microcosm trial were sealed with crimped lids with silicone septa. The second microcosm trial utilized plastic lids with sample tube holes drilled into the top. All gaps were sealed with a silicone sealant. The third microcosm trial utilized small 40 mL glass vials with plastic caps and silicon septa.

Assembly of the first microcosm trial was performed as follows: First, 500 mL autoclaved glass bottles were filled with 30 g of prepared electron donor and 250 mL combined amended groundwater (described subsequently) and biomass consortium, described below, to achieve target nutrient and biomass concentrations of 50 mg NO₃⁻-N/L, 229 mg bicarbonate/L, 5 mg PO₄³⁻-P, and 500 mg VSS/L (Tong et al., 2017). Determination of the concentration volatile suspended solids (VSS) within the acclimation microcosm was performed using standard methods 2540 E. Second, the bottles were sealed with silicone septa which were pierced with plastic micropipettes, one in the headspace to prevent over-
pressurization, and one in the liquid to allow for sampling. A 5” piece of Masterflex tubing was added to the external end of the micropipette and sealed with a clamp. Return activated sludge (RAS) was obtained from Falkenburg Advanced Wastewater Treatment Plant (Hillsborough County, FL) was used as a biomass consortium to inoculate the microcosms with an active biological community (Tong et al., 2017). Third, each microcosm was flushed with \( \text{N}_2 \) gas for 5 minutes (15 psi) to create an anaerobic environment, sealed further with foam, and set in a 22 °C constant temperature room (Liu et al., 2018).

In total, fourteen microcosms were prepared. Pyrite, sphalerite, and pyrrhotite microcosms were each prepared in triplicate. Additionally, five control microcosms were constructed to provide a positive control using zero-valent sulfur, a mineral-free control, and biomass-free controls for each mineral.

The initial 21-days for all microcosms was operated as an acclimation phase in order to remove available organic carbon in the sludge and allow for chemolithotrophs to thrive. Once per week, the microcosms were manually mixed for 30 seconds to allow for mixing and gas release from the solids. In order to prevent pressure build up, 5 mL of gas was removed after mixing. At the end of the 21-day acclimation period, all microcosms were sampled for nitrate and re-spiked with amended groundwater to the target concentration.

### 3.3 Microcosm Analysis

After the initial 21-day acclimation period, the microcosms were analyzed three times per week for pH (Oakton probe on an Orion 5 Star benchtop system) and alkalinity (Standard Methods 2320) as well as major anions & cations. During the duration of the microcosm trial, major anions and cations were measured by filtering sample water through a 0.45 μm nitrocellulose membrane and analyzing the filtrate via ion chromatography (USEPA, 1997) on a Metrohm 881 Compact IC Pro (Metrohm AG, Switzerland). The exact procedure for this analysis can be found in section 3.6. When the NO\(_3\)N concentrations dropped below 5 mg/L, the microcosms were re-spiked to the appropriate volume and nitrate concentration.
Microcosm Trial #2 followed a similar procedure to that of Trial #1 in terms of construction, though the VSS target concentration was lowered to 150 mg/L to reduce endogenous decay. Furthermore, Trial #2 included all available electron donor candidates, except for S\(^0\): pyrite, sphalerite, pyrrhotite, molybdenite, and FeS. Operation of Trial #2 included a 21-day acclimation period and a re-spike procedure similar to Trial #1.

Microcosm Trial #3 was performed in 40 mL glass vials with tight-fitting septa capable of accommodating 35 mL of spiked groundwater and RAS as well as 4.2 g of electron donor. These microcosms were removed and sampled to limit the risk of oxygen intrusion due to sampling. Furthermore, Trial #3 did not include molybdenite, pyrrhotite, uninoculated controls, or zero-valent sulfur controls as sufficient information was obtained through the previous trials.

Table 3.1: Components and general layout for each microcosm trial.

<table>
<thead>
<tr>
<th></th>
<th>Trial #1</th>
<th>Trial #2</th>
<th>Trial #3</th>
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<tbody>
<tr>
<td>Liquid Volume (mL)</td>
<td>250</td>
<td>250</td>
<td>35</td>
</tr>
<tr>
<td>Water Source</td>
<td>El Rancho Mexicano</td>
<td>USF Botanical Gardens</td>
<td>USF Botanical Gardens</td>
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<tr>
<td>VSS (mg/L)</td>
<td>500</td>
<td>100</td>
<td>250</td>
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<tr>
<td>Innoculum Base Source</td>
<td>Falkenburg Advanced</td>
<td>Northwest Regional</td>
<td>Northwest Regional</td>
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<td></td>
<td>Water Treatment Plant</td>
<td>Water Reclamation</td>
<td>Water Reclamation</td>
</tr>
<tr>
<td>Electron Donor Mass (g)</td>
<td>30</td>
<td>30</td>
<td>4.2</td>
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<tr>
<td>Mineral-Free Control</td>
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<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Uninnoculated Control</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>S(^0) Control</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Pyrite</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Sphalerite</td>
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<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Pyrrhotite</td>
<td>Yes</td>
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Table 3.1 Continued

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<tr>
<th></th>
<th>Molybdenite</th>
<th></th>
<th>Yes</th>
<th></th>
<th>FeS</th>
<th></th>
<th>Yes</th>
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<th>Yes</th>
</tr>
</thead>
</table>

3.4 Spiked Groundwater Preparation

Groundwater for the first microcosm trial was obtained from a groundwater well behind El Rancho Mexicano, a bodega, in Polk County, FL. This location historically had NO$_3^-$-N levels at or above the National Primary Drinking Water Regulation (NPDWR) and was thus chosen as an ideal source of groundwater from which a spiked nutrient solution could be created. However, the distance from USF to El Rancho Mexicano was inconvenient when performing extended studies, so groundwater from the USF Botanical Gardens was used for microcosm trials #2 and #3 and for the column study.

For microcosm trial #1, the spiked groundwater had target concentrations of 50 mg/L NO$_3^-$-N, 5 mg/L PO$_4^{3-}$-P, and over 229 mg/L bicarbonate (Zhang et al., 2015). These concentrations were obtained by adding KNO$_3$ (≥99.0%), KH$_2$PO$_4$ (≥99%), K$_2$HPO$_4$ (98-100.5%), and NaHCO$_3$ (99.7-100.3%) obtained from Fisher Scientific (Pittsburgh, PA). The actual masses added to the groundwater varied based on the background concentrations of target nutrients in the groundwater. The second microcosm trial had similar target concentrations for the aforementioned species, though a target of 5 mg/L NH$_4^+$-N was included to account for the microbes preferred form of nitrogen for biosynthesis. The NH$_4$Cl (≥99.0%) used to obtain this target concentration was also a product of Fisher Scientific (Pittsburgh, PA).

The third microcosm trial increased the target concentrations of NO$_3^-$-N to 100 mg/L, PO$_4^{3-}$-P to 10 mg/L, NH$_4^+$-N to 10 mg/L to mimic highly-nitrified groundwater. The minimum bicarbonate concentration of 229 mg/L was adhered to throughout all microcosm trials as well as the column study; excess bicarbonate was used to ensure sufficient inorganic carbon for biomass growth. The feed solution for the column study used the same target concentrations as the third microcosm trial.
3.5 Column Study Construction and Operation

3.5.1 Column System Construction

The column study involved two side-by-side up-flow packed-bed reactors (UPBR). The column housing utilized 500 mL/min KoFlo calibration columns that were capable of housing ~ 750 mL total water (Cary, IL). The columns had a height of approximately 40.64 cm with three sampling ports drilled equidistant from one another along the midsection of the column (Fig. 1). A rubber stopper with a plastic sample nozzle was fit into the top of the column to provide both a seal and a location to attach an effluent line.

Each column was packed with homogenized mixtures of mineral/pumice in ten layers and inoculated with a biological consortium taken from the RAS line at the Northwest Regional Water Reclamation Facility (Hillsborough Country, FL). Prior to column inoculation, the RAS was analyzed for VSS to determine the necessary volume to add into the columns to achieve a target VSS concentration of 250 mg/L. Between each layer of homogenized media, 13.7 mL of RAS was added to obtain a total volume of 137 mL and thus the appropriate concentration. In the pyrite column, 634.32 g of mineral was added alongside 187.29 g of pumice to act as the electron donor and biofilm carrier, respectively. The sphalerite column contained 634.27 g of mineral, but only 172.37 g of pumice due to the lower density of sphalerite (3.9-4.1 g/cm³) compared to pyrite (4.8-5 g/cm³). This approximate mass of mineral was added to provide sufficient electron donor over an extended period of time. Additionally, an initial layer of 20 g of expanded clay was added to the bottom of the column and covered with a fine-mesh geotechnical membrane to maintain consistent packing and to prevent the influent line from clogging (Sierra-Alvarez, 2007).
Figure 3.1: Column study schematic. A central feed stock was pumped through independent high-pressure piston pumps into columns packed with mineral, pumice, and a base layer of expanded clay. The effluent from these columns was then retained into separate waste containers.

3.5.2 Column Operation

During phase 1 (P1) of the column study, the flow was recycled from the effluent of each column to the influent to promote biofilm attachment to the pumice (Table 3.2). In phase 2 (P2) of the column study, the columns were operated under up-flow packed-bed reactor (UPBR) conditions with an approximate hydraulic residence time (HRT) of 12 hours. Phase 3 (P3) included a line maintenance procedure, manual removal followed by 10% bleach rinse, that defouled the influent and effluent lines once per week. Phases 1-3 encompassed an acclimation and testing period, where the operational conditions were improved and maintenance became regular. Additionally, phases 1-3 all used a dual flow peristaltic pump that utilized size 16 Masterflex® peristaltic tubing (Vernon Hills, IL).
Table 3.2: General layout for the six different phases in the column experiment.

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
</tr>
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<tr>
<td>HRT (hr)*</td>
<td>Recycled Flow</td>
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<td>12</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
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<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Groundwater</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Line Maintenance</td>
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<td>Yes</td>
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<tr>
<td>Yeast Extract</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Hydraulic residence times are approximate

During P3, it was determined that autoclaving the groundwater would control for any microbes found naturally in the USF Botanical Gardens groundwater. This was performed to reduce the likelihood of biological contamination of the feed bottles and denitrification prior to the column. Therefore, phases 4 (P4) & 5 (P5) included autoclaving the groundwater pre-filter to the list of procedures. P4 and P5 both operated with an approximate HRT of 24 hours by utilizing two independent Eldex Optos 2SM piston pumps (Napa, CA, USA). Throughout all phases, samples were filtered through 0.45 μm nitrocellulose filters and analyzed via IC for major anions and cations.

