Spring 2023

Application of Nitrate to an Anaerobic Subsurface Bioremediation

Cassie Marie Roberts
Missouri University of Science and Technology

Department:

APPLICATION OF NITRATE TO AN ANAEROBIC SUBSURFACE BIOREMEDIATION

by

CASSIE MARIE ROBERTS

A THESIS

Presented to the Graduate Faculty of the

MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE IN APPLIED AND ENVIRONMENTAL BIOLOGY

2023

Approved by:

Dr. Melanie Mormile, Advisor
Dr. Glenn Ulrich
Dr. David Borrok
Dr. Dev Niyogi

PUBLICATION THESIS OPTION

This thesis consists of the following two articles, formatted in the style used by the Missouri University of Science and Technology:

Paper I, found on pages 10-36, to be published at a later date.

Paper II, found of pages 37-61, to be published at a later date.

ABSTRACT

The highly contaminated subsurface matrix of the Baird and McGuire Superfund site is currently threatening the health and safety of the surrounding environment of Holbrook, MA. Contaminants of significant concern due to high concentration are inorganic arsenite and petroleum hydrocarbons, such as naphthalene. Parsons Corporation and the Massachusetts Department of Environmental Protection have implemented a bioremediation pilot to attempt to degrade the hydrocarbons and arsenic with the application of nitrate. The nitrate would act as an electron acceptor for biodegradation of the hydrocarbon contaminants, produce nitrite that would oxidize reduced iron, and iron oxides would sequester arsenic. Preliminary data showed that nitrate was utilized quickly compared to lab rates and was not distributed to the entire contaminant plume. Additionally, arsenic that was sequestered began to be released into the aqueous phase again over time. The purpose of this study was to investigate nitrate utilizing metabolisms to determine how nitrate is being used by the microorganisms in the subsurface as well as determine what treatments create iron minerals that are capable of long-term arsenic sequestration. It was found that the addition of a labile carbon/electron source such as lactate can facilitate rapid denitrification and when the only source of carbon/electrons are the hydrocarbon contaminants, many metabolisms take place. The iron oxide mineral goethite is primarily produced under nitrate reducing conditions with an added carbon/electron source and is capable of arsenic sequestration. When an abundance of iron is present under nitrate reducing conditions, arsenic will be sequestered and will not be released over a six-month period.

ACKNOWLEDGMENTS

I first want to thank my advisor Dr. Mormile. We have been working together since my sophomore year of undergraduate school. Dr. Mormile is an amazing mentor and scientist that I am lucky to have learned from and worked with. I found frequently that just a few minutes of conversation with her in passing made me feel better about whatever I was stressing over at the time.

I want to thank Dr. Glenn Ulrich for being a great advisor and the liaison between my lab and Parsons Corporation and Massachusetts Department of Environmental Protection. His interest in studying this site further catalyzed this thesis and all of the work I have done over the past two years. I greatly appreciate the opportunity to work on an active bioremediation and contribute to a newer discipline that will hopefully become the status quo for environmental clean-up. I also thank Dr. David Borrok and Dr. Dev Niyogi for their contributions and advice during this thesis process as members of my committee.

I thank all who contributed financially to this thesis project. The S&T department of biological sciences for GTA support and a lab supply grant that went to good use buying chemicals needed for this research. Dr. David Borrok funded the x-ray diffraction analyses. Brookside Capital funded the ICP-MS analyses for arsenic and iron concentrations in a set of experiments.

I thank Dr. Eric Bohannan for performing XRD analyses for me. Also, Dr. Nathan Leigh for performing GCMS analyses of hydrocarbons present in my samples.

Lastly, I want to thank my fellow graduate students that kept me sane.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

PAPER I Page

PAPER II

LIST OF TABLES

NOMENCLATURE

Symbol	Description
mM	Millimolar
ml	Milliliter
g	Grams

1. INTRODUCTION

1.1. BACKGROUND

The Baird and McGuire superfund site located in Holbrook, MA is currently an EPA national hazardous waste priority site. Waste produced by this chemical manufacturing facility from 1912-1983 has contaminated the surrounding groundwater table due to negligence and lack of regulations. While there are many types of contaminants present in the subsurface of this site, the main contaminants of concern include naphthalene (an aromatic polycyclic hydrocarbon) and inorganic arsenite due to their high concentration and persistence. This site is situated on top of a hill leading the groundwater to flow downhill and directly intersects with the nearby Cochato River. Sustained reducing conditions are present in the subsurface due to the stable presence of hydrocarbon contaminants. Outside of the contamination plume, conditions are naturally oxidizing and iron oxides are ubiquitous. However, the biodegradation of these contaminants leads to strong reducing conditions that dissolve any iron oxides present that would have already begun to bind arsenic, thereby mobilizing the arsenic (Cozzarelli et al., 2016).

A pump and treat system was installed as a remediation tactic, however, arsenic concentration was predicted to be sustained nearly indefinitely at an operating cost of approximately 1 million dollars annually. Parsons Corporation and Massachusetts Department of Environmental Protection have developed and implemented a pilot in-situ bioremediation studies to attempt to sequester arsenic and degrade the naphthalene employing the bacterial community naturally present in the subsurface of this site.

Separate sulfate and nitrate pilot tests are each being implemented to stimulate the native subsurface bacterial community into degrading naphthalene and sequestering arsenic via the development of iron oxides.

Nitrate depletion data from the site has shown that the nitrate injected was reduced quickly around the injection site. This is puzzling because the rate of nitrate utilization in the field did not match that found in lab studies. As a result, the nitrate was not delivered to the whole pilot area of the contamination plume. To account for this discrepancy, nitrate metabolisms present at the site were enriched in the laboratory to determine how the nitrate being injected is being utilized.

The goal of injecting nitrate into the subsurface of the Baird and McGuire superfund site is to stimulate nitrate reducing bacteria and generate iron oxide minerals that are capable of arsenic sequestration, thereby immobilizing the arsenic and protecting the Cochato river. Preliminary results from the pilot study showed that iron oxides were being generated and arsenic was being sequestered. However, as the wells were being monitored over time after the injection of nitrate, the levels of arsenic began to increase again, in some areas higher than it was before nitrate injection. The following goals were created to gain a better understanding of how to manipulate the conditions at the site and develop an effective nitrate amended bioremediation.

1.2. OBJECTIVES AND GOALS

The following goals were created to gain a better understanding of how to manipulate the conditions at the Baird and McGuire Superfund site for development of an effective nitrate amended bioremediation.

1.2.1. Identify Possible Nitrate utilizing Bacterial Metabolisms Present at the Baird and McGuire Superfund Site. Due to the observation of nitrate being utilized quickly in the field at the Baird and McGuire superfund site, it is hypothesized that many nitrate reducing metabolisms are contributing to this rapid depletion. Nitrate reducing metabolisms that were speculated to be present include autotrophic nitrate dependent iron oxidation, denitrification, dissimilatory nitrate reduction to ammonia, and anaerobic ammonia oxidation.

1.2.2. Determine if Differing Treatments Affect Generation of Iron Minerals that are Capable of Arsenic Sequestration. Groundwater and soil from the Baird and McGuire superfund site were aliquoted into anaerobic serum bottles and amended with various treatments. Differing carbon sources were tested to create varying nitrate reducing rates based on carbon source complexity. Thereby possibly creating differing iron minerals and affecting arsenic sequestration. Differing concentrations of ferrous iron were tested to identify if iron concentration would influence arsenic sequestration. Iron treatments include iron present from site, no groundwater iron present, 1 mM added ferrous chloride, and 10 mM added ferrous chloride. Carbon source treatments include: no added carbon source, bicarbonate, lactate, or naphthalene. Nitrate and nitrite concentrations in the aqueous phase were recorded once a week for four months. Samples for determining aqueous arsenic and iron concentrations over four months were taken once a week and analyzed later via ICP-MS.

1.2.3. Analyze the Iron Minerals Formed in Differing Carbon-Source-Amended Groundwater. Iron minerals that are formed due to nitrate reduction are the basis of the bioremediation strategy at the Baird and McGuire superfund site for arsenic

sequestration. Differing treatments of groundwater may have an influence on what iron minerals precipitate, their stability, and ability to sorb or incorporate arsenic. The iron minerals that developed were analyzed via x-ray diffraction.

2. IN SITU ANAEROBIC BACTERIAL BIOREMEDIATION FOR ARSENIC CONTAMINATED GROUNDWATER- A REVIEW

Arsenic (As) is a toxic metalloid element that is a leading groundwater contaminant around the world. It is considered by The World Health Organization (WHO) to be one of the top ten chemicals of major public health concern. The current concentration limit for arsenic in drinking water is 10 µg/L; however, many people around the world have been exposed to concentrations much higher than this limit (WHO, n.d.). One of the most arsenic contaminated areas of the world is Bangladesh, where arsenic in groundwater can be found in mg/L concentrations (Harvey et al., 2002; Nickson et al., 1998). Consumption of contaminated groundwater with hazardous levels of arsenic can lead to many diseases such as cancer and other chronic conditions. While arsenic is naturally found in the earth's crust, its problem as a contaminant is exacerbated by anthropogenic activity. Arsenic has been widely used in wood preservation and the agricultural industry as a component of insecticides and herbicides since the 1970s (Mandal & Suzuki, 2002).

Characteristics of contaminants such as toxicity, bioavailability, speciation, and mobility all are dependent on the surrounding redox state and biogeochemical processes of the environment (Borch et al., 2010). Arsenic is a metalloid element, but it is usually

included with the heavy metal contaminants because it acts similarly in the environment and can be remediated with similar tactics. However, arsenic is different from heavy metals as it can be found in inorganic and organic forms. Inorganic forms of arsenic are significantly more toxic to living organisms than the organic forms and are commonly found in two oxidation states, arsenate (As(V)) and arsenite (As(III)). Arsenate is generally found in aerobic environments where it is insoluble, while arsenite is generally found in anoxic environments where it is mobile/soluble (Zhuang et al., 2023).

While physical methods of remediating arsenic have been employed extensively in the past, new in situ methods of remediating arsenic have been developed that are more environmentally benign. Native microbial communities can have a profound impact on the behavior of arsenic at contaminated sites. Bacteria have been noted oxidizing arsenite to arsenate to decrease toxicity and bioavailablity and methylating arsenite to volitize it, removing it from the area (Laha et al., 2022).. These and many more bacterial interactions between contaminants and the surrounding environment can be harnessed and manipulated anthropogenically to remediate and restore sites.

2.1. EX-SITU ARSENIC REMEDIATION METHODS

The following describes common ex-situ methods for arsenic sequestration that are typically more expensive and environmentally harmful compared to bioremediation.

2.1.1 Incineration. Incineration is an ex-situ remediation method that can be utilized for sites contaminated with a wide range of pollutants. The application of incineration may be effective in ridding the soil of contaminants such as petroleum hydrocarbons, but metal and metalloid contaminants cannot be destroyed by high

temperatures (Vidonish et al., 2016). This approach is very expensive due to the temperatures that must be reached between 600 to 1600 degrees Celsius. It is also protocol to dump the ash back onto site as backfill.

2.1.2 Pump and Treat. Pumping and treating contaminated groundwater is one of the most common methods of ex situ remediation. This approach works to remove contaminated groundwater, remove pollutants, and return the newly cleaned water back to the aquifer from which it was taken. The physical pumping of the water also prevents contaminant from spreading further in the subsurface, protecting environmental features such as rivers or lakes. The geology of the site can also play a large role in a pump and treat set up. Depending on the hydraulic conductivity of the geological sections in the subsurface, pump and treat may not be affective at all (Mercer et al., n.d.).

Pumping and treating is only successful to an extent. This approach is most effective when used on contaminants that do not become chemically and/or physically bound to the subsurface matrix. Contaminants such as non-aqueous phase liquids (NAPL) become trapped between soil particles in pores due to capillary attraction and are not able to be pumped out. Under reducing conditions seen in anaerobic aquifers (especially those contaminated with NAPL), high concentrations of arsenic can be sustained for long periods of time. The application of a pump and treat system would not be best for this situation because of how long the system would need to be active to obtain acceptable levels of arsenic. Pumping and treating can become expensive quickly if implemented for long periods of time (Sharma, 2019).

2.2. NEW REMEDIATION TECHNOLOGY- BACTERIALLY MEDIATED ARSENIC BIOREMEDIATION

Bioremediation methods have been employed more recently due to increased research and positive outcomes with treatments. This method of remediation works by mineralizing, degrading, and detoxifying contaminants. However, metals/metalloids such as arsenic cannot be broken down any further than their elemental form as opposed to how carbon-based contaminants can be mineralized to inert carbon dioxide. Two methods in bioremediation include changing the speciation of the contaminant to decrease toxicity or manipulate the surrounding environment to encourage sequestration and immobilization of the contaminant (Laha et al., 2022). Specifically, anaerobic bioremediation methods involve providing electron acceptors and/or a liable carbon/electron source to the bacteria that are present in the contaminated subsurface.

A common approach for arsenic remediation in anaerobic groundwater is inducing the formation of iron minerals. Ferric iron and insoluble ferrous iron minerals are known sorbents of arsenic. Other sorbates found in the environment can compete for iron mineral surfaces including phosphate, bicarbonate, and natural organic matter (Borch et al., 2010). This competition leaves less sorption sites for contaminants such as arsenic and must always be considered when designing a bioremediation of this nature. Depending on the contaminant and the subsurface environment, specific amendments can be chosen for the remediation of arsenic.

2.2.1 Application of Nitrate. The addition of nitrate as in anaerobic subsurface bioremediation can provide many benefits. Nitrate can act as an electron acceptor for bacteria in denitrification and be reduced to nitrite, followed by nitric oxide, nitrous oxide, and ending with dinitrogen gas (Kuypers et al., 2018). All the intermediates of

denitrification are reactive with the surrounding environment and are capable of creating remediating conditions when manipulated correctly. With the application of nitrate, there are many metabolic pathways that can be utilized by bacteria such as autotrophic, heterotrophic, dissimilatory nitrate reduction to ammonia (DNRA), and anaerobic ammonia oxidation (anammox) (Francis & Casciotti, 2016; Kuypers et al., 2018).

It is known that nitrate reducing bacteria are capable of iron oxidation that produce iron minerals capable of adsorbing contaminants, such as arsenic (Sun et al., 2009). There are two ways that nitrate reducing bacteria can cause iron oxidiation. Ferrous iron can act as an electron donor for bacteria and directly oxidize iron to ferric iron. The formation of nitrite via biotic nitrate reduction can also lead to abiotic oxidation of ferrous iron to ferric iron. However, there is a concern when developing iron oxides for arsenic sequestration as they are easily dissolved under reducing conditions. Nitrate reducing conditions have been shown to produce magnetite, a mixed valence iron mineral, that is capable of long-term arsenic sequestration due to its stability under reducing and oxidizing conditions (Sun et al., 2016). However, more research is still needed to identify exactly how to reliably produce magnetite under nitrate reducing conditions.

2.2.2 Application of Sulfate. In the development of anaerobic subsurface bioremediation, sulfate can be added as an electron acceptor for sulfate reducing bacteria. Sulfate is a preferred electron acceptor to inject into contaminated sites because it is not toxic in this form. However, it can lead to the possibility of buildup of toxic sulfides if conditions are right. The sulfate reducing bacteria reduce sulfate to hydrogen sulfide and this compound is highly reactive. Usually, these free sulfides react with reduced iron in

the anaerobic subsurface quickly and form iron sulfide minerals such as pyrite (FeS_2) (Acton & Barker, 1992; Ehrlich, 2016). Many other iron sulfide minerals can develop depending on the environmental conditions such as greigite (Fe_3S_4) and amorphous iron sulfide (FeS). Iron sulfide minerals are stable under reducing conditions and are capable of arsenic sequestration (Teclu et al., 2008).

