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Evaluation of Electron Donors for Perchlorate and Nitrate Biodegradation in Contaminated Groundwater Applications

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EVALUATION OF ELECTRON DONORS FOR PERCHLORATE AND NITRATE BIODEGRADATION IN CONTAMINATED GROUNDWATER APPLICATIONS

by

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ABSTRACT

by

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In the United States, perchlorate contamination has been widely reported, including in Las Vegas, Nevada, where perchlorate has been detected at concentrations of 34.7 mg/kg in vadose zone soil and 0.18-3.7 g/L in groundwater. Once this groundwater reaches the Las Vegas Wash, there is potential for widespread contamination of drinking water sources throughout the Southwest, including in Nevada, Arizona, and California. This issue is becoming increasingly important because even at low perchlorate concentrations, sensitive populations such as infants and pregnant women can be potentially impacted due to perchlorate's ability to hinder iodine uptake into the thyroid glands, which leads to inhibition of hormone production. Biodegradation is generally recognized as the most cost effective treatment strategy for perchlorate mitigation. The use of *in situ* bioremediation is common in vadose zone soils, while *ex situ* bioremediation has been employed in groundwater and saturated soil applications. For remediation of vadose zone soils, organic or inorganic electron donors can be added to stimulate the native microbial community, specifically perchlorate reducing bacteria, to reduce perchlorate to chloride through a series of redox reactions. However, co-occurring electron acceptors, particularly nitrate, may compete with perchlorate and hinder the bioremediation process.

This study evaluated the efficacy of four electron donors, specifically two emulsified soybean oils (EOS-100 and EOS-Pro), glycerol, and a compost/mulch extract, for biological reduction of nitrate and perchlorate using batch microcosm testing. These electron donors were evaluated in two different test matrices: (1) vadose zone soil mixed with surface water from Lake Mead and (2) saturated soil mixed with groundwater. Samples were analyzed to evaluate nitrate and perchlorate removal kinetics, the effects of phosphate addition, and the effects of varying soil to water ratios. Results indicated that EOS-100 and glycerol achieved similar overall reduction of nitrate and perchlorate in the vadose zone soil application, although EOS-100 exhibited faster kinetics. In the saturated soil experiments, EOS-Pro was superior to EOS-100. The evaluation of soil to water ratios demonstrated that the most significant variable limiting nitrate and perchlorate reduction was the availability of electron donor rather than water volume. Finally, phosphate addition indirectly improved perchlorate reduction by increasing the rate of nitrate biodegradation, particularly for samples with a mass-based nitrogen to phosphorus ratios higher than 0.22:1. The results from this study can be used to better inform bioremediation efforts at perchlorate-contaminated sites, thereby improving treatment efficacy and decreasing risks to downstream drinking water sources

TABLE OF CONTENTS

ABSTRACT III

CHAPTER 1. INTRODUCTION 1

CHAPTER 2. LITERATURE REVIEW 7

- Perchlorate Biodegradation 7
- Perchlorate Bioremediation in Surface and Groundwater 10
- Perchlorate Biodegradation in Vadose Zone Soil 14
- Applied Electron donors (Microcosm Studies) 17
- Knowledge Gaps in the Literature Related to Groundwater and Soil Perchlorate Bioremediation 19

CHAPTER 3. PERCHLORATE BIOREMEDIATION IN SURFACE WATER AND VADOSE ZONE SOIL: MICROCOSMS STUDY 23

- Introduction 23
- Materials and Methods 27
  - Perchlorate Reducing Bacteria (PRB) 27
  - Electron Donors Source for Vadose Zone Soil and Surface Water Microcosms 28
  - Vadose Soil and Surface Water Samples 29
  - Microcosms Experimental Setup 31
  - Electron Donor Dose 32

[redacted] Nitrate and Perchlorate Biodegradation in Microcosms with Varied Soil to Water Ratios 35

[redacted] Analytical Methods 36

[redacted] Results and Discussion 37

[redacted] Chemical Oxygen Demand in Vadose Zone Soil and Surface Water Microcosms 37

[redacted] Biodegradation of Nitrate in Vadose Zone Soil and Lake Mead Water Microcosms 39

[redacted] Biodegradation of Perchlorate in Vadose Zone Soil and Lake Mead Water Microcosms 41

[redacted] Nitrate and Perchlorate Reduction Kinetics in Vadose Zone Soil and Lake Mead Water Microcosms 44

[redacted] Changes in pH and Reduction of Sulfate in Vadose Zone Soil and Lake Mead Water Microcosms 47

[redacted] Abiotic Controls in Vadose Zone Soil and Lake Mead Water Microcosms 48

[redacted] Phosphate Amendment in Vadose Zone Soil and Lake Mead Water Microcosms 50

[redacted] Soil to Water Ratios Second Set of Microcosms in Vadose Zone Soil and Lake Mead Water Microcosms with Glycerol as Electron Donor.....53

3. 3. 8. 1 Biodegradation of Nitrate as a Function of Soil to Water Ratios 56

3. 3. 8. 2 Biodegradation of Perchlorate as a Function of Soil to Water Ratios 58

Summary 61

CHAPTER 4. NITRATE AND PERCHLORATE BIOREMEDIATION IN GROUNDWATER AND SATURATED SOIL: MICROCOSMS STUDY 63

4. 1. Introduction 63

4. 2. Material and Methods 65

4. 2. 1. Groundwater and Saturated Soil Samples 66

4. 2. 2. Electron donors Source in Groundwater and Saturated Soil Microcosms 67

4. 2. 3. Groundwater and Saturated Soil Microcosm Setup 68

4. 2. 4. Analytical Methods 71

4. 3. Results and Discussion 72

4. 3. 1. Chemical Oxygen Demand in Groundwater and Saturated Soil Microcosms Augmented with EOS-100 72

4. 3. 2. Biodegradation of Nitrate in Groundwater and Saturated Soil Microcosms-EOS-100 73

4. 3. 3. Biodegradation of Perchlorate in Groundwater and Saturated Soil Microcosms-EOS-100 77

4. 3. 4. Biodegradation of Sulfate in Groundwater and Saturated Soil Microcosms-EOS-100 79

4. 3. 5. Abiotic Controls in Groundwater and Saturated Soil Microcosms-EOS-100 80

4. 3. 6. Nitrate Reduction Kinetics in Groundwater and Saturated Soil Microcosms-EOS-100 82

4. 3. 7. Phosphate Amendment in Groundwater and Saturated Soil Microcosms-EOS-100 84

4. 3. 8. Nitrate and Perchlorate Biodegradation in Groundwater and Saturated Soil Microcosms-EOS-Pro 86

4. 4. Summary 92

CHAPTER 5. CONCLUSIONS 95

REFERENCES 100

CURRICULUM VITAE 111

LIST OF TABLES

Table 1. Summary of Studies of Electron Donors Used in Bioremediation of Perchlorate……22

Table 2. Chemical and Physical Properties of EOS-100, Glycerol and Compost/mulch Electron Donors.....29

Table 3. Vadose Zone Soil and Lake Mead Water Initial Quality Parameters.....31

Table 4. EOS-100 demand considering the Stoichiometric Reaction of the Electron Donors and Hydrogen.....32

Table 5. EOS-100 Demand.....33

Table 6. Experimental Design Matrix for Preliminary Microcosms Experiments.....35

Table 7. Experimental Design Matrix for Secondary Microcosm Experiments.....36

Table 8. Analytical Methods.....37

Table 9. Summary Rate Constants for Nitrate and Perchlorate Reduction with EOS-100 and Glycerol at 21±2°C.....46

Table 10. Sulfate Concentrations in the Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors.....48

Table 11. Results from Abiotic Control Microcosms for Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors.....49

Table 12. Phosphate Concentrations in Nutrient Controls.....52

Table 13. Perchlorate and Nitrate Reduction in Nutrient-Amended in Vadose Zone Soil and Lake Mead Water Microcosms.....53

Table 14. Saturated Soil and Groundwater Initial Quality Parameters.....67

Table 15. EOS-100 and EOS-Pro Properties.....68

Table 16. Microcosms Electron Donor Volumes EOS-100/EOS-Pro.....69

Table 17. Experimental Design Matrix for the Preliminary Experiments with EOS-100.70

Table 18. Experimental Design Matrix for the Secondary Experiments with EOS-Pro ...71

Table 19. Analytical Methods.....72

Table 20. Nitrate Biodegradation Rates in Saturated Soil and Groundwater Microcosms-EOS-100.....76

Table 21. Nitrate Reduction in Abiotic vs. Biotic Microcosms - EOS-100 81

Table 22. Nitrate and Perchlorate Biodegradation Rates in Microcosms with EOS-100 (140X Stoichiometric COD) and 65 mg- PO_4^{-3}/L of Phosphate. 85

Table 23. Nitrate and Perchlorate Biodegradation Rates in Groundwater and Saturated Soil Microcosms Augmented with EOS-100 and EOS-Pro 89

LIST OF FIGURES

Figure 1. Perchlorate Reduction Pathway.....12

Figure 2. Sequence of Utilization of Electron Acceptors (ITRC, 2008) 13

Figure 3. Chemical Oxygen Demand Concentrations in Microcosms Augmented with EOS-100, glycerol and Compost/mulch Electron Donors.....39

Figure 4. Nitrate Reduction in Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors 40

Figure 5. Perchlorate Reduction in Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors 43

Figure 6. Nitrate Reduction Kinetics in Vadose Zone Soil and Lake Mead Water Microcosms at 21±2°C 45

Figure 7. Perchlorate Reduction Kinetics in Vadose Zone Soil and Lake Mead Water Microcosms at 21±2°C 46

Figure 8. pH and Sulfide Comparison for Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors 48

Figure 9. Phosphate as PO_4^{-3} Concentrations-No Additional Phosphate Added for Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors.....51

Figure 10. Chemical Oxygen Demand as a Function of Soil to Water Ratios.....56

Figure 11. Nitrate Reduction Soil to Water Ratios. 57

Figure 12. Maximum Nitrate Biodegradation Rates as a Function of Soil to Water ..... Ratios.....58

Figure 13. Perchlorate Reduction as a Function of Soil to Water Ratio.....59

Figure 14. Perchlorate Degradation Rates as Function of Soil to Water Ratios. 60

Figure 15. Chemical Oxygen Demand in Groundwater and Saturated Soil Microcosms-EOS-100.....73

Figure 16. Nitrate Reduction in the Groundwater and Saturated Soil Microcosms - EOS-100 76

Figure 17. Perchlorate Reduction in the Groundwater and Saturated Soil Microcosms - EOS-100.....78

Figure 18. Sulfate Reduction in the Groundwater and Saturated Soil Microcosms - EOS-100 80

Figure 19. Nitrate and Perchlorate Concentrations in Abiotic Control Microcosms with EOS-100 at 140X Stoichiometric COD.....82

Figure 20. Nitrate Reaction Kinetics in Saturated Soil and Groundwater Microcosms - EOS-100 at 21±2°C.. 83

Figure 21. Nitrate and Perchlorate Concentrations in Microcosms with EOS-100 (140X Stoichiometric COD) and 65 mg- PO_4^{-3}/L of Phosphate. 84

Figure 22. Chemical Oxygen Demand in Groundwater and Saturated Soil - EOS-Pro....87

Figure 23. Nitrate Reduction in Groundwater and Saturated Soil Microcosms - EOS-Pro……..88

Figure 24. Perchlorate Reduction in Groundwater and Saturated Soil Microcosms - EOS-Pro.....90

Figure 25. Sulfate Reduction in Groundwater and Saturated Soil Microcosms - EOS-Pro……..91

Figure 26. Phosphate Concentrations in Groundwater and Saturated Soil Microcosms - EOS-Pro 92

CHAPTER 1. INTRODUCTION

Perchlorate (ClO_4^-) can be generated through anthropogenic or natural processes, and it is considered a contaminant of concern due to its potential human health effects. Perchlorate interferes with iodine uptake into the thyroid gland, leading to inhibition of thyroid hormone production in the body (Motzer, 2001). In 2005, The United States Environmental Protection Agency (USEPA) included perchlorate on the Contaminant Candidate List (CCL) and identified a chronic oral reference dose (RFD) of 0.7 µg/kg-day. This RFD corresponds with a drinking water equivalent level (DWEL) of 24.5 µg/L, assuming drinking water is the only source of perchlorate consumption (USEPA, 2014). The USEPA then identified a more stringent 15-µg/L threshold for adverse noncarcinogenic effects after a lifetime exposure. This new interim health advisory level accounted for additional exposure to perchlorate from contaminated food. In 2011, the USEPA declared its intent to regulate perchlorate in drinking water, but by 2016, no regulation had been established. Meanwhile, some states have adopted safety advisory levels (e.g., Nevada at 18 µg/L and Arizona at 14 µg/L) until a national standard is established (Water Research Foundation, 2014).

The biophysicochemical properties of perchlorate facilitate its accumulation and transport in soil and groundwater. Perchlorate is a persistent contaminant in water because it is highly soluble, non-volatile, and kinetically inert (X. Xu et al., 2015). The high solubility and mobility of perchlorate contribute to its spreading from the source of contamination to other distant locations (Karimi & Rezaee, 2014a). The stability of perchlorate due to its high activation energy (120 kJ/mol) contributes to its accumulation, and because of its low adsorption onto soil, infiltration mobilizes perchlorate present in the vadose zone, thereby generating a constant

source of perchlorate contamination in groundwater sources (Evans & Trute, 2006). As a result, contamination of soils, groundwater, and surface water has been widely reported in the last decade (Motzer, 2001).

Southern Nevada is the site of one of the most severe examples of perchlorate contamination in the environment. Concentrations in the vadose zone soil of the Las Vegas Wash have been reported at 34,700 µg/kg of soil (Batista et al., 2005), and this contamination has been linked to detection of perchlorate in local drinking water source, Lake Mead, —ranging from 18 to 280 µg/L. In fact, Las Vegas groundwater have even reached concentrations of 180 to 3,700 mg/L in heavily contaminated areas and 8 to 21 µg/L in less contaminated areas (Motzer, 2001;)

Because perchlorate mitigation is challenging, diverse technologies have been developed and tested for their efficacy in cleaning soil, surface water, and groundwater. Physical/chemical technologies for contaminated surface water and groundwater include ion exchange, membrane filtration technologies, adsorption with granular activated carbon (GAC), and chemical and electrochemical reduction (ITRC, 2008). Biological reduction has also been implemented for in-situ and ex situ bioremediation. Soil treatment includes in situ bioremediation with bioventing, phytoremediation, and soil flushing, while ex situ bioremediation generally relies on thermal or excavation treatment technology. These technologies have proven to be efficient on cleaning perchlorate contaminations in vadose zone soils, and many studies in the literature agree that one of the most economically viable and environmentally friendly treatment options is in-situ biodegradation treatments.

In-situ bioremediation has been specially applied in contaminated vadose zone soils, for example to clean contaminated-perchlorate soils. This technique has been applied to overcome bioremediation limitations such as the insufficient nutrients, electron donors, and water contain

in the soil that inhibit the natural biological reduction of contaminants (i.e., nitrate and perchlorate). Therefore, in-situ bioremediation is a technique applied to stimulate the microbial activity in soils and provide high contamination removals. Two different techniques can be implemented during in-situ perchlorate bioremediation treatments: (1) water is injected to flush the perchlorate into saturated zones in which the high availability of water (groundwater) facilitate subsequent treatments and (2) a mix of an enhanced water with an organic or inorganic electron donor is applied into the vadose zone soil to treat the contaminated zone while flushing the perchlorate into the saturated zone (groundwater). During in-situ biodegradation of contaminated-perchlorate vadose zone soils, the native microbial communities use perchlorate as the electron acceptor and the organic or inorganic electron donors to catalyze the reactions in contaminated zones.

Perchlorate reducing bacteria (PRB) are ubiquitous in the environment, and under anaerobic conditions and sufficient electron donor concentrations, PRB can degrade perchlorate. Laboratory research is often required to characterize the efficacy of a particular treatment approach before it is implemented at full-scale. Using microcosms, previous studies have evaluated different sources of carbon as potential electron donors, and these studies have also assessed potential interferences between perchlorate and co-contaminants. Further examination of alternative electron donors may improve perchlorate bioremediation. For example, electron donors capable of improving perchlorate removal kinetics, reducing operational costs and complexity. More specifically, there is a need to identify a suitable, cost-effective, slow-release electron donor for soil and groundwater remediation applications.

Furthermore, the use of microcosm batch tests can determine crucial parameters to increase the efficiency of in-situ perchlorate bioremediation in contaminated vadose zone soils

through (1) the identification of the adequate water content to warranty complete mobilization of perchlorate into saturated soils (groundwater), (2) the identification of competitive electron acceptors that could delay perchlorate reduction, and (3) an adequate contact time to ensure complete perchlorate biodegradation.

The objective of this research was to evaluate the potential use of diverse electron donors and the associated degradation kinetics for perchlorate and nitrate biodegradation in vadose and saturated soils from a contaminated site. Specifically, the electron donor glycerol, commercially available emulsified oils (EOS®-100 and EOS-PRO), and a compost/mulch extract were evaluated. The characterization of the nitrate and perchlorate biodegradation in both of the zones is an innovative approach due to the chemical and physical properties of the soils and the application of specific aforementioned electron donors. This research would provide new perspectives to improve the application of different electron donor/carbon sources to perchlorate and nitrate biodegradation treatments. The kinetics of perchlorate reduction were characterized through the use of laboratory microcosms in two phases: (1) use of surface water and vadose zone soil to simulate in-situ bioremediation treatment (i.e., adding Lake Mead water to evaluate the maximum perchlorate releases or mobilization from the soil into the source of water) and (2) use of groundwater and saturated soil to evaluate the efficiency of the applied electron donors in the contaminated groundwater. Thus, a comparison of perchlorate biodegradation in the vadose zone vs. the saturated zone is warranted.

The first phase of this research (Chapter 3) involved the use of surface water and vadose zone soil samples, and experiments were performed to quantify the release and subsequent reduction of nitrate and perchlorate. Specific tasks included the following:

- Evaluate perchlorate reduction kinetics with electron donors in vadose zone soils

- Evaluate perchlorate attenuation in abiotic controls
- Evaluate competitive reduction of nitrate
- Investigate the effect of phosphate addition
- Evaluate different soil/water ratios to evaluate the amount of water needed to mobilize perchlorate contamination into saturated soils.

The second phase of this research (Chapter 4) involved the use of contaminated groundwater and saturated soil. The experimental objectives of this phase are provided below:

- Evaluate perchlorate reduction kinetics with electron donors in saturated zone soils
- Evaluate perchlorate reduction in abiotic controls
- Evaluate competitive reduction of nitrate
- Investigate the effect of phosphate addition
- Determine the impact of soil moisture content on kinetics

Both phases assessed nitrate interference and the potential benefits of phosphate augmentation. All microcosms were tested for additional contaminants such as phosphate, sulfate, and sulfide, as well as pH, hardness, and conductivity.

The primary hypothesis of this research is that proving an adequate electron donor will catalyze the natural microbial degradation of nitrate and perchlorate in contaminated soils by simulating in-situ biodegradation treatments using microcosms batch test. The evaluation of nitrate and perchlorate biodegradation in both vadose zone and saturated soils is an innovative approach due to the chemical and physical variabilities of the soils (i.e., water, carbon, nutrients, pH, and temperature) that can lower the efficacy of the selected electron donor during in-situ biodegradation treatments. Considering the chemical and physical conditions of the soil at the

site of the study, this research will determine the most effective electron donor evaluating suitable conditions such as moisture content, soil to water ratios, and nutrient availability. As a result of this research, stakeholders interested in remediating perchlorate-contaminated soils can have a wider selection of electron donors that adapting to different conditions can lead to mitigate perchlorate plumes into groundwater sources.

CHAPTER 2. LITERATURE REVIEW

Perchlorate Biodegradation

Perchlorate (ClO_4^-) is an inorganic contaminant resulting from the dilution of various ion salts such as perchloric acid, ammonium perchlorate, potassium perchlorate, and sodium perchlorate in water. The most important sources of perchlorate contamination include sites manufacturing solid rocket fuel propellants, pyrotechnics, fertilizers, munitions, and car air bags (Gullick et al., 2001), as well as releases from medical and chemical laboratory facilities (Motzer, 2001). Additionally, natural sources of perchlorate contamination have been reported. These natural sources have been associated with photochemical atmospheric reactions. It has been suggested that perchlorate can be generated naturally from the reaction between sodium chloride present on land and sea surfaces with ozone found in the atmosphere. The sodium chloride is blown into the atmosphere where it reacts with ozone, thereby resulting in the generation of perchlorate salts. Consequently, the natural accumulation of perchlorate in soils and water results from precipitation (Karimi & Rezaee, 2014a).