P5 included the addition of 4.1 mg/L of yeast extract (Alfa Aesar, Tewksbury, MA) in the column feed stock in order to promote partial biosynthesis by providing a limited amount of organic carbon. This quantity was used as an overestimate, assuming that the yeast extract consisted of 50% organic carbon. Further through the study, the organic carbon in the yeast extract determined by dissolving a 10 mg of yeast extract in 250 mL of water (40 mg/L) and analyzing the solution on a TOC analyzer (Shimadzu TOC-V CSH). It was determined that the yeast extract was approximately 40% organic carbon. Should future studies require sufficient organic carbon to account for complete cell biosynthesis, then the required dose would be approximately 24.6 mg/L of yeast extract to account for the organic carbon used to form new biomass when denitrifying 100 mg/L NO3-N when performing chemolithotrophic denitrification utilizing pyrite.

During P4 and P5, two chemical snapshots were taken to measure several parameters in the influent, the first column ports, and the column product waters. Alkalinity and pH were measured to
ensure that the conditions within the reactors were conducive to the growth of chemoautotrophs (Oakton probe on an Orion 5 Star benchtop system; Standard Methods 2320). Turbidity was measured on a handheld turbidimeter (HACH) to determine the reactors capability of removing turbidity (Standard Methods 2130 B). Chemical Oxygen Demand (COD) was measured using Hach ULR kits in P5 to act as an analogue for oxidizable organic carbon (Standard Method 5220 D). Ferrous and ferric iron were measured using HACH powder pillows to determine if either mineral was releasing iron species into solution or if iron precipitates were forming (Standard Methods 8146; Standard Methods 8008). Measurements of COD, ferrous and ferric iron, and turbidity were all performed using the kit instructions. Total nitrogen (TN) and total organic carbon (TOC) were measured using a TOC/TN analyzer (Shimadzu TOC-V CSH and TNM-1). Finally, sulfide was measured in P4 to observe if any sulfide was available for chemolithotrophs to perform denitrification (Standard Methods 4500-S2). The precise method of measuring sulfide is presented in section 3.6. Additionally, when the columns were deconstructed, solids and aqueous portions were tested for biomass content. The solids were analyzed via extraction according to Lynn et al. (2013) while the aqueous portions were analyzed via VSS (Standard Methods 2540). This is presented in detail in section 3.6.

The column influent, both column product waters, and the raw groundwater were measured for elements necessary to fulfill the NPDWR and NSDWR on an ICP-MS (Perkin Elmer Elan II DLC) at the USF Center for Geochemical Analysis.

3.6 Analytical Methods

During the microcosm operation, both pH and alkalinity were occasionally measured. The methodology regarding pH measurements was presented above. Alkalinity measurements were taken according to Standard Methods 2320 (APHA, 2012).

Throughout all microcosm trials and column phases, concentrations of nitrate, nitrite, ammonium, and sulfate were quantified by ion chromatography with a Metrohm 881 Compact IC Pro (Metrohm AG, Switzerland). Prior to analysis, water samples thought to contain ions of interest were filtered through a
0.45 μm nitrocellulose filter to remove large particles and most bacteria. Anions and cations were analyzed separately. For analysis of NO$_3^-$, NO$_2^-$, Cl$^-$, SO$_4^{2-}$, PO$_4^{3-}$, Ca$^{2+}$, Mg$^{2+}$, NH$_4^+$, K$^+$, and Na$^+$, the ion chromatograph was calibrated using mixed standards, one for the anions and one for the cations, at the following concentrations: blank (DI water), 1 mg/L, 2.5 mg/L, 5 mg/L, 15 mg/L, 50 mg/L and 100 mg/L. These standards are diluted, serially, from a 500 mg/L stock solution. Microcosm samples were analyzed in triplicate, but the column samples were analyzed in duplicate. Triplicate would be preferable, but due to the large number of samples that had to be analyzed in a short period of time, duplicate samples were utilized. Measurements of the intermediate sulfur oxyanions was performed by adding 0.5 mL of hydrogen peroxide (3% v/v) for each 2 mL of filtered sample (Pu et al., 2014). These samples were analyzed via ion chromatography for sulfate prior to oxidation with hydrogen peroxide and after oxidation. The difference between the two analysis would be the concentration of intermediate sulfur oxyanions

During analysis of the “snapshot” samples, the first analysis conducted was sulfide, because of sulfide’s tendency to volatilize. In the future, I recommend creating a series of sulfide standards left open to the atmosphere to quantify volatilization rates. Following sulfide measurements, I analyzed Fe$^{2+}$ and total Iron concentrations were measured. Then the samples were prepared for TOC and COD analysis while the pH meter was equilibrating.

Collected samples were diluted prior to analysis. Each analysis listed in Table 3.3 might require a different dilution factor. To determine the appropriate dilution factor for each analysis, I tested several different possible dilution factors and observed which dilution factor yielded a measured concentration within the desired range of the analytical method.

The method used by Lynn et al. (2013) to measure biomass necessitated extracting the protein from 0.03 g of mineral in a 1.5 mL bead-beater tube by combining a buffer consisting of 10 mM Tris-Cl, 0.5 mM ethylenediaminetetraacetic acid and 60 μL of C1 solution from the Power Soil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, California). This was then vortexed for approximately 30 seconds before beating for 40 seconds. The samples were then refrigerated at 4 °C for 30 min and the supernatant...
was collected. Approximately 1.0 mL of supernatant was collected and standardized according to the Micro BCA Protein Assay Kit instructions obtained from Thermo Scientific (Rockford, IL). Once the test tubes had cooled to room temperature, protein was measured via a pre-calibrated Hach spectrophotometer alongside Albumin Standards to act as an analog for biomass concentrations.

Table 3.3: Analytical Methods

<table>
<thead>
<tr>
<th>Target Analysis</th>
<th>Method Number or Source</th>
<th>Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anions/Cations</td>
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<td>Metrohm 881-C</td>
</tr>
<tr>
<td>pH</td>
<td>Standard Methods 4500-H&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Orion 5-Star Probe</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>Standard Methods 2320&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Titration w/ H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>Sulfide</td>
<td>Standard Methods 4500-S&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (methylene blue)</td>
<td>HACH DR 2700 Spectrophotometer</td>
</tr>
<tr>
<td>TOC</td>
<td>Standard Methods 5310&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Shimadzu TOC-V CSH</td>
</tr>
<tr>
<td>COD</td>
<td>Standard Methods 5220&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HACH DR 2700 Spectrophotometer</td>
</tr>
<tr>
<td>TN</td>
<td>Standard Methods 4500-N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Shimadzu TNM-1</td>
</tr>
<tr>
<td>Turbidity</td>
<td>Standard Methods 2130 B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HACH Turbidimeter</td>
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<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Standard Methods 3500-Fe&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HACH DR 2700 Spectrophotometer</td>
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<tr>
<td>Total Iron</td>
<td>Standard Methods 3500-Fe&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HACH DR 2700 Spectrophotometer</td>
</tr>
<tr>
<td>Metals</td>
<td>Standard Methods 3125&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ICP-MS Perkin Elmer Elan II DLC</td>
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<td>Suspended Biomass</td>
<td>Standard Methods 2540&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mass Balance/Oven</td>
</tr>
<tr>
<td>Fixed Biomass</td>
<td>Lynn et al. (2013)</td>
<td>Micro BCA Protein Assay Kit</td>
</tr>
</tbody>
</table>

<sup>a</sup> All Standard methods refer to the APHA (2012) reference.
CHAPTER 4: RESULTS AND DISCUSSION

4.1 Microcosm Experiments

4.1.1 Microcosm Trial #1

The first microcosm study was carried out in four phases over 82 days. This microcosm study was inoculated with RAS from Falkenburg Wastewater Treatment Facility to achieve a VSS concentration of 500 mg/L as is consistent with Tong et al. 2017. The endogenous control microcosm was set-up as a singular unit and consisted of spiked groundwater and RAS inoculum without any added electron donors. Additionally, the sulfur control consisted of a single microcosm and included spiked groundwater, RAS inoculum, and a well-studied electron donor control in the form of sulfur pastilles. Though the endogenous decay and sulfur controls were performed as single samples, each mineral was examined in triplicate with an abiotic accompaniment. From day 0 to day 21 the biological community in the RAS was allowed time to acclimate to the environment within the microcosms. Unfortunately, after the measurements following days 22 and 24, the ion chromatograph experienced technical difficulties and ammonium analysis coupled with nitrite analysis was used to obtain concentrations of TIN species. Consequently, sulfate data was not obtained for this first microcosm period.

All microcosms were able to fully remove nitrate during the 21-day acclimation period, likely due to a mixture of chemolithotrophic denitrification and heterotrophic denitrification fueled by dissolved organic carbon (DOC) released during cell lysis. After re-spiking with the feed solution, a concentration around 30 mg/L NO₃⁻-N was measured in all microcosms, which is less than the target concentration of 50 mg/L. Nonetheless, the pyrrhotite and the sulfur control exhibited relatively rapid removal of nitrate-nitrogen compared to the mineral-free control, pyrite, and sphalerite microcosms (Fig. 4.1). Though the pyrite and sphalerite microcosms tracked closely with the mineral-free control in terms of nitrate removal,
the mineral-free control produced considerably more ammonium and nitrite (Fig. 4.2 & 4.3). One possible explanation of this phenomena is that the microbes used what little organic carbon remained from cell lyses to perform partial denitrification, prior to lysing and releasing further ammonium. Ultimately, this indicates that the minerallogically active microcosms performed complete denitrification and were able to remove more TIN than the mineral-free control. The pyrrhotite and sulfur control microcosms obtained NO$_3^-$-N levels below the MCL within six days and required a re-spark with the feed stock (50 mg/L NO$_3^-$-N, 10 mg/L PO$_4^{3-}$-P, and >300 mg/L alkalinity). Prior research has shown that zero valent sulfur, used in the sulfur-control microcosms, promote relatively rapid denitrification compared to pyrite as an electron donor (Tong et al., 2017).