2.3 WHY BIOREMEDIATION RESEARCH IS IMPORTANT

With increasing population, industrialization, and lack of regulations it was inevitable that our environment would become a dumping ground for polluting wastes. Once the environmental protection agency (EPA) was created within the United States, we started to become conscious of our impact on the environment with regulations in place to prevent contamination events. Over the past 50 years, the EPA has been working to clean up past pollution events and prevent future incidents (US EPA, 2020). The first use of bioremediation was for an oil spill in 1972, and since then bioremediation has been a large research focus in environmental science (National Research Council, 1993).

Bioremediation is more environmentally friendly than other methods of remdiation because it employs naturally occurring reactions and pathways to break down and detoxify contaminants. Due to this, bioremediation tends to be more cost effective for long term treatments. Bioremediation can be implemented at sites that have been contaminated for decades as well as those that have been recently contaminated. With increased research and implementation, bioremediation is one of the most preferred methods of remediation.

PAPER

I. NITRATE REDUCING METABOLISMS PRESENT IN AN ANAEROBIC SUBSURFACE BIOREMEDIATION

ABSTRACT

Nitrogen is an integral part of the living world. It is not only required for many cellular functions, but in other organic and inorganic forms it can be utilized as a source of energy. There are many pathways within a microbial soil community that compete for nitrogen, regardless of the form it is found in. Within an anaerobic subsurface environment, nitrate is a preferred electron acceptor for microorganisms that have the means to utilize it in their metabolic processes. The tangled web of microbial nitrogen metabolisms is still under investigation by microbiologists. During a bioremediation effort, it is important to understand exactly how nitrate is being utilized to know how to best treat a contaminated site. Nitrate can be utilized as a terminal electron acceptor to enable microorganisms to degrade hydrocarbon contaminants. The resulting nitrite can oxidize surrounding iron minerals to cause arsenic sequestration. Within this study, heterotrophic nitrate reduction was seen to be the easiest metabolism to kickstart creation of iron oxides with liable carbon sources such as lactate. Autotrophic metabolism was not detected via enrichments but does not rule out their presence at the site. Dissimilatory nitrate reduction to ammonia (DNRA) and anaerobic ammonia oxidation (anammox) are both suspected to be present based on nitrate concentrations increasing during an experiment. This work was performed to determine the possible nitrogen-utilizing

metabolisms present in the anaerobic subsurface environment found at the Baird and McGuire superfund site in Holbrook, MA.

1. INTRODUCTION

The Baird and McGuire superfund site is located in Holbrook, MA. The main contaminants of concern include naphthalene and inorganic arsenite. Due to the stable presence of the naphthalene in the pores of the subsurface matrix, sustained reducing conditions are created and allow the normally immobilized arsenic to become mobilized with the groundwater. Parsons Corporation and Massachusetts Department of Environmental Protection have developed and implemented a pilot in-situ bioremediation to attempt to sequester arsenic and degrade the naphthalene by employing the bacterial community naturally present in the subsurface of this site. Nitrate was injected into the subsurface to stimulate anaerobic nitrate reducing bacteria. The goal in doing this was to generate iron oxide minerals that are capable of arsenic sequestration, thereby immobilizing the arsenic (Dixit & Hering, 2003).

Preliminary results from the pilot study showed that iron oxides were being generated and arsenic was being sequestered. However, as the nitrate wells were being monitored over time after the injection of nitrate, the concentration of arsenic began to increase again. It was also noted that the injected nitrate was not traveling far beyond the injection point because it was utilized by the microbial community quickly compared to rates observed in the lab.

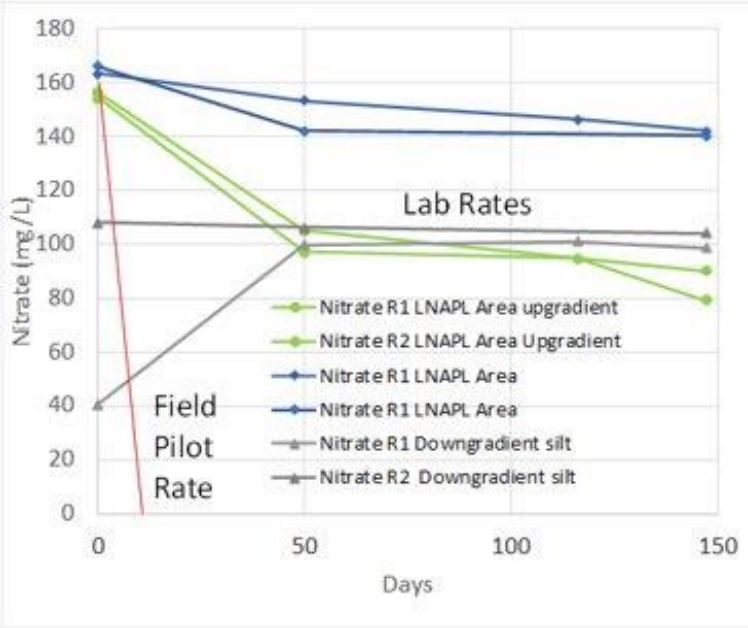

Figure 1. Lab rates of nitrate depletion vs. field pilot rates of nitrate depletion as recorded by Parsons Corporation

This was a problem because it meant that nitrate was not being distributed to the whole contaminated area of interest. This dilemma inspired a hypothesis that the reason for the quick usage of nitrate is the consortia of microorganisms present are capable of many nitrate-utilizing metabolisms.

1.1. DENITRIFICATION

Denitrification is one of the most studied and widespread nitrogen reducing metabolisms. The pathway of denitrification involves the reduction of nitrate to nitrogen gas, forming reduced/electron-accepting intermediates along the way. The reaction that reduces nitrate to nitrite is called dissimilatory nitrate reduction and adheres to the following equation (Kuypers et al., 2018):

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad (1)$$

The intermediate nitrite is reactive and can abiotically oxidize ferrous iron to ferric iron rapidly (Coby & Picardal, 2005; Picardal, 2012). Not all bacteria capable of denitrification can perform every reaction in the pathway due to genetic limitations. Some can only complete dissimilatory nitrate reduction, while others can reduce the gaseous intermediates further along this pathway including nitric oxide, nitrous oxide, and nitrogen gas (Francis & Casciotti, 2016). Most microorganisms that are capable of denitrification are heterotrophic and require an organic carbon source to facilitate metabolism (Kuypers et al., 2018).

1.2. AUTOTROPHIC NITRATE-DEPENDENT IRON OXIDATION

Iron plays an important and complex role in anaerobic environments as an electron donor and /or electron acceptor depending on what valence state it is in. As a result of being one of the most abundant redox-active metals, iron is a major source of energy for microorganisms that have the mechanisms to take advantage of it (Kappler et al., 2016). Microbial oxidation of ferrous iron was first thought to be only completed with oxygen as the terminal electron acceptor, but in the 1990s it was discovered that other electrons acceptors such as nitrate and sulfate can be utilized. Straub et al. (1996) isolated cultures of anaerobic ferrous iron oxidizers that were capable of strict lithotrophic metabolism following this stoichiometric equation:

$$10Fe^{2+}+2NO_3^-+24H_2O \rightarrow 10Fe(OH)_3+N_2+18H^+ \quad (2)$$

However, under nitrate-reducing conditions it is difficult to distinguish between iron being directly oxidized autotrophically through microbial metabolism or the intermediate nitrite abiotically oxidizing the iron.

If autotrophic denitrification is a viable metabolism to create iron oxides at this site, there may be no need to introduce an organic carbon source to stimulate the native microorganisms. This pathway may be the most benign to the environment because the addition of a carbon source can lead to secondary contamination depending on the carbon source's intermediates (Zhang et al., 2014).

1.3. DISSIMILATORY NITRATE REDUCTION TO AMMONIA (DNRA)

Dissimilatory nitrate reduction to ammonia (DNRA) involves an alternate fate for nitrate; it is microbially transformed into ammonia. The reaction is as follows (Francis & Casciotti, 2016):

$$NO_3^- + 9H^+ + 8e^- \rightarrow NH3 + 3H_2O \quad (3)$$

This metabolism has only been studied for the past few decades and is still under investigation to truly understand its scope and role in the environment (Francis & Casciotti, 2016). It is known that conditions that favor denitrification such as no oxygen, high organic carbon, and nitrate also favor DNRA. Because of these similar favorable conditions, the DNRA pathway competes with the denitrifying pathway for resources (Tiedje, 1988). The determining factor of whether DNRA or denitrification is most prevalent in an environment depends heavily on the availability of labile carbon sources (Valiente et al., 2022). There is evidence that an environment that would favor DNRA over denitrification would contain a low nitrate and high organic carbon concentrations (van den Berg et al., 2015a). In fact, DNRA is one of the most favorable reactions in an environment that is electron acceptor-limited because eight electrons can be accepted by nitrate to nitrite to ammonia.

1.4. ANAEROBIC AMMONIA OXIDATION (ANAMMOX)

Anaerobic ammonium oxidation (anammox) is one of the most elusive microbial nitrogen metabolisms. In this chemoautotrophic pathway, ammonium acts an electron donor and nitrite as the electron acceptor as seen in this reaction (Strous et al., 1998; van de Graaf et al., 1996):

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow$$
$$1.02N_2 + 0.26NO_3 + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (4)$$

While nitrate is not the major product of anammox, it can be about 10% of the total product with the rest being nitrogen gas (Strous et al., 1998; van de Graaf et al., 1996). Not much is known about this process, mostly as a result of not having isolated a pure culture of an organism that is capable of anammox. With the small amount of knowledge that has been gathered, it is thought that anammox is important to the nitrogen cycle of environments with low levels of labile carbon and excess nitrate (Burgin & Hamilton, 2007).

The presence of these metabolisms within the subsurface environment at the Baird and McGuire Superfund site was investigated with two experiments: artificial medium enrichments and soil/groundwater enrichments. Nitrate and nitrite concentrations were monitored over time as evidence of metabolic activity. The redox dye resazurin was utilized to indicate redox conditions within artificial enrichments to inform whether nitrate reduction was taking place. These results then informed whether the suspected nitrate utilizing metabolisms were present at the site.

2. MATERIALS AND METHODS

2.1. SAMPLING SITE AND PROCEDURES

The Baird and McGuire Superfund site is located in Holbrook, MA. The groundwater and soil were collected prior to nitrate amendment at the site. Ground water was collected, with and without sediment in nitrogen purged 2.5L amber jars, overfilled, and capped with no headspace. Soil samples were collected with a direct push Geoprobe rig. Cores were collected in plastic sleeves and processed in the field by cutting the sleeves to expose soil. Grab and composite samples were placed in glass jars with no headspace and frozen until shipped under ice and overnight from Holbrook, MA to Missouri University of Science and Technology, Rolla MO.

2.2. TREATMENT STOICHIOMETRY

Microbial nitrate reducing and iron oxidizing metabolism adheres to the stoichiometric equation seen in equation 2. This equation applies to nitrate reducing iron oxidizing metabolisms regardless of the carbon source provided. Most microorganisms that are capable of this metabolism use organic sources of carbon, such as acetate, lactate, and more specifically aromatic hydrocarbons such as naphthalene (Rockne et al., 2000).

Based on this equation it was determined that 5mM of ferrous iron is required to reduce 1mM of nitrate to 1mM nitrite within the enrichments in this study. Furthermore, the abiotic reaction between nitrite and ferrous iron fulfills the following stoichiometry equation (Melton et al., 2014):

$$4Fe^{2+}+2NO_2+5H_2O \rightarrow 4FeOOH+N_2O+6H^+ \quad (5)$$

The following stoichiometric equation details sulfate reduction with naphthalene as a carbon source (Kümmel et al., 2015):

$$C_{10}H_8+6SO_4^{2-}+2H^+ +6H_2O \rightarrow 10HCO_3^- +6H_2S \quad (6)$$

From this equation, it has been determined that 48 electrons can be donated through the mineralization of naphthalene. Therefore, the electron ratio for naphthalene mineralization under nitrate reducing conditions is calculated to be 0.042mM of naphthalene to reduce 1mM of nitrate.

2.3. ARTIFICIAL MEDIUM ENRICHMENT SETUP

Enrichment base medium recipe: ATCC Medium 2672: Modified Wolfe's Mineral Medium (MgCl2 0.2 g, K_2HPO_4 0.05 g, Wolfe's mineral medium 1 mL, distilled H_2O 1 L). This medium recipe was modified to not contain any sulfur compounds that would interfere with the goal of enrichment for nitrate reducing bacteria. All enrichments were set up within dishwasher-cleaned 100 ml glass serum bottles. All bottles were set up in an anaerobic glove bag under 90% nitrogen gas and 10% hydrogen gas.

For autotrophic nitrate dependent iron oxidizing enrichments, 1 L of the enrichment base medium was mixed in a 2 L flask. The medium was then dispensed into four 500 ml flasks, 200 ml each except for one flask (nitrate+ferrous iron+bicarbonate treatment was also used as a control treatment) which received 400 ml. The treatments in each flask included nitrate+bicarbonate, nitrate+ferrous iron, nitrate+ferrous iron+bicarbonate, and ferrous iron+bicarbonate. The concentrations of each treatment were 10 mM nitrate, 10 mM sodium bicarbonate, and 15 mM ferrous chloride.

For heterotrophic enrichments with naphthalene as the carbon source, 1 L of enrichment base medium was mixed in a 2 L flask. The base medium was then distributed to three 1 L flasks, two with 200 ml and one with 400 ml. The treatments in the 200 ml flasks include iron and iron+nitrate. The 400 ml flask received only nitrate. The concentrations of each treatment were 10 mM nitrate and 15 mM ferrous chloride. For naphthalene delivery, 1 ml of a 200 mg/50 ml concentration naphthalene/acetone solution was added to empty sterile serum bottles. The acetone was allowed to evaporate, leaving behind naphthalene residue at the concentration of 4mg /50 ml of medium (Dou et al., 2009).

The redox indicator dye resazurin was added to each culture media to visually show redox conditions within the media where pink indicated oxidizing conditions and clear indicated reducing conditions. All media was degassed under a steady stream of nitrogen gas. The media was brought into the anaerobic glove bag along with all of the serum bottles. Media was dispensed according to treatment in triplicate, 50 ml per serum bottle. Serum bottles were then sealed with butyl rubber stoppers, removed from the anaerobic glove bag, and crimp sealed with aluminum caps. The headspace in each serum bottle was exchanged with 100% nitrogen gas. The bottles were then autoclaved at 121º C for 30 minutes to sterilize the medium. Once cooled, 5 ml of sediment-containing groundwater was used as inoculum for each bottle. All serum bottles were stored at room temperature in the dark. After a month, one bottle out of each triplicate of treatments was amended with 10 mM lactate and allowed to continue incubating.

2.4. SOIL AND GROUNDWATER ENRICHMENT SETUP

Treatments were set up in 100 ml glass serum bottles that were cleaned in a laboratory dishwasher and autoclaved for 30 minutes at 121º C to sterilize. All bottles were set up in an anaerobic glove bag under 90% nitrogen gas and 10% hydrogen gas. 50 ml of sediment-containing groundwater from the Baird and McGuire Superfund site was pipetted into each serum bottle.

Table 1. Soil and groundwater experiment treatments.