Perchlorate salts produce adverse effects on human health, particularly interference of iodine uptake into the thyroid gland leading to an inhibition of hormone production. The production of thyroid hormones is important because they assist and regulate the metabolism and normal growth in the human body (Motzer, 2001). In fact, perchlorate exposure can inhibit the development of fetuses, the central nervous system, and the skeletal system of infants (USEPA, 2014). After perchlorate was linked to these adverse public health outcomes, evaluating potential exposure to perchlorate in water sources became critically important.

To date, the most used perchlorate detection method is ion chromatography (IC) with conductivity detection (Federal Facilities Forum, 2005). Standard IC was initially capable of detecting perchlorate at concentrations above 100 µg/L, but in 1997, the California Department of Health Services improved the method and achieved a detection limit of 4 µg/L. This method is now recognized as United States Environmental Protection Agency (USEPA) Method 314.0 and is applicable to perchlorate detection in drinking water, groundwater, and surface water (USEPA, 2014). More recently, the USEPA developed an alternative method for perchlorate that relies on detection by a more sensitive mass spectrometer. These new methods (i.e., 314.1, 314.2, 331.0, and 332.0) can achieve perchlorate detection down to 30 ng/L, 12-18 ng/L, 8 ng/L, and 20 ng/L, respectively (Karimi & Rezaee, 2014a).

In 2005, the USEPA identified a chronic oral reference dose (RfD) for perchlorate of 0.7 µg/kg-d and included perchlorate on the Contaminant Candidate List (CCL) (USEPA, 2014). Based on the reported RfD, the USEPA identified a corresponding drinking water equivalent level (DWEL) of 24.5 µg/L, which assumed that water was the only source of perchlorate consumption. The USEPA then identified 15 µg/L as the no observed adverse effects level (NOAEL) for noncarcinogenic effects over a lifetime of exposure. The reduction in the interim health advisory level (i.e., 24.5 µg/L down to 15 µg/L) was intended to account for additional exposure to perchlorate from contaminated food. Subsequently, in 2006, Massachusetts set a Maximum Contaminant Level (MCL) of 2 µg/L, and then California set an MCL of 6 µg/L in 2007 (Water Research Foundation, 2014). In 2011, the USEPA decided to regulate perchlorate in drinking water at the federal level, but by early 2016 (the time of this research), no regulation had been established yet. Some states decided to adopt safety advisory levels (e.g., Nevada at 18 µg/L and Arizona at 14 µg/L) until a federal standard was established (USEPA, 2014).

Contamination of soils, groundwater, and surface water has been widely reported in the last decade. Perchlorate concentrations have even been detected in edible products in different locations around the world. Perchlorate concentrations were reported in fruits, vegetables, milk (Karimi & Rezaee, 2014a), and bottled and tap water, among other products in the United States, Canada, Japan, China, and India (Kumarathilaka et al., 2016). Perchlorate concentrations in soils in Texas, New Mexico, Nevada, and Utah have been reported at relatively low concentrations ranging from 1.6 to 13 μg/kg of soil. On the other hand, soil concentrations vary from 290 to 2,565 μg/kg of soil in the Atacama Desert in northern Chile, although this is considered a natural occurrence of perchlorate (Kumarathilaka et al., 2016).

In northern and central New Mexico, aqueous perchlorate has been documented from 0.12 μg/L to 1.8 μg/L in groundwater (Plummer et al., 2006). In the United States, high perchlorate contamination was detected in the Southwest in 2005. Accordingly, the sources of this contamination were investigated, and the results showed that different companies were responsible, particularly the former Kerr-McGee Chemical Corporation (Tronox), Pacific Engineering & Production Company of Nevada (PEPCON), the American Potash and Chemical Corporation (AP & CC), the Western Electrochemical Company, and the U.S. Navy (Batista et al., 2005; Gullick et al, 2001; Nevada Division of Environmental Protection, 2011; Zhu., 2016). In response, these companies developed projects to reduce the perchlorate contamination to levels as low as 18 μg/L in their discharges. The technique most frequently used has been in-situ biodegradation (Nevada Division of Environmental Protection, 2011).

Henderson, Nevada is the site of one of the most severe examples of anthropogenic perchlorate contamination in the environment (Nevada Division of Environmental Protection, 2011). Concentrations in the vadose zone soil of the Las Vegas Wash have been reported at

34,700 µg/kg of soil (Smith et al., 2004), and perchlorate has been detected in drinking water in Las Vegas. The Southern Nevada Water Authority reported perchlorate concentrations ranging from 18 to 280 µg/L (Nzengung et al., 1999). Concentrations in Las Vegas groundwater have even been reported to range between 1.8×10^5 to 3.7×10^6 µg/L in highly contaminated areas and 8 µg/L to 21 µg/L in less contaminated areas (Motzer, 2001).

The biophysicochemical properties of perchlorate facilitate its accumulation and transport in soil and groundwater. Perchlorate is a persistent contaminant in water due to its high solubility, low volatility, and kinetically inert properties (X. Xu et al., 2015). The high solubility (e.g., ammonium perchlorate, 200 g/L; perchloric acid, 100 g/L) and mobility of perchlorate contribute to its rapid spreading from the source of contamination to other distant locations (Karimi & Rezaee, 2014b). The Gibbs free energy of formation of perchlorate in aqueous solution is -8.5 kJ/mol, which indicates that perchlorate has a low association with cations and high solubility in aqueous and nonaqueous media (Urbansky, 1998). The stability of perchlorate due to its high activation energy (120 kJ/mol) contributes to its accumulation, and because of its low adsorption onto soil, infiltration mobilizes any perchlorate present in the vadose zone (Evans & Trute, 2006).

Perchlorate Bioremediation in Surface and Groundwater

Because perchlorate mitigation is so challenging, diverse technologies have been developed and tested for their efficacy in cleaning surface water and groundwater. Physical/chemical technologies include ion exchange, membrane filtration, adsorption with granular activated carbon (GAC), and chemical and electrochemical reduction. Biological reduction has also been implemented for in situ and ex situ bioremediation (ITRC, 2008).

Ion exchange technology is one of the most effective processes to remove perchlorate from water. Treatment by ion exchange occurs through the adsorption of dissolved perchlorate anions onto engineered resins or natural zeolites. The efficiency of perchlorate removal through ion exchange is affected by the presence of co-contaminants such as nitrate, sulfate, bicarbonate, carbonate, and bromide, which are competing anions usually present in contaminated perchlorate groundwater. Ideal resins are highly specific for a target contaminant, and they can generally be regenerated and used repeatedly. Ion exchange is not effective for the removal of high concentrations of perchlorate due to saturation limitations of the resins. Therefore, perchlorate remediation applications require continuous monitoring to quickly identify and respond to perchlorate breakthrough (ITRC, 2008).

Darracq et al. (2014) compared anion removal with five different commercial resins (A532E, A520E, A400E, PWA-5, and PSR-2) through kinetics and isotherm batch tests with synthetic water. Results showed that these resins were highly effective for the target anions, but that removal efficiencies, including for perchlorate, decreased in the presence of competing anions, such as nitrate, sulfate, and chloride. The study noted that PSR-2 and A532E had the highest specificity for perchlorate with first and second order sorption models with removal rate constants of 1.52×10^{-2} min^{-1} for PSR-2 and 2.3×10^{-3} g $mg^{-1}min^{-1}$ for A532E. Although these resins proved to be efficient in removing perchlorate, they are not regenerable. Although ion exchange is a promising method for perchlorate removal, resin replacement is potentially cost-prohibitive, and the regeneration of the resin, when feasible, results in a highly contaminated brine requiring disposal (Ye et al., 2012)

Membrane filtration using reverse osmosis (RO) has also been shown to be effective for perchlorate removal (Kumarathilaka et al., 2016). This technology is considered a physical

separation method in which the perchlorate-contaminated water passes through a semipermeable membrane under pressure (ITRC, 2008). However, RO systems continuously generate a concentrated brine solution that requires further treatment or disposal (Srinivasan & Sorial, 2009). Broad implementation of RO for perchlorate remediation is hindered by its exceptionally high capital and operational costs as well as the costs and effort associated with brine disposal (ITRC, 2008).

Electrochemical reduction has also been demonstrated for perchlorate removal without the generation of significant byproducts (e.g., perchlorate brines) (Rusanova et al., 2006; D. M. Wanget al., 2009). However, Kumarathilaka et al. (2016) suggested that further study is needed in order to extend laboratory research to field applications.

Many studies in the literature agree that one of the most economically viable and environmentally friendly treatment options is biodegradation or biological reduction (Srinivasan & Sorial, 2009). Because biological reduction is an energy intensive process for bacteria, they require enzymes capable of lowering the activation energy of the reactions (Bardiya & Bae, 2011). The principal cell-bound enzymes responsible for biological reduction of perchlorate are perchlorate reductase and chloride dismutase. Figure 1 shows the perchlorate reduction pathway and the field of action of each enzyme (Frankenberge, 2003).

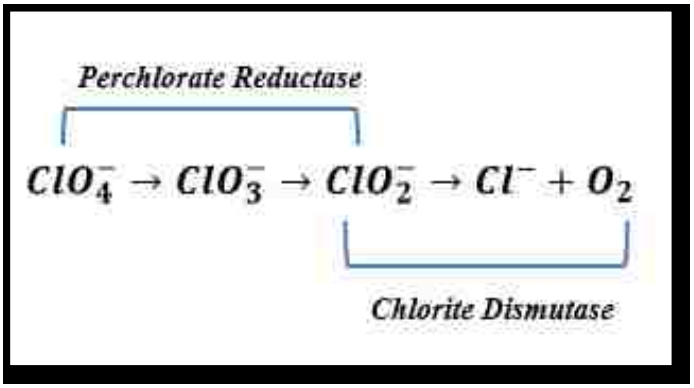

Figure 1. Perchlorate Reduction Pathway

Perchlorate reducing bacteria (PRB) are ubiquitous in natural environments (Bruce & Coates, 1999). PRB are facultative anaerobes which have been classified as Gram-negative and of the Proteobacteria class. Relevant genera include *Dechloromonas* and *Azospira* (formerly *Dechlorosoma*), which are able to reduce perchlorate and chlorate. Enzymatic competition between perchlorate and other electron acceptors decreases the efficiency of biological reduction. It has been demonstrated that in the presence of oxygen and nitrate, PRB have an affinity to reduce oxygen and nitrate before perchlorate due to the thermodynamic favorability of the competing electron acceptors (Bardiya & Bae, 2011). In addition, perchlorate reduction can also be hindered by sulfate and carbon dioxide. Figure 2 shows the preferred utilization of electron acceptors for PRB based on their redox potentials. Perchlorate reduction generally occurs between 0 and -110 mV, while oxygen and nitrate reduction occurs at higher redox potentials. In addition, PRB can be limited by high salinity (conductivity) environments, low perchlorate concentrations, and a lack of electron donors (Batista et al., 2005; Nozawa-Inoue et al., 2011). Therefore, understanding the enzymatic reactions and potential competition by other species within the target matrix are important factors in improving the efficiency of this treatment approach (Srinivasan & Sorial, 2009).

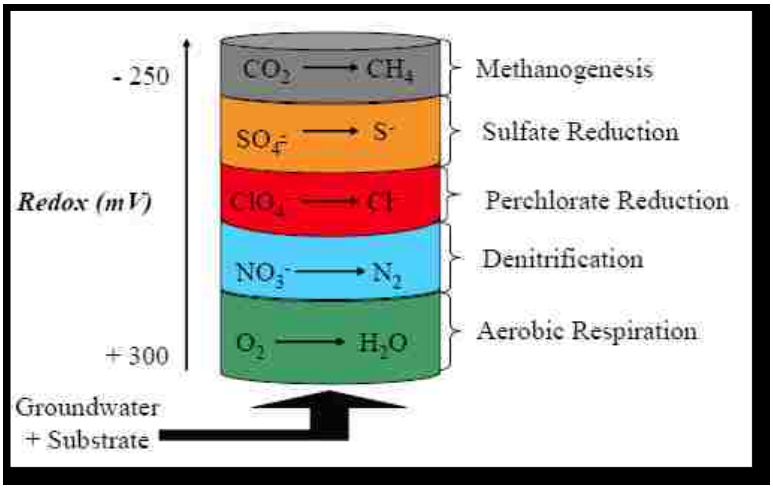

Figure 2. Sequence of Utilization of Electron Acceptors (ITRC, 2008)

Perchlorate Biodegradation in Vadose Zone Soil

The depth of the soil formation where the pores are not saturated with water is name the vadose zone. The depth of a formation where the pores are permanently saturated with water is called the saturated zone. Because perchlorate contamination generally occurred by discharge of perchlorate containing wastes into the ground, these wastes percolated through the vadose zone and reached the saturated zone, where groundwater resides. Therefore, in many areas, both the vadose zone and the groundwater are contaminated with perchlorate (Holden, Patricia A, 2005). Due to the presence of bacteria in the vadose zone, bioremediation technologies are capable of enhance the conditions of the vadose zone soil through the injection of water/moisture, electron donors, and nutrients into the soil to promote perchlorate degradations. Based on this principle, new research has been performed to improve treatment in the vadose zone and reduce groundwater contamination during infiltration and mobilization of adsorbed perchlorate. Soil treatment includes in situ bioremediation with bioventing, phytoremediation, and soil flushing, while ex situ bioremediation generally relies on thermal or excavation treatment technology (ITRC, 2008).

Bioventing typically involves the injection of oxygen to stimulate natural in situ biodegradation within native microbial communities (Evans & Trute, 2006), but this is ineffective for the remediation of highly oxidized contaminants such as perchlorate (ITRC, 2008). However, bioventing, which involves either gas injection or soil vapor extraction (SVE), can be adapted to target reduced or oxidized contaminants. For example, during gas injection, nitrogen gas can be amended with a gaseous electron donor and then injected into the vadose zone. The electron donor serves as the electron source for biological reduction, and the gaseous nitrogen displaces some of the dissolved oxygen present in the contaminated soil, thereby

enhancing perchlorate reduction kinetics. With SVE, soil vapor is extracted from the contaminated site, amended with a gaseous electron donor, and the mixture is then injected back into the contaminated vadose zone (Khan et al., 2004). Bioventing is applicable for sites with perchlorate contamination at depths greater than 1.5 m because of its easy installation and operation (ITRC, 2008), but it can be hindered by soils with low permeability (high water content) or high clay composition due to the inability of the air to pass through these zones (Khan et al., 2004).

Bioventing has been widely used for petroleum contamination (Höhener & Ponsin, 2014; Khan et al., 2004), and it has also been used for nitrate and perchlorate removal in microcosm and column studies (Evans et al., 2011; Evans & Trute, 2006). Cai et al. (2010) used microcosm experiments to demonstrate the effectiveness of various gaseous electron donors in supporting perchlorate bioremediation. The microcosms were amended with hydrogen, 1-hexene, ethyl acetate, and liquefied petroleum gas (propane) as the electron donors. Different concentrations of the electron donors and two different soil moisture contents (high soil moisture = 16% w/w and low soil moisture = 13% w/w) were analyzed. Results indicated that with high soil moisture content, hydrogen achieved complete perchlorate degradation, liquefied petroleum gas (LPG) and 1-hexene achieve partial perchlorate degradation, and ethyl acetate did not achieve any perchlorate degradation. In addition, the experiments indicated first order kinetics with rate constants ranging from 0.13 d^{-1} to 0.20 d^{-1} for hydrogen, 0.005 d^{-1} for LPG, and 0.11 d^{-1} for 1-hexene.

Phytoremediation of perchlorate and nitrate in soil and groundwater involves the use of plants. A variety of terrestrial plants have been demonstrated to be effective for perchlorate removal, including black willow (*Salix nigraand, Salix caroliniana*), eastern cottonwood

(*Populus deltoides*), eucalyptus (*Eucalyptus cinerea*), loblolly pine (*Pinus taeda*), French tarragon (*Artemisia dracunculus*), and spinach (*Spinacia oleracea*). Likewise, a variety of wetland species, such as *Typha latifolia* (cattail), *Spirodela polyrhiza*, Shield (duck weed), microbial mats, and *Myriophyllum aquaticum* (parrot feather), have also been shown to be effective (ITRC, 2008). Phytoremediation involves three different mechanisms: uptake and phytodegradation, uptake and phytoaccumulation, and rapid rhizodegradation. These mechanisms differ according to the action zone in the plant (i.e., leaves, stem, or roots). Phytodegradation takes place in the leaves of the plants. This process takes longer than the uptake process, so phytoaccumulation (above the surface zone of the plant) is likely to occur simultaneously. Rhizodegradation takes place in the root zone of the plant. This process utilizes anaerobic microbes and exudates (ethanol, acetate, glucose) present in the roots and in the soil to reduce perchlorate to chloride. Nitrogen and oxygen have been reported to inhibit perchlorate biodegradation in phytoremediation applications but can be overcome by applying high concentrations of electron donors in the root zone. This technology is cost effective, ecofriendly, and it garners significant public support. However, climate conditions limit plant growth in some areas, thereby hindering perchlorate removal (Khan et al., 2004). In addition, this mechanism can take long periods of time, sometimes requiring several growing seasons to reach perchlorate removal standards, and the process has limited applicability for contaminated soil and groundwater at depth (Khan et al., 2004).

Soil flushing is another alternative that consists of passing fluid through a soil to mobilize target contaminants. The fluid is then captured, treated (potentially with electron donor addition and bioremediation) according to design or regulatory criteria, and discharged. Similar to

bioventing, this approach can be hindered if the soil contains high amounts of clay, which restricts fluid flow (Khan et al., 2004).

Finally, thermal excavation is one of the most invasive remediation options because it involves the excavation of contaminated soil followed by heating to 500-1100°F. Although this approach achieves complete destruction of perchlorate, it is generally infeasible because of the high cost, energy consumption, and logistical difficulties (ITRC, 2008).

Applied Electron donors (Microcosm Studies)

Laboratory research is often required to characterize the efficacy of a particular treatment approach before it is implemented at full-scale. Using microcosms or columns, previous studies have evaluated different sources of electron donors, and they have also evaluated potential interferences between perchlorate and co-contaminants. Evans & Trute (2006) used gaseous electron donor injection in a microcosm configuration to evaluate the effectiveness of hydrogen and ethanol for nitrate and perchlorate removal. Results showed that under adequate soil moisture content, the electron donors would be able to induce reduction of nitrate and perchlorate in the vadose zone. Gal et al. (2008) examined the potential for native soil microbes recovered from different vadose zone depths to reduce perchlorate and nitrate. The results showed that perchlorate can be completely removed after 134 days of incubation without external sources of carbon due to ambient electron donor (i.e., natural organic matter) availability in the soil, although the kinetics are hindered by low concentrations of natural organic matter. The natural organic matter demonstrated a perchlorate reduction rate of 0.45 mg day^{-1}, whereas acetate demonstrated 7.2 mg day^{-1} of perchlorate reduction. In addition, the limitations of perchlorate reducing bacteria were also evaluated during the research. Results showed that high

concentrations of perchlorate (10,000 – 20,000 mg/L) did not affect perchlorate reducing bacteria, presumably due to long periods of adaptation to high concentrations prior to the experiments. However, when higher water content (e.g., close to the water table) and nutrients were available, perchlorate reducing bacteria exhibited higher efficiencies. Likewise, Shrout & Parkin (2006) studied the biodegradation of perchlorate at different molar ratios of lactate (as the electron donor) to perchlorate (i.e., 1:1, 2:1, and 4:1) with batch microcosm testing. These ratios were based on the stoichiometric electron equivalent basis in which 8 electrons per mole of perchlorate are required for biodegradation. Results showed that perchlorate reduction rates were 0.038 mgClO_4^-/mgVSS-h for the 1:1 ratio and 0.045 mgClO^{-4}/mgVSS-h for the 2:1 and 4:1 ratio, which were reported as 25 times the initial rate of perchlorate degradation in the absence of spiked lactate as the electron donor.

Nozawa-Inoue et al. (2005) evaluated the efficiency of two electron donors—acetate and hydrogen—for perchlorate contaminated vadose zone soil samples (110,000 g of perchlorate per kg of soil) in microcosm batch tests. Results showed that acetate was faster than hydrogen for perchlorate degradation with lag periods of 14 days and 41 days, respectively. The maximum perchlorate degradation rates for acetate and hydrogen were 2.7 mg/kg dry soil per day and 1.68 mg/kg dry soil per day, respectively. Wang et al. (2013) evaluated the potential use of emulsified oil substrate (EOS®598), EHC® (patented combination of controlled-release, integrated carbon and zero valent iron), and a compost/mulch mixture for perchlorate-contaminated groundwater (500 µg/L) and soil (26 µg/kg) with microcosm batch tests. Microcosms were supplemented with diammonium phosphate ($(NH_4)_2 HPO_4$) to enhance perchlorate biodegradation. Results showed that EHC achieved a reduction rate of 314 µg/L-d, EOS achieved 142 µg/L-d, and the compost/mulch mixture yielded 40 µg/L-d without nutrient addition. Nutrient addition yielded

greater reduction in perchlorate for the EOS and compost/mulch electron donors (250 µg/L-d and 90 µg/L-d, respectively), but no benefits were observed for EHC, which actually dropped to 263 µg/L-d.