Throughout the microcosm study, the mineral-free microcosms steadily released NH$_4^+$-N to a maximum of ~6.5 mg/L at day 37 before declining (Fig.4.2). This is likely due to ammonium release from cell lysis. Meanwhile, the pyrite and sphalerite microcosms produced low and inconsistent NH$_4^+$-N concentrations likely due to the combined effects of cell lysis from high concentrations of VSS and assimilation into growing cells. The pyrrhotite and sulfur-control microcosms produced more ammonium throughout all periods of the trial than the other microcosms.

The nitrite-nitrogen production within the microcosms follows a quick reduction from NO$_3^-$ to NO$_2^-$ via denitrification and the nitrate reductase enzyme followed by a brief period of nitrite accumulation visible, most notably after the 21-day acclimation period (Fig. 4.3). Nitrite remains relatively low in all mineralogically active microcosms except for a gradual rise in pyrrhotite after the second re-spark at day 34. Throughout the first microcosm trial, the mineral-free control microcosm produced large amounts of nitrite, reaching a maximum value of 11.8 mg/L NO$_2^-$-N, which is quite above the NPDWR MCL of 1 mg/L. Combined with the ammonium results, this could indicate simultaneous denitrification/nitrification.
Figure 4.1: Nitrate concentrations for the Microcosm Trial #1 biotic reactors. The black bars represent nutrient spike points where the liquid portion in the microcosms were brought back up to 250 mL with a target concentration of 50 mg/L NO$_{3}^{-}$-N. The mineralogically active microcosms are shown above (top) while the control microcosms are shown below (bottom).
Figure 4.2: Nitrite concentrations for the Microcosm Trial #1 biotic reactors. The black bars represent nutrient spike points where the liquid portion in the microcosms were brought back up to 250 mL with a target concentration of 50 mg/L NO$_3$-N. The bottom graph shows the active mineral microcosms, pyrite, sphalerite and pyrrhotite, while the top graph shows the control microcosms.
Figure 4.3: Ammonium concentrations for the Microcosm Trial #1 biotic reactors. The black bars represent nutrient spike points where the liquid portion in the microcosms were brought back up to 250 mL with a target concentration of 50 mg/L NO$_3$-N. The top graph shows the active mineral microcosms, pyrite, sphalerite and pyrrhotite, while the lower graphs shows the control microcosms.

4.1.2 Microcosm Trial #2

The second microcosm trial was carried out over 72 days and a wider range of potential electron donors was examined. Instead of being inoculated with RAS like the first microcosm trial, RAS was added to a 1L microcosm containing sulfur pastilles for four weeks. TSS and VSS analysis was performed
on the water from the sulfur microcosm, and an appropriate volume was added to each of the microcosms to obtain a VSS concentration of 150 mg/L. This concentration was chosen over the 500 mg/L from the first microcosm in an attempt to reduce the amount of organic carbon which promoted denitrification via endogenous decay. Of these 72 days, the first 21 days of the study were allocated to acclimating the microbial communities to utilizing sulfur-bearing minerals rather than S\(^0\) as electron donors for chemolithotrophic denitrification. These microcosms were not re-spiked at any point due to limited TIN removal.

The only microcosms to remove all NO\(_3^-\) during the 21-day acclimation period were those that contained FeS (Fig. 4.4). The other microcosms, including the endogenous control, removed relatively little NO\(_3^-\) during the acclimation period. Once the microcosms were re-spiked with amended groundwater, it became clear that the nitrate removal capabilities of FeS microcosms greatly exceeded all others. The other microcosms displayed no significant difference from one another.

As the FeS microcosms removed nitrate, the produced ammonium and nitrite indicated that DNRA was the dominant process (Fig. 4.5). The other microcosms produced minimal amounts of NH\(_4^+\)-N and NO\(_3^-\)-N while sulfate generally increased in all microcosms. This suggests that sulfate was created via aerobic oxidation of the sulfur-bearing minerals due to the inability to maintain an anaerobic environment. That is to say, this study was likely compromised due to oxygen intrusion into the microcosms. Previous research has suggested that nitrite reductase and nitrous oxide reductase are sensitive to elevated concentrations of dissolved oxygen (Oh et al., 1999; Sabba et al., 2016). These are necessary to completely reduce nitrate to nitrogen gas, and may have resulted in the small amount of nitrite accumulation observed (Fig. 4.6, bottom). Furthermore, oxygen intrusion could replace nitrate as the terminal electron acceptor, thus limiting overall denitrification. Though the microcosms were purged with N\(_2\) gas, the seal on the microcosms could have been compromised, thus leading to oxygen intrusion (He et al., 2018).

It is notable, however, that even with oxygen contamination, the FeS microcosms were still able to undergo DNRA, indicating that there were ample amounts of oxidizable materials, either Fe\(^{2+}\) or S\(^2^-\), to
consume all of the available dissolved oxygen. The post-acclimation re-spike resulted in a NO$_3$-N concentration of 36.9 mg/L before reaching a concentration of 0.52 mg/L at day 61.

Figure 4.4: Nitrate concentrations for the Microcosm Trial #2 biotic reactors. The black bars represent nutrient spike points where the liquid portion in the microcosms were brought back up to 250 mL with a target concentration of 50 mg/L NO$_3$-N. The top graph shows the FeS, molybdenite, and mineral-free control microcosm data, while the lower graphs shows the pyrrhotite, sphalerite, and pyrite microcosm data.
Figure 4.5: Nitrite concentrations for the Microcosm Trial #2 biotic reactors. The black bars represent nutrient spike points where the liquid portion in the microcosms were brought back up to 250 mL with a target concentration of 50 mg/L NO$_3$-N. The top graph shows the FeS, molybdenite, and mineral-free control microcosm data, while the lower graphs shows the pyrrhotite, sphalerite, and pyrite microcosm data.
Figure 4.6: Ammonium concentrations for the Microcosm Trial #2 biotic reactors. The black bars represent nutrient spike points where the liquid portion in the microcosms were brought back up to 250 mL with a target concentration of 50 mg/L NO$_3$-N. The top graph shows the FeS, molybdenite, and mineral-free control microcosm data, while the lower graphs shows the pyrrhotite, sphalerite, and pyrite microcosm data.

FeS produced intermittent amounts of NH$_4^+$-N during and after the acclimation period reaching a maximum concentration of 13.5 mg/L at day 41 following a residual re-spike concentration of 5.23 mg/L. Afterwards, the ammonium concentration steadily decreased to levels below the MDL on day 5.

While the nitrate concentrations decreased throughout the FeS microcosm, ammonium and nitrite accumulated. Though the ammonium began to decline around day 41, the nitrite steadily rose to a
maximum of 40.1 mg/L at day 57 (Fig. 4.6). This represents a reasonable nitrogen balance throughout the FeS microcosms in the study with a TIN concentration of 45.31 mg/L on day 24 and 42.06 mg/L on day 57. It is important to note, however, that the TIN concentrations within the FeS microcosm began to decline after day 61 suggesting that the N/S ratio had achieved a value appropriate for denitrification to occur utilizing NO$_2^-$ as the electron acceptor.

Figure 4.7: Sulfate concentrations for the Microcosm Trial #2 biotic reactors. The black bars represent nutrient spike points where the liquid portion in the microcosms were brought back up to 250 mL with a target concentration of 50 mg/L NO$_3^-$-N. The top graph shows the FeS, molybdenite, and mineral-free control microcosm data, while the lower graphs shows the pyrrhotite, sphalerite, and pyrite microcosm data.
Figure 4.8: Uninoculated microcosm concentration profiles for nitrate-nitrogen for Microcosm Trial #2.
Figure 4.9: Uninoculated microcosm concentration profiles for nitrite-nitrogen for Microcosm Trial #2.
Figure 4.10: Uninoculated microcosm concentration profiles for ammonium-nitrogen for Microcosm Trial #2.
Figure 4.11: Uninoculated microcosm concentration profiles for sulfate for Microcosm Trial #2.

The uninoculated microcosms performed in tandem to the biologically inoculated ones provide interesting results. The quick drop in nitrate coupled with a rise in ammonium and nitrite occurred in both the abiotic and biotic FeS microcosms (Fig. 4.7). This suggests that the nitrate reduction was primarily driven by a chemical reduction rather than a biologically mediated one. However, the abiotic FeS microcosm experienced prolonged high ammonium concentrations along with a later sharper increase in nitrite. Furthermore, the nitrate in the other abiotic microcosms remained steady from days 21 to 61. Additionally, the sulfate generated in the abiotic microcosms is roughly equivalent to that in the biologically active microcosms (Fig. 4.10). This supports the conclusion that oxygen intrusion was the primary cause of sulfur oxidation and likely inhibited denitrification in all microcosms.
4.1.3 Microcosm Trial #3

The third microcosm study was constructed in a way to minimize any potential contact with oxygen. Sampling was carried out by sacrificing the microcosm rather than removing and replacing liquid, to limit contamination from sampling. Furthermore, the caps on the microcosms consisted of screw top vials with a fresh rubber septum in lieu of autoclavable lids sealed with foam sealant. In total, this trial was performed for 28 days with a VSS of 250 mg/L which was greater than that of the second microcosm trial and less than the first microcosm trial. The VSS for this study was obtained from a culture grown on sulfur pastilles, in an attempt to by-pass the acclimation phase and acquire meaningful data quickly.