Treatments	50ml sediment-containing groundwater	30g soil	10mg/L Sodium Arsenite	5mM Sodium Bicarbonate	1mM Sodium Nitrate	Ferrous Chloride	5mg/L Naphthalene
unamended	X	X	X	X			
Only nitrate	X	X	X	X	X		
Naphthalene	X	X	X	X	X		X
Groundwater with iron removed +soil	X	X	X	X	X		
Ferrous Chloride 1mM	X	X	X	X	X	X	
Ferrous Chloride 10mM	X	X	X	X	X	X	

Five g of soil from each soil sample (six total samples) obtained from differing wells and depths at the site were added to each bottle. Each bottle was sealed with sterile butyl rubber stoppers crimped in place with aluminum caps. All treatments were added to bottles with nitrogen degassed syringes. All bottles except unamended control received 1 mM sodium nitrate. 10 mg/L of sodium arsenite solution was added to each bottle to assess sequestration over time. 5 mM of sodium bicarbonate was added to every bottle for pH stabilization. The groundwater with no iron + soil treatment was created by oxidizing groundwater and allowing for the resulting oxidized iron to settle. Then, the groundwater was made anaerobic by sitting in the anaerobic glove bag overnight, and subsequently used in bottles for this treatment. 5 mg/L naphthalene was delivered to three sterile serum bottles with acetone for naphthalene treatment (Dou et al., 2009).

2.5. ANALYTICAL METHODS

Nitrate and nitrite concentrations were quantified colorimetrically (Kamphake et al., 1967). The pH of enrichments was analyzed by using pH strips. Naphthalene and other hydrocarbon contaminant concentrations were quantified via gas chromatography mass spectrophotometry (Shimadzu QP-2020). The gas chromatograph was equipped with an AOC-6000 auto-sampler. Separation was performed using a DB-5MS column (30 m long x 0.25 mm ID and film thickness 0.25 µm). The initial oven temperature was 35º C, increased to 195º C over ten minutes with no hold time, followed by an increase to 245º C over the next 25 minutes with a hold time of two minutes. The injection mode was splitless with the injection temperature at 200º C. The pressure in the column was 100 kPa with a total flow of 50 ml/min and helium carrier gas. Sampling of hydrocarbons

took place for one minute via SPME sampling with a 100 µm PDMS film (PAL system). The mass spectrometer had an interface temperature of 250º C and an ion source temperature of 200º C. The solvent cut time was one minute. The analysis began at 1.10 minutes and ended after 22 minutes with a full scan between 10 m/z-280 m/z. The event time was 0.05 seconds. The library utilized to identify compounds of interest was NIST14.

3. RESULTS

3.1. ARTIFICIAL ENRICHMENT EXPERIMENTS

3.1.1. Six-Month Enrichments. Over the course of six months, the autotrophic enrichments did not change in color (Figure 2A, 2B) and there was also no significant decrease in nitrate concentration (Figure 2C), indicating that no nitrate reducing metabolic activity had taken place.

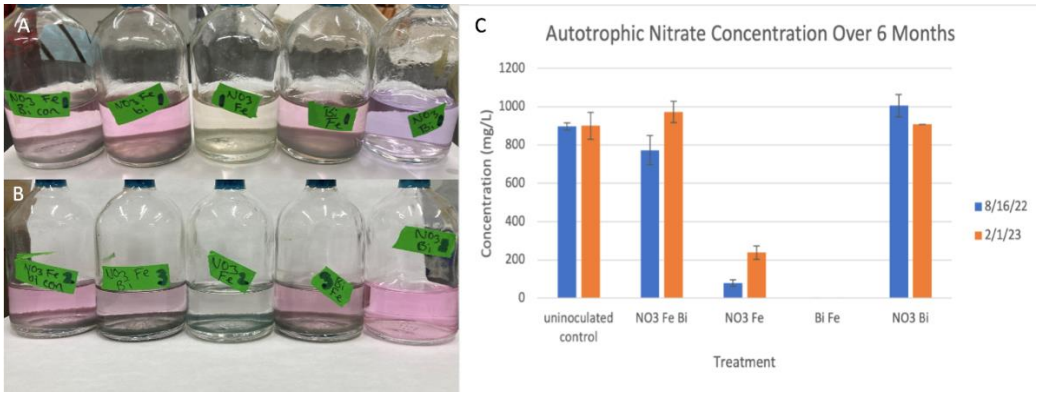

Figure 2. Autotrophic enrichments visual on day one (A) and six months (B). Nitrate concentrations on day one and six months for each treatment. including nitrate (NO3), bicarbonate (Bi), and ferrous chloride (Fe) (C).

Naphthalene was provided at the start of incubation in the heterotrophic enrichments to demonstrate that it could be used as a carbon source under nitrate reducing conditions. There was no change in nitrate concentration over the six months that this experiment took place. There also was no nitrite development in the enrichments at either time point. GC-MS was used to identify if naphthalene was present after six months. The data revealed that there was no difference in naphthalene concentration between the treated bottles and the control bottles with no inoculum.

The ferrous iron nitrate enrichment shows almost immediate reduction of nitrate on day one (Figure 2C) due to the absence of bicarbonate and the addition of ferrous iron, bringing the pH down to ~4.5 (Table 2). All other treatments contained bicarbonate as a source of carbon dioxide for potential carbon fixation but also acted as a buffer to keep the pH 6-7 (Table 2).

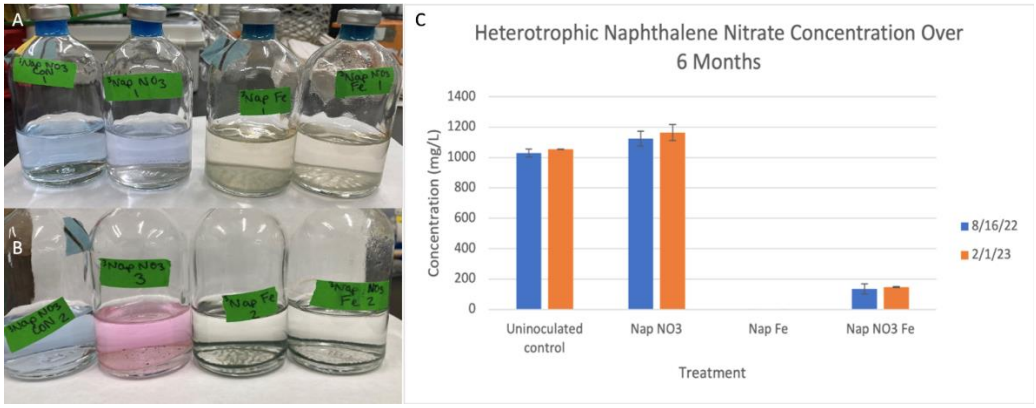

Figure 3. Naphthalene amended heterotrophic enrichments visual on day one (A) and after six months (B). Nitrate concentrations on day one and six months for each treatment, including naphthalene (nap), nitrate (NO3), and ferrous chloride (Fe) (C).

The naphthalene nitrate ferrous iron treatment again showed a decreased nitrate concentration with low pH (Figure 3C, Table 2).

Table 2**.** pH readings of each enrichment treatment on day one and after six months, including lactate (L) amended bottles, including nitrate (NO3), bicarbonate (Bi), ferrous chloride (Fe), and naphthalene (nap).

Treatments	pH day 1	pH month 6 lactate unamended	pH month 6 lactate amended
NO3 Fe Bi uninoculated	6.5	6	6
NO3 Fe Bi	6.5	6.5	7
NO3 Fe	4	4	4
Fe Bi	6	6	6
NO3 Bi	7	7	7
Nap NO3 uninoculated	8.5	7	7
Nap NO3	8.5	7	8
Nap Fe	4.5	4	5
Nap NO3 Fe	4.5	4	5

3.1.2. Addition of Lactate to Artificial Enrichments. After a month of incubation, nothing appeared to be changing in the autotrophic or heterotrophic naphthalene enrichments. To compare non-labile to labile carbon sources, 10 mM lactate was injected into one replicate of each triplicate treatment.

Within 48 hours of adding lactate, nitrate was completely utilized, and the resulting nitrite oxidized the iron that was present in bottle 1 (Figure 4A). The same trend was seen in bottle 3 (Figure 4B) when lactate was added, but with no iron oxidation because no iron was present. In both bottles, 1 and 3, the resazurin changed from pink to clear due to shift in redox conditions to reducing. Over the course of six months, bottles 2 and 4 (Figure 4A, 4B) showed no orange iron precipitation or resazurin color change.

This is also corroborated by Figure 4C as it shows the depletion of nitrate concentrations of treatments nitrate ferrous iron bicarbonate and nitrate bicarbonate with the addition of lactate.

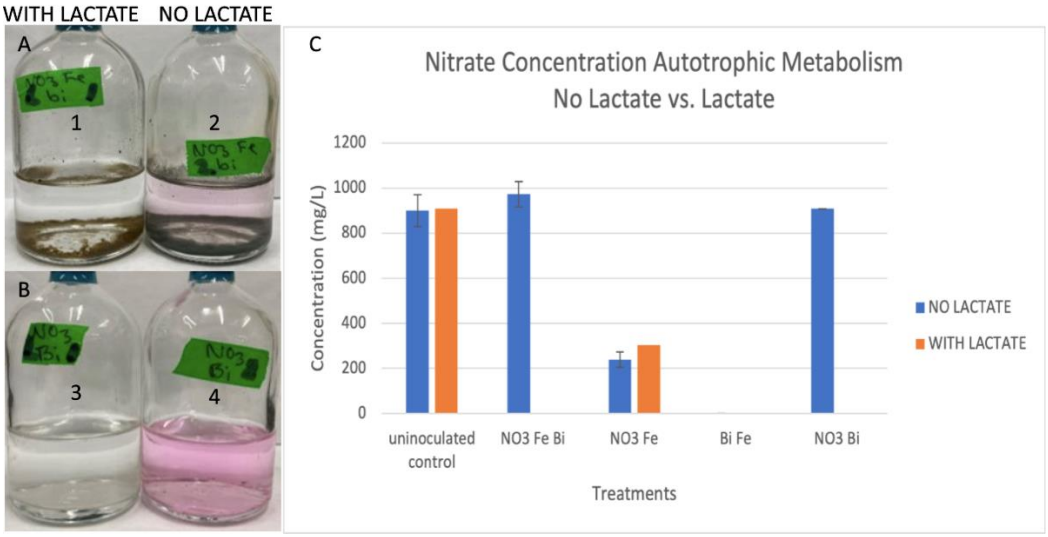

Figure 4**.** Autotrophic enrichments vs. lactate amended autotrophic enrichments. Visual resazurin and iron evidence (A,B). Nitrate concentrations in lactate amended vs. not amended, including nitrate (NO3), bicarbonate (Bi), and ferrous chloride (Fe) (C).

Within 48 hours of adding lactate to bottle 1, some iron had precipitated, resazurin had gone clear, and turbidity had developed (figure 5A). Over six months, the resazurin in bottle 2 and 3 had not changed. Figure 5B corroborates what is seen in Figure 5A because there is significant nitrate reduction in the naphthalene nitrate treatment amended with lactate as compared to the nitrate concentration in the same treatment not amended with lactate.

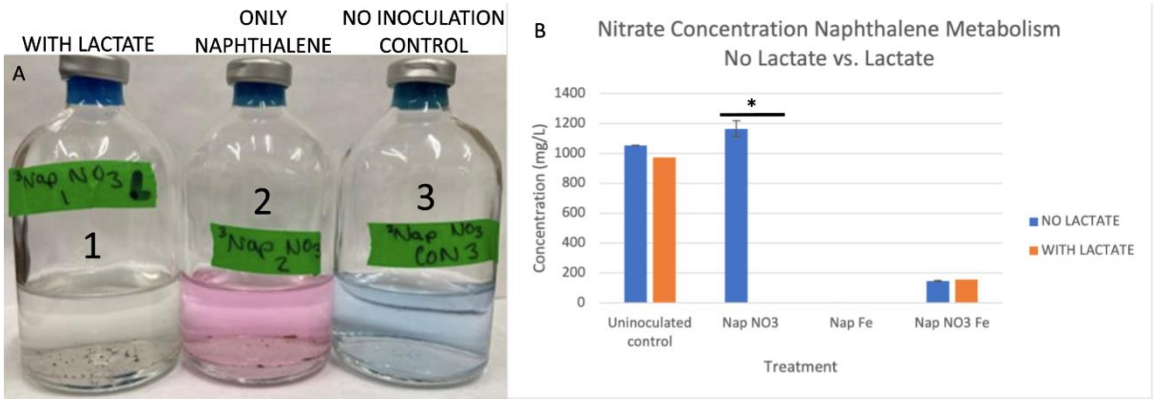

Figure 5. Naphthalene heterotrophic enrichments vs. lactate amended naphthalene heterotrophic enrichments. Visual resazurin and iron evidence (A,B). Nitrate concentrations lactate amended vs. not amended, including naphthalene (Nap), nitrate (NO3), and ferrous chloride (Fe) (C).

3.2. SOIL AND GROUNDWATER EXPERIMENTS

3.2.1. Hydrocarbon Contaminant Biodegradation. A triplicate set of soil and groundwater bottles were amended with naphthalene to assess naphthalene biodegradation over time with microorganisms found in the soil samples (Figure 6). After six months, GC-MS was used to analyze naphthalene and other hydrocarbon contaminants concentration compared to unamended (no added naphthalene) frozen samples.

Naphthalene- the highest concentration contaminant seen at the Baird and McGuire superfund site- demonstrated the most biodegradation over 6 months with 96% depletion. Other hydrocarbon contaminants at this site demonstrated less biodegradation over the course of six months compared to naphthalene likely due to increased complexity and steric hinderance.

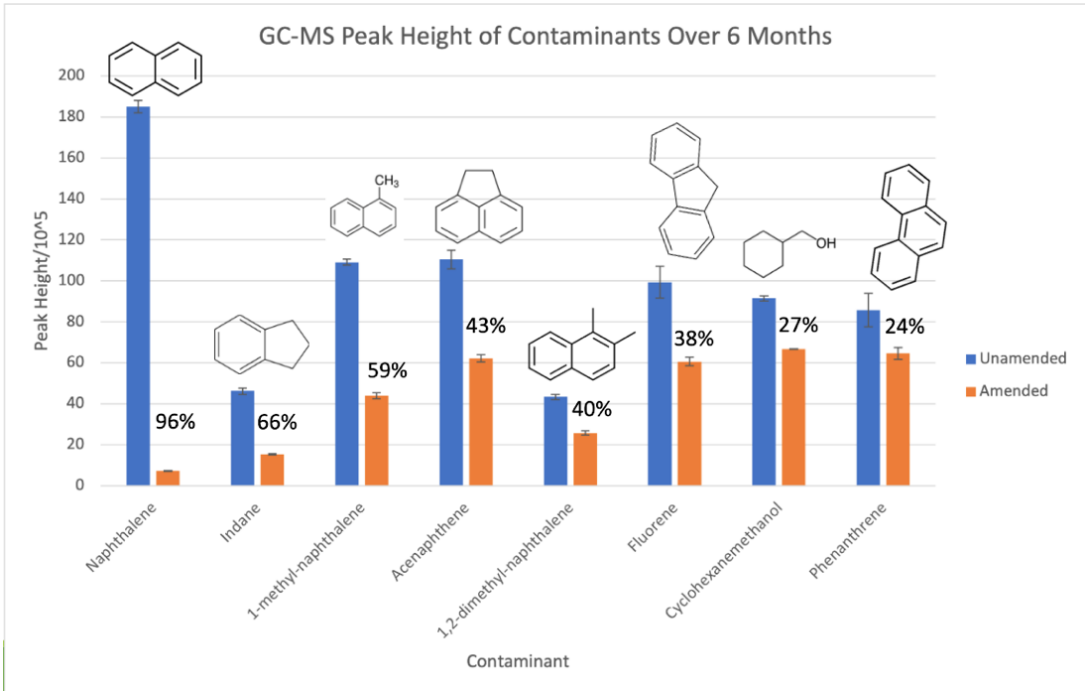

Figure 6**.** Percent biodegradation of hydrocarbon contaminants over six months between naphthalene unamended bottles and naphthalene amended bottles.