Knowledge Gaps in the Literature Related to Groundwater and Soil Perchlorate Bioremediation

To date, different potential remediation technologies have been developed to cleanup nitrate and perchlorate contaminated surface and groundwater. These technologies include ion exchange, membrane filtration, adsorption with granular activated carbon (GAC), chemical and electrochemical reduction, and ex-situ biological reduction. Similar technologies for nitrate and perchlorate contaminated soils have also been implemented (ITRC, 2008). These technologies mainly include in-situ biological treatment reduction due to their high efficacy and low operational cost compared with ex-situ bioremediation treatments in which post-treatment of the extracted soils resulted in higher operational costs (ITRC, 2008).

Commonly, biological reduction of perchlorate has been applied in contaminated vadose zone soils, especially in-situ bioremediation reduction (ITRC, 2008). One of the most used perchlorate in-situ bioremediation treatments in soils is recognized as “soil flushing”. Soil flushing involves the addition of water into the vadose zone soils to flush or mobilize the perchlorate into deeper soils or into the saturated zone. Once the perchlorate reaches the saturated zone is mixed with the groundwater present in this zone, and then the contaminated water is pumped to the surface for subsequent treatments. Other application during soil flushing involves the addition of enhanced water with electron donor addition to mobilize and treat the perchlorate in the saturated zone.

In bioremediation treatments, native microbial communities are stimulated with electron donors and nutrients to breakdown contaminants. During in-situ biodegradation of perchlorate, perchlorate reducing bacteria ubiquitous in natural environments (e.g., vadose zone soil) utilize perchlorate as the electron acceptor and organic or inorganic compounds as electron donors to catalyze the reactions in contaminated zones. Therefore, the identification of a suitable electron donor benefits the implementation and operation of bioremediation treatments.

The vadose zone has been widely studied because the contaminants contained in this zone are directly related with groundwater contamination (e.g., perchlorate). The vadose zone is an aerated zone (i.e., the spaces between the soil particles are occupied by air), characterized by lower water/moisture content. While in the saturated zone, the water/moisture content is considerable higher due to the present of groundwater occupying the spaces between the soil particles. The characteristics of these two zones limit full-scale bioremediation applications. For example, high oxygen content, low amount of water, variability of pH and temperature, high salinity, and low nutrient and electron donor contents are the main limiting factors in perchlorate biodegradation.

To improve biological reduction of nitrate and perchlorate, laboratory research has also been performed to assess different sources of carbon in microcosm and column testing. For example, acetate (Batista et al., 2005), hydrogen (Evans & Trute, 2006), glycerol (X. Xu et al., 2015), ethanol (Evans & Trute, 2006) succinate, glucose, and benzoate (X. Xu et al., 2015) are some of the electron donors previously studied. These electron donors proved to be effective for nitrate and perchlorate reduction, but the lag time to achieve perchlorate reduction and/or the rapid consumption/mobilization of the electron donors may be limiting factors in some full-scale applications.

More sophisticated electron donors have been investigated for various test matrices (e.g., vadose zone vs. saturated soil) and water sources (e.g., surface water, groundwater, or synthetic water) as shown in Table 1, but there are few studies that directly compare bioremediation efficacy across a wide range of variables, including (1) nitrate vs. perchlorate reduction efficacy and kinetics, (2) standard vs. slow-release electron donors, (3) surface water vs. groundwater matrices, and (4) vadose zone soil vs. saturated soil environments. This study evaluates competitive reduction of nitrate and perchlorate using four different electron donors: (1) EOS-100 (a slow-release emulsified oil with large droplet size), (2) EOS-Pro (a slow-release, nutrient-amended emulsified oil with small droplet size), (3) glycerol (a standard, highly soluble electron donor), and (4) compost/mulch extract (a low cost alternative that repurposes used materials). This study also evaluates the efficacy of these electron donors in both vadose zone and saturated soil applications and surface water and groundwater matrices simulating in-situ bioremediation of nitrate and perchlorate in two test matrices (i.e., vadose zone soil and saturated soil/groundwater). Ultimately, this expanded knowledge base will further reduce risks associated with the consumption of perchlorate-contaminated drinking waters by improving the efficacy of bioremediation efforts.

Table 1. Summary of Studies of Electron Donors Used in Bioremediation of Perchlorate

Electron Donor	Bioremediation Technology	Source of Perchlorate	Initial perchlorate Concentration	Final Perchlorate Concentrations	Contact Time	Performance	Reference
Acetate	Laboratory batch test, microcosms	Synthetic water	1300 mg/L	(< 4µg/L)*	60 hours	62.72 mg/L/h	Zhu, Yanping, 2016
EOS 598[2] -Di ammonium phosphate	Laboratory batch test, microcosms	Groundwater Soil	0.5 mg/L 0.026 mg/kg-soil	(< 4µg/L)*	7 days	0.25 mg/L/d	Y. Wang et al., 2013
EHC[3] -Di ammonium phosphate	Laboratory batch test, microcosms	Groundwater Soil	0.5 mg/L 0.026 mg/kg-soil	(< 4µg/L)*	5 days	0.263 mg/L/d	
Compost/mulch mixture[4]-Di ammonium phosphate	Laboratory batch test, microcosms	Groundwater Soil	0.5 mg/L 0.026 mg/kg-soil	(< 4 µg/L)*	8 days	0.09 mg/L/d	
Acetate Hydrogen	Laboratory batch test, microcosms	Vadose zone soil	20 mg/kg-soil	---	43 days (acetate) 7 days (hydrogen)	~22% (acetate) >90% (hydrogen)	Nozawa-Inoue,Mamie, 2011
Glycerin-Di ammonium phosphate	Ex-situ Bioremediation	Soil	0.04-10 mg/L	---	15 days	0.2 mg/L/d	Evans, Patrick J. 2008
EOS[1]	Field pilot test	Groundwater	3.1-20 mg/L	(< 4 µg/L)*	5 days	---	Borden, Robert. C, 2007
EOS[1]	Laboratory batch test, microcosms	Groundwater	50 mg/L	8 mg/L	14 days	~3 mg/L/day**	Solution-IES, 2006
Acetate	Laboratory batch test, microcosms	Soil	$5x10^{-4}$ mg/kg-soil	---	> 4 months	>0.2 mg/day	Batista et al., 2005

[1]Emulsified Soybean Oil
[2]Emulsified Soybean Oil EOS-Pro previously call EOS 598
[3]Mix of integrated carbon and zero valent iron electron donor
[4]100% wood mulch electron donor
* Below detection limit
** Determined based on the data provided in the journal paper
---Data no reported

CHAPTER 3. PERCHLORATE BIOREMEDIATION IN SURFACE WATER AND VADOSE ZONE SOIL: MICROCOSMS STUDY

Introduction

Perchlorate (ClO_4^-) is an inorganic contaminant resulting from the dilution of various ion salts such as perchloric acid, ammonium perchlorate, potassium perchlorate, and sodium perchlorate in water. Perchlorate interferes with iodine uptake into the thyroid gland, leading to inhibition of hormone production (Motzer, 2001). As a result of the potential adverse human health effects, a drinking water equivalent level of 15 μg/L has been identified, and the USEPA has decided to regulate perchlorate at the federal level, although a maximum contaminant level (MCL) has not yet been established.

In 1997, high perchlorate contamination was detected in the southwestern United States (Zhu et al., 2016), and it has been reported that more than 15 million people within the region consume some level of perchlorate-contaminated water (Nevada Division of Environmental Protection, 2011). Specifically, Las Vegas is the site of one of the most severe examples of perchlorate contamination in the environment. Concentrations in the vadose zone soil of the Las Vegas Wash have been reported at 34,700 μg/kg of soil (Smith et al., 2004), and perchlorate has been detected in drinking water in Las Vegas. In fact, the Southern Nevada Water Authority reported perchlorate concentrations ranging from 18 to 280 μg/L (Nzengung et al., 1999). Concentrations in Las Vegas groundwater have even been reported to range between 180 and 3,700 mg/L in heavily contaminated areas and between 8 and 21 μg/L in less contaminated areas (Motzer, 2001).

The accumulation and transport of perchlorate in soils have been widely reported (Tipton et al., 2003). Perchlorate-contaminated soils are one of the most significant sources of groundwater contamination (Gal et al., 2009). As a result, remediation technologies have been developed to improve treatment in the vadose zone, groundwater, and surface water. Physicochemical technologies to clean groundwater and surface water include ion exchange, membrane filtration technologies, adsorption with granular activated carbon (GAC), and chemical and electrochemical reduction. Biological reduction has also been implemented for in situ and ex situ bioremediation (Bardiya & Bae, 2011). Soil treatment includes in situ bioremediation with bioventing, phytoremediation, and soil flushing, while ex situ bioremediation generally relies on thermal or excavation treatment technology (Caliman et al., 2011).

Many studies in the literature agree that one of the most economically viable and environmentally friendly treatment options is biodegradation, specifically biological reduction (Srinivasan & Sorial, 2009) . Because biological reduction requires bacterial enzymes capable of lowering the activation energy of the reactions. The principal cell-bound enzymes responsible for biological reduction of perchlorate are perchlorate reductase, which degrades perchlorate (ClO_4^-) to chlorate (ClO_3^-) and then to chlorite (ClO_2^-), and chloride dismutase, which degrades chlorite (ClO_2^-) to chloride (Cl^-) and oxygen (O_2) (Frankenberge, 2003). Perchlorate reducing bacteria (PRB) are ubiquitous in natural environments (Bruce et al., 1999). PRB are capable of reducing perchlorate and chlorate under anaerobic conditions using perchlorate as the electron acceptor and diverse organic (e.g., acetate, lactate, methanol, ethanol, and vegetable oils) or inorganic (e.g., hydrogen, reduced iron, and hydrogen sulfide) substrates as electron donors.

Biological reduction of perchlorate has been applied in contaminated vadose zone soils, especially in-situ bioremediation reduction (ITRC, 2008). In situ bioremediation in contaminated vadose zone soils involves the injection of water into the vadose zone soils to mobilize the perchlorate into the saturated zone or groundwater zone. After the perchlorate is concentrated in the saturated zone, the groundwater is then pumped to the surface for subsequent treatments. Other application during soil flushing involves the addition of enhanced water with electron donor to mobilize and treat the perchlorate in the saturated zone.

The vadose zone is a zone characterized by the high oxygen and lower water/moisture content. These characteristics are factors that limited bioremediation treatments. However, the injection of water, nutrients, and electron donors into the vadose zone soil promotes the efficiency of biological reduction treatments by increasing the natural bacteria activity in the contaminated soil. Therefore, there is a need for identification and further investigation of electron donors that persist in the contamination zone, exhibit more rapid kinetics, and can compete with low-cost electron donors.

To date, there are a variety of electron donors that have previously been evaluated for the reduction of perchlorate salts in different water or soil matrices. Zhu, Yanping (2016) used acetate to reduce perchlorate from a contaminated synthetic water through microcosms batch test. Results indicated that acetate reduces perchlorate to levels lower than the detection limit of 4 μg/L within 28 to 60 hours of incubation at a rate of reduction of 62.72 mg/L/h. In a separated research, acetate demonstrated lower perchlorate reduction rates in a contaminated soil (~ 0.2 mg/day). Results indicated that the rate constants may have resulted due to lower perchlorate contamination present in site of study ($5x10^{-4}$ mg/kg-dry soil) (Batista et al., 2005). More sophisticated electron donors have been also investigated for perchlorate and chlorinated

solvents. For example, EOS was employed in a permeable reactive barrier to treat groundwater contaminated with perchlorate and 1,1,1-trichloroethane (1,1,1-TCA). Microcosm batch tests were performed before field implementation. Microcosm results showed that within 14 days of incubation, perchlorate was reduced from 50 mg/L to ~8 mg/L, and 1,1,1-TCA was reduced from 2.5 mg/L to 0.3 mg/L within 140 days (Solution-IES, 2006). In a field pilot test, perchlorate and 1,1,1-TCA concentrations were observed at different distances from the permeable reactive barrier. Perchlorate was ~100% and 99% removed at 10 and 20 feet, respectively, from the permeable reactive barrier within 5 days of installation (Borden, Robert. C, 2007). Glycerol (Evans et al., 2008) and a variety of compost/mulch extracts have also been utilized as electron donors for perchlorate biodegradation (Fox et al., 2014; Y. Wang et al., 2013).

In the past decade, several electron donors have been identified for perchlorate bioremediations, but a few research has involved the evaluation of nitrate and perchlorate reductions with standard vs. slow-release electron donors. The objective of this phase of the research was to evaluate the potential efficacy and kinetics of diverse electron donors for nitrate and perchlorate biodegradation in a contaminated vadose zone soil. Emulsified oil, EOS-100 (a slow-release emulsified oil), glycerol (a standard, highly soluble electron donor), and compost/mulch extract (a low cost alternative that repurposes used materials) were used as the experimental electron donors. The microcosm batch test was designed to simulate in-situ bioremediation of perchlorate in a contaminated vadose zone soil. Microcosms were augmented with the aforementioned electron donors to stimulate the ubiquitous microbial communities present in the soil at the site of study and to decrease the contact time during full-scale applications (i.e., faster nitrate and perchlorate reduction rates). Additionally, Lake Mead water

was used to simulate the moisture/water content in the vadose zone soil and the mobilization of perchlorate from the vadose soil to the Lake Mead water.

Alternative, the study also evaluates the impact of soil to water ratios on perchlorate kinetics, co-contaminant interference, and the effects of macronutrient augmentation. This expanded knowledge base will further reduce risks associated with the consumption of perchlorate-contaminated drinking waters by improving the efficacy of bioremediation efforts and reducing the operational cost related with long-lasting electron donors.

Materials and Methods

Microcosm batch tests were performed to achieve the objectives of this research. Microcosms were built with vadose zone soil from a perchlorate-contaminated site and surface water from Lake Mead. Glycerol, EOS-100, and a compost/mulch extract were used as the electron donors for perchlorate biodegradation, and native bacteria from the vadose zone soil were used as the source of perchlorate reducing bacteria. Additionally, the effects of macronutrient augmentation, the impact of soil to water rations on perchlorate mobilization and kinetics were evaluated.

Perchlorate Reducing Bacteria (PRB)

Using a standard plate count technique, Batista et al. (2003) reported that the concentration of PRB in Lake Mead fluctuated from <1 to 1000 CFU/mL. The bacterial counts were specific to the genera *Shewanella spp.* and *Rahnella aquatilus.* Therefore, it was assumed that PRB were present in the surface water from Lake Mead and in the vadose zone soil used in this microcosm batch test. Thus, no additional bacteria were spiked into the microcosms.

[redacted] Electron Donors Source for Vadose Zone Soil and Surface Water Microcosms

Microcosms were augmented with three different electron donors to stimulate nitrate and perchlorate reductions in the vadose zone soil and Lake Mead water. Glycerol, EOS-100, and a compost/mulch extract were used as the electron donors for perchlorate biodegradation.

EOS-100 is a mixture of organic carbon in the form of refined and bleached U.S. soybean oil (85% by weight), intended to enhance perchlorate biodegradation (EOS Remediation LLC, Raleigh, NC., 2016; Zawtocki et al., 2004). Anaerobic conditions result in the hydrolysis of EOS-100, which releases glycerol and long chain fatty acids (LCFAs). For in situ biodegradation applications, LCFAs are adsorbed onto soil sediments due to their lower solubility in water and are then converted to acetate and hydrogen (electron donors) via fermentation. Because hydrogen production generally exceeds acetate production, contaminant reduction (e.g., perchlorate and nitrate) is generally attributed to hydrogen release (R. C. Borden, 2007). EOS-100 can theoretically generate 156 moles of hydrogen, as shown in Eq. 1 (Solutions-IES, 2010). However, inefficiencies in the fermentation process (i.e., conversion to less desirable products) limits the actual hydrogen yield (Rittmann & McCarty, 2001).

$$C_{56}H_{100}O_6(oil) + 106H_2O \xrightarrow{fermenting\ bacteria} 56CO_2 + 156H_2 \qquad (1)$$

Glycerol and the compost/mulch extract were also derived from commercially available products. The compost/mulch extract was a mixture of biocomponents (i.e., recycled branches, logs and trees) obtained from a local composting company. The compost/mulch extract solution was obtained by washing 1 lb of soil compost/mulch with recirculated deionized water at a flow rate of 150 mL/min. Glycerol was obtained from Sigma Aldrich Corporation. Glycerol is a stable

and low cost nontoxic alcohol. The reductive pathway of glycerol always results in the production of 1,3-propanediol (1,3-PDO) assisted by the enzymatic action of 1,3-propanediol dehydrogenase. The oxidative pathway of glycerol starts with dehydration of dihydroxyacetone and ends in the production of succinate. Both processes occur by the action of two enzymes, glycerol dehydrogenase and dihydroxyacetone kinase, respectively. The succinate is then converted to propionate or to pyruvate. Finally, depending on which reducing bacteria are present and the environmental conditions, the pyruvate is converted to additional subproducts (i.e., acetate, butyric acid, CO_2, n-butanol, ethanol, lactic acid) and hydrogen (da Silva et al., 2009; Viana et al., 2012). Table 2 summarizes the chemical and physical properties of commercially available EOS-100 and glycerol, as described by the manufacturers.

Table 2. Chemical and Physical Properties of EOS-100, Glycerol and Compost/mulch Electron Donors

Parameter	EOS-100	Glycerol	Compost/mulch*
Chemical Oxygen Demand (mg/L)	2.07×10^6	1.21×10^6 mg	250
Organic Carbon (% by Weight)	100	N/A	N/A
Refined and Bleached U.S. Soybean Oil (% by	85	N/A	N/A
Slow Release Organics (% by Weight)	15	N/A	N/A
Mass of Hydrogen Produced (lb H_2 / lb EOS-100)	0.40	N/A	N/A
Solubility in water	Miscible with	Miscible with	N/A
Melting point (°C)	N/A	20	N/A
Flash Point (°C)	N/A	199	N/A
Viscosity (% by Weight)	Low	N/A	N/A
Relative Density	0.92-0.93	1.26	N/A

(N/A: no data available)

*Measured in the Water and Environmental Laboratory, University of Nevada Las Vegas (UNLV)

Vadose Soil and Surface Water Samples

Soil samples were collected from the vadose zone in four different locations and two different profile depths (0-12 feet and 14-26 feet) at a perchlorate-contaminated site. The

samples were mixed in equal volumes (3 L) in serial partitions to obtain a homogeneous mixture. The soil mixture was preserved in a refrigerator at 4°C before experiments.

Initial contaminant concentrations in the vadose soil were determined through a sequential extraction process (described below), and the concentrations were calculated on a dry weight basis. The moisture content of the soil was determined by weighing 20 g of soil before and after drying in an oven at 105°C for 12 hours. The analysis was performed in duplicate, and the average moisture content was 7.7%.

The extraction process was performed in duplicate across multiple stages. For each stage, two 50-mL centrifuge tubes, each containing 20 g of wet soil and 20 mL of nanopure water, were centrifuged at 9,000×g and 4°C (Solvall Legent-GT) for 10 min. This procedure was repeated nine times until perchlorate and nitrate were not detected in the resulting extract (i.e., the contaminants had been completely transferred from the soil to the extraction water). The final extracts were aggregated (final volume of ~68 mL per duplicate) and analyzed for perchlorate, nitrate, and other water quality parameters, as shown in Table 3. On average, the perchlorate and nitrate concentrations in the combined extracts were 48.1 and 91.2 mg/L (as NO_3), respectively. Based on the measured moisture content of 7.7%, the adsorbed perchlorate and nitrate concentrations on the soil were determined to be 0.18 and 0.34 mg/g-dry weight soil, respectively. Therefore, the soil-bound nitrate concentration was almost twice the concentration of perchlorate. This is significant because nitrate competes with perchlorate as an electron acceptor in bioremediation applications. In fact, nitrate is the thermodynamically preferred electron acceptor, which means its presence adversely impacts the kinetics of perchlorate bioremediation.