As with the second microcosm trial, FeS had the highest nitrate removal efficiency, yet had the worst TIN removal efficiency. Essentially, all nitrate converted to other nitrogen species was retained, negating any possible benefits (Fig. 4.11). Pyrite, sphalerite, and the endogenous control all performed similarly in terms of nitrate removal and TIN removal, eliminating an average of ~20% and ~33% respectively. Between day 0 and day 3, ~60 mg/L of sulfate was produced in all microcosms corresponding to a ~20 mg/L removal of nitrate which is higher than the expected stoichiometric ratio. This could indicate that the inoculum still had some organic carbon, which could have inhibited the growth of chemoautotrophs, or, more likely, encouraged facultative autotrophs to become accustomed to organic carbon rather than zero-valent sulfur.
Figure 4.12: Microcosm trial #3 plots for nitrate (top), nitrite (middle-top), ammonium (middle-bottom) and sulfate (bottom).
4.1.4 Overall Microcosm Results and Ideal Candidates

Molybdenite had physical characteristics which made it non-ideal for microcosm studies. While attempting to crush, it became evident that molybdenite would rather cleave into thin sheets rather than particles. Though they would pass through the 2 mm sieve and not the 1 mm sieve, this indicates that two of the dimensions were appropriate while the other was not. Molybdenite has a hexagonal crystal system with perfect cleavage on the \( \{0 \ 0 \ 1\} \) plane and therefore tends to form hexagonal sheets. This in turn limits the available surface area for biofilm growth and mineral utilization and therefore the denitrification process (Torrento 2012; Tong et al. 2017). As a result of these physical characteristics combined with the chemical information obtained from the second microcosm study, it would not be recommended to use molybdenite.

Pyrrhotite, \( \text{Fe}_{1-x}\text{S} \ (0 < x < 0.125) \), showed promising results within the first microcosm study, exhibiting excellent nitrate removal at the expense of nitrite and ammonium accumulation. However, in the second microcosm study, the difference in removal efficiencies from the other microcosms was not statistically significant. This could be due to a few factors, namely the possibility of oxygen intrusion and low biomass. If the former is the case, then the sulfate data supports the conclusion that pyrrhotite is more vulnerable to chemical oxidation than the other minerals. While sulfate is produced, the surface of the mineral becomes oxidized, retaining iron-hydroxides such as \( \text{Fe(OH)}_3 \) on the surface. This limits the specific surface area corresponding to exposed sulfur sites and thus the ability of microbes to obtain sulfur for chemolithotrophic denitrification. However, iron-hydroxides have an affinity to adsorb heavy metals such as arsenic, potentially creating a more suitable effluent in contaminated regions (Li et al., 2016). Nonetheless, once its absorptive capabilities have been exhausted, it will leach these contaminants into the effluent, requiring more frequent maintenance and replacement, and therefore a higher operational cost. Thus, pyrrhotite is not recommended for use in long-term chemoautotrophic denitrifying reactors that run the risk of oxygen exposure.

The synthetic FeS used in microcosm trials #2 and #3 exhibited a strong affinity for dissimilatory nitrate reduction to ammonium and effectively removed a negligible amount of total inorganic nitrogen
(TIN). This is likely due to an excess of Fe$^{2+}$ and/or S$^{2-}$ released due to chemical decomposition of FeS relative to the available nitrate (Kraft et al., 2014; Ettwig et al., 2016). Furthermore, the FeS microcosms in both studies had a dark discoloration in the water, which could prove to be a tenacious secondary drinking water concern. To these points, the synthetic FeS should not be utilized as an electron donor for chemolithotrophic denitrification due to its instability and rapid formation of electron donors which encourages DNRA.

Pyrite, FeS$_2$, and sphalerite, ZnS, had similar performance in all three microcosm trials. However, there are some notable differences, particularly in the first two trials. The first trial suggested that sphalerite reduces slightly more nitrate than pyrite, while achieving similar consumption and production of ammonium and nitrite, respectively. The second trial elucidated that sphalerite produced more sulfate, ~32 mg/L, than pyrite over a 36-day period, suggesting that sphalerite is more prone to oxidative processes. Due to its lower iron content than pyrrhotite, sphalerite will produce less iron-hydroxides per mole of sulfur oxidized to sulfate and will therefore foul the surface much slower.

Ultimately, though, pyrite and sphalerite were chosen as ideal candidates for the column study as they had the most consistent denitrification results, the least production of sulfate, and the least aesthetic impacts.

4.2 Column Study

4.2.1 Column Study Phases One, Two, and Three

Phase one (P1) of the column study was carried out for 15 days, during which, the columns were operated under recirculating flow to promote biofilm growth. Samples were taken every three to four days during P1 to monitor the acclimation. Nitrate decreased below 50 mg NO$_3^-$-N/L within eight days for the sphalerite column and five days for the pyrite column. Nitrate was observed to be BDL in the recirculation fluid within 12 days for the sphalerite column and eight days for the pyrite column. Nitrite concentrations in the sphalerite column during P1 were consistently below 0.5 mg NO$_2^-$-N/L, though concentrations in the pyrite column reached a maximum of 1.5 mg NO$_2^-$-N/L on day eight. This may be
attributable to the faster observed denitrification in the pyrite column during P1. The ammonium concentration during P1 was 25.4 mg NH₄⁺-N/L in the sphalerite column and 44.1 mg NH₄⁺-N/L in the pyrite column after one day of operation. Ammonium levels declined rapidly in the pyrite column, reaching a minimum of 0.3 mg NH₄⁺-N/L on day eight before rising to 1.5 mg NH₄⁺-N/L on day 12. Ammonium in the sphalerite column was 25.4 mg NH₄⁺-N/L on day one and declined steadily to BDL by day 15. Sulfate rose by approximately 100 mg/L in both columns, which is much lower than the expected sulfate concentrations predicted by equation 7 (500 mg/L sulfate produced per 100 mg/L NO₃⁻-N reduced). This could be due to biomass decay from the biological inoculum contributing organic carbon for heterotrophic denitrifiers. The acclimation phase was carried out until day 15 to ensure complete total inorganic nitrogen removal in both columns.

Phase two (P2) of the column study was carried out between days 15 and 36, during which, both columns exhibited nitrate reduction, ammonium consumption, minimal nitrite production, and insignificant sulfate production. During P2, the columns were operated as UPBR with an approximate HRT of 12 hours. Preventative maintenance during P2 was limited to manually cleaning the influent and product water lines to remove fouling, likely thought to be scale. Average NO₃⁻-N concentrations in the influent and product waters of the sphalerite and pyrite columns were 89 ± 7 (reported as average ± standard deviation), 63 ± 12, and 58 ± 13 respectively (Table 4.1). Nitrite concentrations between the influent, pyrite column product water, and sphalerite column product water did not differ significantly (p > 0.05), indicating no accumulation occurred (Table 4.2). Ammonium decreased from 8.9 ± 1.1 mg NH₄⁺/L in the influent to 6.5 ± 2.2 mg NH₄⁺/L in the pyrite column product water and 6.6 ± 1.7 mg NH₄⁺/L in the sphalerite column product water, indicating that some ammonium was oxidized either by biological processes or abiotic oxidation. No significant SO₄²⁻ (p > 0.05) was produced during P2, which could indicate either incomplete oxidation of sulfur derived from the mineral electron donors, thus creating intermediate sulfur oxyanions, or heterotrophic denitrification from consumption of decaying biomass. P2 instilled an operating procedure that including more stringent maintenance by reducing the fouling in both the influent and product water lines. This maintenance reduced the likelihood that
nutrients were being removed in the lines, which would effectively increase the HRT of the columns while obscuring the nitrate removal efficiency.

Phase three (P3) of the column study was carried out between days 36 and 71. The approximate HRT during P3 was the same as P2, though additional regular maintenance was added to the operational procedures as described in Chapter 3. Nitrate concentrations in the influent and column product waters were comparable to those in P2 (Table 4.1). However, nitrite accumulation was observed in both columns. The influent had an average NO$_2^-$-N concentration of 1 ± 1 mg/L, while the pyrite and sphalerite column product waters had concentrations of 6 ± 5 and 5 ± 4 mg/L respectively (Table 4.2). The large standard deviation shows the high variability in the NO$_2^-$-N concentrations of both columns. Ammonium and sulfate concentrations of the influent and both column product waters during P3 closely mirrored the trend observed in P2 (Table 4.3; Table 4.4) Notably, the large standard deviation observed in the sulfate concentrations in both the influent and column product waters could be due to changes in the groundwater concentrations.

Overall, the sulfate concentration in the product water of both columns was much lower than anticipated. Some studies have observed lower than expected sulfate concentrations in natural aquifer systems to an excess of sulfur to the nitrate concentrations. This could lead to the production of intermediate sulfur oxyanions (Miotlinski, 2008; Zhang et al., 2009). Other studies have found that utilizing pyrite in controlled environments often results in lower-than-expected sulfate concentrations, likely due to the same explanation as the previous example (Tong et al., 2017).

Furthermore, the overall nitrate removal efficiency during this period was much lower than studies where a similar hydraulic retention time was used (Li et al., 2016; Tong et al., 2018; Zhang et al., 2019). In the studies where sodium bicarbonate was used, between 96.2% and 99% of NO$_3^-$-N was removed. The discrepancy observed between prior research to these results could be due to insufficient biomass inoculum used, which could limit the maximum nitrate removal efficiency. Prior studies used biomass concentrations of 1250 mg VSS/L when denitrifying synthetic wastewater, though this
concentration may present a risk when treating nitrate-contaminated waters for drinking water purposes (Tong et al., 2018).

4.2.2 Column Study Phase Four

Phase four (P4) of the column experiment began after day 70 and was carried out for a total of 152 days. During this time, both columns were operated at an approximate hydraulic residence time (HRT) of 24 hours. Throughout P4, the average nitrate-nitrogen concentration in the pyrite and sphalerite column product waters were 48 ± 21 and 43 ± 19 mg/L which are reported as the average ± the standard deviation (Table 4.1).