3.2.2. Nitrate and Nitrate Concentration over Time. Soil and groundwater experiments were set up with treatments not amended with lactate (Figure 7). Nitrate concentrations steadily declined, and nitrite concentrations steadily increased from day 17 (addition of 10 mM nitrate) to day 31. On days 38 and 45, the nitrate concentration significantly increases to levels above what was initially added on day 17 (Figure 7A). Nitrite concentrations increased only in treatments with no added iron on day 45 (Figure 7B). Nitrate and nitrite concentrations sharply decreased by day 52 in all bottles that previously had significant accumulations and leveled out from day 59 on (Figure 7).

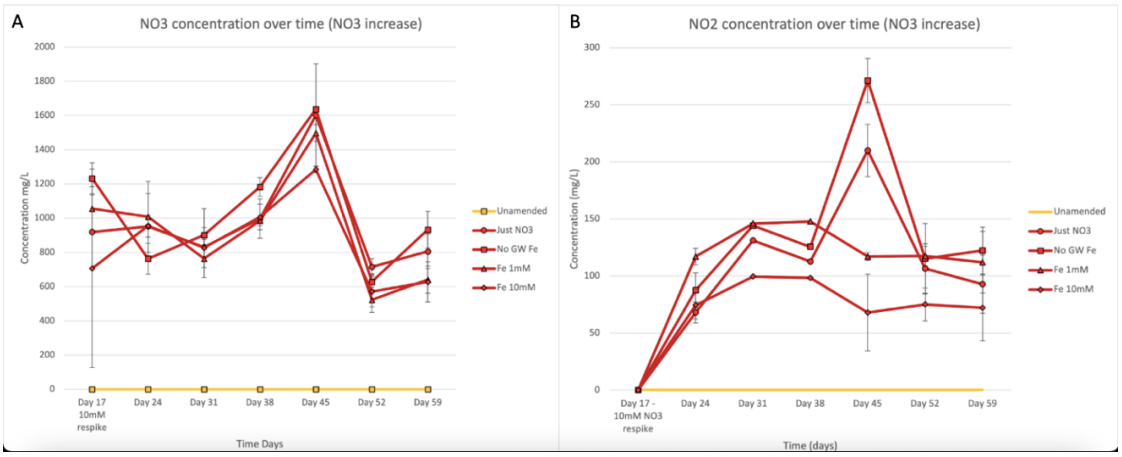

Figure 7. Soil and groundwater enrichments with no added carbon source. Nitrate concentration over time (A). Nitrite concentration over time (B).

4. DISCUSSION

4.1. PRESENCE OF AUTOTROPHIC NITRATE REDUCING IRON OXIDIZING METABOLISM

Autotrophic nitrate dependent iron oxidation enrichment experiments did not indicate nitrate reduction and development of iron oxides as typically demonstrated by a population of autotrophic nitrate reducing iron oxidizing bacteria in the literature (Straub et al., 1996). No autotrophic metabolic activity was detected within the enrichments in this study over the course of six months (Figures 2, 3). This may be due to conditions not being conducive and/or the population of microbes that are capable of this metabolism was too small. Microorganisms that are capable of autotrophic metabolism are most likely present at this site because they are known to be ubiquitous throughout the environment, but they could not be enriched in the laboratory.

4.2. PRESENCE OF HETEROTROPHIC NAPHTHALENE NITRATE REDUCING METABOLISM

Naphthalene amended enrichments over six months demonstrated no reduction of nitrate (Figure 3C) as well as no degradation of naphthalene as indicated by GC-MS data. Other experiments investigating the ability of nitrate reducing bacteria to utilize naphthalene as a carbon source show that naphthalene concentration begins to decrease significantly within two weeks to a month (Dou et al., 2009; Zhang et al., 2019). If naphthalene was being utilized, the resazurin in the enrichments would have lost color and the nitrate concentration would have decreased. No other electron acceptor or carbon source was present, so if nitrate and naphthalene were not being depleted then there was no significant metabolic activity taking place. Naphthalene degradation was most likely not seen in the enrichment cultures because the population of microbes was not large enough to cause a significant decrease in naphthene or nitrate concentration over six months.

Figure 6 demonstrates that under conditions more closely resembling the Baird and McGuire superfund site, hydrocarbons are readily used by nitrate reducing microorganisms. The addition of soil to the enrichment cultures provided the concentration of bacteria needed to cause naphthalene biodegradation over six months. The percentile of hydrocarbon depletion seen in Figure 6 follows the expected trend for compounds with increased complexity and steric hinderance to be more difficult for microorganisms to degrade (Varjani, 2017). These hydrocarbons can be utilized as the carbon and electron sources for nitrate reduction, with subsequent nitrite production leading to iron oxidation for sorption of materials, such as arsenic. , but however, there is

a tradeoff as the hydrocarbon biodegradation is less time-efficient than addition of a more liable carbon source such as lactate.

4.3. LACTATE UTILIZATION LEADS TO NITRATE REDUCTION AND IRON OXIDATION AT RATE USEFUL FOR BIOREMEDIATION

After three months of incubating the enrichments, lactate was added to one replicate of each treatment. This was done to demonstrate and compare how the enrichments would change if a readily available source of electrons was present. Lactate enabled the microorganisms present in the bottles to become metabolically active with nitrate depletion and resazurin becoming clear went clear within 48 hours (Figures 4, 5). After six months of incubating the non-lactate amended bottles, it can be concluded that a labile carbon source such as lactate is necessary for nitrate reduction to occur at a rate useful for bioremediation and even with a dilute population of microorganisms, lactate is readily and quickly utilized for nitrate reduction.

Enrichments that did not contain bicarbonate or naphthalene but had ferrous chloride added appeared to experience rapid nitrate reduction within the day of inoculation (Figures 2, 3). The pH of these enrichments was measured to be stably 4-4.5 due to the addition of ferrous chloride (Table 2). However, it is not thermodynamically favorable for nitrate to be abiotically reduced with ferrous iron unless under high temperatures or in the presence of a copper catalyst (Liu et al., 2019). It is suspected that this sudden reduction of nitrate is an artifact of the colorimetric nitrate assay combined with the low pH of the samples.

4.4. DNRA AND ANAMMOX HYPOTHESIS IN SOIL AND GROUNDWATER EXPERIMENTS

Without metagenomic and mRNA analyses of the bacteria present at this site, it is difficult to pinpoint exactly how the nitrate is being utilized. Figure 7 provides preliminary evidence that DNRA and anammox metabolisms are present at the Baird and McGuire superfund site. This conclusion was based on nitrate and nitrite data collected over the course of 59 days within the soil and groundwater experiments not amended with a labile carbon source. After the 10 mM nitrate respike on day 17, denitrification and DNRA are both speculated to be active metabolisms until day 31. Equation 1 and 2 show stoichiometrically how microbes complete denitrification and DNRA, respectively. During this process, nitrate is being reduced to nitrite and eventually nitrogen gas by denitrification, while DNRA uses nitrite to create ammonia. Both processes are heterotrophic and utilize the hydrocarbons present in the soil as carbon sources. When easy to degrade contaminants run out on day 31, denitrification and DNRA starts to slow down due to the process of degrading more complex hydrocarbons (Figure 7). Anammox microbes can become active and begin to oxidize the ammonia with nitrite produced from denitrification and bicarbonate as a carbon source. This process is speculated to take place between day 31 and day 45 (Figure 7A). Equation 3 demonstrates how anammox metabolism can lead to the production of nitrate. It is possible that DNRA and anammox metabolisms could have utilized the nitrite and forming nitrogen gas faster than nitrate can be replenished for denitrification to use it and create more nitrite (Figure 8). Both nitrate and nitrite concentrations become stable after day 59 around 500 mg/L and 100 mg/L respectively.

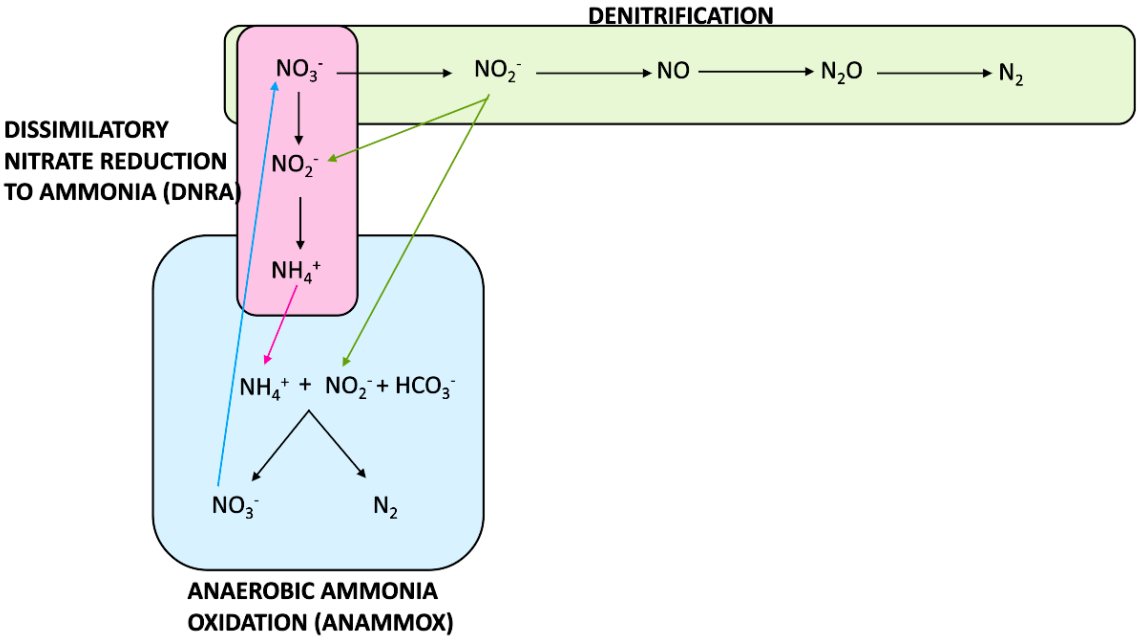

Figure 8. Visual map of how denitrification, DNRA, and anammox are hypothesized to interact. Black arrows indicate pathways. Green arrows indicate where nitrite from denitrification can be utilized. The blue arrow indicates where nitrate from anammox can be used. The pink arrow indicates where ammonia from DNRA is used by anammox. (Hu et al., 2013; Kartal et al., 2013; Kuypers et al., 2018; Strous et al., 1998; van de Graaf et al., 1996).

5. CONCLUSION

Based on the data that has been collected in this study, conclusions can be drawn about how nitrate is being utilized by capable bacteria at the Baird and McGuire superfund site. Artificial medium enrichments are not the most efficient way to select for autotrophic and/or naphthalene utilizing nitrate reducing metabolisms. Heterotrophic metabolism dominates when labile carbon sources are present such as lactate or an easily biodegradable hydrocarbon. The addition of lactate to the enrichment cultures resulted in nitrate reduction at a pace useful for bioremediation. Hydrocarbon contaminants can be subjected to biodegradation under nitrate reducing conditions, but this process is much

slower and may not be useful for bioremediation. DNRA and anammox metabolisms are hypothesized to be present under specific denitrifying conditions with no labile carbon sources and can cause a rebound in nitrate concentration.

It appears that the addition of a labile carbon source such as lactate led to faster depletion of nitrate compared to treatments with only hydrocarbons as a carbon source and many nitrate utilizing metabolisms taking place. It is likely that denitrification is taking place at the Baird and McGuire superfund site and is the reason why nitrate was depleted quickly after injection. The application of nitrate in the context of an anaerobic subsurface bioremediation for the purpose of inducing iron-oxidation conditions requires more research to make the technology more efficient, but this data is a starting point in characterizing exactly how microorganisms utilize the nitrate that is injected for bioremediation.

REFERENCES

Burgin, A. J., & Hamilton, S. K. (2007). Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. *Frontiers in Ecology and the Environment*, *5*(2), 89–96. https://doi.org/10.1890/1540-9295(2007)5[89:HWOTRO]2.0.CO;2

Coby, A. J., & Picardal, F. W. (2005). Inhibition of NO3- and NO2- reduction by microbial Fe(III) reduction: Evidence of a reaction between NO2- and cell surface-bound Fe2+. *Applied and Environmental Microbiology*, *71*(9), 5267–5274. https://doi.org/10.1128/AEM.71.9.5267-5274.2005

Dixit, S., & Hering, J. G. (2003). Comparison of Arsenic(V) and Arsenic(III) Sorption onto Iron Oxide Minerals: Implications for Arsenic Mobility. *Environmental Science & Technology*, *37*(18), 4182–4189. https://doi.org/10.1021/es030309t

Dou, J., Liu, X., & Ding, A. (2009). Anaerobic degradation of naphthalene by the mixed bacteria under nitrate reducing conditions. *Journal of Hazardous Materials*, *165*(1–3), 325–331. https://doi.org/10.1016/j.jhazmat.2008.10.002

Francis, C., & Casciotti, K. (2016). Geomicrobiology of Nitrogen. In *Ehrlich's Geomicrobiology* (6th ed.). Taylor and Francis Group.

Hu, Z., Lotti, T., de Kreuk, M., Kleerebezem, R., van Loosdrecht, M., Kruit, J., Jetten, M. S. M., & Kartal, B. (2013). Nitrogen Removal by a Nitritation-Anammox Bioreactor at Low Temperature. *Applied and Environmental Microbiology*, *79*(8), 2807–2812. https://doi.org/10.1128/AEM.03987-12

Huang, Y. H., & Zhang, T. C. (2004). Effects of low pH on nitrate reduction by iron powder. *Water Research*, *38*(11), 2631–2642. https://doi.org/10.1016/j.watres.2004.03.015

Kamphake, L. J., Hannah, S. A., & Cohen, J. M. (1967). Automated analysis for nitrate by hydrazine reduction. *Water Research*, *1*(3), 205–216. https://doi.org/10.1016/0043-1354(67)90011-5

Kappler, A., Emerson, D., Gralnick, J. A., Roden, E. E., & Muehe, E. M. (2016). Geomicrobiology of Iron. In *Ehrlich's Geomicrobiology* (6th ed.). Taylor and Francis Group.

Kartal, B., de Almeida, N. M., Maalcke, W. J., Op den Camp, H. J. M., Jetten, M. S. M., & Keltjens, J. T. (2013). How to make a living from anaerobic ammonium oxidation. *FEMS Microbiology Reviews*, *37*(3), 428–461. https://doi.org/10.1111/1574-6976.12014

Kümmel, S., Herbst, F.-A., Bahr, A., Duarte, M., Pieper, D. H., Jehmlich, N., Seifert, J., von Bergen, M., Bombach, P., Richnow, H. H., & Vogt, C. (2015). Anaerobic naphthalene degradation by sulfate-reducing Desulfobacteraceae from various anoxic aquifers. *FEMS Microbiology Ecology*, *91*(3). https://doi.org/10.1093/femsec/fiv006

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nature Reviews Microbiology*, *16*(5), Article 5. https://doi.org/10.1038/nrmicro.2018.9

Liu, T., Chen, D., Li, X., & Li, F. (2019). Microbially mediated coupling of nitrate reduction and Fe(II) oxidation under anoxic conditions. *FEMS Microbiology Ecology*, *95*(4), fiz030. https://doi.org/10.1093/femsec/fiz030

Melton, E. D., Swanner, E. D., Behrens, S., Schmidt, C., & Kappler, A. (2014). The interplay of microbially mediated and abiotic reactions in the biogeochemical Fe cycle. *Nature Reviews Microbiology*, *12*(12), 797–808. https://doi.org/10.1038/nrmicro3347

Picardal, F. (2012). Abiotic and Microbial Interactions during Anaerobic Transformations of Fe(II) and NOX-. *Frontiers in Microbiology*, *3*, 112. https://doi.org/10.3389/fmicb.2012.00112

Postma, D. (1990). Kinetics of nitrate reduction by detrital Fe(II)-silicates. *Geochimica et Cosmochimica Acta*, *54*(3), 903–908. https://doi.org/10.1016/0016-7037(90)90384-W

Rockne, K. J., Chee-Sanford, J. C., Sanford, R. A., Hedlund, B. P., Staley, J. T., & Strand, S. E. (2000). Anaerobic Naphthalene Degradation by Microbial Pure Cultures under Nitrate-Reducing Conditions. *Applied and Environmental Microbiology*, *66*(4), 1595–1601.