Table 3. Vadose Zone Soil and Lake Mead Water Initial Quality Parameters

Parameter	Extract (mg/L)[1]	Soil (mg/g)[2]	Lake Mead Water (mg/L)
Perchlorate	48.1	0.18	0.0106
Nitrate (as NO_3)	91.2	0.34	2.04
Nitrate (as N)	20.9	0.76	0.45
Hardness (as $CaCO_3$)	---	---	294
Total Dissolved Solids	377	---	619
Chlorate	48.1	0.18	ND
Chloride	150	0.55	81.6
Phosphate (as PO_4^{-3})	0.4	0.0015	---
Iron	1.9	0.010	ND
Sulfate	105	0.39	238
pH (unitless)	7.3	---	7.7

[1]Concentrations in the aggregated extract (total volume of ~68 mL)
[2]Calculated based on 20 g of wet soil with a moisture content of 7.7%
---: Not analyzed
ND: non-detect

Microcosms Experimental Setup

The microcosm experiments were conducted in 150-mL borosilicate glass bottles. The microcosms contained contaminated soils, water, electron donor, and nutrients. For the EOS-100 and glycerol microcosms, soil and surface water from Lake Mead were added to each bottle at a ratio of 30 g of vadose zone soil to 100 mL of water (i.e.,1:3 soil to water ratio). The microcosms were then augmented with 0.5 mL of EOS-100 or 7 mL of 10-fold diluted glycerol. For the compost/mulch extract samples, 40 mL of compost extract was combined with 60 mL of surface water and 30 g of soil. The dosages of the electron donors were based on two parameters: (1) the hydrogen generated during the fermentation process and (2) the chemical oxygen demand (COD) of each donor. Both parameters impact the amount of donor needed to remediate the observed concentrations of the target electron acceptor (i.e., perchlorate) and other competing acceptors (e.g., oxygen, nitrate, and iron). The additional electron acceptors must be included when

calculating the amount of electron donor required because of the preferred redox state of some of these competing acceptors, as shown in the aforementioned redox tower (Figure 2).

[redacted] Electron Donor Dose

As explained in section 3. 2. 2, EOS-100 and glycerol generate sub-products as part of their degradation pathways. EOS-100 releases glycerol and long chain fatty acids (LCFAs). LCFAs are then converted to acetate and hydrogen (electron donors) via fermentation. Likewise, glycerol releases propionate or pyruvate, and depending on which reducing bacteria are present and the environmental conditions, the pyruvate is converted to additional subproducts, including acetate and hydrogen. Given that 0.4 lb of H_2 is generated per lb of EOS-100 (Table 1), it is possible to estimate the amount of EOS-100 required to reduce the various electron acceptors typically found at perchlorate-contaminated sites, as summarized in Table 4.

Table 4. EOS-100 demand considering the Stoichiometric Reaction of the Electron Donors and Hydrogen

Electron Acceptor	Reduction Equation	Moles H_2 / Moles Acceptor	lb Acceptor / lb H_2	lb Acceptor / lb EOS-100[1]	lb EOS-100 / lb Acceptor
Oxygen	$O_2 + 2\ H_2 \rightarrow 2\ H_2O$	2.0	7.9	3.2	0.31
Nitrate	$2\ NO_3^- + 2\ H^+ + 5\ H_2 \rightarrow N_2 + 6\ H_2O$	2.5	12	4.9	0.20
Perchlorate	$ClO_4^- + 4\ H_2 \rightarrow Cl^- + 4\ H_2O$	4.0	13	5.0	0.21
Chlorate	$ClO_3^- + 3H_2 \rightarrow Cl^- + 3\ H_2O$	3.0	14	5.6	0.10
Iron III	$2\ Fe^{+3} + H_2 \dashrightarrow 2\ Fe^{+2} + 2\ H^+$	0.5	55	22	0.05

[1]**Assumes 0.4 lb H_2 per lb EOS-100 (Table 1)**

Based on the concentrations of the electron acceptors present in the vadose zone soil (Table 3) and the amount of EOS-100 per electron acceptor required (Table 5), the estimated amount of EOS-100 required for the experimental testing can be determined (Table 6).

Table 5. EOS-100 Demand

Electron Acceptor	lb EOS-100/ lb Acceptor	Electron acceptor in vadose zone soil, mg/g soil	EOS Demand g oil/g soil	EOS-100 Demand for 30 g of wet soil, mg
Oxygen	0.31	0.017	$5.2x10^{-6}$	0.16
Nitrate	0.20	0.340	$6.9x10^{-5}$	2.06
Iron III	0.21	0.010	$4.5x10^{-7}$	0.01
Chlorate	0.10	0.180	$3.2x10^{-5}$	0.97
Perchlorate	0.05	0.180	$3.6x10^{-5}$	1.09
Total	---	---	$1.4x10^{-4}$	4.28

The total EOS-100 demand is ~4.28 mg per microcosm (i.e., per 30 g of wet soil and 100 mL of Lake Mead water). Given the relative density of the EOS-100 of 0.93, the total stoichiometric oil demand (i.e., 1X) for each microcosm would be 0.0045 mL. To achieve a 100x stoichiometric excess, 0.5 mL of EOS-100 was used in each microcosm. The stoichiometric excess is used to account for all the electron donors use for the bacteria present in the contaminated zone and due to the soil constituents that could limit the electron donor availability.

The amount of glycerol added was based on achieving a chemical oxygen demand (COD) equivalent to the aforementioned EOS-100 addition. The average COD concentrations of the pure EOS-100 and glycerol were 2.07×10^6 mg/L and 1.21×10^6 mg/L, respectively. Therefore, the COD of EOS-100 is about 1.7 times higher than glycerol. Therefore, the volume of glycerol required for each microcosm was assumed to be 1.7 times the EOS-100 volume (i.e., 0.0045 mL of EOS-100 × 1.7 = 0.0077 mL of pure glycerol). To achieve a 100x stoichiometric excess, 0.77 mL of glycerol needed to be dose. However, the glycerol stock was diluted ten-fold because glycerol is a highly viscous compound. Therefore, to facilitate the dosing or pipetting of the glycerol, the actual glycerol addition was 7 mL of 10-fold diluted glycerol in 100 mL of Lake Mead water.

The equivalent COD required for a complete reduction of the electron acceptors present in the contaminated vadose zone soil (i.e., 1X) would theoretically be ~103.50 and ~84.70 mg/L for EOS-100 and glycerol, respectively. Based on the equivalent COD requirements for EOS-100, to account for a 100x stoichiometric excess approximately 10,350 mg/L would be assumed. Therefore, based on the COD concentrations of the electron donors, the aforementioned volumes of EOS-100 and 10-fold diluted glycerol resulted in ~100X stoichiometric excess, while the 40 mL of compost extract resulted in ~1.0X stoichiometric excess. Nutrient requirements, specifically phosphorus, were calculated assuming a typical bacterial composition ($C_5H_7O_2NP_{0.1}$).

Initially, the microcosms were divided into four groups: (1) microcosms amended with EOS-100, (2) microcosms amended with glycerol, (3) microcosms amended with compost extract, and (4) control microcosms (i.e., 6.5 mg-P/L of phosphate addition, blanks (no electron donor added), and abiotic controls (autoclaved soil mixture)). After preparing the microcosms, the bottles were closed with a butyl rubber cap, crimped sealed with aluminum rings, and incubated at 21°C and 70 rpm for up to 25 days in the dark. At the time of analysis (i.e., after the specified incubation periods), the microcosms were opened, and the liquid and soil mixtures were transferred to 250 mL centrifuge bottles. The samples were then centrifuged at 4000×G for 20 minutes until the soil mixture was completely separated from the solution. The supernatants were transferred into different vials and then analyzed for perchlorate, nitrate, COD, sulfate, phosphate, sulfide, and pH. All microcosms were analyzed in duplicate at predetermined time intervals. The experimental matrix is summarized in Table 6 .

Table 6. Experimental Design Matrix for Preliminary Microcosms Experiments

Microcosm Sets	Days of Incubation							
	2	**6**	**8**	**12**	**16**	**20**	**24**	**25**
EOS (**E**)	E2	E6	E8	E12	E16	E20	E24	--
	E2-D	E6-D	E8-D	E12-D	E16-D	E20-D	E24-D	--
Glycerol (**G**)	G2	G6	G8	G12	G16	G20	G24	--
	G2-D	G6-D	G8-D	G12-D	G16-D	G20-D	G24-D	--
Compost Extract (**C**)	C2	C6	C8	C12	C16	C20	--	--
	C2-D	C6-D	C8-D	C12-D	C16-D	C20-D	--	--
Nutrient Buffer (**NB**)	--	E6-NB	E8-NB	--	--	C20-NB	E24-NB	C25-NB
	--	C6-NB	C8-NB	--	--	C20-NB-D	E24-NB-D	C25-NB-D
	--	--	--	--	--	--	G24-NB	--
	--	--	--	--	--	--	G24-NB-D	--
Blanks (**BK**)	--	--	--	--	--	BK20	--	BK25
	--	--	--	--	--	BK20-D	--	BK25-D
Abiotic Controls (**AC**)	--	--	--	--	--	--	E24-AC	C25-AC
	--	--	--	--	--	--	E24-AC-D	C25-AC-D
	--	--	--	--	--	--	G24-AC	--
	--	--	--	--	--	--	G24-AC-D	--

- EOS-100: 0.5 mL of EOS (COD equivalent of about 10,350 mg/L), 100 mL Lake Mead water, 30 g wet soil
- Glycerol: 7 mL of 10-fold diluted glycerol (COD equivalent of 8,470 mg/L), 100 mL Lake Mead Water, 30 g wet soil.
- Compost: 40 mL compost extract (COD equivalent of 101 mg/L), 60 mL Lake Mead water, 30 g of wet soil
- Wet soil = 7.7% moisture content

Notation:
Electron Donors: E = EOS-100 oil, G = Glycerol, C = Compost
AC = Abiotic Control (autoclaved soil and water mixture with electron acceptor)
BK = Blank (No electron donor nor phosphate added)
D = Duplicate
NB = nutrient buffer (addition of nutrient at 6.5 mg P/L)
--: No sample

Nitrate and Perchlorate Biodegradation in Microcosms with Varied Soil to Water Ratios

In a separate set of experiments, microcosms were prepared with only glycerol as the electron donor. These experiments were intended to evaluate the kinetics of two different doses of glycerol and two different soil to water ratios. Two different soil to water ratios were evaluated (i.e., 30g of soil to 100 mL of water (1:3) and 30g of soil to 60 mL of water (1:2)) to identify the adequate amount of water needed to mobilize the nitrate and perchlorate from the contaminated vadose soil into the aqueous phase.

The selection of the electron donor (glycerol) was based on acceptable kinetic rates observed during the initial testing and the fact that standard chemical properties are known for

this electron donor. In contrast, the full chemical properties of EOS-100 are unknown because they are proprietary. In this second set of experiments, the microcosms were prepared with either 0.35 mL or 0.70 mL of 100-fold diluted glycerol. These doses resulted in 0.5X and 1X stoichiometric COD (i.e., ~42.4 mg/L and ~84.7 mg/L, respectively). Recall that the initial experiments were performed with 100X stoichiometric COD (i.e., 10,350 mg/L). As shown in Table 7, these samples were incubated under similar conditions as the initial experiments but for up to 40 days.

Table 7. Experimental Design Matrix for Secondary Microcosm Experiments

Microcosm Sets	Days of Incubation						
	5	**13**	**18**	**24A**	**24B**	**35**	**40**
0.5X Glycerol – Water 100mL	0.5X-W100	0.5X-W100	0.5X-W100	0.5X-W100	0.5X-W100	0.5X-W100	0.5X-W100
	0.5X-W100-D	0.5X-W100-D	0.5X-W100-D	0.5X-W100-D	0.5X-W100-D	0.5X-W100-D	0.5X-W100-D
0.5X Glycerol – Water 60 mL	0.5X-W60	0.5X-W60	0.5X-W60	0.5X-W60	0.5X-W60	0.5X-W60	0.5X-W60
	0.5X-W60-D	0.5X-W60-D	0.5X-W60-D	0.5X-W60-D	0.5X-W60-D	0.5X-W60-D	0.5X-W60-D
1X Glycerol – Water 100 mL	1X-W100	1X-W100	1X-W100	1X-W100	1X-W100	1X-W100	1X-W100
	1X-W100-D	1X-W100-D	1X-W100-D	1X-W100-D	1X-W100-D	1X-W100-D	1X-W100-D
1X Glycerol – Water 60 mL	1X-W60	1X-W60	1X-W60	1X-W60	1X-W60	1X-W60	1X-W60
	1X-W60-D	1X-W60-D	1X-W60-D	1X-W60-D	1X-W60-D	1X-W60-D	1X-W60-D
Blanks (NC)	--	W-60 NC	W-60 NC	W-60 NC	--	--	W-60 NC
	--	W-100 NC	--	--	W-100 NC	W-100 NC	--

- All microcosms contain 30 g of soil and either 100 mL or 60 mL of water (1:3 and 1:2 soil to water ratios, respectively)

Notation:
0.5X = 0.5X stoichiometric COD = 0.35 mL glycerol 100-fold diluted glycerol
1X = 1X stoichiometric COD = 0.70 mL of 100-fold diluted glycerol
W100 = 100 mL of surface water from Lake Mead
W60 = 60 mL of surface water from Lake Mead
NC = No carbon source added (i.e., no glycerol added)
D = Duplicate
--: No sample

Analytical Methods

Perchlorate concentrations were determined with ion chromatography (IC) using US EPA Method 314. Other analyses were performed according to EPA-approved methods, as summarized in Table 8.

Table 8. Analytical Methods

Analysis	EPA Method
Nitrate	Hach EPA 10206 and EPA 352.1
COD	Hach 8000
Sulfate	IC and Hach EPA 8051
Phosphate	EPA 365.1
Iron	Hach 8008 and 8147-ferrover
Chloride	Hach 8225
pH	Hach EPA 8156

Results and Discussion

Chemical Oxygen Demand in Vadose Zone Soil and Surface Water Microcosms

The chemical oxygen demand (COD) originating from the vadose zone soil and the water from Lake Mead was assumed to be the same as the blank control since no nutrients or substrates were added to these microcosms. Thus, the initial COD originating from the vadose zone soil and Lake Mead was approximately 16 mg/L. Based on the stoichiometry of nitrate and perchlorate reduction, this COD concentration is insufficient to achieve complete removal of these contaminants, thereby warranting amendment with electron donors.

For samples amended with the experimental electron donors (i.e., EOS-100, glycerol, and compost/mulch), the COD was used as an indirect measurement of the electron donor concentrations. As indicated earlier, the average COD concentrations of neat solutions of EOS-100, glycerol, and compost extract were 2.07×10^6 mg/L, 1.21×10^6 mg/L, and 253 mg/L, respectively. With the exception of the compost/mulch extract, which had a significantly lower stock concentration, the electron donors were added to the microcosms to achieve 100-fold stoichiometric excess for the preliminary experiments.

During the incubation period, the COD concentrations exhibited interesting patterns, presumably due to differing biophysicochemical properties. Although a sufficient quantity of EOS-100 was added to achieve an initial COD concentration of approximately 10,350 mg/L, COD decreased rapidly to ~270 mg/L. It is assumed that a majority of the EOS-100 adsorbed onto the soil in the microcosm and that the ~270 mg/L was the amount remaining dissolved in solution early in the incubation period. This highlights the potential use of EOS-100 as a slow-release electron donor in long-term soil remediation applications. Aqueous glycerol concentrations remained relatively constant at ~10,000 mg/L (slightly above the expected value of 8,470 mg/L) during the entire incubation period. The COD concentrations for the compost/mulch extract also remained relatively constant at the spiking level of ~100 mg/L. Therefore, there was minimal adsorption of the glycerol or compost/mulch extract compounds onto the soil, thereby indicating these alternatives may not be appropriate for long-term remediation applications requiring slow release substrates. The minimal decrease in COD concentration over the incubation period suggests that the spiked quantities were in fact in stoichiometric excess, or that there was minimal reduction of the electron acceptors (discussed later).

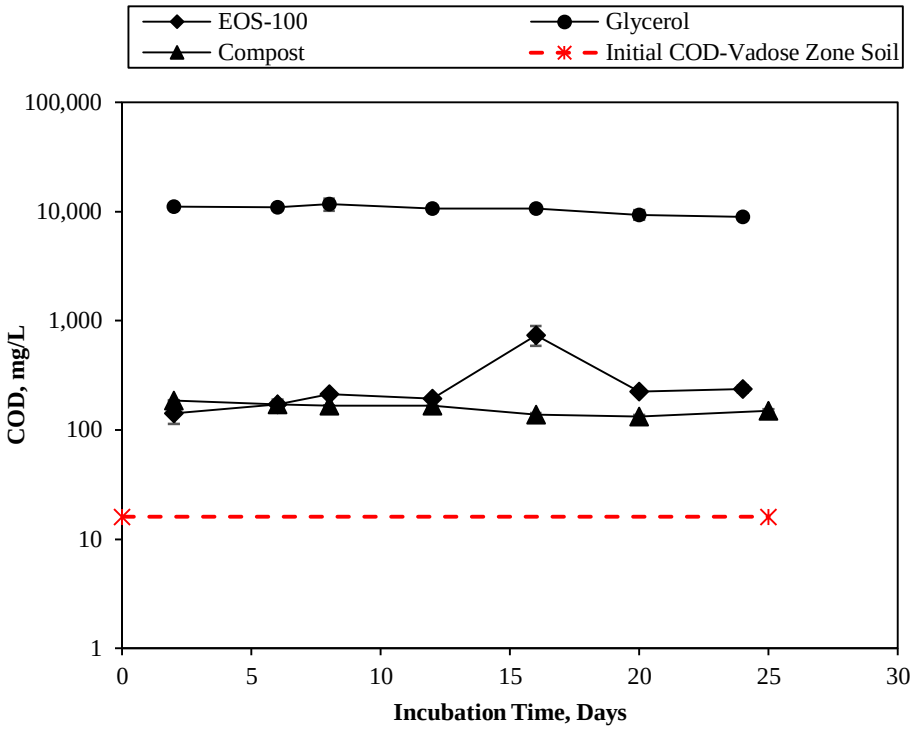

Figure 3. Chemical Oxygen Demand Concentrations in Microcosms Augmented with EOS-100, glycerol and Compost/mulch Electron Donors. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

Biodegradation of Nitrate in Vadose Zone Soil and Lake Mead Water Microcosms

The initial nitrate contributions from the soil and surface water were 20.9 mg-N/L and 0.46 mg-N/L, respectively. Nitrate is a more favorable electron acceptor compared to perchlorate in perchlorate bioremediation systems. As shown earlier in

Figure 2, the sequence of electron acceptors indicates the relative preferences of PRB. Specifically, the redox potentials indicate that oxygen and nitrate are preferred over perchlorate as a terminal electron acceptor. Therefore, perchlorate bioremediation is hindered by the presence of these competing species.

Figure 4 shows nitrate reduction in the presence of the three electron donors. EOS-100 and glycerol demonstrated rapid nitrate reduction over the first 6-8 days of incubation. In fact, this reduction matches the lag in perchlorate reduction observed between days 2 and 8 (described later in Figure 5). Again, this supports the statement that nitrate is preferred over perchlorate as an electron acceptor. These results are consistent with previous research (Coates & Achenbach, 2004; Gal et al., 2008; ITRC, 2008; Zhu et al., 2016). After day 8 of incubation EOS-100 and glycerol achieved concentrations closed to the detection limit of the Hach assay (< 0.2 mg/L). Nevertheless, EOS-100 demonstrated higher maximum nitrate degradation rates than glycerol with maximum degradation rates of 3.42 mg-N/L/d and 2.75 mg-N/L/d, respectively between the period of predominant nitrate reduction activity (i.e., days 0 to day 6 of incubation).

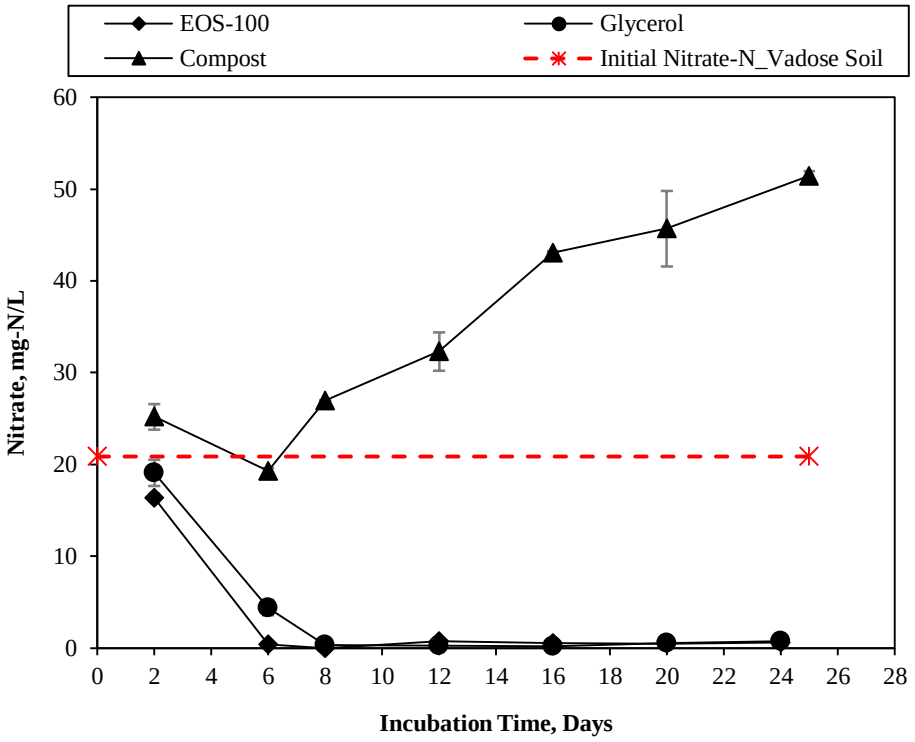

Figure 4. Nitrate Reduction in Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

In contrast with EOS-100 and glycerol, the microcosms amended with compost/mulch did not demonstrate any nitrate reduction; instead, nitrate actually increased in concentration over time, perhaps due to further decomposition of the extract. Considering that compost/mulch often contains varying concentrations of nitrate, ammonium, and other nitrogen-containing compounds (Kazuto et al., 2013; USDA, 2010), it is possible that the inorganic or organic nitrogen was converted to nitrate over time. This is particularly problematic considering that nitrate inhibits perchlorate reduction in bioremediation applications.