However, Figure 4.12 indicates that the high standard deviations likely stem from a decrease in nitrate removal efficiency after day 121. From days 79-121, the nitrate concentrations in both column product waters indicated an average removal efficiency above 50%, reaching a maximum removal efficiency of 88% on day 121 for the sphalerite column and 91% on day 111 for the pyrite column (Fig. 4.12). Average nitrite-nitrogen concentrations during P4 for the pyrite and sphalerite column product waters were 6 ± 6 and 5 ± 6 mg/L respectively (Table 4.2). The large standard deviation for these averages can be attributed to variability and a general decrease in nitrite production, as well as a likely decrease in denitrification, around day 121 (Fig 4.12).
### Table 4.1: Nitrate-nitrogen concentrations for each phase throughout each column.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Days</th>
<th>Influent&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
<th>Pyrite Product Water&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
<th>Average Pyrite Column Removal Rate (mg/L/d)</th>
<th>Sphalerite Product Water&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
<th>Average Sphalerite Column Removal Rate (mg/L/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1*</td>
<td>0-15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P2</td>
<td>15-35</td>
<td>89 ± 7</td>
<td>58 ± 13</td>
<td>62</td>
<td>63 ± 12</td>
<td>52</td>
</tr>
<tr>
<td>P3</td>
<td>36-70</td>
<td>97 ± 4</td>
<td>64 ± 15</td>
<td>66</td>
<td>66 ± 16</td>
<td>62</td>
</tr>
<tr>
<td>P4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71-120</td>
<td>95 ± 2</td>
<td>37 ± 21</td>
<td>58</td>
<td>36 ± 20</td>
<td>59</td>
</tr>
<tr>
<td>P4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>121-122</td>
<td>95 ± 6</td>
<td>63 ± 16</td>
<td>32</td>
<td>56 ± 15</td>
<td>39</td>
</tr>
<tr>
<td>P5</td>
<td>223-312</td>
<td>90 ± 4</td>
<td>69 ± 8</td>
<td>21</td>
<td>68 ± 12</td>
<td>22</td>
</tr>
</tbody>
</table>

*Concentrations for P1 were omitted in this table due to recycled flow conditions.

<sup>b</sup>Concentrations for all phases are based on the mean and standard deviations for samples collected three times per week.

<sup>c</sup>Phase four (P4) was split into two subsections to better elucidate the anomaly on day 120. The first P4 is from days 71-120 while the second is from days 121-122.
Table 4.2: Nitrite-nitrogen concentrations in the influent and product waters of pyrite and sphalerite for all phases of the column study.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Days</th>
<th>Influent(^a) (mg/L)</th>
<th>Pyrite Product Water(^b) (mg/L)</th>
<th>Average Pyrite Column Rate (mg/L/d)</th>
<th>Sphalerite Product Water(^b) (mg/L)</th>
<th>Average Sphalerite Column Rate (mg/L/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0-15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P2</td>
<td>15-35</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>0</td>
<td>1 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>P3</td>
<td>36-70</td>
<td>1 ± 1</td>
<td>6 ± 5</td>
<td>10</td>
<td>5 ± 4</td>
<td>8</td>
</tr>
<tr>
<td>P4(^c)</td>
<td>71-120</td>
<td>1 ± 0</td>
<td>8 ± 8</td>
<td>7</td>
<td>9 ± 9</td>
<td>8</td>
</tr>
<tr>
<td>P4(^c)</td>
<td>121-222</td>
<td>1 ± 1</td>
<td>5 ± 8</td>
<td>4</td>
<td>3 ± 3</td>
<td>2</td>
</tr>
<tr>
<td>P5</td>
<td>223-312</td>
<td>0 ± 0</td>
<td>1 ± 2</td>
<td>1</td>
<td>2 ± 2</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) Concentrations for P1 were omitted in this table due to recycled flow conditions
\(^b\) Concentrations for all phases are based on the mean and standard deviations for samples collected three times per week.
\(^c\) Phase four (P4) was split into two subsections to better elucidate the anomaly on day 120. The first P4 is from days 71-120 while the second is from days 121-122.
After day 121, the nitrate concentrations in both columns gradually rose to a removal efficiency around 30-40% and plateaued until day 222. This average nitrate removal efficiency is The nitrate removal rates in this phase were approximately 58 mg/L/d in the pyrite column and 59 mg/L/d in the sphalerite column. Furthermore, nitrite concentrations in both column product waters remained much lower than those observed prior to day 121, though there were several instances when the treated water contained nitrite above the NPDWR limit. This decline in nitrate removal efficiency could be due to a number of factors. One possible explanation could be that the hard-water (>300 mg CaCO$_3$/L) used as the base for the feed stock, could cause some precipitation in the packed-bed reactors (Dries et al., 1988). This could effectively reduce the available surface area of the minerals, and thus, the microbial access to electron donor. However, since bicarbonate was utilized as an inorganic carbon source rather than limestone, scale formation should have been limited (Sahinkaya and Dursun, 2012). Another explanation is that the increase in phosphate in the influent during this period was due to an unknown substance in the groundwater.

Between days 70-121, both column product waters exhibited nitrite-nitrogen concentrations that greatly exceed the NPDWR of 1 mg/L as nitrogen (Fig. 4.12). When compared with the nitrate removal during this time frame, the nitrite production visible in the column product waters can be attributed to the rate limiting step of denitrification involving the production and utilization of nitrite reductase.

Average ammonium concentrations in the pyrite and sphalerite column product waters during P4 were 5.2 ± 2.6 and 5.3 ± 2.3 respectively (Table 4.3). Limited ammonium utilization was observed during this period, though delayed uptake can be observed after nitrate removal, notably on days 79 and 87 (Fig. 4.12).

No significant sulfate production was observed in either the pyrite or sphalerite column product waters during P4 (Table 4.4). The average sulfate concentrations in the pyrite and sphalerite column product waters did not differ significantly from that of the influent (p>0.05). Prior research has suggested that even in neutral pH waters, precipitation of jarosite could occur, which could act as a sink for sulfate
(Pauwels et al., 2000). Other research has suggested that lower-than expected sulfate production could occur from the presence of sulfate-reducing bacteria (SRB) that can result in the measurement of intermediate sulfur oxyanions (Tong et al., 2017).

This lack of observed sulfate production indicates that either the sulfur derived from the minerals was not fully oxidized to sulfate during chemolithothrophic denitrification, an alternative autotrophic electron donor was utilized in lieu of reduced sulfur, or that chemolithothrophic denitrification did not occur and that heterotrophic denitrification was the primary removal mechanism for nitrate.

During P4, a concentration profile was developed for each column, by collecting samples from the influent, port 1, and the product water. In addition to the major anions and cations, intermediate sulfur oxyanions, pH, alkalinity, turbidity, sulfide, Fe^{2+}, Fe^{3+}, TON, and TOC were also measured (Table 7). Of note, the alkalinity was recorded as 630 ± 14 mg/L as HCO_3^- within the column influent (CI) and decreased throughout the length of the pyrite column, which is consistent with the alkalinity consumption predicted by the reaction stoichiometry. Both the sphalerite and pyrite columns were able to remove some turbidity, though the pyrite column removed ~ 2.3 NTU compared to the sphalerite column’s 0.8 NTU. This decrease can likely be attributed to the tightly packed configuration between the <0.25 mm pumice grains and the 1-2 mm mineral grains, which acted as a physical filter. Ferrous and ferric iron concentrations in both columns and the influent was negligible during this snapshot, suggesting that the denitrification observed could not be the sole result of iron oxidation. The total organic nitrogen (TON) within the CI was recorded as 10.6 mg/L and rapidly increased throughout the entire sphalerite column, reaching an average concentration of 44.3 mg/L in the product water. The pyrite column had much less TON throughout the entire column than what was observed in the sphalerite column, reaching a product water concentration of 14.7 mg/L.
### Table 4.3: Ammonium-nitrogen concentrations for each phase in the influent and product waters of pyrite and sphalerite.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Days</th>
<th>Influent&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
<th>Pyrite Product Water&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
<th>Average Pyrite Column Rate (mg/L/d)</th>
<th>Sphalerite Product Water&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
<th>Average Sphalerite Column Rate (mg/L/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0-15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P2</td>
<td>15-35</td>
<td>8.9 ± 1.1</td>
<td>6.5 ± 2.2</td>
<td>4.8</td>
<td>6.6 ± 1.7</td>
<td>4.6</td>
</tr>
<tr>
<td>P3</td>
<td>36-70</td>
<td>7.8 ± 1.2</td>
<td>5.3 ± 1.6</td>
<td>5.0</td>
<td>5.2 ± 1.7</td>
<td>5.2</td>
</tr>
<tr>
<td>P4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71-120</td>
<td>7.9 ± 2.2</td>
<td>5.5 ± 2.4</td>
<td>2.4</td>
<td>5.6 ± 2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>P4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>121-222</td>
<td>6.5 ± 3.8</td>
<td>4.2 ± 2.7</td>
<td>2.3</td>
<td>4.3 ± 2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>P5</td>
<td>223-312</td>
<td>8.9 ± 2.5</td>
<td>5.3 ± 1.8</td>
<td>3.6</td>
<td>6.1 ± 2.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

a. Concentrations for P1 were omitted in this table due to recycled flow conditions.

b. Concentrations for all phases are based on the mean and standard deviations for samples collected three times per week.

c. Phase four (P4) was split into two subsections to better elucidate the anomaly on day 120. The first P4 is from days 71-120 while the second is from days 121-122.
Table 4.4: Average sulfate concentration during each phase of the column experiment for the influent and product waters of both the pyrite and sphalerite columns

<table>
<thead>
<tr>
<th>Phase</th>
<th>Days</th>
<th>Influent</th>
<th>Pyrite Product</th>
<th>Average Pyrite Column</th>
<th>Sphalerite Product</th>
<th>Average Sphalerite Column</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mg/L)</td>
<td>Rate (mg/L/d)</td>
<td>Water (mg/L)</td>
<td>Rate (mg/L/d)</td>
</tr>
<tr>
<td>P1</td>
<td>0-15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P2</td>
<td>15-35</td>
<td>115 ± 71</td>
<td>102 ± 36</td>
<td>26</td>
<td>102 ± 34</td>
<td>26</td>
</tr>
<tr>
<td>P3</td>
<td>36-70</td>
<td>119 ± 32</td>
<td>116 ± 48</td>
<td>6</td>
<td>123 ± 60</td>
<td>4</td>
</tr>
<tr>
<td>P4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71-120</td>
<td>111 ± 6</td>
<td>112 ± 8</td>
<td>1</td>
<td>116 ± 12</td>
<td>5</td>
</tr>
<tr>
<td>P4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>121-222</td>
<td>117 ± 9</td>
<td>114 ± 9</td>
<td>3</td>
<td>123 ± 16</td>
<td>6</td>
</tr>
<tr>
<td>P5</td>
<td>223-312</td>
<td>116 ± 7</td>
<td>118 ± 5</td>
<td>2</td>
<td>122 ± 8</td>
<td>6</td>
</tr>
</tbody>
</table>

a. Concentrations for P1 were omitted in this table due to recycled flow conditions.
b. Concentrations for all phases are based on the mean and standard deviations for samples collected three times per week.
c. Phase four (P4) was split into two subsections to better elucidate the anomaly on day 120. The first P4 is from days 71-120 while the second is from days 121-122.
Figure 4.13: Concentration profiles of nitrate, nitrite, ammonium, and sulfate (top to bottom) during Phase 4 (days 70-222) of the column experiments for the influent and product waters of the pyrite and sphalerite columns.