Straub, K. L., Benz, M., Schink, B., & Widdel, F. (1996). Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Applied and Environmental Microbiology*, *62*(4), 1458–1460. https://doi.org/10.1128/aem.62.4.1458-1460.1996

Strous, M., Heijnen, J. J., Kuenen, J. G., & Jetten, M. S. M. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Applied Microbiology and Biotechnology*, *50*(5), 589–596. https://doi.org/10.1007/s002530051340

Tiedje, J. (1988). Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties* (Vol. 717, pp. 179–244).

Valiente, N., Jirsa, F., Hein, T., Wanek, W., Prommer, J., Bonin, P., & Gómez-Alday, J. J. (2022). The role of coupled DNRA-Anammox during nitrate removal in a highly saline lake. *Science of The Total Environment*, *806*, 150726. https://doi.org/10.1016/j.scitotenv.2021.150726

van de Graaf, A. A., Bruijn, P., Robertson, L., Jetten, M., & Kuenen, J. G. (1996). Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology 142(8), 2187-2196. (1996)*, *142*. https://doi.org/10.1099/13500872-142-8-2187

van den Berg, E. M., van Dongen, U., Abbas, B., & van Loosdrecht, M. C. (2015). Enrichment of DNRA bacteria in a continuous culture. *The ISME Journal*, *9*(10), 2153–2161. https://doi.org/10.1038/ismej.2015.26

Varjani, S. J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresource Technology*, *223*, 277–286. https://doi.org/10.1016/j.biortech.2016.10.037

Zhang, M., Zhang, F., Ma, Z., & Wan, Y. (2019). Nitrogen Biogeochemistry of Anaerobic Biodegradation of Naphthalene. *Water, Air, & Soil Pollution*, *230*(9), 222. https://doi.org/10.1007/s11270-019-4276-9

Zhang, M., Zheng, P., Wang, R., Li, W., Lu, H., & Zhang, J. (2014). Nitrate-dependent anaerobic ferrous oxidation (NAFO) by denitrifying bacteria: A perspective autotrophic nitrogen pollution control technology. *Chemosphere*, *117*, 604–609. https://doi.org/10.1016/j.chemosphere.2014.09.029

II. IRON MINERALS AND ARSENIC SEQUESTRATION VIA ANAEROBIC NITRATE AMENDED GROUNDWATER BIOREMEDIATION

ABSTRACT

Arsenic is a leading groundwater contaminant at superfund sites in the United States and around the world. One of these sites is the Baird and McGuire superfund site located in Holbrook, MA. The main contaminants of concern include naphthalene and inorganic arsenite. This site is situated on top of a hill leading the groundwater to flow downhill and directly intersects with the nearby Cochato river. Due to the presence of naphthalene in the pores of the subsurface matrix, sustained reducing conditions are created that allows arsenic to become mobilized with the groundwater. Nitrate was being injected by Parsons Co. into the subsurface to stimulate anaerobic nitrate reducing bacteria to generate iron oxide minerals that are capable of arsenic sequestration, therefore, immobilizing the arsenic. It was found that under nitrate reducing conditions with labile carbon source present, the iron oxide mineral goethite is predominantly formed. Arsenic will be sequestered under nitrate reducing conditions, but it will be released easily back into the aqueous phase with changing redox conditions over a six-month period. However, if an abundance of ferrous iron is present, arsenic will not be released back into the aqueous phase over a six-month period. The following study assesses iron mineral development under nitrate reducing conditions and their arsenic sequestration capabilities.

1. INTRODUCTION

While arsenic is a common element found in low levels in the environment, it is one of the leading groundwater contaminants in the world (WHO, n.d.). In some areas of the world, such as Bangladesh, wells created to access anoxic and reduced aquifers tend to have high concentrations of arsenic naturally (Harvey et al., 2002; Nickson et al., 1998). However, arsenic contamination in groundwater is mainly a result of poor management of anthropogenic activities over time, mostly before the establishment of the EPA in the United States. Products that contain high concentrations of arsenic include pesticides, herbicides, desiccants, and wood preservatives (Mandal & Suzuki, 2002). The manufacturing process of these materials produces waste that is heavily contaminated with arsenic. The maximum allowed concentration of arsenic in drinking water is 0.01 mg/L by the EPA (US EPA, 2015) and before the EPA, there were no strict regulations for disposal of such waste, so it commonly found its way to the environment surrounding such facilities (US EPA, 2013).

The bioremediation of metalloids such as arsenic usually involves redox changes to the contaminated environment. This can be achieved biologically with microorganisms such as bacteria that are native to the contaminated site (Adriano et al., 2004). To remedy arsenic groundwater contamination, nitrate can be anthropogenically injected to act as an oxidant for the reduced subsurface. The application of nitrate keeps the environment under oxidizing conditions without amendments with oxygen.

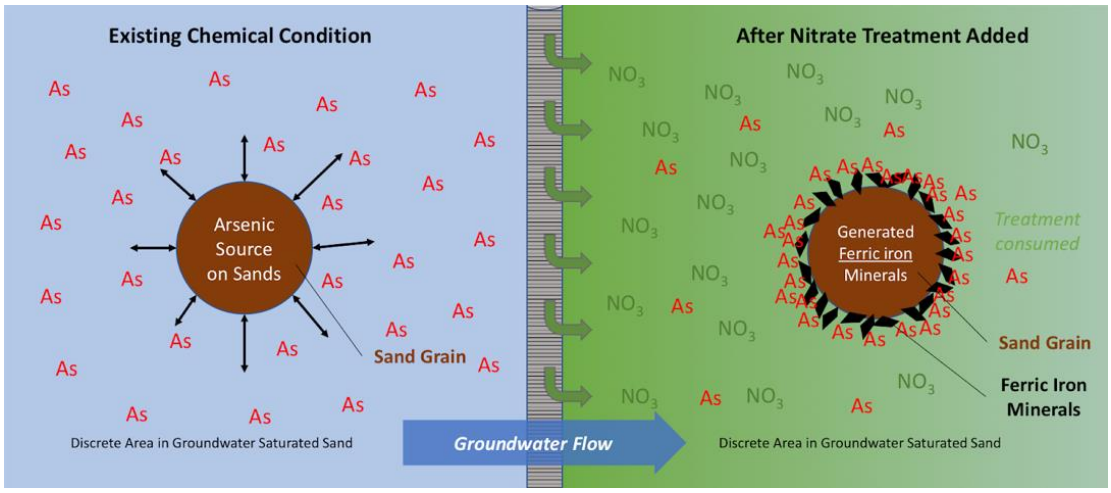

Figure 1. Nitrate bioremediation mechanism demonstrating how nitrate application can lead to iron oxidation and arsenic sequestration (Parsons Co. and MassDEP, 2022)

The available nitrate will act as an electron acceptor for microorganisms present, while ferrous iron (Fe^{2+}) will be oxidized into its ferric form (Fe^{3+}) (Figure 1). There are two pathways in nitrate reduction that can lead to the oxidation of iron: nitrite from denitrification can oxidize iron abiotically or bacteria can use ferrous iron as an electron donor (Coby & Picardal, 2005; Picardal, 2012; Schaedler et al., 2018; Straub et al., 1996). Under anaerobic conditions, the majority of iron oxidiation takes place biotically via autotrophic denitrification and a minority is caused by chemodenitrification- abiotic nitrite reaction with ferrous iron (Liu et al., 2019; Schaedler et al., 2018).

In an anaerobic subsurface environment, such as that found at the Baird and McGuire Superfund site, it is important to understand how conditions in the subsurface can be manipulated to generate iron minerals that will persist over time. If iron minerals are produced that are not stable over time, any arsenic that is sequestered will be mobilized again. There are a wide variety of iron minerals that can form under differing conditions in the subsurface. The form and stability of iron largely depends on the pH,

redox conditions, concentration of iron, availability of reducing or oxidizing compounds, and microbial community of the surrounding environment (Dixit & Hering, 2003; Salas et al., 2010). Ferrous iron minerals (iron sulfides, siderite, and pyrite) are stable in reducing conditions and ferric iron minerals (ferrihydrite, goethite, and hematite) are stable in oxidizing conditions (Kappler et al., 2016). It is known that reduced iron minerals such as ferrous sulfides are capable of lowering aqueous arsenic concentration (Jingtai & Fyfe, 2000).

The Baird and McGuire superfund site poses a unique situation where the subsurface is reducing and anoxic, but the addition of nitrate creates oxidizing conditions for a short period of time before it reverts to reducing when nitrate is depleted. Creating only ferric iron minerals would not be useful because any arsenic that had been bound would easily be released when conditions become reducing again and the minerals dissolve. An ideal iron mineral for this site is a mixed valence iron mineral such as magnetite because it would be stable under both oxidizing and reducing conditions (Mayo et al., 2007). Therefore, it would not be subjected to dissolution or other transformation that would lead to the release of any sequestered arsenic. Goethite has been shown to be the predominant iron mineral that develops under nitrate reducing conditions (Klueglein et al., 2014).

A nitrate amended pilot study was developed and implemented by Parsons Corporation at the Baird and McGuire superfund site to monitor how arsenic concentration changed over time. A series of nitrate injection pulses were administered in four wells. It was noted that nitrate was utilized very quickly under field conditions and as a result, nitrate was only delivered to shallow wells and did not disseminate to deeper

wells (Figure 2). Over time, arsenic concentrations began to decrease as seen in Figure 2 on days 10/11/21 and 11/30/21.

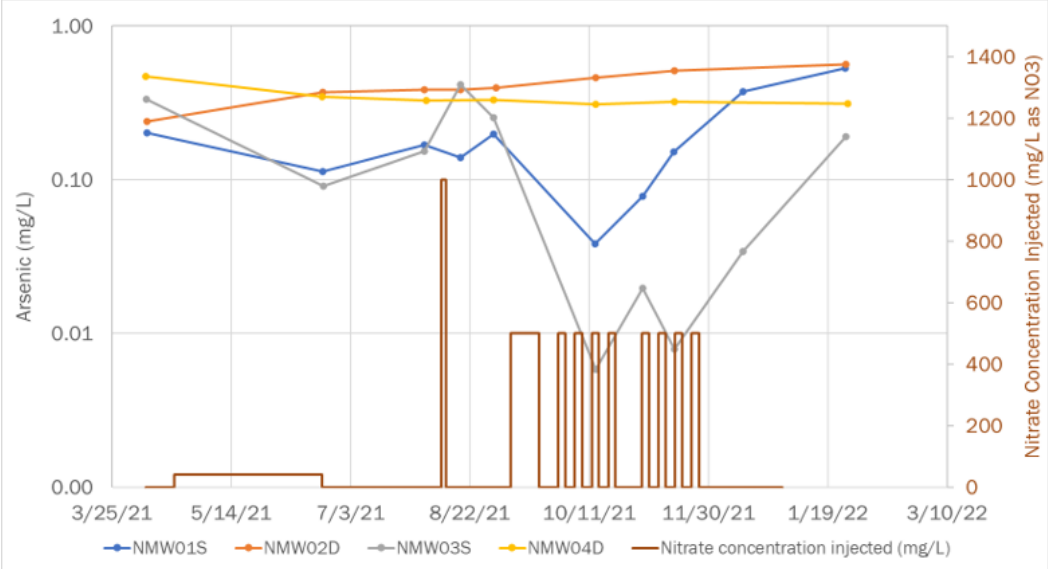

Figure 2. Arsenic concentration over time in wells treated with nitrate at the Baird and McGuire superfund site; preliminary results. Wells NMW01S and NMW03S are shallow in depth. Wells NMW02D and NMW04D are deep wells (Parsons Co. MassDEP, 2022).

However, by 1/19/22 arsenic concentrations had returned to their original concentration. This is hypothesized to be due to iron oxides developing, sequestering arsenic, and over time dissolving due to the reductive conditions of the aquifer. Thus, the arsenic is mobilized again. The following study investigates how arsenic reacts to differing carbon/electron sources and iron concentration treatments over time with nitrate amendment and what iron minerals predominantly develop under nitrate reducing conditions.

2. MATERIALS AND METHODS

2.1. SAMPLING SITE AND PROCEDURES

The Baird and McGuire superfund site is located in Holbrook, MA. The groundwater and soil were collected prior to nitrate amendment at the site. Two bottles of groundwater, with and without sediment, was collected in nitrogen purged 2.5 L amber jars, overfilled, and capped with no headspace. Soil samples were collected by using a direct push Geoprobe rig. Cores were collected in plastic sleeves and processed in the field by cutting the sleeves to expose soil. Grab and composite samples were placed in glass jars with no headspace and frozen until shipped under ice and overnight from Holbrook, MA to Missouri University of Science and Technology, Rolla MO.

2.2. TREATMENT STOICHIOMETRY

Microbial nitrate reducing and iron oxidizing metabolism adheres to the following stoichiometric equation (Straub et al., 1996):

$$10Fe^{2+}+2NO_3^-+24H_2O \rightarrow 10Fe(OH)_3+N_2+18H^+ \quad (1)$$

This equation applies to nitrate reducing iron oxidizing metabolisms regardless of the carbon source provided. Most microorganisms that are capable of this metabolism use organic sources of carbon, such as acetate, lactate, and more specifically aromatic hydrocarbons such as naphthalene (Rockne et al., 2000).

Based on this equation it was determined that 5 mM of ferrous iron is required to reduce 1 mM of nitrate to 1 mM nitrite within the enrichments in this study. Furthermore,

the abiotic reaction between nitrite and ferrous iron fulfills the following stoichiometry equation (Melton et al., 2014):

$$4Fe^{2+}+2NO_2+5H_2O \rightarrow 4FeOOH+N_2O+6H^+ \quad (2)$$

The following stoichiometric equation details sulfate reduction with naphthalene as a carbon source (Kümmel et al., 2015):

$$C_{10}H_8+6SO_4^{2-}+2H^++6H_2O \rightarrow 10HCO_3^-+6H_2S \quad (3)$$

From this equation, it has been determined that 48 electrons can be donated through the mineralization of naphthalene. Therefore, the electron ratio for naphthalene mineralization under nitrate reducing conditions is calculated to be 0.042 mM of naphthalene to reduce 1 mM of nitrate.

2.3. SOIL AND GROUNDWATER EXPERIMENT SETUP

Treatments were set up in 100 ml glass serum bottles that were cleaned in a laboratory dishwasher and autoclaved for 30 min at 121º C to sterilize. All bottles were set up in an anaerobic glove bag under 90% nitrogen gas and 10% hydrogen gas. 50 ml of groundwater with sediment from the Baird and McGuire Superfund site was pipetted into each serum bottle.