[redacted] Biodegradation of Perchlorate in Vadose Zone Soil and Lake Mead Water Microcosms

As determined by the sequential extractions described earlier, the initial perchlorate concentrations in the vadose zone soil and in the surface water from Lake Mead were 48.1 mg/L and 0.012 mg/L, respectively. The extraction process was assumed to yield the maximum aqueous concentration, but the true initial perchlorate concentration in the microcosms (i.e., day 2; >50 mg/L) was higher than the concentrations found during the soil extraction. This unexpected increase in perchlorate concentration may have resulted from the extended contact time (i.e., 2 days of incubation), heterogeneity in the soil samples, or simply experimental error. Nevertheless, the two concentrations were relatively similar, and the difference did not pose any significant issues for data interpretation.

Perchlorate reduction in the microcosms amended with different electron donors is shown in Figure 5. EOS-100 and glycerol demonstrated a lag period during the first 6 days of incubation. Between day 6 and 12, however, EOS-100 demonstrated ~87% perchlorate removal, while glycerol achieved just ~74% removal for the same period of incubation. By day 20, EOS-100 and glycerol achieved similar reductions in perchlorate concentration. In contrast, the

compost/mulch solution demonstrated minimal perchlorate removal, even after 25 days of incubation, presumably because of the significantly lower initial COD concentration, the inability for the compost/mulch extract to reduce nitrate, and the fact that the compost/mulch extract actually released additional nitrate into solution.

Furthermore, based on the initial perchlorate concentration in the blank control microcosms (i.e., no electron donor added) of 52 mg/L, the maximum perchlorate degradation rates were calculated as 3.21 mg/L/d and 2.85 mg/L/d for EOS-100 and glycerol, respectively. The degradation rates obtained in this study are similar to than the degradation rates reported by Solution-IES (2006) and Evans, Patrick J (2008) of ~3 mg/L/d and 0.2 mg/L/d for EOS-100 and glycerol, respectively. But compared with perchlorate biodegradation rates reported by Batista et al., (2005) and Nozawa-Inoue, Mamie (2011) when using acetate as the source of electron donor (Table 1), EOS-100 and glycerol demonstrated higher perchlorate degradation rates.

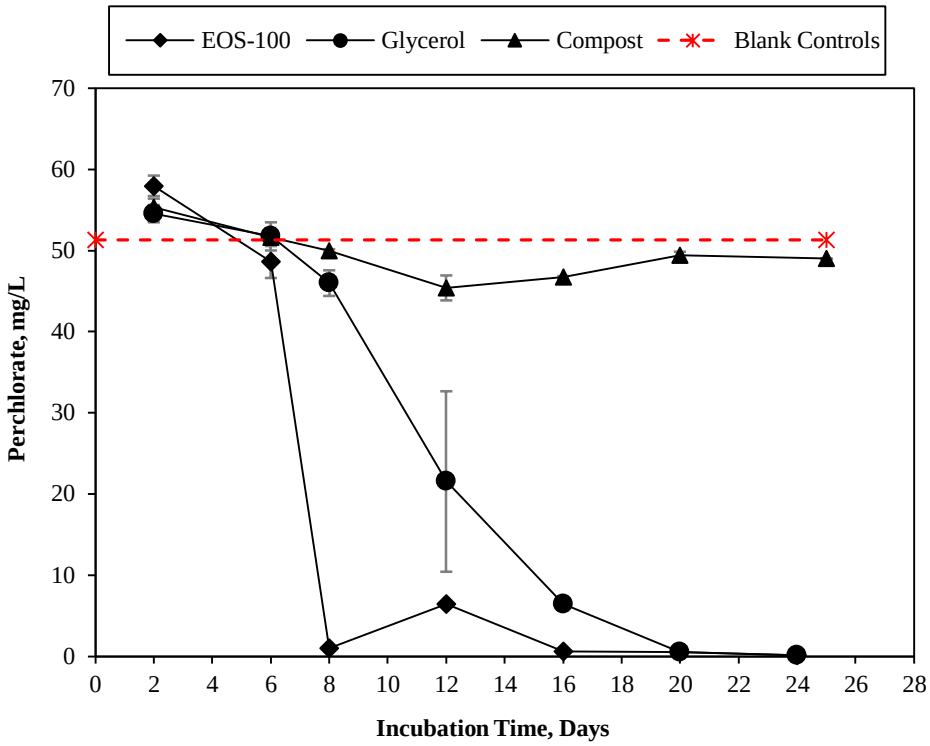

Figure 5. Perchlorate Reduction in Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

The blank microcosms (i.e., no electron donor added) achieved minimal perchlorate reduction. The limited removal observed may have been due to the presence of natural electron donors (e.g., natural organic matter) present in the vadose zone soil, but clearly the lack of sufficient electron donor limits degradation This result is similar to that of Gal et al. (2008), which found that natural organic matter was able to induce perchlorate reduction but at significantly slower rates than other carbon sources (or higher concentrations of carbon). Therefore, with a longer period of incubation, blank microcosms may have achieved greater perchlorate reduction but not at an acceptable rate or extent compared to engineered bioremediation applications.

[redacted] Nitrate and Perchlorate Reduction Kinetics in Vadose Zone Soil and Lake Mead Water Microcosms

Using the aforementioned data for EOS-100 and glycerol, pseudo first order rate constants describing the reduction of nitrate and perchlorate were determined based on linear regression over defined incubation periods. More specifically, nitrate reduction was evaluated over the first 6 and 8 days of incubation for microcosms amended with EOS-100 and glycerol, respectively. These periods were chosen based on the observed nitrate reduction during experimentation (Figure 4). Similarly, perchlorate reduction was generally characterized after nitrate had been completely removed (i.e., between 6 and 20 days of incubation). The reduction kinetics for the compost/mulch were not determined since no perchlorate or nitrate reduction was observed with that electron donor.

The nitrate rate constants were determined based on data in which nitrate reduction was prominent (i.e., from days 6 and 8 days for microcosms amended with EOS-100 and glycerol, respectively). By day 12, the nitrate had essentially reached the detection limit of the Hach assay. Figure 6 shows the linear regression of nitrate reduction over the defined incubation period. The rate constants for EOS-100 and glycerol were 0.60 d^{-1} and 0.42 d^{-1}, respectively, at 21±2°C. Because of the limited data collected during the nitrate reduction period, the rate constants should be used with caution, as they may include significant experimental error. Nevertheless, the rate constants confirm the observation from Figure 5 that EOS-100 achieves more rapid reduction of nitrate.

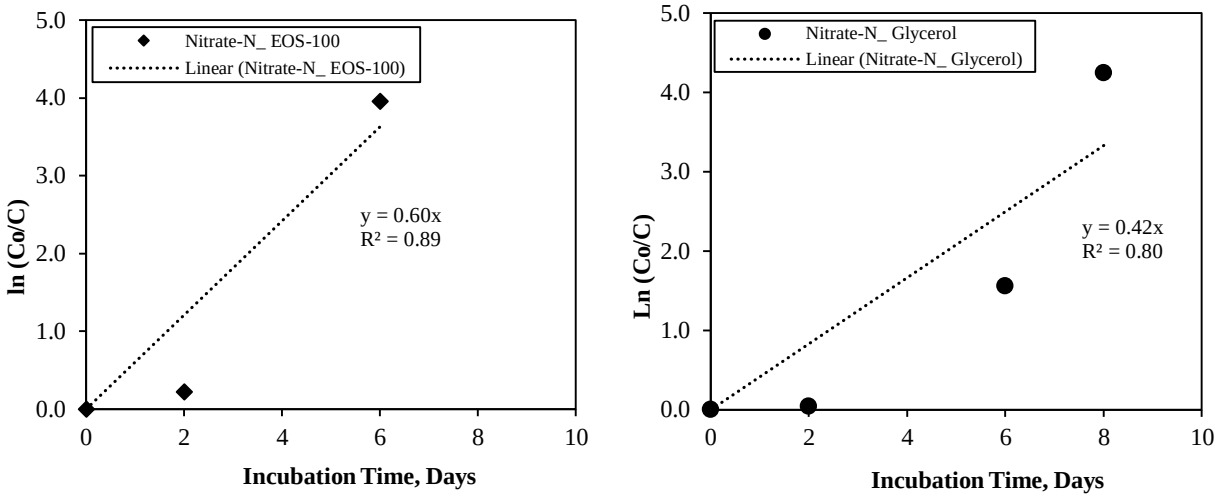

Figure 6. Nitrate Reduction Kinetics in Vadose Zone Soil and Lake Mead Water Microcosms at 21±2°C

The rate constants for perchlorate reduction were also calculated. But in contrast to nitrate, perchlorate reduction was characterized between days 6 and 20 of incubation (i.e., after nitrate had been removed), thereby resulting in more data points to calculate more reliable rate constants, as shown in Figure 7. Perchlorate reduction also followed a pseudo first order reaction when the microcosms were amended with EOS-100 or glycerol at ~100X stoichiometric COD. The slopes of the linear regressions represent the first order rate constants describing the biological reduction of perchlorate in the absence of nitrate for EOS-100 (0.36 day^{-1}) and glycerol (0.31 day^{-1}) at 21±2°C. These results are consistent with the perchlorate biodegradation observed during experimentation (Figure 5). Therefore, EOS-100 is also slightly faster than glycerol for perchlorate biodegradation.

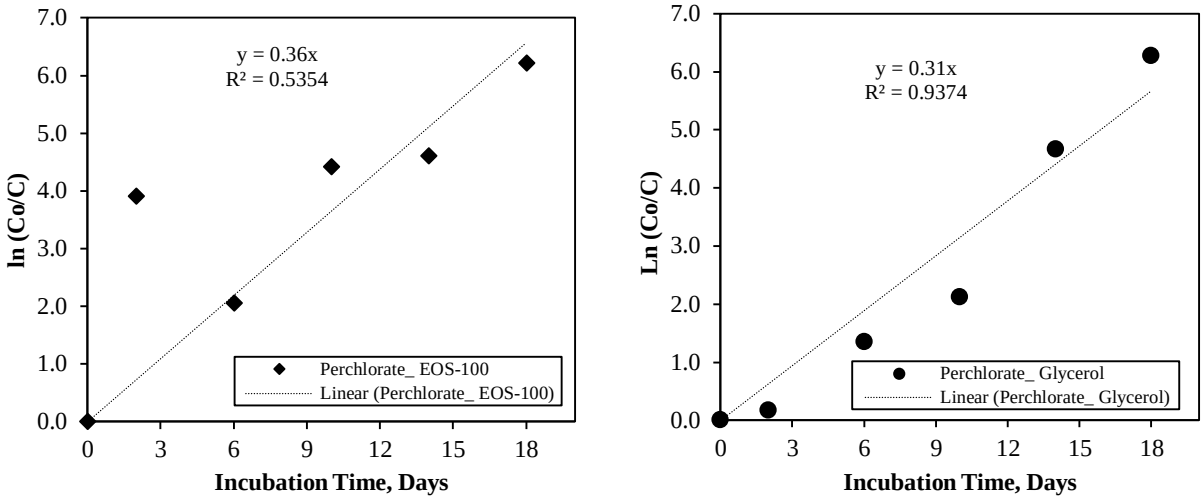

Figure 7. Perchlorate Reduction Kinetics in Vadose Zone Soil and Lake Mead Water Microcosms at 21±2°C

The objective of the first part of this research was to evaluate the potential use of diverse electron donors, specifically EOS-100, glycerol, and a compost/mulch extract, for perchlorate biodegradation in vadose zone soil and surface water from Lake Mead. Based on the aforementioned data, EOS-100 appears to be the best electron donor because it exhibited the fastest kinetics for nitrate and perchlorate reduction as shown in Table 9, and did not contribute significant quantities of competing species (e.g., nitrate release from the compost/mulch extract).

Table 9. Summary Rate Constants for Nitrate and Perchlorate Reduction with EOS-100 and Glycerol at 21±2°C

Electron Donor	Electron Acceptor	First Order Rate Constant
EOS-100	Nitrate	0.60 d^{-1}
Glycerol	Nitrate	0.42 d^{-1}
EOS-100	Perchlorate	0.36 d^{-1}
Glycerol	Perchlorate	0.31 d^{-1}

Changes in pH and Reduction of Sulfate in Vadose Zone Soil and Lake Mead Water Microcosms

The initial pH of the microcosms was ~7.7, which was consistent with the pH of the Lake Mead water, and during the incubation period, the pH in the microcosms decreased to ~7.3 (Figure 8). This is contrary to the increase in pH expected when sulfate is reduced to sulfite and then sulfide. The reduction of sulfate to sulfide is summarized in Table 10 and Figure 8b. Interestingly, the compost extract resulted in the highest sulfide concentration, but this was most likely due to the higher sulfate content of the compost extract.

The maximum degradation rates of sulfate were evaluated during the period of high sulfate reduction (i.e., between day 2 and day 12). As mention before, the compost/mulch solution demonstrated the higher sulfate reduction with a degradation rate of 34mg/L/d compared with EOS-100 and glycerol, which demonstrated lower sulfate reduction with degradation rates of 1 mg/L/d and 3 mg/L/d, respectively. This high sulfate reduction in the compost/mulch extract, as described earlier, may have resulted due to lower efficiencies of the compost extract in reducing nitrate and perchlorate, and this may have been caused—at least in part—by the fact that the compost/mulch extract contributed a significant quantity of competing electron acceptors (i.e., nitrate and sulfate).

Sulfate depletion occurs in the redox range of -120 mV and -180 mV, while perchlorate occurs in the range from 0 mV to -100 mV. However, the concentrations of the various competitors also impact the thermodynamic favorability of the various reactions. Nevertheless, high sulfate concentrations are undesirable because of the adverse effects of competitive reduction and the potential for odor formation as sulfate is reduced to sulfide. The pka of H_2S/HS^- is 6.99 so at the experimental pH value of ~7.3, the distribution of the species would be

33% as H_2S and 67% as HS^-. This suggests a portion of the sulfide will volatilize and generate noxious odors during the bioremediation process.

Table 10. Sulfate Concentrations in the Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors

Electron Donor	Sulfate, mg/L	
	Day 2	Day 12
EOS-100	390	360
Glycerol	350	340
Compost	670	300

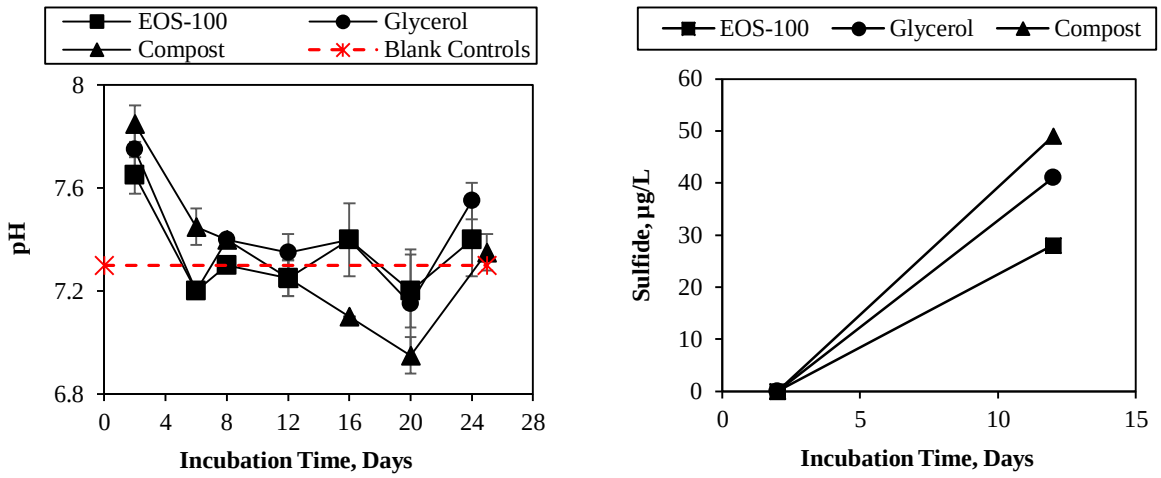

Figure 8. pH and Sulfide Comparison for Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

Abiotic Controls in Vadose Zone Soil and Lake Mead Water Microcosms

The abiotic controls also achieved decreases in nitrate and, to a lesser extent, perchlorate. These results were not expected because the vadose zone soil samples and water from Lake Mead had been autoclaved to inactivate any native PRB. Nitrate concentrations were reduced to the detection limit of the Hach assay in the abiotic controls amended with EOS-100 and glycerol, but perchlorate concentrations exhibited only slight decreases (Table 11).

Table 11. Results from Abiotic Control Microcosms for Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors

Electron Donor	Nitrate			Perchlorate		
	Day 0	Day 24	Day 25	Day 0	Day 24	Day 25
EOS-100	20.9 mg-N/L	0.5 mg-N/L	---	48.1 mg/L	47.1 mg/L	---
Glycerol	20.9 mg-N/L	0.3 mg-N/L	---	48.1 mg/L	46.1 mg/L	---
Compost	20.9 mg-N/L	---	17.2 mg-N/L	48.1 mg/L	---	51.1 mg/L

--- Not analyzed

It is possible that the autoclaving procedure was unable to inactivate the entire microbial community, particularly spore-forming microorganisms, thereby allowing for some degree of biological reduction during the batch tests (Su & Puls, 2007). The duration of the autoclave cycle was 30 minutes at the standard temperature of 250°F (121°C). Previous studies suggested that autoclaving soil may not be as effective as alternative sterilization techniques, including exposure to dilute formaldehyde or mercuric chloride, but these procedures are less common (Trevors, 1996; Wolf et al., 1989). On the other hand, physicochemical methods of nitrate reduction may also contribute to nitrate removal, particularly as a result of the autoclaving process (Trevors, 1996). For example, Dail et al. (2001) investigated the abiotic and biotic reduction of nitrate and nitrite in sterile and non-sterile forest soils. Their results demonstrated that abiotic reduction of nitrate and nitrite can occur during autoclaving.

The actual cause of the nitrate reduction in the abiotic controls is still unclear for the current study because further testing was not performed to assess whether nitrogen compounds were generated or if any bacteria survived the autoclaving process. Based on the literature review, longer autoclave cycles or repeated autoclaving might be warranted to achieve complete sterilization of soils. Regardless, minimal perchlorate reduction was observed in the abiotic

controls so the perchlorate reduction observed in the other experimental samples can be attributed to biodegradation.

Phosphate Amendment in Vadose Zone Soil and Lake Mead Water Microcosms

Because bacteria need essential macronutrients to grow (e.g., phosphorus, carbon, nitrogen), higher concentrations or manual augmentation of these nutrients should presumably lead to more efficient biodegradation. For the samples containing EOS-100 or glycerol, the phosphate concentrations ranged from 0.3-2.4 mg/L and 0.2-0.5 mg/L as phosphate, respectively, during the incubation period. The phosphate concentration in the blank control (i.e., soil and water only; no electron donor or nutrient addition) was similar to that of glycerol, which indicates that the higher phosphate concentrations in the EOS-100 samples were likely originating from the emulsified oil solution. This additional phosphate could be a contributing factor to the faster nitrate and perchlorate degradation kinetics for EOS-100. Figure 9 illustrates the phosphate concentrations over time for the various microcosms.