The total organic carbon (TOC) within the influent during the snapshot was approximately 1.8 mg/L. The first port of the pyrite column (PP1) had a TOC concentration of 5.6 mg/L while the product water had a concentration of 2.1 mg/L. Assuming that the TOC can represent biomass, the aqueous phase of PP1 could be considered relatively rich in biomass compared to the remainder of the column. This is
likely due to the UPBR nature of the columns resulting in the nutrients entering through the bottom and thus creating a bias as to where the biomass would be concentrated. Similar conclusions were drawn from previous studies (Li et al., 2016). The TOC within the sphalerite column had the inverse trend; in the first sphalerite port (SP1), the concentration drops from 1.8 mg/L to 1.0 mg/L and then rises to 2.3 mg/L throughout the length of the column. This insinuates that the organic carbon was utilized in some regard at SP1 and slowly released throughout the rest of the column. This could be due to an increase in the combination of live and dead cells and extracellular material over the length of the column.

The major ions that are indicative to chemolithotrophic denitrification that relies on sulfur-bearing minerals were measured during the P4 snapshot (Table 8). Notably, the sphalerite column exhibits higher levels of nitrite and nitrate removal than the pyrite column, which indicate that denitrification is occurring via the predicted pathway. Furthermore, ammonium concentrations decrease steadily throughout the profile of the column, which can be attributed to denitrifying microbes preferentially utilizing ammonium for biomass synthesis.

Though there was evidence of denitrification, little sulfate was produced during this P4 snapshot. However, roughly 20-25 mg/L of intermediate sulfur oxyanions were produced during this snapshot, likely indicating excess levels of electron donors compared to the nitrate concentrations. Tong et al. (2017) also observed limited sulfate production in their pyrite and oyster shell packed-bed reactors. They found no evidence of intermediate sulfur oxyanions in the influent, but roughly 10-15 mg/L of intermediate sulfur oxyanions in their pyrite columns. This would not fully explain the amount of nitrate removal they observed (~40-60 mg/L at an EBCT of 2.92 hrs). They suggested that the low observed production sulfate and intermediate sulfur oxyanions could be due to the presence of sulfate-reducing bacteria (SRB). Should this be the case, it would be expected that nitrate would be completely removed prior to sulfate reduction occurring; however, this is not the case. Similar results were observed in our study, with some production of intermediate sulfur-oxyanions and little to no sulfate production in either column.
Table 4.5: Chemical analysis performed on the column effluents & first ports during Phase 4.

<table>
<thead>
<tr>
<th>Snapshot #1 Chemical data</th>
<th>ALK (mg/L HCO$_3^-$)</th>
<th>Turbidity (NTU)</th>
<th>Sulfide (µg/L)</th>
<th>Fe$^{2+}$ (mg/L)</th>
<th>Fe$^{3+}$ (mg/L)</th>
<th>TN (mg/L)</th>
<th>TON$^a$ (mg/L)</th>
<th>TOC$^b$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent</td>
<td>8.2 ± 0.0</td>
<td>630 ± 14</td>
<td>7 ± 3</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>117 ± 1</td>
<td>10.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Pyrite Port 1</td>
<td>8.2 ± 0.0</td>
<td>540 ± 170</td>
<td>11 ± 1</td>
<td>0.02 ± 0.01</td>
<td>0.00 ± 0.01</td>
<td>105 ± 2</td>
<td>14.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Pyrite Effluent</td>
<td>8.0 ± 0.0</td>
<td>540 ± 85</td>
<td>8 ± 1</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.01</td>
<td>100 ± 1</td>
<td>14.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Sphalerite Port 1</td>
<td>8.3 ± 0.0</td>
<td>590 ± 14</td>
<td>5 ± 2</td>
<td>0.02 ± 0.01</td>
<td>0.00 ± 0.01</td>
<td>105 ± 0</td>
<td>30.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Sphalerite Effluent</td>
<td>8.1 ± 0.0</td>
<td>640 ± 0</td>
<td>7 ± 4</td>
<td>0.01 ± 0.01</td>
<td>0.00 ± 0.00</td>
<td>104 ± 2</td>
<td>44.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

a. TON analysis were performed in true duplicate on the TOC analyzer with pseudo triplicates obtained for each sample. However, every other result reported values below detection limits. Therefore, no standard deviation was reported for the average concentrations.

b. TOC analysis were performed in true duplicate on the TOC analyzer with pseudo triplicates obtained for each sample. However, every other result reported values below detection limits. Therefore, no standard deviation was reported for the average concentrations.

Table 4.6: Anion & cation results obtained from the IC analysis during Phase 4.

<table>
<thead>
<tr>
<th>Snapshot #1 Anion &amp; Cation data</th>
<th>NO$_3^-$-N (mg/L)</th>
<th>NO$_2^-$-N (mg/L)</th>
<th>PO$_4^{3-}$-P (mg/L)</th>
<th>SO$_4^{2-}$ (mg/L)</th>
<th>Sulfate and Sulfur Intermediates$^a$ (mg/L)</th>
<th>NH$_4^+$-N (mg/L)</th>
<th>TIN (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent</td>
<td>0 ± 0</td>
<td>97 ± 1</td>
<td>3 ± 0</td>
<td>116 ± 0</td>
<td>129 ± 5</td>
<td>10 ± 1</td>
<td>107 ± 1</td>
</tr>
<tr>
<td>Pyrite Port 1</td>
<td>0 ± 0</td>
<td>83 ± 1</td>
<td>3 ± 0</td>
<td>115 ± 0</td>
<td>153 ± 2</td>
<td>8 ± 1</td>
<td>90 ± 1</td>
</tr>
<tr>
<td>Pyrite Effluent</td>
<td>0 ± 0</td>
<td>78 ± 1</td>
<td>3 ± 0</td>
<td>114 ± 2</td>
<td>150 ± 1</td>
<td>7 ± 0</td>
<td>85 ± 1</td>
</tr>
<tr>
<td>Sphalerite Port 1</td>
<td>4 ± 1</td>
<td>64 ± 0</td>
<td>2 ± 0</td>
<td>117 ± 1</td>
<td>151 ± 2</td>
<td>6 ± 0</td>
<td>74 ± 1</td>
</tr>
<tr>
<td>Sphalerite Effluent</td>
<td>4 ± 1</td>
<td>55 ± 0</td>
<td>1 ± 0</td>
<td>118 ± 2</td>
<td>156 ± 0</td>
<td>2 ± 0</td>
<td>60 ± 1</td>
</tr>
</tbody>
</table>

a. Sulfur Intermediates refers to any sulfur oxyanion which could be oxidized to sulfur. The sulfate and sulfur intermediates is thus a measure of the sulfate and any extra oxidizable sulfur oxyanions present in the water.
4.2.3 Column Study Phase Five

Phase five (P5) of the column study began on day 223 and ended on day 312, essentially operating for a total of 89 days. P5 operated under the same approximate hydraulic residence times as P4 (24 hrs), though ~4.1 mg/L of yeast extract, as described in Chapter 2, was added to supply enough organic carbon to promote biosynthesis without interfering with the chemolithotrophic denitrification process.

Throughout P5, the average nitrate concentrations in the influent, pyrite product water, and sphalerite product water were 90 ± 4, 69 ± 8, and 68 ± 12 mg/L respectively (Table 3). These results, and all similar results forthcoming, are recorded as averages from triplicate samples acquired during a single day while the error is reported as the standard deviation. These values indicate that pyrite and sphalerite exhibited similar tendencies to promote denitrification in the presence of yeast extract. However, the values presented in Table 3 suggest that the addition of yeast extract failed to improve the overall denitrification efficiency of both columns. Tong et al. (2017) found that the addition of small amounts of organic carbon increased the denitrification efficiency to approximate 89.7% at an EBCT of 5.83 hrs and ~15 mg/L COD. Our results, however, are not as promising. This could be due to a number of factors, though the column autopsy results suggest that little to no biomass remained in the columns. Adding a small amount of organic carbon to the system was thought to improve overall denitrification efficiency (Kiskira et al., 2017; Tong et al., 2018). However, little improvement occurred between P4 and P5.

Comparison between Figures 4.12 and 4.13 elucidates that while the average nitrate concentrations in both column product waters during P5 were higher than during P4, the results observed during P5 are similar to those observed during the latter half of P4. This could indicate that a severe disruption in operating conditions occurred during P4 around day 121.

Average nitrite-nitrogen concentrations during P5 were 1 ± 2 mg/L in the pyrite column product water and 2 ± 2 mg/L in the sphalerite column while the influent concentrations were BDL (Table 4). These values are much lower than those reported for P4, further indicating a disruption in the denitrification process. The influent initially had a target phosphate-phosphorus concentration of 10 mg/L.
with typical product water concentrations averaging 5 mg/L, though a surge in the ambient groundwater concentration around day 121 resulted in product water concentrations around 34 mg PO$_4^{3-}$-P/L.

Concentrations of sulfate within both column product waters during P5 was roughly similar to those observed in P4, further indicating that removing the microbial stress of fixing carbon dioxide via the addition of yeast extract did not improve chemolithotrophic denitrification.