Five g of soil from each soil sample (six total samples) obtained from differing wells and depths at the site were added to each bottle, except for the groundwater only treatment. Each bottle was sealed with sterile butyl rubber stoppers and aluminum caps. All treatments were added to bottles with nitrogen degassed syringes. Each bottle was treated with 5 mM sodium bicarbonate as a pH buffer. All bottles except unamended

control received 1mM sodium nitrate. 10 mg/L of sodium arsenite solution was added to each bottle to assess sequestration over time as ferric iron minerals were formed. 0.2 ml of naphthalene/acetone stock solution was added to the naphthalene treatment bottles.

Table 1. Soil and groundwater experiment treatments.

Treatments	50ml sediment-containing groundwater	30g soil	10mg/L Sodium Arsenite	5mM Sodium Bicarbonate	1mM Sodium Nitrate	Ferrous Chloride	.5mM Sodium Lactate	5mg/L Naphthalene
unamended	X	X	X	X				
Only NO3	X	X	X	X	X			
Groundwater only	X		X	X	X			
Naphthalene	X	X	X	X	X			X
Lactate	X	X	X	X	X		X	
Groundwater with Fe removed +soil	X	X	X	X	X			
FeCl3 1mM	X	X	X	X	X	X		
FeCl3 1mM + lactate	X	X	X	X	X	X	X	
FeCl3 10mM	X	X	X	X	X	X		
FeCl3 10mM + lactate	X	X	X	X	X	X	X	

2.4. IRON MINERAL DEVELOPMENT EXPERIMENT SETUP

Treatments were set up in 25 ml glass serum bottles. Serum bottles were cleaned in a laboratory dishwasher and autoclaved for 30 min at 12º C to sterilize. All bottles were set up in an anaerobic glove bag under 90% nitrogen gas and 10% hydrogen gas. 5.3 mg/L naphthalene was delivered to six serum bottles with acetone (Dou et al., 2009). Stock solutions of 100 mM ferrous chloride, 500 mM nitrate, 1 M bicarbonate, and 500 mM lactate were used to amend each treatment. Unfiltered sediment-free groundwater was added to each bottle along with components of each treatment to a total volume of 20 ml. Treatments are as seen in table 2. A 10% inoculum was added from corresponding soil and groundwater enrichments. Controls of each treatment (except unamended) were created without inoculation.

All serum bottles were sealed with butyl rubber stoppers and crimp sealed with aluminum caps. Head spaces were exchanged with 100% nitrogen gas. All bottles were incubated at room temperature in the dark.

Table 2. Iron mineral experiment treatments

Treatments	Sediment-free groundwater	5mM ferrous chloride	5mM sodium bicarbonate	1mM sodium nitrate	10% inoculum	5.3mg/L naphthalene	2mM lactate
Unamended	**X**	**X**	**X**				
Just NO3	**X**	**X**	**X**	**X**	**X**		
Naphthalene	**X**	**X**	**X**	**X**	**X**	**X**	
Lactate	**X**	**X**	**X**	**X**	**X**		**X**

2.5. ANALYTICAL METHODS

Nitrate and nitrite concentrations were quantified colorimetrically (Kamphake et al., 1967). Total arsenic and ferrous iron in the liquid phase were quantified by ICPMS. For sampling, borosilicate glass HPLC vials were acid-washed with 20% nitric acid before use. Then vials were loaded with 200 µl of 2% nitric acid for sample preservation. 800 µl samples from each experimental serum bottle were filtered through 0.2 µm nylon filters to remove microorganisms and sediment and then added to the HPLC vials, crimped to seal. X-ray diffraction patterns were obtained utilizing a PANalytical X'Pert Multipurpose Diffractometer utilizing a copper source and a PIXcel detector.

3. RESULTS

3.1. IRON MINERAL DEVELOPMENT RESULTS

Over the course of three months, iron minerals precipitated in the serum bottles due to native bacteria utilizing differing carbon source treatments. Figure 3 depicts treatments unamended and just nitrate. All bottles exhibited the same fine and orange iron minerals that did not adhere well to the glass cover slips. Figure 6A depicts the iron minerals that developed in the nitrate-amended cultures under magnification. After six months of incubation, nitrate and nitrite concentrations were analyzed in each bottle. Nitrate was still present in all of the bottles and did not significantly decrease over the course of incubation and no nitrite was detected (Figure 3C). XRD analysis confirmed that the major iron mineral that precipitated in all of the bottles was siderite, $FeCO_3$, a ferrous iron carbonate.

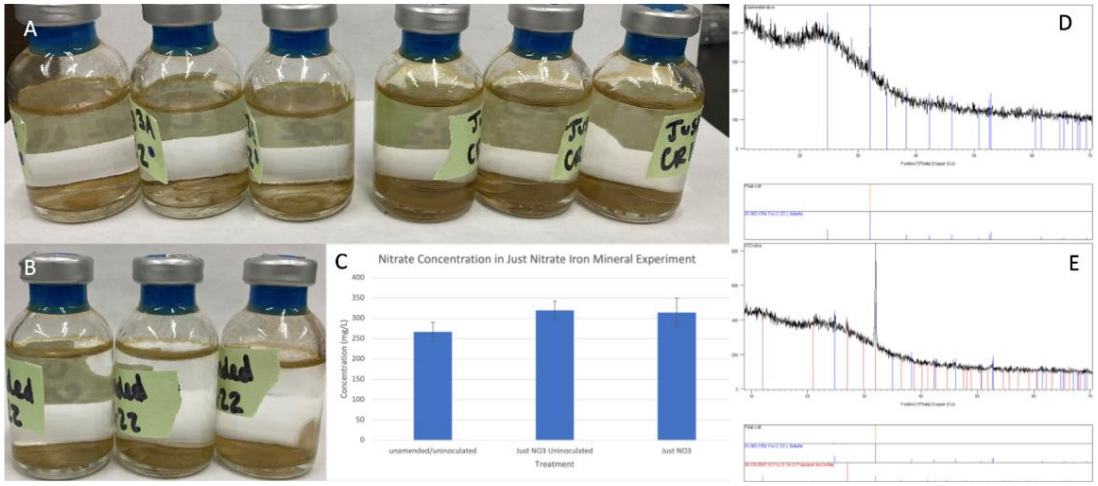

Figure 3. Iron mineral experiment unamended and nitrate-only treatments. Uninoculated just nitrate (left three bottles) and inoculated just nitrate treatments (right three bottles) (A), unamended uninoculated treatment (B), nitrate concentrations after six months of incubation (C), X-ray diffraction analysis results from unamended (D) and just nitrate inoculated (E).

Figure 4 shows results from the naphthalene treated iron mineral experiments. The left three bottles are uninoculated naphthalene bottles and the right three bottles are inoculated naphthalene bottles (Figure 4A). The iron minerals that developed were orange, stuck to the glass cover slips, and formed a thick layer at the bottom of the bottles. Figure 6B depicts the iron minerals that developed in the naphthalene amended bottles under magnification. After three months of incubation, nitrate and nitrite concentrations were analyzed from each bottle. Nitrate was utilized fully in the inoculated naphthalene bottles and was not utilized in the uninoculated bottles with no nitrite present. The XRD analysis indicated that the iron mineral present in the inoculated naphthalene bottles was goethite FeO(OH), a ferric iron oxide-hydroxide (Figure 4C).

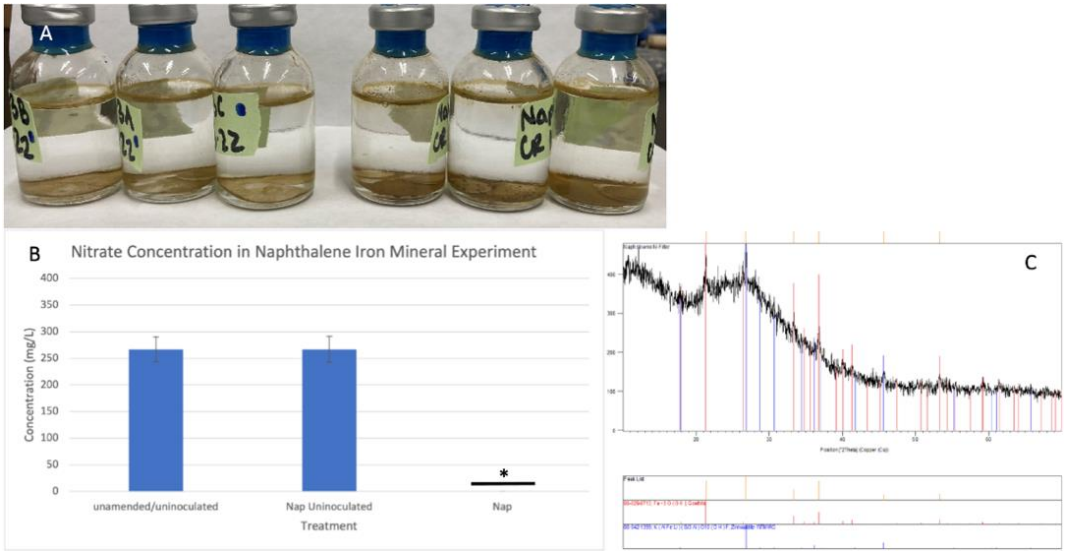

Figure 4. Naphthalene amended iron mineral experiment. Uninoculated naphthalene amended (left three bottles) and inoculated naphthalene amended (right three bottles) (A), nitrate concentrations in unamended, uninoculated naphthalene, and inoculated naphthalene bottles after six months (B), x-ray diffraction results from inoculated naphthalene treatment.

Figure 5 includes results from the lactate amended iron mineral experiments. The left three bottles are uninoculated lactate bottles and the right three bottles are inoculated lactate bottles. Within these two treatments, the color of the minerals is strikingly different. Both sets of bottles developed orange iron minerals first, but over the course of three months, the inoculated lactate treatment iron minerals turned black (Figure 5A). Figure 6C depicts the iron minerals that developed in the lactate amended bottles. After three months, nitrate and nitrite concentrations were analyzed in all bottles. All the bottles amended with lactate showed a complete utilization of nitrate and no nitrite was present (Figure 5B).

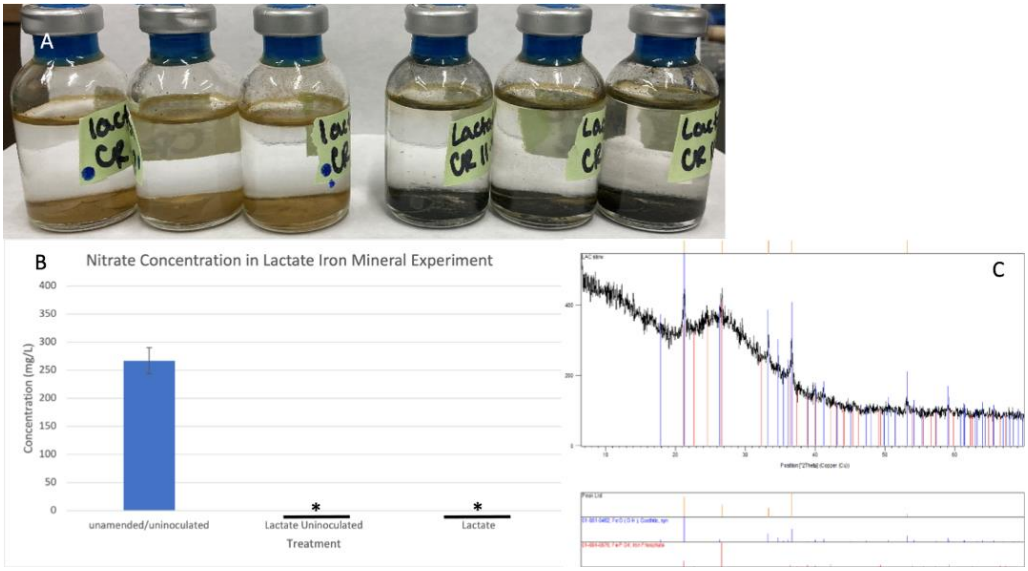

Figure 5. Lactate amended iron mineral experiment. Uninoculated lactate amended (left three bottles) and inoculated lactate amended (right three bottles) (A), nitrate concentrations in unamended, uninoculated lactate, and inoculated lactate bottles after six months (B), x-ray diffraction results from inoculated lactate treatment (C).

XRD analysis indicated that the iron mineral present within the inoculated lactate bottles was goethite, just like what was seen in the naphthalene amended treatment.

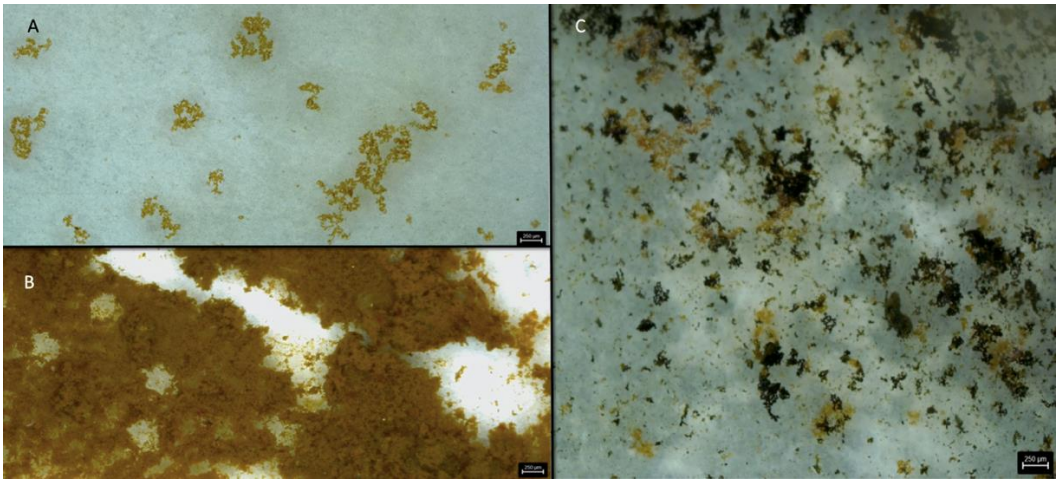

Figure 6. Iron minerals under 150x magnification. Developed in just nitrate treatment (A), naphthalene amended treatment (B), and lactate amended treatment (C) Images were taken after 3 months of incubation.

3.2. SOIL AND GROUNDWATER EXPERIMENT RESULTS

3.2.1. Nitrate-Only Amended Versus Lactate Amended Treatments. Nitrate concentrations over time decreased in both treatments, but at differing rates. When a labile carbon/electron source is present such as lactate, nitrate is reduced readily and quicker than if only hydrocarbon contaminants were present as carbon/electron sources. The initial concentration of arsenic was over 10 mg/L**,** but within the first day only two mg/L was left in the aqueous phase in both treatments. The arsenic that was sequestered was done so quicker in the nitrate-only treatment compared to the lactate treatment. The lowest concentration of arsenic in the nitrate-only treatments is seen on day one and the lowest seen in the lactate treatment is day 17 (Figure 7). The nitrate-only treatment experienced quicker arsenic release compared to the lactate treatment. By the end of six months, about seven mg/L of arsenic was released into the aqueous phase in the nitrate-only bottles (Figure 7A), but only about one mg/L of arsenic had been released over six months in the lactate amended bottles (Figure 7B). Dissolved iron concentration stays relatively constant in the nitrate-only treatment (Figure 7A). Iron concentration in the aqueous phase decreases about 0.5 mM from day one to day 17 in the lactate amended bottles, but rebounds back to 1mM after 6 months.