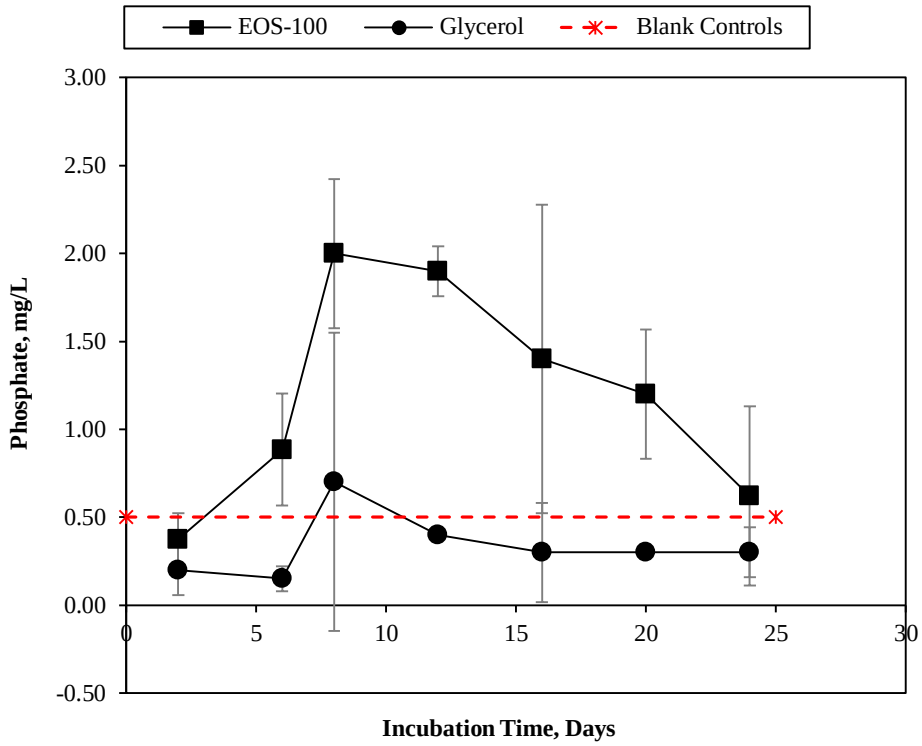

Figure 9. Phosphate as PO_4^{-3} Concentrations-No Additional Phosphate Added for Microcosms Augmented with EOS-100, Glycerol, and Compost/mulch Electron Donors. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

To test the relationship between phosphate and biodegradation kinetics, the experimental microcosms were amended with ~6.5 mg-P/L of phosphate and incubated for up to 25 days. Table 12 summarizes the residual phosphate concentrations over time during this experiment. The data indicate that phosphate removal (i.e., uptake) was more apparent in the microcosms containing EOS-100 or glycerol, which were also the samples with faster nitrate and perchlorate reduction kinetics.

Table 12. Phosphate Concentrations in Nutrient Controls

Days	Electron Donor		
	EOS-100	Glycerol	Compost/mulch
6	---	0.4 mg- PO_4^{-3}/L	---
8	1.4 mg- PO_4^{-3}/L	---	---
20	---	---	7.7 mg- PO_4^{-3}/L
24	1.6 mg- PO_4^{-3}/L	0.6 mg- PO_4^{-3}/L	---
25	2.3 mg- PO_4^{-3}/L	1.8 mg- PO_4^{-3}/L	8.4 mg- PO_4^{-3}/L

--- No Sample

However, perchlorate and nitrate reductions in the nutrient-amended microcosms were similar to those achieved without nutrient amendment, as shown in Table 13. These results are similar to those reported in the literature, which indicated the addition of phosphate did not enhance perchlorate removal rates. Evans & Trute (2006) stated that nutrient addition is not necessary to enhance perchlorate removal. The authors found that the use of ethanol and hydrogen as electron donors did not achieve total perchlorate removal with nutrient addition (i.e., amendment with $(NH_4)_2HPO_4$), but changes in moisture content did have a significant effect on perchlorate reduction. Conversely, Wang et al. (2013) demonstrated that nutrient addition enhanced perchlorate removal when using emulsified vegetable oil (EOS-598) and a compost/mulch substrate, which contradicts the results from the current study. However, the nutrient amendment in Wang et al. (2013) consisted of 1,000 mg/L of $(NH_4)_2HPO_4$, which was 50 times higher than the concentration added during the current study (i.e., 20 mg-PO_4^{-3}/L). The concentration of phosphate used in the current study was based on the typical bacterial composition ($C_5H_7O_2NP_{0.1}$) in which the mass ratio of nitrogen to phosphorus (N:P) should be 4.5:1. Therefore, with an initial nitrate concentration of ~20.9 mg-N/L, the minimum amount of phosphorus needed in the microcosms for an effectively biological reduction would approximately be 4.6 mg-P/L. However, to improve perchlorate biological reductions during the batch test 6.5 mg-P/L of phosphate was provided.

Table 13. Perchlorate and Nitrate Reduction in Nutrient-Amended in Vadose Zone Soil and Lake Mead Water Microcosms

Electron Donor	Day	Perchlorate (mg/L)		Nitrate (mg-N/L)	
		Nutrient Added	No Nutrient	Nutrient Added	No Nutrient
EOS-100	8	10.0	1.0	**	**
	4	0.1	0.1	1.4	1
	25	**	---	1.1	---
Glycerol	6	53.7	53.0	2.1	5.0
	24	0.1	0.1	7.5	0.9
	25	**	---	0.7	---
Compost	20	50.1	49.7	48.2	48.6
	25	49.6	49.0	47.2	51.8

Initial concentrations: nitrate = 20.9 mg-N/L and perchlorate = 48.2 mg/L
** below detection limit
--- No Sample

[redacted] Soil to Water Ratios Second Set of Microcosms in Vadose Zone Soil and Lake Mead Water Microcosms with Glycerol as Electron Donor

The objective of this component of the study was to evaluate the impact of varying soil to water ratios on nitrate and perchlorate. For these experiments, only a single electron donor (glycerol) was used, but the concentration was decreased to 0.5X and 1.0X stoichiometric COD, as compared with the ~100X stoichiometric COD in the previous experiments. The amount of glycerol used in this phase was lower than that of phase I because excessive quantities of glycerol were still present at the end of the initial batch experiments. Although the excess electron donor presumably improved perchlorate reduction kinetics, the excess glycerol might be viewed as a waste and an unnecessary cost in full-scale applications. Therefore, this phase of the research also evaluated the impact of reduced electron donor addition.

The soil quantities were held constant at 30 grams, but the amount of water added to each microcosm varied between 60 and 100 mL, giving to different soil to water ratios to evaluate (i.e., 1:3 and 1:2, respectively). This allowed for an analysis of varying soil to water ratios, which could impact mobilization of perchlorate from the contaminated vadose zone soil. In a full-scale

'soil flushing' application, water is injected into the contaminated vadose zone to mobilize perchlorate and make it available for downstream treatment in a permeable reactive barrier. In one scenario, perchlorate mobilization might be hindered if less water is injected into the ground, thereby slowing remediation efforts. Alternatively, the reduced water volumes might mobilize the same amount of perchlorate, thereby resulting in a higher effective perchlorate concentration. This could potentially improve biodegradation kinetics.

In summary, these experiments were intended to evaluate the impacts of lower electron donor concentrations and varying soil to water ratios (i.e., varying soil-flushing volumes). In addition to the primary experimental samples, control microcosms were prepared to evaluate the impacts of electron donor blanks (i.e., no glycerol added). The experimental matrix was summarized previously in Table 7.

The initial nitrate and perchlorate concentrations in the microcosms were affected by the different volume of water utilized (60 mL and 100 mL) (i.e., soil to water ratios 1:2 and 1:3, respectively). When using 100 mL of water, the initial nitrate and perchlorate concentrations were the same as in the first phase of the research: ~20.9 mg-N/L and ~48.1 mg/L, respectively. But when using 60 mL of water, the concentrations of nitrate and perchlorate increased due to lower dilution ratios (i.e., ~49.1 mg-N/L and ~85.8 mg/L, respectively, based on the concentrations observed in the blank control microcosms). This indicates that the reduced 'soil flushing' volume would still be adequate to mobilize the same amount adsorbed perchlorate.

In addition to the increase in nitrate and perchlorate concentrations, other effects can be generated in lower dilution ratios such as high salinity or high total dissolve soils contents. These increments could affect the microbial community present in the contaminated vadose soil and Lake Mead water by reducing the number of bacteria or decreasing their activity.

During the first three weeks of batch testing (i.e., day 0 to day 14), there was no consistent reduction in perchlorate concentration, as shown in Figure 13. To expedite the reaction, the amount of glycerol was increased to target ~2.5X and ~11X stoichiometric COD. Figure 10 shows the COD observed during experimentation. During the first few days of incubation, the COD concentration remained relatively constant at a spiking level of ~17 mg/L, which correlates with the lower initial spiking level of glycerol.

Interestingly, after spiking the additional glycerol, the COD concentration in the ~2.5X stoichiometric COD samples remained at ~20 mg/L, this is unexpected because these microcosms were designed to achieve an initial COD concentration of ~212 mg/L and ~ 424 mg/L when using 100 mL and 60 mL of Lake Mead water, respectively. This low COD stoichiometric excess is presumable due to absorption of the glycerol into the soil during the incubation period. Similarly, the COD concentration in the 11X stoichiometric COD samples were designed to achieve an initial COD concentration of ~932 mg/L and ~1,864 mg/L when using 100 mL and 60 mL of Lake Mead water, respectively. However, the stoichiometric COD excess in these samples remained at ~1,000 mg/L. The COD stoichiometric excess in these samples is more evident due to the saturation of the soil by the absorption of the glycerol and the higher dosing in these microcosms. These effects are more noticeable in the samples with lower water content (i.e., 60 mL).

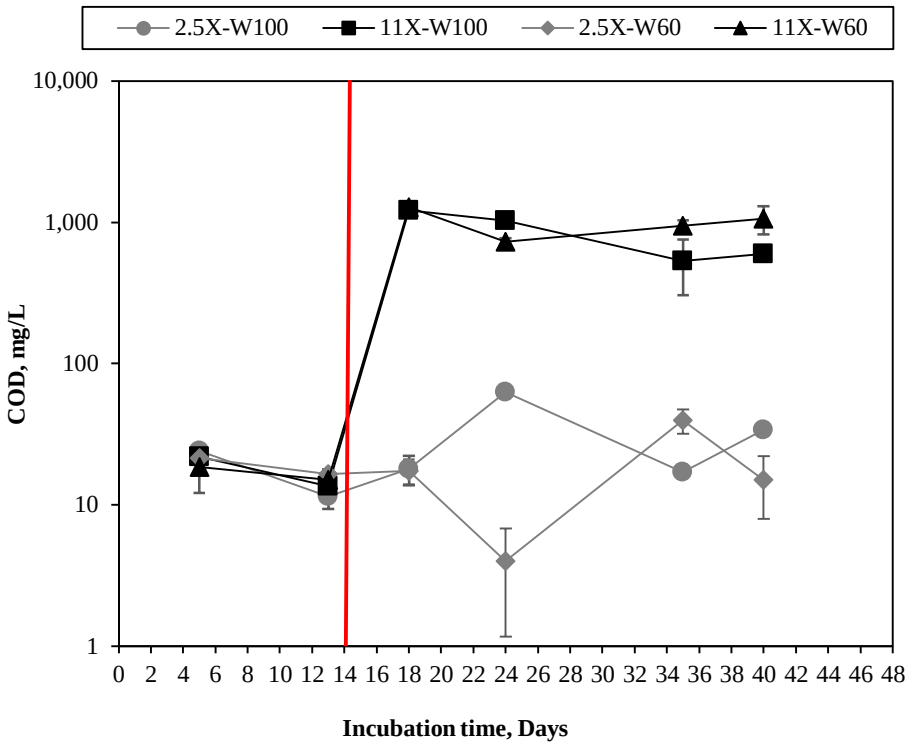

Figure 10. Chemical Oxygen Demand as a Function of Soil to Water Ratios. The red line indicates the glycerol dosage increment in the microcosms. Until day 14, stoichiometric COD were 0.5X and 1X. After glycerol addition (day 14), the stoichiometric COD were 2.5X and 11X. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

3. 3. 8. 1 Biodegradation of Nitrate as a Function of Soil to Water Ratios

After the additional injection of glycerol, most of the microcosms demonstrated significant nitrate reduction, except microcosms with 2.5X stoichiometric COD in 60 mL of water (i.e., 0.5X-W60 designation previous to glycerol addition), as shown in Figure 11. In other words, microcosms with 2.5X stoichiometric COD and 1:3 soil to water ratio (30g soil/100 mL water) or both soil to water ratios at 11X stoichiometric COD demonstrated comparable nitrate reductions (~98%) with comparable degradation rates as shown in Figure 12 .

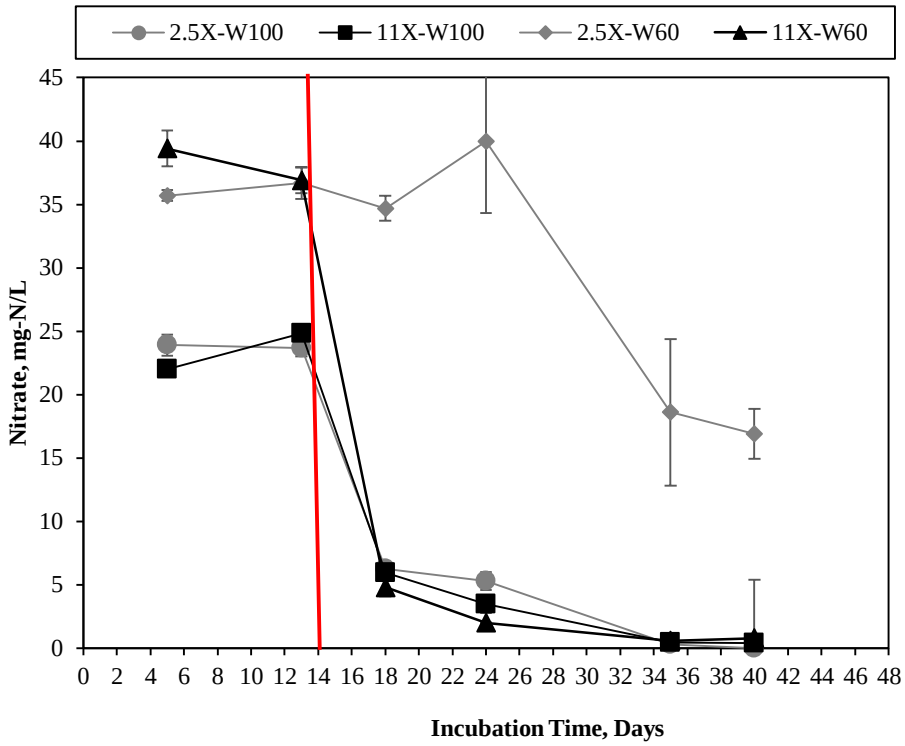

Figure 11. Nitrate Reduction Soil to Water Ratios. The red line indicates the glycerol dosage increment in the microcosms. Until day 14, stoichiometric COD were 0.5X and 1X. After glycerol addition (day 14), the stoichiometric COD were 2.5X and 11X. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

Based on these results, stoichiometric equivalent concentrations (i.e., ~1X) are inadequate for reliable nitrate reduction, and the efficacy of nitrate reduction with limited excess COD (i.e., ~2.5X) appears to vary based on soil flushing volume. When the electron donor addition reaches ~11X stoichiometric COD, reliable and complete nitrate reduction can be achieved with the soil to water ratios tested in this research, with comparable nitrate degradation rates of 0.091 mg-N/d for 1:3 soil to water ratio and 0.080 mg-N/d for 1:2 soil to water ratio. This potentially represents a significant cost savings compared to donor addition at ~100X stoichiometric COD, although perchlorate reduction must be verified under the modified conditions.

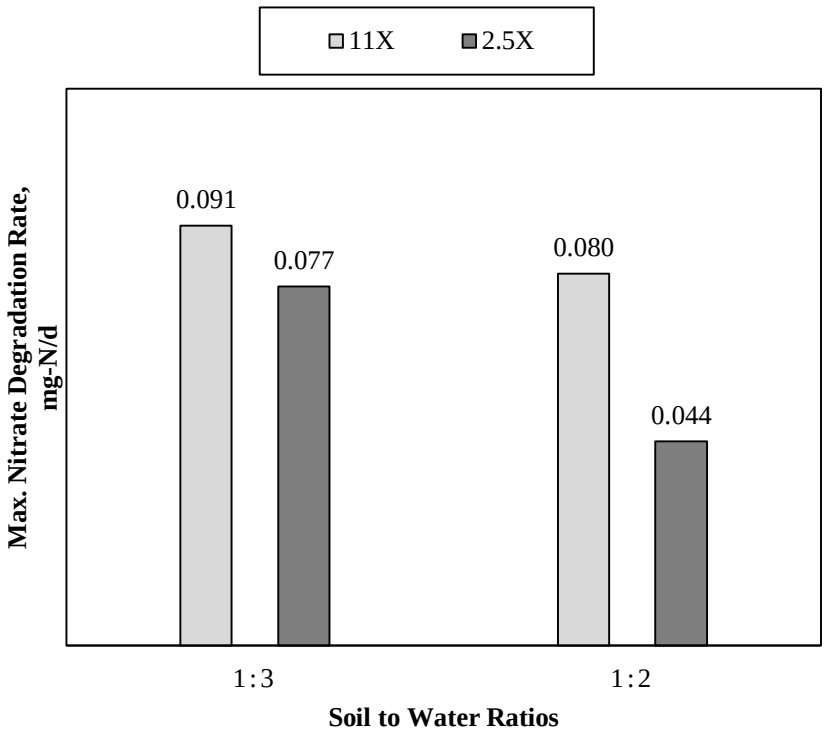

Figure 12. Maximum Nitrate Biodegradation Rates as a Function of Soil to Water Ratios. 1:3 (30g of soil/100 mL of water), 1:2 (30g of soil/60 mL of water). The degradation rates are calculated based on the degradation observe after the additional injection of glycerol (period of incubation of ~27 days)

3. 3. 8. 2 Biodegradation of Perchlorate as a Function of Soil to Water Ratios

Although significant nitrate reduction was achieved during the incubation period, perchlorate reduction was significantly inhibited compared to the first set of microcosms (Figure 12). The lack of perchlorate reduction was most likely due to a combination of the lower glycerol concentration (up to ~11X instead of 100X) and the apparently insufficient incubation period after the additional glycerol injection (~27 days). In addition, the low initial glycerol concentration prevented nitrate reduction, which extended the lag period during which perchlorate reduction was thermodynamically unfavorable.

As shown in Figure 13, microcosms with 2.5X stoichiometric COD achieved ~2% perchlorate reduction with 60 mL of water (1:2 soil to water ratio) and ~8% perchlorate reduction with 100 mL of water (1:3 soil to water ratio). The microcosms with 11X stoichiometric COD achieved perchlorate reductions of ~5 % for 60 mL and ~18 % for 100 mL. The 2.5X-W100 and 11X-W60 microcosms exhibited an increase in perchlorate concentration at the end of the incubation period (i.e., day 40). These increases were attributed to experiment error, as each day represents a different microcosm, and no additional analyses were performed to investigate this anomaly.

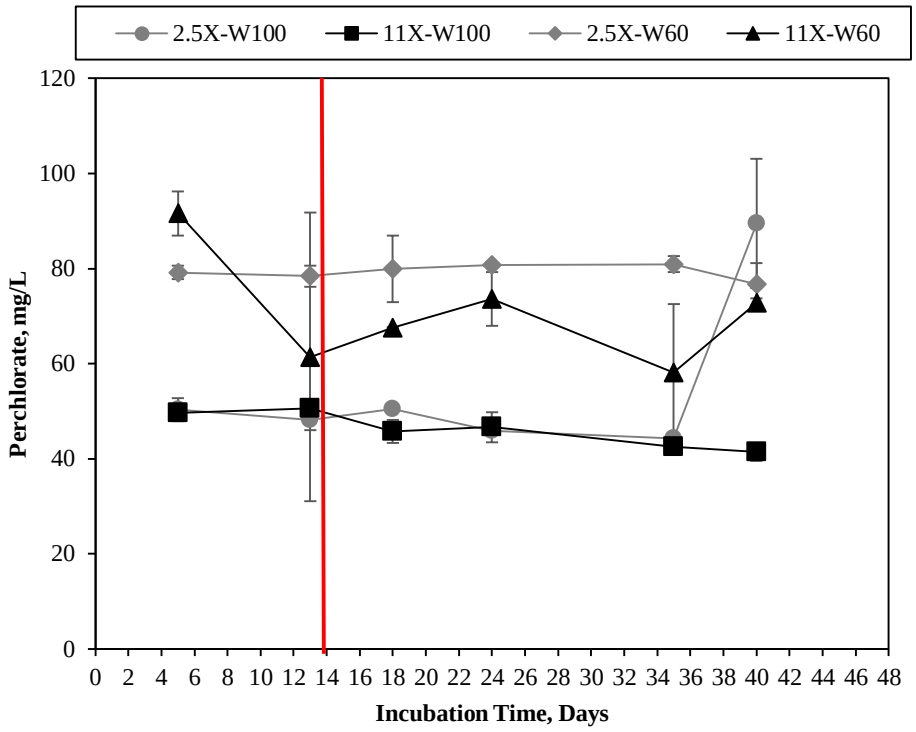

Figure 13. Perchlorate Reduction as a Function of Soil to Water Ratio. The red line indicates the glycerol dosage increment in the microcosms. Until day 14, stoichiometric COD were 0.5X and 1X. After glycerol addition (day 14), the stoichiometric COD were 2.5X and 11X. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

The perchlorate percentage reductions are comparable with the maximum perchlorate degradation rates calculated after the additional injection of glycerol as shown in Figure 14. For

both 11X and 2.5X stoichiometric COD, the 1:3 ratio demonstrated higher perchlorate biodegradation rates of 0.034 mg/d and 0.014 mg/d, respectively compared with the 1:2 soil to water ratios of 0.004 mg/d for 2.5X stoichiometric excess and 0.007 mg/d for 11X stoichiometric excess. Additional perchlorate reduction could likely have been achieved with longer incubation periods considering the samples contained sufficient COD concentrations to drive the biological reactions. However, it is clear that the lower COD concentrations in the second set of microcosms severely inhibited the kinetics of nitrate and perchlorate reduction. In summary, nitrate and perchlorate reduction were similar for 11X stoichiometric COD in 60-mL and 100-mL water volumes, while the 2.5X stoichiometric COD microcosms appeared to be inhibited by the lower water volume of 60 mL. The 11X stoichiometric COD was adequate for nitrate reduction, but perchlorate reduction required significantly longer incubation times.