![Graph](image)

**Figure 4.14:** Concentration profiles of nitrate, nitrite, ammonium, and sulfate (top to bottom) during Phase 5 (days 223-312) of the column experiments for the influent and product waters of the pyrite and sphalerite columns.
Another snapshot was taken during P5, measuring the same constituents as those in the P4 snapshot with the inclusion of COD (Table 9). The pH decreased throughout both columns compared to the pH 8 influent, though each product water remained above a pH of 7.5. Tong et al. (2018) performed a series of batch microcosms, of which, the acid-pretreated microcosms exhibited a similar pH drop over the acclimation period. Furthermore, a drop in alkalinity was observed in both columns, which, when combined with the drop in pH, could be indicative of chemolithotrophic denitrification. Both columns exhibited similar turbidity removal efficiencies as those seen in P4, suggesting that the turbidity is a function of the initial column packing rather than a shift in operating procedure. COD and TOC were recorded at concentrations significantly higher than that of the influent in both SP1 and PP1, then declined below the CI values in the column product waters. Notably, the pyrite column exhibited a higher affinity for removing/utilizing both COD and TOC than the sphalerite column.

During this snapshot, major ions and intermediate sulfur oxyanions were measured via IC (Table 10). Most of the nitrate utilization in both columns takes place at or before the first port, while the remainder of the column removes less than 10% of the nitrate found in the CI feed stock. This likely corresponds to the biomass concentrating near the inflow of nutrients. Though the influent during this snapshot recorded elevated nitrite levels, both columns exhibited complete utilization of nitrite around the first port. Throughout the remainder of the column, nitrite slowly accumulated, likely due to lack of biomass within the later sections which resulted in low nitrite-reductase levels. Sulfate levels throughout both columns did not vary significantly (p>0.05) than the influent, though the intermediate sulfur oxyanions in both columns registered roughly 20-25 mg/L higher than the influent. Alternative theories suggest that, since the inoculum was based on a mixed culture, that the presence of sulfur reducing bacteria could result in lower-than-expected sulfate concentrations in the product water (Tong et al., 2017). The lack of observed denitrification in conjunction with the production of intermediate sulfur oxyanions and incomplete chemolithotrophic denitrification products could therefore be indicative of low overall biomass during the second snapshot.
Table 4.7: Chemical analysis performed on the column product waters & first ports during Phase 5.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>ALK (mg/L)</th>
<th>Turbidity (NTU)</th>
<th>COD (mg/L)</th>
<th>Fe$^{2+}$&lt;sup&gt;a&lt;/sup&gt; (mg/L)</th>
<th>Fe$^{3+}$ (mg/L)</th>
<th>TN (mg/L)</th>
<th>TON (mg/L)</th>
<th>TOC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent</td>
<td>8.0 ± 0.0</td>
<td>520 ± 28</td>
<td>5.8 ± 1.8</td>
<td>22.9 ± 1.3</td>
<td>BDL</td>
<td>0.04 ± 0.00</td>
<td>122 ± 1</td>
<td>12.5 ± 1.6</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td>Pyrite Port 1</td>
<td>8.0 ± 0.1</td>
<td>450 ± 14</td>
<td>2.8 ± 0.2</td>
<td>43.6 ± 2.0</td>
<td>BDL</td>
<td>0.02 ± 0.03</td>
<td>104 ± 1</td>
<td>17.0 ± 0.2</td>
<td>10.7 ± 4.0</td>
</tr>
<tr>
<td>Pyrite Effluent</td>
<td>7.6 ± 0.1</td>
<td>470 ± 42</td>
<td>0.8 ± 0.0</td>
<td>10.6 ± 5.8</td>
<td>BDL</td>
<td>0.03 ± 0.01</td>
<td>96 ± 4</td>
<td>15.1 ± 1.0</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>Sphalerite Port 1</td>
<td>8.0 ± 0.0</td>
<td>510 ± 14</td>
<td>3.8 ± 0.7</td>
<td>29.8 ± 7.1</td>
<td>BDL</td>
<td>0 ± 0.00</td>
<td>102 ± 1</td>
<td>15.1 ± 0.1</td>
<td>9.7 ± 0.9</td>
</tr>
<tr>
<td>Sphalerite Effluent</td>
<td>7.8 ± 0.1</td>
<td>495 ± 7</td>
<td>2.0 ± 0.7</td>
<td>18.4 ±19.5</td>
<td>BDL</td>
<td>0.05 ± 0.01</td>
<td>92 ± 0</td>
<td>13.5 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a.</sup> Ferrous iron was BDL during this snapshot.

Table 4.8: Anion & cation results from the IC analysis of both columns during Phase 5.

<table>
<thead>
<tr>
<th></th>
<th>NO$_3$-N (mg/L)</th>
<th>NO$_2$-N (mg/L)</th>
<th>NH$_4$+-N (mg/L)</th>
<th>Sulfate (mg/L)</th>
<th>Sulfate and Sulfur Intermediates&lt;sup&gt;a&lt;/sup&gt; (mg/L)</th>
<th>PO$_4$³⁻-P (mg/L)</th>
<th>TIN (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent</td>
<td>92 ± 1</td>
<td>6 ± 0</td>
<td>12 ± 1</td>
<td>112 ± 1</td>
<td>125</td>
<td>8 ± 0</td>
<td>109 ± 0</td>
</tr>
<tr>
<td>Pyrite Port 1</td>
<td>79 ± 1</td>
<td>0 ± 0</td>
<td>8 ± 0</td>
<td>109 ± 1</td>
<td>144</td>
<td>6 ± 0</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>Pyrite Effluent</td>
<td>73 ± 3</td>
<td>1 ± 0</td>
<td>7 ± 0</td>
<td>108 ± 0</td>
<td>148</td>
<td>6 ± 0</td>
<td>81 ± 3</td>
</tr>
<tr>
<td>Sphalerite Port 1</td>
<td>78 ± 1</td>
<td>1 ± 0</td>
<td>9 ± 0</td>
<td>110 ± 1</td>
<td>148</td>
<td>5 ± 0</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>Sphalerite Effluent</td>
<td>70 ± 2</td>
<td>2 ± 2</td>
<td>7 ± 0</td>
<td>109 ± 1</td>
<td>147</td>
<td>5 ± 0</td>
<td>79 ± 0</td>
</tr>
</tbody>
</table>

<sup>a.</sup> Sulfur Intermediates refers to any sulfur oxyanion which could be oxidized to sulfur. The sulfate and sulfur intermediates is thus a measure of the sulfate and any extra oxidizable sulfur oxyanions present in the water.
4.2.4 Column Autopsy

Once the columns reached 312 days of operation, the pumps were turned off and the column autopsy began. The homogenized mineral/pumice mixture from each column were exhumed in 15 cm sections around sampling ports 1-3, separated into mineral and pumice portions of 3 g, and 0.3 g were analyzed for fixed biomass according to Lynn et al. (2013). Standards were run between 0.5-200 μg/L using Albumin (BSA) Standard, though the method suggests a linear working range of 0.5-20 μg/L. The pyrite, sphalerite, and pumice samples all returned values BDL for protein concentrations on solid media surfaces.

The aqueous biofilm at each port in each column was approximated by TSS and VSS methods. Both columns reported small amounts of suspended biomass. In the pyrite column, approximate biomass concentrations were 0.02, 0.285, and 0.075 mg/L for ports 3, 2, and 1 respectively. In the sphalerite column, the approximate biomass concentrations in the aqueous portion were 0.045, 0.075, and 0.075 mg/L for ports 3, 2, and 1 respectively. Once converted to the approximate volume contained by the boundaries of each port (<0.14L), the total approximate biomass in the pyrite and sphalerite columns becomes 0.052 mg and 0.027 mg respectively, which is much less than the 137 mg of enriched biomass used to initially inoculate each column. This insinuates that, over the 312-day operational period, significant biomass was lost. One possible explanation for the decline in biomass could be an unforeseen chemical disturbance within the groundwater source.

Looking at Figure 4.12, a gradual decline in nitrate removal from days 121-130 occurred which was preceded by a nitrite spike (day 121) in the sphalerite and pyrite column product waters of 9 and 28 mg NO$_2$-N/L respectively. Furthermore, a phosphate spike of 31 mg PO$_4^{3-}$-P/L in the column influents on day 121 which could provide uncommon microbial access to phosphate. Should the microbes make use of this phosphate, they may be able to increase their rates of denitrification, leading to a period of intense biomass growth. However, the sharp decline in denitrification efficiency following day 120 could not be attributed to phosphate limitation, at least in the case of the pyrite column. The sphalerite column,
however, was phosphate limited from days 110-130. Furthermore, microbes in both columns still had access to both the electron donor and acceptor.

In addition to the biological data collected during the column autopsy phase, aqueous samples were taken from the feed stock and both column product waters to measure for the following analytes: chromium, manganese, iron, copper, zinc, arsenic, selenium, cadmium, antimony, barium, mercury, thallium, and lead (Table 4.9). These elements were chosen as they were likely to exist at above normal concentrations due to minerals leaching into the water, some of which are regulated as NPDWR, and the others as NSDWR.

The sphalerite column product water would violate the secondary maximum contaminant level for manganese, the maximum contaminant level goal (MCLG) for arsenic (0 mg/L), approached the secondary maximum contaminant level for cadmium (5 μg/L), and breached the maximum contaminant level (MCL) and action level for lead (Treatment Technique and 15 μg/L respectively). However, the increase in arsenic in the sphalerite column product water was roughly 0.4 μg/L compared to the influent feed solution. The sphalerite column product water increased the concentrations of a few analytes compared to the initial feed solution: approximately 1.23 μg/L of total iron, 582.98 μg/L of zinc, 1.50 μg/L of antimony, and 0.07 μg/L of thallium. Notably, the sphalerite column product water had approximately 16.1 μg/L of barium and 0.54 μg/L of selenium less than the influent. The decrease in barium could be due to the precipitation insoluble barium sulfate and could thus act as a sink for a small amount of sulfate.

Meanwhile, the pyrite column product water would violate the MCLG for arsenic and the MCL for lead. Notably, the feed solution (nutrient amended groundwater) was already in violation of the MCLG for arsenic and lead. The pyrite column product water showed an increase of approximately 4.9 μg/L of copper from the initial feed stock and approximately 0.8 μg/L of arsenic. Minimal increases in antimony, cadmium, manganese, and zinc were also observed. Much like the sphalerite column product water, the pyrite column product water also exhibited a decrease of barium compared to the feed solution (approximately 12.95 μg/L).
The majority of the metals analyzed in this experiment have been studied, though not extensively, for their inhibitory effects on denitrification. However, copper concentrations have been shown to reduce denitrification efficiency by 48% at soluble copper concentrations of 0.7 mg/L (Kiskira et al., 2018). Other studies have shown that 50 μg/L of Cu can inhibit denitrification to a degree (Bollag and Barabasz, 1978). However, this concentration is much higher than those reported in this study. Nonetheless, both columns had elevated concentrations of copper, though the pyrite column product water had a concentration of approximately 24 μg/L. This could have partially inhibited denitrification in both columns. Others have noted that copper concentrations above 0.05 mg/L could inhibit nitrification, rather than denitrification by 50% (You et al., 2009).