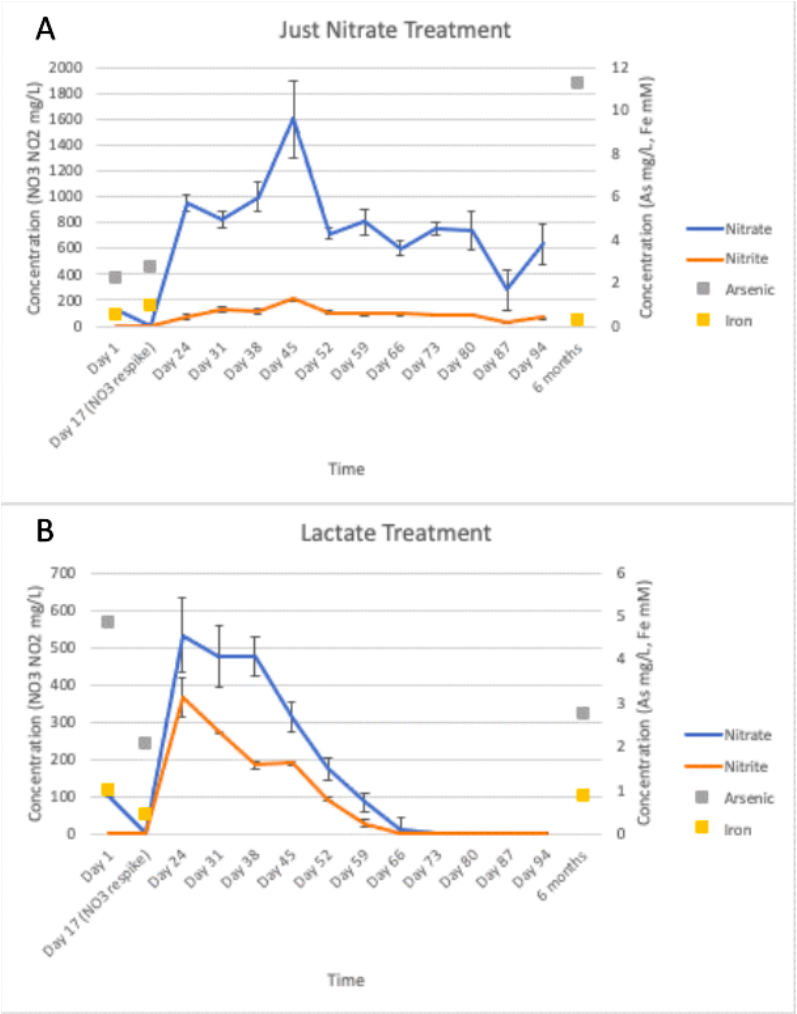

Figure 7. Nitrate-only (A) versus lactate (B) amended NO_3, NO_2, As, and Fe concentrations.

3.2.2. Ferrous Iron 10 mM Versus Ferrous Iron 10 mM + Lactate Amended Treatments. The nitrate and nitrite concentrations in the 10 mM ferrous iron treatments were similar in pattern to those in Figure 7, with major differences caused by the availability of lactate or not (Figure 8).

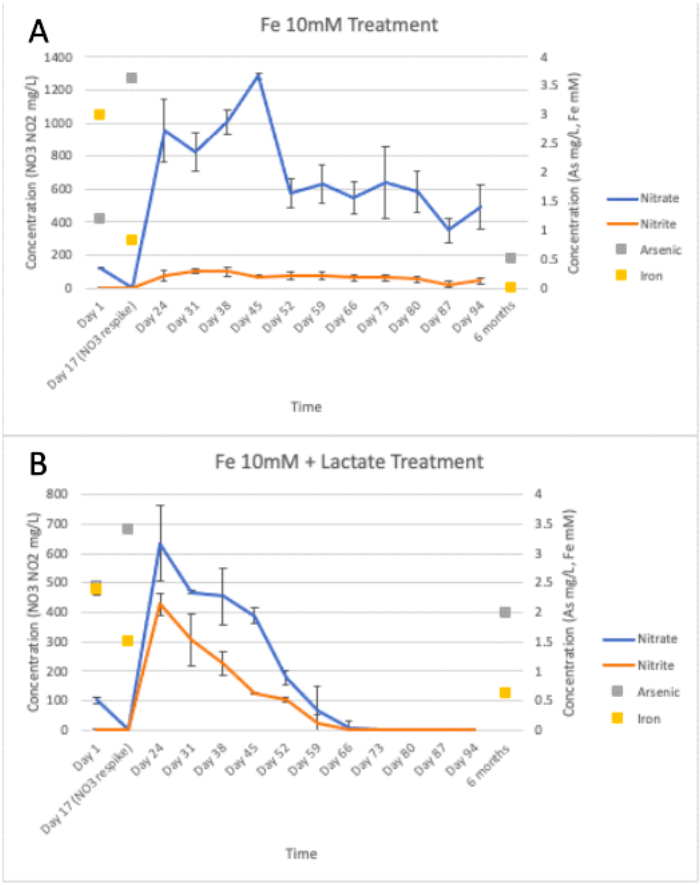

Figure 8. Fe^{2+} 10 mM (A) and Fe^{2+} 10mM + lactate (B) NO_3, NO_2, As, and Fe concentrations.

Arsenic concentration in both treatments initially decreased from the added 10 mg/L to one mg/L and 2.5 mg/L, respectively. After the 10 mM respike of nitrate on day 17, arsenic increased in both treatments to about 3.5 mg/L. Both treatments experienced iron oxidation from the initially added 10 mM concentration to about one mg/L after 17 days. Once the bottles were spiked with 10 mM nitrate, aqueous iron concentration continued to decrease over six months. The non lactate amended bottles dissolved iron reached zero mM after six months.

3.2.3. Unamended Treatment. Nitrate and nitrite concentrations were zero throughout the experiment because neither were added to any of the bottles. Dissolved iron concentration decreased from day one to day 17. After day 17, the iron concentration increased by four mg/L and arsenic concentration increased by nine mg/L (Figure 9).

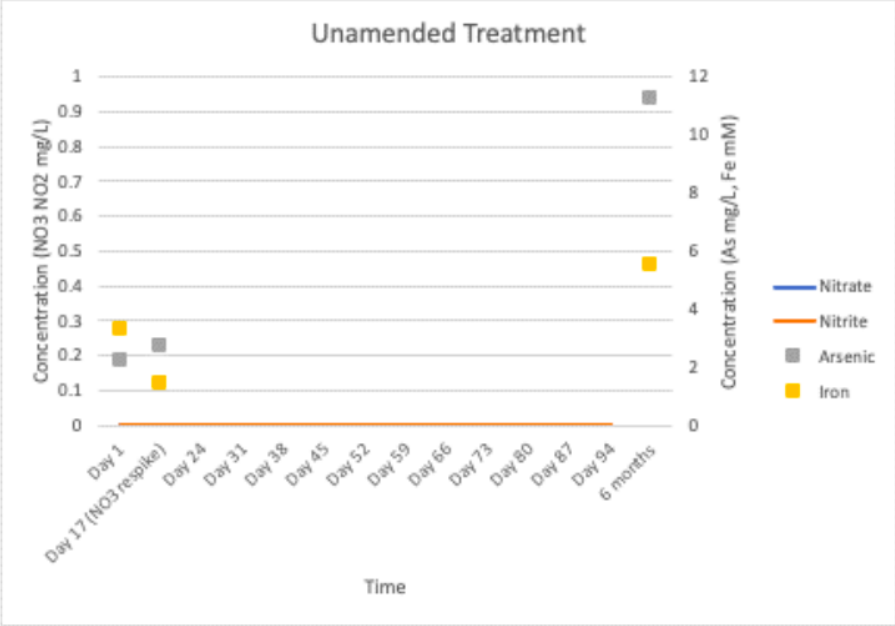

Figure 9. Unamended treatment NO_3, NO_2, As, and Fe concentrations.

4. DISCUSSION

4.1. NITRATE UTILIZATION AND IRON MINERAL DEVELOPMENT

The iron mineral that developed within the nitrate-only and unamended bottles was a ferrous iron carbonate, siderite $FeCO_3$ (Figure 3A and B). This iron mineral can develop abiotically if enough bicarbonate and ferrous iron are present. Microbial activity did not bring about the development of siderite because the nitrate that was provided was

not utilized. Nitrate concentration did not decrease because no carbon/electron source was added and indicates that sediment-free groundwater from the Baird and McGuire superfund site does not contain enough hydrocarbon contaminants to facilitate microbial activity. This also implies that the hydrocarbons found at this site are sorbed to soil particles and are not dissolved in the aqueous phase at concentrations useful for microbial metabolism.

The naphthalene amended bottles only exhibited nitrate reduction in the bottles inoculated with a higher concentration of bacteria from the site. It is not surprising that nitrate reduction did not occur in the bottles with only groundwater and no inoculum because microorganisms that are acclimated to using naphthalene as a carbon source would most likely be found in soil where naphthalene is present. By inoculating the naphthalene bottles with a sample from the naphthalene amended soil and groundwater experiment, microbes were added to this iron mineral experiment that were already enriched for their capacity to biodegrade naphthalene. It is noted that the groundwater without any amendments contained too few bacteria to significantly reduce the nitrate concentration over time. The main iron mineral that developed in these bottles was goethite, the same iron mineral that developed in the lactate amended bottles.

All nitrate added to the bottles amended with lactate was depleted by the time sampling occurred after three months. The bacterial concentration was different in the bottles between uninoculated and inoculated, but despite this, all nitrate was reduced. The groundwater was not filter sterilized, so a small number of bacteria found in the undisturbed groundwater naturally was in every bottle. This nitrate data reinforces that as

a labile carbon/electron source, lactate can facilitate nitrate reduction fully even with a small population of microorganisms present.

4.2. IRON SULFIDE DEVELOPMENT

The difference in iron mineral color between the naphthalene and lactate amended bottles was surprising, especially because the XRD analysis indicated that the minerals formed in each were goethite. Because of this, it was speculated that the black color was due to the development of a ferrous iron mineral—iron sulfide. The inoculum for the lactate amended bottles came from its corresponding lactate amended soil and groundwater experiment that was also amended with lactate. Under nitrate reducing conditions with lactate as a carbon source, iron sulfides will readily oxidize to ferric iron and sulfate (Haaijer et al., 2007). With free sulfate and lactate present, any bacterium that is capable of sulfate reduction can take advantage of these conditions and reduce sulfate to hydrogen sulfide. This gas will then abiotically react with ferrous iron and create black iron sulfides again. This cycle will keep taking place as long as nitrate and lactate are present (Zhang et al., 2009). The soil experiments are known to already contain iron sulfides in the soil naturally, so some of the sulfate must have transferred over to the iron mineral experiment during inoculation to drive iron sulfide development over three months. As a result, iron sulfide coated the goethite and turned the orange mineral black. This can be seen in figure 6C where black and orange minerals appear together under magnification.

4.4. ARSENIC AND IRON CONCENTRATIONS OVER SIX MONTHS

Over six months, the lactate bottles released less arsenic than the nonlactate amended bottles (Figure 7B). The presence of lactate causes not only rapid nitrate reduction and iron oxidation but also slower arsenic release over time compared to bottles with hydrocarbon contaminants as the only carbon source. The slower release of arsenic is most likely due to the development of iron minerals that are more stable under the conditions that are present such as goethite as shown in Figure 5. The iron minerals that developed in the nitrate-only bottles were most likely siderite and/or amorphous iron oxides as shown in Figure 3. Arsenic was initially sequestered to about 2 mg/L on day one, after the initial 10 mg/L addition of arsenic. However, arsenic was released over six months to a concentration slightly above what was added. The arsenic data in the nitrate-only treatments is similar to what was seen in the Parsons Co. nitrate pilot study. In both studies arsenic was initially sequestered, but over time was released to concentrations higher than they were before nitrate amendment (Figure 2).

When an abundance of reduced iron is available, a significant amount of arsenic can be sequestered over six months (Figure 8). After the 10 mM respike of nitrate on day 17, iron oxidation continued and lead to significant decrease in dissolved arsenic concentration. The bottles not treated with lactate ended up sequestering more arsenic than if lactate was present. This may be due to competing iron reducing bacteria that would also utilize the ferric iron resulting from ferrous iron oxidation from any produced nitrite. Based on this data, when iron is abundantly available, arsenic will be sequestered and not be released back into the aqueous phase over a six-month period. It is suspected

that this is due to a binding site to an iron mineral not being the limiting factor for arsenic sequestration.

The unamended bottles received only bicarbonate as a pH buffer and arsenic. The arsenic concentration being lower than the initial 10 mg/L that was added indicates that arsenic sequestration took place even when no electron acceptor was added (Figure 9). There is less iron in the aqueous phase overall in all treatments compared to the unamended control treatment confirming that iron oxidation was taking place in all bottles, but at different capacities due to differing treatments. The sediment in the unamended bottles turned black over time perhaps due to iron sulfide development due to potential sulfate reduction or black goethite development due to potential iron oxidiation. There may have been an autotrophic metabolism taking place because only bicarbonate was added to the groundwater. Iron sulfides have been shown to be used as electron donors for nitrate utilizing autotrophic metabolisms (Haaijer et al., 2007). No nitrate was present in the unamended bottles, so perhaps any iron oxides that were present were utilized as electron acceptors.

5. CONCLUSIONS

Nitrate reducing conditions leads to arsenic sequestration onto oxidized iron minerals, but the release rate of that arsenic from the iron minerals depends on the carbon/electron source available and the abundance of iron. The addition of lactate slows the rebound in arsenic concentrations. An abundance of ferrous iron leads to significant

arsenic sequestration with no release over the course of six months. This is most likely due to plenty of oxidized iron minerals for arsenic to sorb to.

The development of iron sulfides may be a viable option for subsurface arsenic bioremediation. Iron sulfides are capable of arsenic sequestration and are stable under reducing conditions. However, they are susceptible to dissolution under oxidizing conditions that would lead to the release of any sequestered arsenic. Goethite is shown to be the predominant iron minerals that develops under nitrate reducing conditions. This ferric iron mineral capable of arsenic sequestration, however, it is not an ideal solution for retaining arsenic and preventing subsequent groundwater migration at the Baird and McGuire Superfund site. Once nitrate is depleted, conditions will go from oxidizing (goethite is stable) back to reducing (goethite is not stable) and any arsenic that was bound to goethite will be released as the mineral dissolves. Due to this, goethite would not be an efficient arsenic sequestering iron mineral over a long period of time.

REFERENCES

Adriano, D. C., Wenzel, W. W., Vangronsveld, J., & Bolan, N. S. (2004). Role of assisted natural remediation in environmental cleanup. *Geoderma*, *122*(2), 121–142. https://doi.org/10.1016/j.geoderma.2004.01.003

Coby, A. J., & Picardal, F. W. (2005). Inhibition of NO3- and NO2- reduction by microbial Fe(III) reduction: Evidence of a reaction between NO2- and cell surface-bound Fe2+. *Applied and Environmental Microbiology*, *71*(9), 5267–5274. https://doi.org/10.1128/AEM.71.9.5267-5274.2005

Dixit, S., & Hering, J. G. (2003). Comparison of Arsenic(V) and Arsenic(III) Sorption onto Iron Oxide Minerals: Implications for Arsenic Mobility. *Environmental Science & Technology*, *37*(18), 4182–4189. https://doi.org/10.1021/es030309t

Dou, J., Liu, X., & Ding, A. (2009). Anaerobic degradation of naphthalene by the mixed bacteria under nitrate reducing conditions. *Journal of Hazardous Materials*, *165*(1–3), 325–331. https://doi.org/10.1016/j.jhazmat.2008.10.002

Ehrlich, H. L. (2016). Biogenesis and Biodegradation of Sulfide Minerals in the Earth's Surface. In *Ehrlich's Geomicrobiology* (6th ed., pp. 517–541). CRC Press.