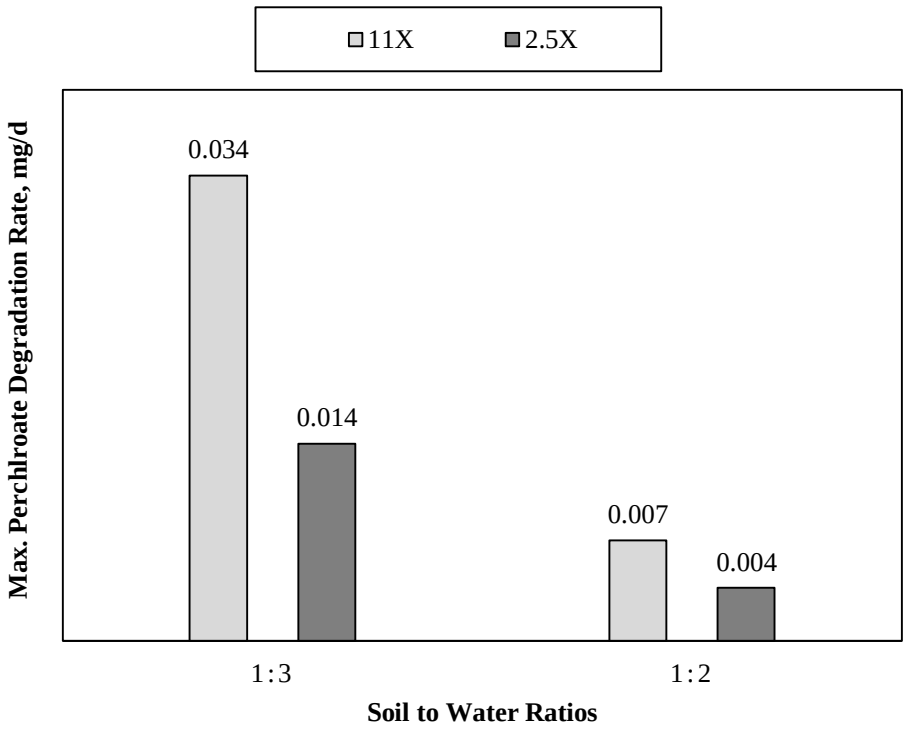

Figure 14. Perchlorate Degradation Rates as Function of Soil to Water Ratios. 1:3 (30g of soil/100 mL of water), 1:2 (30g of soil/60 mL of water). The degradation rates are calculated based on the degradation observe after the additional injection of glycerol (period of incubation of ~27 days)

Summary

For the initial comparison of electron donors, EOS-100 and glycerol achieved nitrate concentrations close to the detection limit (<0.2 mg-N/L), while the microcosms amended with compost/mulch did not demonstrate any significant nitrate reduction. In fact, the nitrate concentrations in the compost/mulch extract microcosms actually increased over time, presumably due to decomposition and release of nitrogen-containing compounds. Although EOS-100 and glycerol achieved similar overall nitrate reductions, the maximum nitrate degradation rates for EOS-100 were higher than for glycerol with corresponding values of 3.42 mg-N/L/d and 2.75 mg-N/L/d, respectively. These degradation rates describe similar trends than the determined first order rate constants of 0.60 d^{-1} and 0.42 d^{-1} for EOS-100 and glycerol, respectively.

Perchlorate reduction followed similar trends to those observed for nitrate, although there was a lag period of approximately 6 days corresponding with the preceding nitrate reduction period. This was expected because nitrate is the preferred electron acceptor for biological reduction. EOS-100 and glycerol achieved similar overall perchlorate reduction, but EOS-100 demonstrated more rapid kinetics. In the absence of nitrate (i.e., after the initial lag period), the pseudo first order rate constants for perchlorate reduction were determined to be 0.36 d^{-1} for EOS-100 and 0.31 d^{-1} for glycerol. Furthermore, the maximum perchlorate biodegradation rates confirm that EOS-100 degrades perchlorate faster than glycerol with degradation rates of 3.21 mg/L/d and 2.85 mg/L/d, respectively.

The compost/mulch extract was ineffective for perchlorate removal over the 25-day incubation period. This is partially due to the fact that the compost/mulch extract was unable to reduce nitrate, which is thermodynamically preferred over perchlorate. Although less favorable

than both nitrate and perchlorate based on the redox tower, the microcosms appeared to achieve some sulfate reduction to sulfide, although there were still significant sulfate concentrations present at the end of the incubation period. Lastly, the nutrient-amended microcosms achieved similar levels of treatment as the microcosms without phosphate addition. Thereby, the amount of phosphate added (6.5 mg-P/L) did not generated improvements for nitrate or perchlorate removals. As a result, higher phosphate dosages are recommended

For the evaluation of soil to water ratios, the most significant variable proved to be glycerol concentration rather than water volume. Both soil to water ratios were effective in mobilizing nitrate and perchlorate, but there were minor impacts on bioremediation. The two soil to water ratios achieved similar reduction of nitrate and perchlorate for 11X stoichiometric COD with maximum nitrate degradation rates of 0.091 mg-N/d and 0.080 mg-N/d for 1:3 and 1:2 soil to water ratios, and maximum perchlorate degradation rates of 0.034 mg/d for 1:3 soil to water ratio and 0.007 mg/d for 1:2 soil to water ratio. But, the lower water volume (i.e., 60-mL) hindered nitrate and perchlorate reduction for 2.5X stoichiometric COD. The 11X stoichiometric COD was adequate for nitrate reduction, but perchlorate reduction required significantly longer incubation times based on the observed kinetics. Furthermore, the preliminary glycerol dosing with 0.5X and 1.0X stoichiometric COD was entirely inadequate to initiate biological reduction. Therefore, more comprehensive cost analyses are warranted to determine if higher electron donor concentrations (e.g., 100X) are justifiable when considering the more rapid kinetics of nitrate and perchlorate reduction.

CHAPTER 4. NITRATE AND PERCHLORATE BIOREMEDIATION IN GROUNDWATER AND SATURATED SOIL: MICROCOSMS STUDY

4. 1. Introduction

Perchlorate has been detected in groundwater, surface water, and drinking water in the United States and abroad (Batista et al., 2005; Karimi & Rezaee, 2014a; Karimi & Rezaee, 2014b; Kumarathilaka et al., 2016). Perchlorate is a particularly persistent contaminant because it is highly stable, mobile, and soluble, and standard drinking water treatment technologies are generally ineffective (Logan, 2002). Perchlorate exposure is a concern for public health because it interferes with iodide uptake into the thyroid, which hinders hormone production, fetal development, skeletal growth, and may even cause mental retardation in infants (Motzer, 2001). Currently, there are no federal drinking water standards for perchlorate, but in 2005, the United States Environmental Protection Agency (USEPA) set an interim health advisory level of 15 μg/L, and some states have adopted safety advisory levels ranging from 1 to 18 μg/L (Srinivasan & Sorial, 2009)

Groundwater contamination often results from mobilization of perchlorate in contaminated soil (Tipton et al., 2003). In the United States, perchlorate contamination is particularly prevalent in the Southwest (Zhu et al., 2016). In Las Vegas, perchlorate concentrations range from 1.8×10^5 to 3.7×10^6 μg/L in contaminated groundwater and up to 34,700 μg/kg in contaminated vadose zone soil (Smith et al., 2004).). Without remediation efforts, the perchlorate is transported to Lake Mead via the Las Vegas Wash and ultimately contaminates drinking water sources in Arizona, California, and Mexico (Batista et al., 2005).

In situ bioremediation of perchlorate-contaminated sites generally involves the injection of a carbon source into the saturated or vadose zone soil to enhance bioremediation by perchlorate reducing bacteria (PRB) (Hatzinger & Diebold, 2009). PRB use the carbon source as an electron donor, and the perchlorate (and chlorate or chlorite) serves as the electron acceptor, ultimately leading to the production of chloride and water (ITRC, 2008). Biological perchlorate reduction demonstrates high efficiency at low cost compared to other technologies such as ion exchange, reverse osmosis, adsorption with granular activated carbon (GAC), and chemical and electrochemical reduction. The efficacy of biological reduction is dependent on the geochemical conditions of the soil and the amount of water and electron donor available (Konopka & Turco, 1991). Furthermore, the presence of competitive electron acceptors such as oxygen and nitrate, which are frequently present in perchlorate-contaminated environments, hinder perchlorate reduction (Bardiya & Bae, 2011).

Bioremediation of perchlorate in soils is challenging due to the diverse physicochemical properties of the soil. The soil horizon is compound of three major zones, namely, vadose zone, capillary fringe, and saturated zone. The vadose and saturated zones are hydrologically separate by the capillary fringe. These zones are characterized for the abundance variability of oxygen, nutrients, carbon, and water contents, likewise variability of pH and temperature (Holden & Fierer, 2005; Konopka & Turco, 1991). The saturated zone is characterized by the high water content occupying the spaces between the pores of the soil. Although the amount of water in the saturated zone is considerable high compared with subsurface soils (Vadose soils), biodegradation treatments can be hindered by low electron donor and nutrient contents. Biodegradation in the saturated zone has been applied through ex-situ bioremediation processes, mainly by extracting the contaminated groundwater to the surface for posterior treatments. In

general, after the water is extracted, electron donor and nutrients are added to stimulate bacteria activity. Biological reduction of perchlorate is carryout by perchlorate reducing bacteria (PRB) which are found ubiquitous in natural environments (Bruce & Coates, 1999). During perchlorate biodegradation, perchlorate is used as the electron acceptor during the enzymatic reaction. Therefore, using electron donors in perchlorate-contaminated environments (i.e., groundwater) increases the nourishment of the bacteria benefiting the efficiency of biological reductions.

The objective of this part of the research was to evaluate the potential use and kinetics of two slow release electron donors, specifically the commercially available emulsified oil EOS-100 and EOS-Pro in perchlorate-contaminated groundwater and saturated soil. To achieve the objective of research microcosm batch tests were implemented to simulate the conditions of the saturated zone at the site of study and provided innovative electron donor that will improve biological remediation of perchlorate in full-scale applications. The microcosms were augmented with the aforementioned slow release emulsified oils which provides particular benefits to stimulate the microbial community present in the perchlorate-contaminated groundwater. Moreover, this research identified optimum ratios of the emulsified oils to the contaminated groundwater and saturated soil (e.g., grams electron donor/gram of soil) that stimulate higher and faster nitrate and perchlorate biodegradation.

4. 2. Material and Methods

To achieve the objective of this phase of the research microcosm batch tests were implemented to simulate the conditions of a perchlorate-contaminated saturated zone. Microcosms were built with groundwater and saturated soil from a site of study. The microcosms were augmented with the two slow release emulsified oils; EOS-100 and EOS-Pro. Each of the

slow release emulsified oils provides particular benefits to stimulate the microbial community present in the perchlorate-contaminated groundwater as describe later in this section.

4. 2. 1. Groundwater and Saturated Soil Samples

Microcosms were prepared with a mixture of soil and groundwater from the saturated zone of a contaminates site. samples were drilled and collected in 5-foot increments of depth (20-25, 25-30, 30-35, and 35-40). Soil samples from the four layers were mixed in equal volumes to obtain a homogeneous mixture. The soil samples were transferred aseptically to previously disinfected plastic containers. The containers and instruments were rinsed with a 5% sodium hypochlorite solution and then rinsed 8 times with deionized autoclaved water and allowed to air dry.

The initial contaminant concentrations in the saturated soil were determined through a sequential extraction process in which two 50-mL centrifuges tubes, each containing 40 g of wet soil and 20 mL of nanopure water, were centrifuged at 9,000×g and 4°C (Solvall Legent-GT-fixed angle rotor) for 15 min. This procedure was repeated nine times until perchlorate and nitrate were not detected in the resulting extract. The final extracts were aggregated (final volume of ~81 mL) and analyzed for perchlorate, nitrate, and other quality parameters, as shown in Table 14. The concentrations of the contaminants were determined on a dry weight basis. The moisture content of the soil was determined by weighing 40 g of soil before and after drying in an oven at 105ºC for 12 hours. This analysis was performed in duplicate, and the average moisture content was 15.9 %. On average, the perchlorate and nitrate concentrations in the combined extracts were 1.7 mg/L and 1.6 mg-N/L, respectively.

Table 14. Saturated Soil and Groundwater Initial Quality Parameters

Parameter	Extract (mg/L)[1]	Soil (mg/g)[2]	Groundwater (mg/L)
Perchlorate	1.7	0.0041	22.25
Nitrate (as N)	1.6	0.0039	16
Nitrate (as NO_3)	7.1	0.072	70.86
Hardness (as $CaCO_3$)	---	---	1,800
Total Dissolved Solids	439	---	4,925
Chemical Oxygen Demand	83	0.20	26.5
Chloride	440	1.06	48.34
Phosphate	2.6	0.01	0.95
Chromium	---	---	0.2
Iron	ND	ND	0.3
Sulfate	140	0.34	1,520
pH (unitless)	7.4	---	7.9

[1]Concentrations in the aggregated extract (total volume of ~81 mL)
[2]Calculated based on 40 g of wet soil with a moisture content of 15.9%
---: Not analyzed
ND: non-detect

4. 2. 2. Electron donors Source in Groundwater and Saturated Soil Microcosms

This bench-scale biodegradation test, two different emulsified oils, EOS-100 and EOS-Pro, were tested as potential electron donors. The emulsified vegetable oils EOS-100 and EOS-Pro were supplied by EOS Remediation, Inc. (Raleigh, NC). EOS-100 is a soluble emulsified vegetable oil used to enhance anaerobic perchlorate biodegradation. EOS-100 is a proprietary mixture of refined and bleached U.S. soybean oil (85% by weight). EOS-100 is considered a slow-release electron donor intended to promote biodegradation over extended time periods. EOS-Pro is also a proprietary mixture of refined and bleached U.S. soybean oil (~60% by weight), nutrients, and vitamins, but EOS-Pro specifically contains diammonium phosphate (DAP), which is used to enhance the growth of microbial communities. Under anaerobic conditions, these emulsified oils hydrolyze into glycerol and long chain fatty acids (LCFAs) (Viana et al., 2012). These compounds are further decomposed into hydrogen (H_2), which can be used by bacteria as a direct electron donor for the reduction of perchlorate and nitrate (da Silva

et al., 2009). Table 15 summarizes the properties of EOS-100 and EOS-Pro, including their H_2 yields of 0.40 and 0.25 pounds of H_2 per pound of oil, respectively.

Table 15. EOS-100 and EOS-Pro Properties

Parameter	EOS-100	EOS-Pro
Organic Carbon (% by Weight)	100	74
Refined and Bleached U.S. Soybean Oil (% by Weight)	85	60
Slow Release Organics (% by Weight)	15	10
Other Organics (emulsifiers, food additives) (% by Weight)	----	10
Mass of Hydrogen Produced (lb H_2 / lb EOS)	0.40	0.25
pH (Standard Units)	----	6-7
Viscosity (% by Weight)	Low	Low
Specific Gravity	0.92-0.93	0.96-0.98

(----: no data available)

4. 2. 3. Groundwater and Saturated Soil Microcosm Setup

The microcosms batch tests were performed in 150-mL borosilicate glass bottles. Saturated soil and groundwater were added to each bottle at a ratio of 100 mL of groundwater to 40 grams of saturated wet soil. The microcosms were then augmented with different oil concentrations, as shown in Table 16. These concentrations equate to 0.02 grams of oil per gram of saturated soil, 0.01 g of oil per g of saturated soil, and 0.002 g of oil per gram of saturated soil, as noted in the sample labeling scheme. The average COD concentration of the neat EOS-100 was previously determined to be 2.07×10^6 mg/L. Based on nitrate and perchlorate stoichiometric reduction in the previous set of microcosms (chapter 3), the samples were designed to account for different stoichiometric excess to reduce nitrate and perchlorate concentrations in the groundwater and saturated soil. In which, E-0.002 for ~14 stoichiometric excess, E-0.01 for ~70 stoichiometric excess, and E-0.02 microcosms were designed to account for ~140 stoichiometric excess. In addition, control microcosms [i.e., blank controls (BLK; no electron donor added),

abiotic control (E-ABIO; autoclaved soil mixture), and phosphate addition (E-Phos; 6.5 mg-PO_4^{-3}/L of phosphate).

Table 16. Microcosms Electron Donor Volumes EOS-100/EOS-Pro

Microcosm Sample Label	Detail*	Volume of EOS-100 (mL)**	Phosphate Concentration (mg-PO4-3 /L)
E-0.02	0.02 g of oil/g soil	0.70	----
E-0.01	0.01 g oil/g soil	0.35	----
E-0.002	0.002 g oil/g soil	0.070	----
E-ABIO	0.02 g of oil/g soil	0.70	----
BLK	----	----	----
E-Phos	0.02 g of oil/g soil	0.70	6.5

---: No phosphate added
***Grams of EOS-100 per gram of soil**
****Volume of EOS-100 used in 40 g of saturated soil.**

After preparing the microcosms, the glass bottles were sealed with a butyl rubber cap and crimped closed with an aluminum ring to ensure anaerobic conditions. The bottles were wrapped in black felt and placed horizontally in a shaker at 70 rpm and room temperature. At the time of analysis, the microcosms were opened, and the liquid and soil mixtures were transferred to 500-mL centrifuge bottles and centrifuged at 9,000 rpm for 15 minutes at 4°C. The supernatant was decanted into a 250-mL bottle.

The experimental matrix and microcosm incubation periods are summarized in Table 17. Each sample was analyzed for perchlorate, nitrate, COD, sulfate, and phosphate.

Table 17. Experimental Design Matrix for the Preliminary Experiments with EOS-100

Microcosm Sets	Days of Incubation								
	5	8	12	16	21	28	41	48	62
E-0.02	E-0.02	E-0.02	E-0.02	E-0.02	E-0.02	E-0.02	--	E-0.02	E-0.02
	E-0.02-D	E-0.02-D	E-0.02-D	E-0.02-D	E-0.02-D	E-0.02-D	--	E-0.02-D	E-0.02-D
E-0.01	E-0.01	E-0.01	E-0.01	E-0.01	E-0.01	E-0.01	E-0.01	--	E-0.01
	E-0.01-D	E-0.01-D	E-0.01-D	E-0.01-D	E-0.01-D	E-0.01-D	--	E-0.01-D	E-0.01-D
E-0.002	E-0.002	E-0.002	E-0.002	E-0.002	E-0.002	E-0.002	E-0.002	--	E-0.002
	E-0.002-D	E-0.002-D	E-0.002-D	E-0.002-D	E-0.002-D	E-0.002-D	--	E-0.002-D	E-0.002-D
E-Phos	E-Phos	--	--	E-Phos	E-Phos	--	--	--	--
	E-Phos-D	--	--	E-Phos-D	E-Phos-D	--	--	--	--
Blanks (**BLK**)	BLK	--	--	--	--	--	--	BLK	--
	BLK-D	--	--	--	--	--	--	--	BLK-D
Abiotic Controls (E-ABIO)	E-ABIO	--	E-ABIO	--	--	--	--	E-ABIO	--
	E-ABIO-D	--	E-ABIO-D	--	--	--	--	--	E-ABIO-D

- E-0.02 (0.02 g of oil/g soil): 0.7 mL of EOS-100, 100 mL groundwater, 40 g saturated wet soil
- E-0.01 (0.01 g oil/g soil): 0.35 mL of EOS-100, 100 mL groundwater, 40 g saturated wet soil.
- E-0.002(0.002 g oil/ g soil): 0.07 mL of EOS-100, 100 mL groundwater, 40 g saturated wet soil

Notation:
Electron Donors: E = EOS-100 oil
E-ABIO = Abiotic Control (autoclaved soil and water mixture with electron acceptor-0.7 mL)
BLK = Blank (No electron donor nor phosphate added)
D = Duplicate
E-Phos = nutrient buffer (addition of nutrient at 6.5 mg P/L)
--: No sample

In a separate set of microcosms, the emulsified oil EOS-Pro was used as the electron donor in the microcosms to evaluate commercially available alternatives to EOS-100. The H_2 yield of EOS-Pro is approximately 38% lower than EOS-100 (i.e., 0.25 lb H_2/lb oil vs. 0.40 lb H_2/lb oil), but EOS-Pro contains extra components such as vitamin B-12 and phosphate that accelerate the bacterial growth in substrates (e.g., saturated soil). In addition, EOS-Pro has lower organic releases than EOS-100, ensuring longer periods of biological activity (i.e., 10 % and 15 % by weight, respectively). EOS-Pro dosing was based on the results of the EOS-100 microcosms. As will be described later in relation to the EOS-100 data, the highest reductions in perchlorate and nitrate concentrations were achieved with the higher dosing rates of 0.01 g oil/g soil and 0.02 g oil/g soil (i.e., ~ 70X and ~140X stoichiometric COD). Therefore, the microcosms were amended with 0.2 or 0.4 mL of EOS-Pro (0.005 g oil/g soil and 0.01 g oil/g

soil, respectivelly). A blank sample consisting of saturated zone soil and groundwater without electron donor amendment was used to characterize the level of remediation achieved with ambient conditions. The groundwater to soil ratio was held constant at 100 mL of groundwater to 40 g of saturated soil, as shown in Table 18. Perchlorate, nitrate, and COD were analyzed in each sample, starting with samples collected after three days of incubation, and a subset of the samples were also tested for sulfate and phosphate.