Possible sorption of Zn, Cu, Pb, and Cd could have occurred, though a more detailed concentration profile would be necessary to formally determine that conclusion (Tyagi et al., 2012). Zn and Pb have previously been shown to have little effect on denitrification up to concentrations of 500 μg/L when introduced to a culture in a liquid medium. However, when introduced to bacteria in soil, it was found that Zn inhibited denitrification at that concentration (Bollag and Barabasz, 1978). Similar studies on heterotrophic denitrifying organisms found in saline waters had 92% inhibition of denitrification processes at 490 μg/L of Zn. Particularly, elevated Zn concentrations were found to have an inhibitory effect on the production of NO$_2^-$ for lower concentrations and N$_2$O reductase for higher concentrations. Cadmium concentrations above 14 μg/L were found to have an inhibitory effect on N$_2$O reductase while concentrations lower than that had an inhibitory effect on NO$_2^-$ reductase (Magalhaes et al., 2007). Ultimately, they found that elevated metal concentrations inhibited denitrification in soils with lower organic matter. This could indicate that the zinc concentrations observed in the sphalerite column could have inhibited denitrification and could have led to long-term cell death.

Overall, multiple partial inhibitory effects stemming from zinc, copper, and cadmium could have limited overall denitrification efficiency, particularly in the sphalerite column. Lead has not previously been shown to have any inhibitory effects on denitrification, though the lead produced still poses a serious human health concern.
**Table 4.9: ICP-MS results of product waters and feed solution.**

<table>
<thead>
<tr>
<th></th>
<th>Feed Solution (μg/L)</th>
<th>Sphalerite Column Product Water (μg/L)</th>
<th>Pyrite Column Product Water (μg/L)</th>
<th>Drinking Water Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>0.10</td>
<td>0.09</td>
<td>0.08</td>
<td>MCL: 0.1 mg/L</td>
</tr>
<tr>
<td>Manganese</td>
<td>3.00</td>
<td>105.77</td>
<td>23.71</td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>5.60</td>
<td>6.83</td>
<td>5.50</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>20.18</td>
<td>20.06</td>
<td>24.27</td>
<td>MCLG: 1.3 mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MCL: TT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Action Level: 1.3 mg/L</td>
</tr>
<tr>
<td>Zinc</td>
<td>12.27</td>
<td>595.25</td>
<td>41.35</td>
<td></td>
</tr>
<tr>
<td>Arsenic</td>
<td>0.46</td>
<td>0.87</td>
<td>1.39</td>
<td>MCLG: 0 mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MCL: 0.01 mg/L</td>
</tr>
<tr>
<td>Selenium</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>MCL: 0.05 mg/L</td>
</tr>
<tr>
<td>Cadmium</td>
<td>BDL</td>
<td>4.13</td>
<td>0.30</td>
<td>MCL: 0.005 mg/L</td>
</tr>
<tr>
<td>Antimony</td>
<td>0.11</td>
<td>1.61</td>
<td>0.56</td>
<td>MCL: 0.006 mg/L</td>
</tr>
<tr>
<td>Barium</td>
<td>41.63</td>
<td>25.53</td>
<td>28.68</td>
<td>MCL: 2 mg/L</td>
</tr>
<tr>
<td>Mercury</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>MCL: 0.002 mg/L</td>
</tr>
<tr>
<td>Thallium</td>
<td>0.05</td>
<td>0.12</td>
<td>BDL</td>
<td>MCLG: 0.005 mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MCL: 0.002 mg/L</td>
</tr>
<tr>
<td>Lead</td>
<td>0.11</td>
<td>40.00</td>
<td>8.80</td>
<td>MCLG: 0 mg/L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MCL: TT</td>
</tr>
</tbody>
</table>
CHAPTER 5: CONCLUSIONS

5.1 Conclusions

The capabilities of pyrite, sphalerite, pyrrhotite, molybdenite, and FeS to act as electron donors for the denitrification of nitrified groundwater for drinking water purposes was investigated through a series of batch reactor studies and a long-term UPBR study. The target application of this technology would be to alleviate SCWS’ frequent failure to meet NPDWR due to either inadequate existing treatment capabilities or inconsistent access to appropriate treatment technologies. This purpose aligned with the objectives of this research,

1) Screen for candidates that best achieve removal of nitrate and nitrite through batch reactor studies of sulfur-bearing minerals.

2) Observe the long-term removal efficiencies of the best-performing sulfur-bearing minerals from goal two through a side-by-side up-flow packed-bed reactor study.

Conclusions and recommendations corresponding to each of these objectives are listed below.

This study has determined, under the conditions maintained during the experiments, that:

1) Nitrate removal and product formation from sulfur bearing minerals was most appropriate for SCWS application in the pyrite and sphalerite batch reactors for all three trials.

a. Pyrrhotite exhibited excellent nitrate removal during the first study, though higher than predicted concentrations of sulfate could be produced should the minerals encounter oxygen.
b. Molybdenite performed poorly, removing less nitrate than the uninoculated control, which could be due, in part, to the minerals’ inability to achieve a particulate form when crushed.

c. Iron-sulfide exhibited rapid dissimilatory nitrate reduction to ammonium, likely due to its ability to push the S/N ratio towards one that favors DNRA.

2) Pyrite and sphalerite exhibited similar long-term nitrate removal performances in the UPBR experiment and were ultimately unable to meet NPWDR.

a. With an approximate HRT of 24 hours, pyrite and sphalerite were capable of removing roughly 50% of NO\textsubscript{3}\^- during P4 decreasing to roughly 33% during P5.

b. This is the first study to show that sphalerite can be used as a source of electron donor to promote biological denitrification.

c. Sustained sulfate production was not observed in either column during the entirety of the study, likely due to either incomplete sulfur oxidation or the presence of a non-sulfur related electron donor.

d. Both column product waters exhibited approximately 2-3 mg/L of TOC and minimal biomass concentrations were observed as fixed film or as suspended biomass following the column autopsy.

e. The sphalerite column product water exhibited higher concentrations contaminants than national drinking water standards, notably, the MCL and action level for lead as well as the MCLG for arsenic.

f. The pyrite column product water violated only the MCL for lead and the MCLG for arsenic.

g. Elevated concentrations of zinc, cadmium, and copper could have partially inhibited denitrification, particularly in the sphalerite column.

In summary, the sulfur-bearing minerals examined in this study were unable to treat groundwater contaminated with 100 mg NO\textsubscript{3}\^-/L to NPDWR under the experimental conditions. That being said, 100
mg NO₃⁻N /L is quite high for groundwater. Chemolithotrophic denitrification using sulfur-bearing minerals as the sole electron donor is not as efficient when compared to mixotrophic or heterotrophic systems and thus requires further study should it be relied upon to treat drinking water for SCWS (Koenig and Liu, 2001). Furthermore, the product waters from each column added net concentrations of lead that would breach the NPDWR MCL. Thus, on this condition, sulfur-bearing minerals would require a secondary treatment for the removal of toxic metals. Further study is required to improve the performance of these minerals.

5.2 Recommendations for Future Research

Chemolithotrophic denitrification using sulfur-bearing electron donors to treat nitrate-contaminated drinking water could provide a niche treatment technology for small-scale use. However, understanding the capacities and limitations of each electron donor is necessary.

While pyrite belongs to the disulfide group of electron donors, the monosulfide sulfur-bearing electron donors used in this study included pyrrhotite, sphalerite, and synthetic FeS. The monosulfide group of minerals should be stringently investigated for their roles in performing chemolithotrophic denitrification. Generally, increasing the available surface area of the electron donor will increase the reaction rate. One method of achieving this has been to synthesize minerals, such as pyrrhotite, which has been shown to outperform the natural minerals (Li et al., 2016). Through satisfying the objectives of this research, synthetic FeS was observed to undergo rapid reduction of nitrogen species, likely due to an increased availability of electron donor. Though synthetic FeS exhibited DNRA in the first and second batch trials, determining the ideal ratio of FeS to NO₃⁻N that results in denitrification rather than DNRA could provide a competitive chemolithotrophic electron donor for use in SCWS. Once the threshold for FeS is determined, applications could vary from promoting denitrification with elevated concentrations of nitrate to reducing nitrate into bioavailable ammonium, thus increasing biofilm growth.

When utilizing an UPBR to study denitrification of drinking water derived from groundwater sources, extra care should be taken to maintain column efficiency. Namely, a side-by-side study
comparing the long-term effects of natural groundwater and synthetic groundwater on a porous (>50%) UBPR could elucidate important operational factors. Small grains of pumice (<250 μm) were used as packing material for the UBPR, though previous studies found high denitrification efficiencies when including crushed oyster shells as a packing medium.

Should heavy metals be observed in the water, then adding an adsorptive material to prevent denitrification inhibition would be necessary to achieve high nitrate removal efficiencies. The inhibitory effects of many of the trace elements in these minerals have not been well studied. Future work could focus on examining the capabilities of a mixed chemolithotrophic denitrifying culture to tolerate high metal concentrations.
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APPENDICES
Appendix A: Microcosm Preparations

Figure A.1: Prepared minerals in nitrogen purged bags. From left to right: molybdenite, pyrite, pyrrhotite, sphalerite, and iron-sulfide.
Figure A.2: Microcosm Trial #1 bottles undergoing nitrogen purge.
Figure A.3: Microcosm Trial #2 bottles operating under experimental conditions.
Figure A.4: Minerals ready to add to microcosm trial #2: sphalerite (top left), pyrite (middle left), iron-sulfide (bottom left), molybdenite (top right), pyrrhotite (bottom right)
Figure A.5: Microcosm trial #3 35 mL microcosms. Pictured here are the iron-sulfide microcosms for the first week of sampling. The biologically active samples have floc on top of the mineral