Haaijer, S. C. M., Lamers, L. P. M., Smolders, A. J. P., Jetten, M. S. M., & Op den Camp, H. J. M. (2007). Iron Sulfide and Pyrite as Potential Electron Donors for Microbial Nitrate Reduction in Freshwater Wetlands. *Geomicrobiology Journal*, *24*(5), 391–401. https://doi.org/10.1080/01490450701436489

Harvey, C. F., Swartz, C. H., Badruzzaman, A. B. M., Keon-Blute, N., Yu, W., Ali, M. A., Jay, J., Beckie, R., Niedan, V., Brabander, D., Oates, P. M., Ashfaque, K. N., Islam, S., Hemond, H. F., & Ahmed, M. F. (2002). Arsenic Mobility and Groundwater Extraction in Bangladesh. *Science*, *298*(5598), 1602–1606. https://doi.org/10.1126/science.1076978

Jingtai, H., & Fyfe, W. (2000). *Arsenic removal from water by iron-sulphide minerals*. *45*(15).

Kamphake, L. J., Hannah, S. A., & Cohen, J. M. (1967). Automated analysis for nitrate by hydrazine reduction. *Water Research*, *1*(3), 205–216. https://doi.org/10.1016/0043-1354(67)90011-5

Kappler, A., Emerson, D., Gralnick, J. A., Roden, E. E., & Muehe, E. M. (2016). Geomicrobiology of Iron. In *Ehrlich's Geomicrobiology* (6th ed.). Taylor and Francis Group.

Klueglein, N., Zeitvogel, F., Stierhof, Y.-D., Floetenmeyer, M., Konhauser, K. O., Kappler, A., & Obst, M. (2014). Potential Role of Nitrite for Abiotic Fe(II) Oxidation and Cell Encrustation during Nitrate Reduction by Denitrifying Bacteria. *Applied and Environmental Microbiology*, *80*(3), 1051–1061. https://doi.org/10.1128/AEM.03277-13

Kümmel, S., Herbst, F.-A., Bahr, A., Duarte, M., Pieper, D. H., Jehmlich, N., Seifert, J., von Bergen, M., Bombach, P., Richnow, H. H., & Vogt, C. (2015). Anaerobic naphthalene degradation by sulfate-reducing Desulfobacteraceae from various anoxic aquifers. *FEMS Microbiology Ecology*, *91*(3). https://doi.org/10.1093/femsec/fiv006

Mandal, B. K., & Suzuki, K. T. (2002). Arsenic round the world: A review. *Talanta*, *58*(1), 201–235. https://doi.org/10.1016/S0039-9140(02)00268-0

Mayo, J. T., Yavuz, C., Yean, S., Cong, L., Shipley, H., Yu, W., Falkner, J., Kan, A., Tomson, M., & Colvin, V. L. (2007). The effect of nanocrystalline magnetite size on arsenic removal. *Science and Technology of Advanced Materials*, *8*(1), 71–75. https://doi.org/10.1016/j.stam.2006.10.005

Melton, E. D., Swanner, E. D., Behrens, S., Schmidt, C., & Kappler, A. (2014). The interplay of microbially mediated and abiotic reactions in the biogeochemical Fe cycle. *Nature Reviews Microbiology*, *12*(12), 797–808. https://doi.org/10.1038/nrmicro3347

Nickson, R., McArthur, J., Burgess, W., Ahmed, K. M., Ravenscroft, P., & Rahmanñ, M. (1998). Arsenic poisoning of Bangladesh groundwater. *Nature*, *395*(6700), Article 6700. https://doi.org/10.1038/26387

Picardal, F. (2012). Abiotic and Microbial Interactions during Anaerobic Transformations of Fe(II) and NOX-. *Frontiers in Microbiology*, *3*, 112. https://doi.org/10.3389/fmicb.2012.00112

Rockne, K. J., Chee-Sanford, J. C., Sanford, R. A., Hedlund, B. P., Staley, J. T., & Strand, S. E. (2000). Anaerobic Naphthalene Degradation by Microbial Pure Cultures under Nitrate-Reducing Conditions. *Applied and Environmental Microbiology*, *66*(4), 1595–1601.

Salas, E. C., Berelson, W. M., Hammond, D. E., Kampf, A. R., & Nealson, K. H. (2010). The Impact of Bacterial Strain on the Products of Dissimilatory Iron Reduction. *Geochimica et Cosmochimica Acta*, *74*(2), 574–583. https://doi.org/10.1016/j.gca.2009.10.039

Schaedler, F., Lockwood, C., Lueder, U., Glombitza, C., Kappler, A., & Schmidt, C. (2018). Microbially Mediated Coupling of Fe and N Cycles by Nitrate-Reducing Fe(II)-Oxidizing Bacteria in Littoral Freshwater Sediments. *Applied and Environmental Microbiology*, *84*(2), e02013-17. https://doi.org/10.1128/AEM.02013-17

Straub, K. L., Benz, M., Schink, B., & Widdel, F. (1996). Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Applied and Environmental Microbiology*, *62*(4), 1458–1460. https://doi.org/10.1128/aem.62.4.1458-1460.1996

US EPA, O. (2013, January 29). *EPA History: Resource Conservation and Recovery Act* [Collections and Lists]. https://www.epa.gov/history/epa-history-resource-conservation-and-recovery-act

US EPA, O. (2015, October 13). *Drinking Water Arsenic Rule History* [Collections and Lists]. https://www.epa.gov/dwreginfo/drinking-water-arsenic-rule-history

WHO. (n.d.). *Arsenic*. Retrieved September 7, 2022, from https://www.who.int/news-room/fact-sheets/detail/arsenic

Zhang, Y.-C., Slomp, C. P., Broers, H. P., Passier, H. F., & Cappellen, P. V. (2009). Denitrification coupled to pyrite oxidation and changes in groundwater quality in a shallow sandy aquifer. *Geochimica et Cosmochimica Acta*, *73*(22), 6716–6726. https://doi.org/10.1016/j.gca.2009.08.026

SECTION

3. CONCLUSIONS

Autotrophic nitrate dependent iron oxidizing metabolism could not be selected for under artificial medium conditions. However, this does not mean that this metabolism is not present at the site. It is hypothesized that this metabolism is present but is a small capacity due to being outcompeted by metabolisms that can utilize organic carbon sources e.g., the hydrocarbon contaminants or added labile carbon.

A labile carbon/electron source such as lactate can facilitate rapid nitrate reduction and iron oxidation even if a small population of bacteria is present. Naphthalene can be utilized as a carbon/electron source for nitrate reduction and subsequent iron oxidation, but it will take a longer period of time. There is a tradeoff in the decision to add a carbon source or utilize hydrocarbon contaminants for bioremediation. It may cost more to inject a labile carbon/electron source and nitrate into the subsurface, but results will be seen quickly. It will mostly likely cost less to just inject nitrate into the subsurface but relying on the indigenous bacteria to utilize the hydrocarbon contaminants as carbon/electron sources will slow down the bioremediation. This study demonstrates that biodegradation of many hydrocarbon contaminants can take place in the subsurface of the Baird and McGuire superfund site with the addition of nitrate as an electron acceptor.

Based on nitrate and nitrite data gathered over time from the non-lactate amended soil and groundwater experiment, DNRA and anammox metabolisms are suspected to be present due to an unexpected rebound in nitrate concentration.

Goethite developed under heterotrophic nitrate reducing conditions with an added carbon/electron source such as lactate or naphthalene. The development of goethite as an arsenic sequestration tool is not recommended in the long term because it is stable in oxidizing conditions, while the natural redox conditions of the Baird and McGuire superfund site are reducing. So, over time in a reducing environment goethite will be reduced to ferrous iron. If no easily oxidizable carbon/electron source is present, siderite can form abiotically if enough bicarbonate and ferrous iron is present.

Nitrate-only and nitrate/lactate amended soil and groundwater can sequester arsenic, but readily releases the arsenic back into the aqueous phase over a six-month period. Lactate amended bottles experienced slower arsenic release than the nitrate only treatment. If an abundance of iron is present, all but 1-0.5 mg/L of arsenic will be sequestered over a period of six-months. This is most likely due to binding sites for arsenic not being a limiting factor in sequestration.

This data can be utilized in future studies about the Baird and McGuire superfund site as a baseline. The conditions tested here demonstrate how nitrate is utilized by the microbial population and what iron minerals develop under nitrate reduction. Further studies on how to control what iron minerals develop under nitrate reduction are needed, such as the development of the mixed iron mineral magnetite that would be stable and sequester arsenic for a long period of time.

BIBLIOGRAPHY

Acton, D. W., & Barker, J. F. (1992). In situ biodegradation potential of aromatic hydrocarbons in anaerobic groundwaters. *Journal of Contaminant Hydrology*, *9*(4), 325–352. https://doi.org/10.1016/0169-7722(92)90002-V

Borch, T., R, K., A, K., Pv, C., M, G.-V., A, V., & K, C. (2010). Biogeochemical redox processes and their impact on contaminant dynamics. *Environmental Science & Technology*, *44*(1). https://doi.org/10.1021/es9026248

Cozzarelli, I. M., Schreiber, M. E., Erickson, M. L., & Ziegler, B. A. (2016). Arsenic Cycling in Hydrocarbon Plumes: Secondary Effects of Natural Attenuation. *Groundwater*, *54*(1), 35–45. https://doi.org/10.1111/gwat.12316

Ehrlich, H. L. (2016). Biogenesis and Biodegradation of Sulfide Minerals in the Earth's Surface. In *Ehrlich's Geomicrobiology* (6th ed., pp. 517–541). CRC Press.

Francis, C., & Casciotti, K. (2016). Geomicrobiology of Nitrogen. In *Ehrlich's Geomicrobiology* (6th ed.). Taylor and Francis Group.

Hajji, S., Montes-Hernandez, G., Sarret, G., Tordo, A., Morin, G., Ona-Nguema, G., Bureau, S., Turki, T., & Mzoughi, N. (2019). Arsenite and chromate sequestration onto ferrihydrite, siderite and goethite nanostructured minerals: Isotherms from flow-through reactor experiments and XAS measurements. *Journal of Hazardous Materials*, *362*, 358–367. https://doi.org/10.1016/j.jhazmat.2018.09.031

Harvey, C. F., Swartz, C. H., Badruzzaman, A. B. M., Keon-Blute, N., Yu, W., Ali, M. A., Jay, J., Beckie, R., Niedan, V., Brabander, D., Oates, P. M., Ashfaque, K. N., Islam, S., Hemond, H. F., & Ahmed, M. F. (2002). Arsenic Mobility and Groundwater Extraction in Bangladesh. *Science*, *298*(5598), 1602–1606. https://doi.org/10.1126/science.1076978

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nature Reviews Microbiology*, *16*(5), Article 5. https://doi.org/10.1038/nrmicro.2018.9

Laha, A., Sengupta, S., Bhattacharya, P., Mandal, J., Bhattacharyya, S., & Bhattacharyya, K. (2022). Recent advances in the bioremediation of arsenic-contaminated soils: A mini review. *World Journal of Microbiology and Biotechnology*, *38*(11), 189. https://doi.org/10.1007/s11274-022-03375-5

Mandal, B. K., & Suzuki, K. T. (2002). Arsenic round the world: A review. *Talanta*, *58*(1), 201–235. https://doi.org/10.1016/S0039-9140(02)00268-0

Mercer, J. W., Skipp, D. C., & Giffin, D. (n.d.). *Basics of Pump-and-Treat Ground-Water Remediation Technology*.

National Research Council. 1993. In Situ Bioremediation: When Does it Work?. Washington, DC: The National Academies Press. https://doi.org/10.17226/2131.

Nickson, R., McArthur, J., Burgess, W., Ahmed, K. M., Ravenscroft, P., & Rahmanñ, M. (1998). Arsenic poisoning of Bangladesh groundwater. *Nature*, *395*(6700), Article 6700. https://doi.org/10.1038/26387

Oremland, R. (2016). Geomicrobial Interactions with Arsenic and Antimony. In *Ehrlich's Geomicrobiology* (6th ed.). Taylor and Francis Group.

Sharma, J. & Vice Principal & Assistant Professor, Department of Biotechnology, Vijayaraje Institute of Science and Management, NH-75, Turari, Jhansi Road, Gwalior (MP) 475001, India. (2019). Advantages and Limitations of In Situ Methods of Bioremediation. *Recent Advances in Biology and Medicine*, *5*, 1. https://doi.org/10.18639/RABM.2019.955923

Sun, J., Chillrud, S. N., Mailloux, B. J., & Bostick, B. C. (2016). In Situ Magnetite Formation and Long-Term Arsenic Immobilization under Advective Flow Conditions. *Environmental Science & Technology*, *50*(18), 10162–10171. https://doi.org/10.1021/acs.est.6b02362

Sun, W., Sierra-Alvarez, R., Milner, L., Oremland, R., & Field, J. A. (2009). Arsenite and Ferrous Iron Oxidation Linked to Chemolithotrophic Denitrification for the Immobilization of Arsenic in Anoxic Environments. *Environmental Science & Technology*, *43*(17), 6585–6591.

Teclu, D., Tivchev, G., Laing, M., & Wallis, M. (2008). Bioremoval of arsenic species from contaminated waters by sulphate-reducing bacteria. *Water Research*, *42*(19), 4885–4893. https://doi.org/10.1016/j.watres.2008.09.010

US EPA, (2020, May 20). *Milestones in EPA and Environmental History* [Collections and Lists]. https://www.epa.gov/history/milestones-epa-and-environmental-history

Vidonish, J. E., Zygourakis, K., Masiello, C. A., Sabadell, G., & Alvarez, P. J. J. (2016). Thermal Treatment of Hydrocarbon-Impacted Soils: A Review of Technology Innovation for Sustainable Remediation. *Engineering*, *2*(4), 426–437. https://doi.org/10.1016/J.ENG.2016.04.005

WHO. (n.d.). *Arsenic*. Retrieved September 7, 2022, from https://www.who.int/news-room/fact-sheets/detail/arsenic

Zhuang, F., Huang, J., Li, H., Peng, X., Xia, L., Zhou, L., Zhang, T., Liu, Z., He, Q., Luo, F., Yin, H., & Meng, D. (2023). Biogeochemical behavior and pollution control of arsenic in mining areas: A review. *Frontiers in Microbiology*, *14*. https://www.frontiersin.org/articles/10.3389/fmicb.2023.1043024

VITA

Cassie Marie Roberts was born in North Branford, Connecticut. At the age of 8, she moved with her family to Troy, Missouri, where she spent the rest of her childhood and teenage years. She attended Troy Buchanan High School where she graduated in the top 10% of her class. She then went on to receive a Bachelor of Science degree in Biological Sciences from Missouri University of Science and Technology in May of 2021. During her undergraduate time there she began to work in Dr. Melanie Mormile's environmental microbiology research lab in 2018. She contributed to the characterization of a new halophilic alkaphilic bacterium and completed an OURE (Opportunities for Undergraduate Research Experience) pertaining to desiccation tolerance of halophilic bacteria. She then continued as a graduate student in Dr. Mormile's lab studying anaerobic hydrocarbon and arsenic bioremediation for her thesis project. She also worked on other projects in the lab such as studying economically feasible feed stocks for *Halanaerobium hydrogeniformans* and genetically transforming the bacterium *Halomonas campisalis*. She earned her Master of Science degree in Applied and Environmental Biology from the Missouri University of Science and Technology in May of 2023. After graduation, she planned to obtain a job in environmental consulting/bioremediation.