Table 18. Experimental Design Matrix for the Secondary Experiments with EOS-Pro

Microcosm Sets	Days of Incubation								
	3	4	6	9	11	13	15	16	18
E-0.4	E 0.4	E 0.4	E 0.4	E 0.4	E 0.4	E 0.4	E 0.4	E 0.4	E 0.4
	E-0.4-D	E-0.4-D	E-0.4-D	E-0.4-D	E-0.4-D	E-0.4-D	E-0.4-D	E-0.4-D	E-0.4-D
E-0.2	E-0.2	E-0.2	E-0.2	E-0.2	E-0.2	E-0.2	E-0.2	E-0.2	E-0.2
	E-0.2-D	E-0.2-D	E-0.2-D	E-0.2-D	E-0.2-D	E-0.2-D	E-0.2-D	E-0.2-D	E-0.2-D
Blanks (**BLK)**	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK
	BLK-D	BLK-D	BLK-D	BLK-D	BLK-D	BLK-D	BLK-D	BLK-D	BLK-D

- E-0.4 (0.01 g oil/g soil): 0.4 mL of EOS-Pro, 100 mL groundwater, 40 g saturated wet soil
- E-0.002 (0.005 g oil/g soil): 0.007 mL of EOS-Pro, 100 mL groundwater, 40 g saturated wet soil

Notation:
Electron Donors: E = EOS-Pro oil
BLK = Blank (No electron donor nor phosphate added)
D = Duplicate
--: No sample

4. 2. 4. Analytical Methods

Perchlorate concentrations were determined with ion chromatography (Dionex ICS 2000 IC) using US EPA Method 314. Other analyses were performed according to EPA-approved methods, as summarized in Table 19.

Table 19. Analytical Methods

Analysis	EPA Method
Nitrate	Hach EPA 10206 and EPA 352.1
Hexavalent Chromium	Hach EPA 8023
COD	Hach 8000
Sulfate	IC and Hach EPA 8051
Phosphate	EPA 365.1
Iron	Hach 8008 and 8147-ferrover
Chloride	Hach 8225
pH	Hach EPA 8156

4. 3. Results and Discussion

4. 3. 1. Chemical Oxygen Demand in Groundwater and Saturated Soil Microcosms Augmented with EOS-100

The ambient COD concentration in the saturated soil was ~83 mg/L which is surprisingly higher than the COD measured in the vadose zone soil samples of ~16 mg/L (Chapter 3). These results differ with previous research in which higher organic carbon contents have been reported in superficial soil profiles due to plant rooting cycles that provides carbon and other nutrients to the soil (Konopka & Turco, 1991). However, some other research suggested that due to higher water content in depth soils the abundance of organic carbon could increase (Hickman & Novak, 1989; Holden & Fierer, 2005). Thus, the high COD concentration in the saturated soil may have resulted by soil adsorptions from the groundwater characteristic of this zone.

The chemical oxygen demand (COD) was used as an indirect measure of the aqueous EOS-100 concentrations. Figure 15 compares the COD originating from the saturated soil and groundwater with the COD originating from the EOS-100. The blank controls (i.e., no EOS-100 or nutrient added) demonstrated a relatively low COD concentrations of ~85 mg/L compared with the microcosms augmented with the electron donor (EOS-100). During the first day of

incubation a COD reduction was observed. This reduction could be due to the consumption of the electron donor by the microbial community present in the microcosms. The reduction was observed until day 40 of incubation. After, the COD concentration increased due to oil releases from the saturated soil, which corroborate the slow release properties proper of the electron donor. Therefore, EOS-100 is considered a suitable electron donor for long-term saturated soil and groundwater applications providing long-term application, which may provide a cost-effective field application.

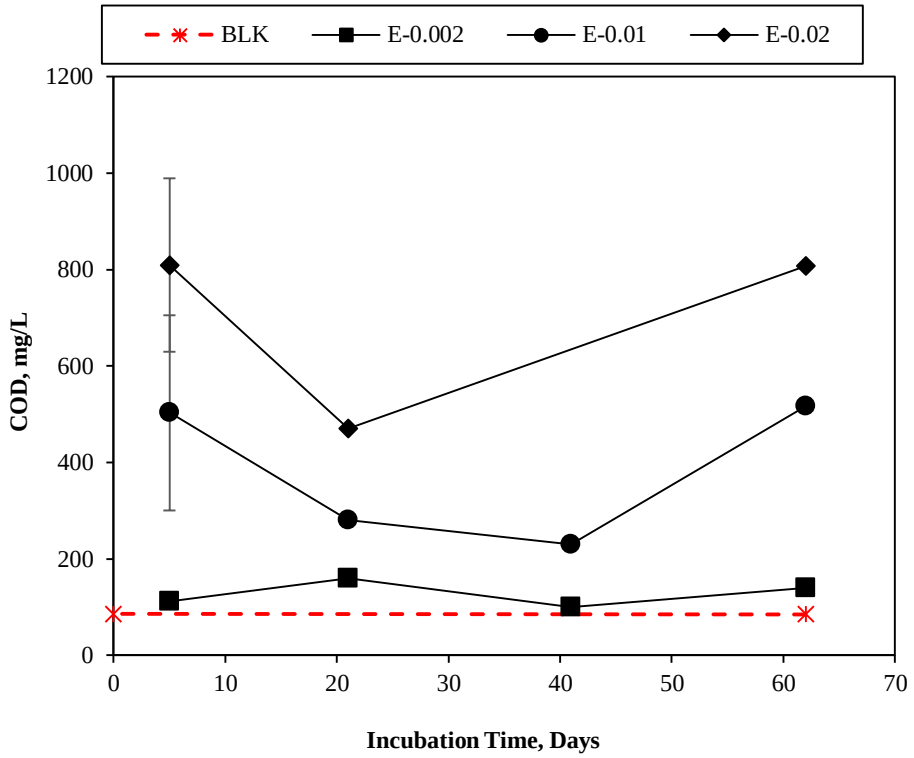

Figure 15. Chemical Oxygen Demand in Groundwater and Saturated Soil Microcosms-EOS-100.
The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

4. 3. 2. Biodegradation of Nitrate in Groundwater and Saturated Soil Microcosms-EOS-100

Nitrate contamination in perchlorate-contaminated environments has been attributed to the nitrification of the ammonium present in one of the most predominant sources of perchlorate

as is the ammonium perchlorate used in air bags, rocket fuel propellants, and in general industrial applications (Urbansky, E. T., 1998). Nitrate is a crucial contaminant that limits perchlorate biodegradation technologies due to bacterial preference for electron acceptors with higher redox potential (e.g., 280~220 mV for nitrate vs. 0~-110 mV for perchlorate). However, these limitations can be overcome by increasing the electron donor concentrations generating faster nitrate degradations in nitrate and perchlorate contaminated environments (Achtnich et al., 1995). Therefore, in this set of microcosms different concentrations of EOS-100 were spiked to evaluate the impact of different electron donor concentrations for nitrate and perchlorate biodegradations.

Based on the soil extractions performed at the beginning of the experiments (Table 14), the nitrate concentrations in the saturated soil and the groundwater used in this set of experiments were ~ 1.6 mg-N/L and ~16 mg-N/L, respectively. Recall the initial nitrate concentration in the vadose zone soil and the presented in chapter 3 were ~20.9 mg-N/L and 0.46 mg-N/L, respectively. The high nitrate concentration in the groundwater can be attributed to nitrate contaminated plumes from superficial contaminated soil horizons (i.e., vadose zone soils) or due to nitrification of ammonium, present in ammonium perchlorate contaminations, into nitrite and its posterior oxidation to nitrate. These concentrations evidenced the variability of the contaminants in the soils and the importance of the evaluation of the enzymatic reactions in in-situ and ex-situ bioremediation technologies in contaminated soils and groundwater, respectively.

The initial nitrate concentration in the saturated soil was found by sequential extractions as described earlier. This concentration was assumed to yield the maximum aqueous nitrate concentration, but the initial concentrations in the microcosms at day 5 of incubation were higher

(E-0.002: 16.75 mg-N/L; E-0.01: 13.45 mg-N/L; and E-0.02: 10.15 mg-N/L) than in the aggregated extract. This higher nitrate concentration may have resulted from heterogeneity in the soil samples, higher nitrate contributions from the groundwater used in the microcosm, or simply experimental error. Nerveless, the nitrate concentrations found in the blank controls microcosms (i.e., no electron donor or phosphate added) of ~19 mg-N/L correlates better with the initial nitrate concentrations at day 5 of incubation. Therefore, for this set of microcosms the initial concentration will be assumed as the concentration found in the blank controls.

Figure 16 illustrates the change in nitrate concentration in the various microcosms as a function of EOS-100 addition and nitrate concentration in the blank control microcosms. Nitrate reduction were observed within the first five days of incubation, microcosms amended with 14X, 70X, and 140X stoichiometric excess (i.e., 0.002 g oil/g soil, 0.01 g oil/g soil, and 0.02 g oil/g soil, respectively) achieved ~12%, ~ 29 %, and 47 % nitrate removals, respectively. In contrast, with the EOS-100 experiments presented in Chapter 3 (vadose zone soil and Lake Mead microcosms), nitrate reduction in the groundwater and saturated soil continued for 28-48 days, depending on the EOS-100 dose. These results are confirmed by the degradation rates calculated during the first 5 days of incubation in the vadose zone soil microcosms (i.e., 100X stoichiometric COD) of 3.42 mg-N/L/d compared with the degradation rates in this set of microcosms shown in Table 20. The degradation rate in microcosms augmented with 140X stoichiometric COD of 1.77 mg-N/L/d is lower than the degradation rate in the previous set of microcosms with 100X stoichiometric COD. These low degradation rates resulted in a longer perchlorate reduction lag period than the first set of EOS-100 experiments, which required 8 days to reach the detection limit of the nitrate assay with similar microcosm conditions. The longer remediation periods (low degradation rates) in the saturated soil may have resulted by the

lower concentrations of nitrate in the saturated soil (0.0039mg-N/g soil) compared with the higher nitrate contents in the vadose zone soil (~0.76 mg-N/g soil). In addition, some research reported that sodium and chlorine concentrations increase in deeper soils (Holden & Fierer, 2005), thereby delating the remediation process.

Table 20. Nitrate Biodegradation Rates in Saturated Soil and Groundwater Microcosms-EOS-100

Stoichiometric COD	Maximum Nitrate Degradation Rate*	Overall Nitrate Degradation Rate**
14X (0.002 g oil/ g soil)	0.45 mg-N/L/day	0.41 mg-N/L/day
70X (0.01 g oil/g soil)	1.11 mg-N/L/day	0.46 mg-N/L/day
140X (0.02 g oil/g soil)	1.77 mg-N/L/day	0.46 mg-N/L/day

*Degradation rate calculated between day 0 and 5 of incubation
**Degradation rate calculated between day 0 and 41 of incubation

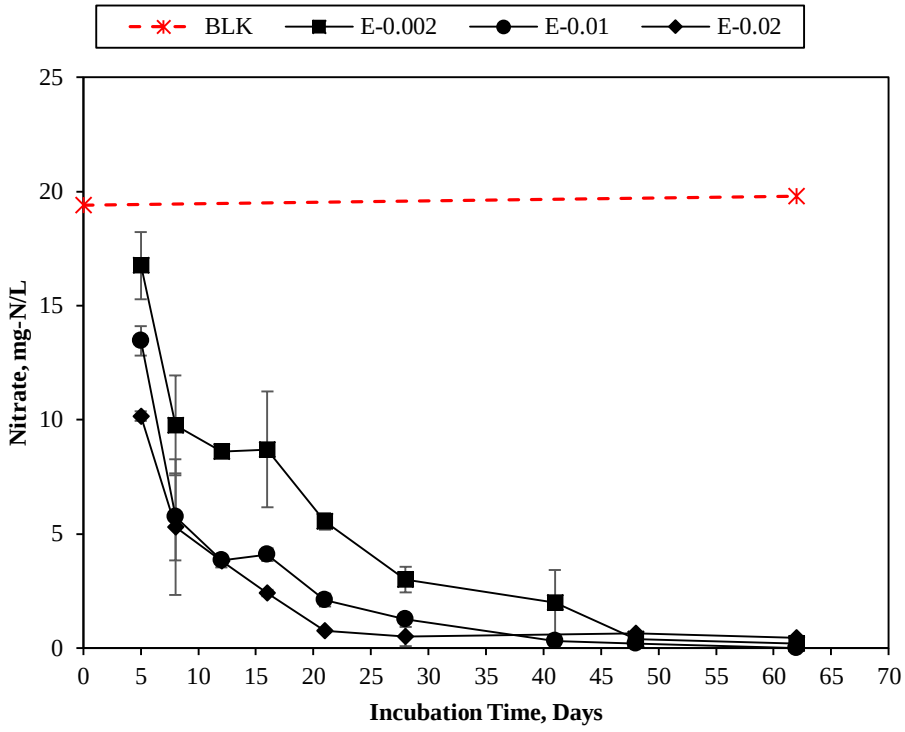

Figure 16. Nitrate Reduction in the Groundwater and Saturated Soil Microcosms - EOS-100. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

4. 3. 3. Biodegradation of Perchlorate in Groundwater and Saturated Soil Microcosms-EOS-100

Figure 17 summarizes the perchlorate concentrations in the microcosms over time as a function of electron donor concentrations. The microcosms with 70X and 140X stoichiometric COD exhibited a 28-day lag period, which was consistent with the amount of time required for complete nitrate reduction (Figure 16). Interestingly, the microcosm with 70X stoichiometric COD achieved the perchlorate method detection limit on day 40, while the microcosm with 140X required ~60 days to achieve the same level of treatment. It is unclear why the microcosm with twice as much electron donor required a longer incubation period, particularly considering that the dissolved COD concentration in that microcosm was also higher. However, there was only one microcosm with 140X stoichiometric COD sampled between days 28 and 62 so the longer treatment time may have simply been attributable to experimental variability. The microcosm with 14X stoichiometric COD did not achieve significant perchlorate reduction. This is attributable to the low COD concentrations and slower kinetics compared with the microcosms with 70X and 140X stoichiometric COD.

In addition, the maximum perchlorate degradation rates (i.e., between day 28 and 62 of incubation) for this set of microcosms are were calculated as 0.10 mg/L/d, 0.95 mg/L/d, and 0.84 mg/L/d for 14X, 70X, and 140X stoichiometric COD, respectively. The degradation rates obtained in this study are considerable lower than the calculated previously in chapter 3 (100X stoichiometric COD) of 3.21 mg/L/d. These low biodegradation rates may have resulted by the low nitrate biodegradation rates reported in the same set of microcosms (Table 20). Furthermore, the 100X stoichiometric COD experiments presented in chapter 3 demonstrated faster perchlorate biodegradation achieving lower detection concentrations by day 8 of incubation. In contrast, saturated soil and groundwater microcosms needed longer periods of incubation with

both 70X and 140X stoichiometric COD excess. This results are attributable to higher perchlorate concentrations in the vadose zone soil than in the saturated soil (0.18 mg/g soil vs 0.0041mg/g soil) resulting in lower availability of the electron acceptor in the enzymatic reactions, thereby delaying the perchlorate biodegradation in the saturated soil.

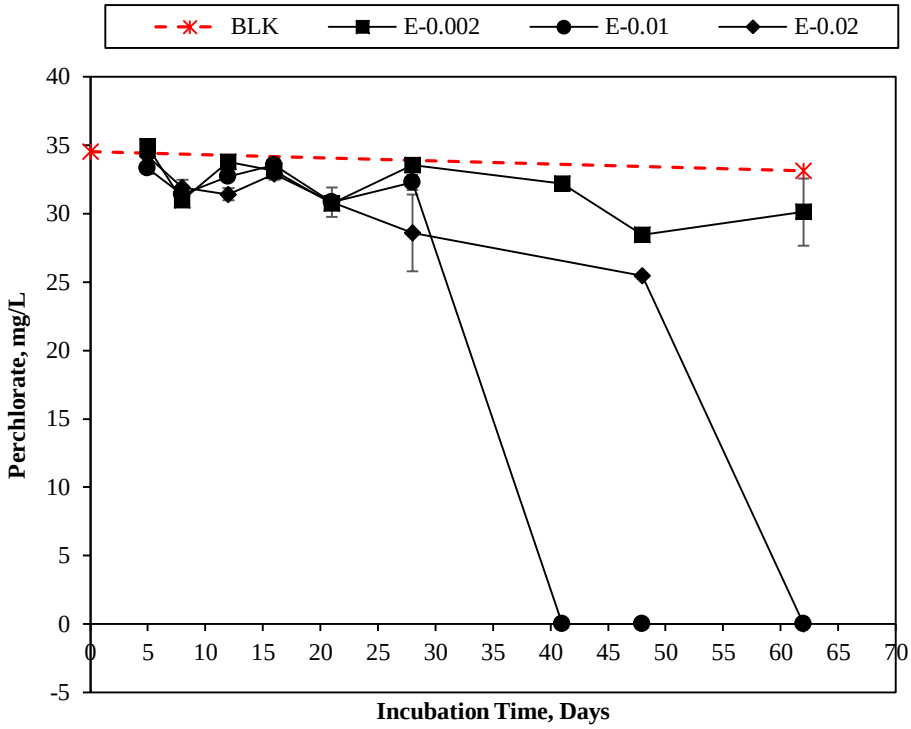

Figure 17. Perchlorate Reduction in the Groundwater and Saturated Soil Microcosms - EOS-100.
The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

The perchlorate concentration in blank microcosms (BLK) were relatively consistent with values between ~33-34 mg/L. Recall that the initial perchlorate concentrations in the groundwater and saturated soil were 22.3 mg/L and 1.7 mg/L, respectively (Table 14). The increase in perchlorate concentration in the blank controls may have resulted from extended contact time during incubation.

4. 3. 4. Biodegradation of Sulfate in Groundwater and Saturated Soil Microcosms-EOS-100

Biodegradation of sulfate was demonstrated for all EOS-100 dosing conditions, but higher sulfate degradations were achieved in microcosms with higher doses of EOS-100 (i.e., 140X and 70X stoichiometric excess), as shown in Figure 18a. During the first days of incubation (i.e., day 5 to day 15), sulfate concentrations were closed to the blank control concentrations (~2,300 mg/L). After day 15, predominant sulfate reductions were observed up to day 28. During this period of incubation sulfate reductions increased producing high concentrations of sulfide in the microcosms, as show in Figure 18b. The highest sulfide concentrations were also demonstrated in microcosms with 140X and 70X stoichiometric COD. Interestingly, the concentration of sulfate increased after day 28 of incubation, this increase may have resulted from soil releases during the extended incubation period. Contrary, sulfide concentration decreased by the end of the incubation period due to the lower sulfate reductions and volatilization of sulfide.

Biodegradation of sulfate in the saturated zone were considerable higher than in the vadose zone soil experiments presented in chapter 3, in which the maximum degradation rate of sulfate in microcosms with 100X stoichiometric COD was 3 mg/L/day. This degradation rate is considerable lower than the calculated in this batch experiments with maximum sulfate degradation rates of 45.7 mg/L/d, 36.9 mg/L/d, and 21.7 mg/L/d for microcosms with 14X, 70X, and 140X stoichiometric COD, respectively. This high sulfate reduction may have resulted by the faster kinetics of the reactions due to the high initial sulfate contribution from the groundwater and saturated soil (i.e., 1,520 mg/L and 140 mg/L, respectively) compared with the initial sulfate concentrations in the Lake Mead water and vadose zone soil (i.e., 238 mg/L and

105 mg/L, respectively) and the stoichiometric COD used in the microcosms study (e.g., 140X vs. 100X stoichiometric excess).

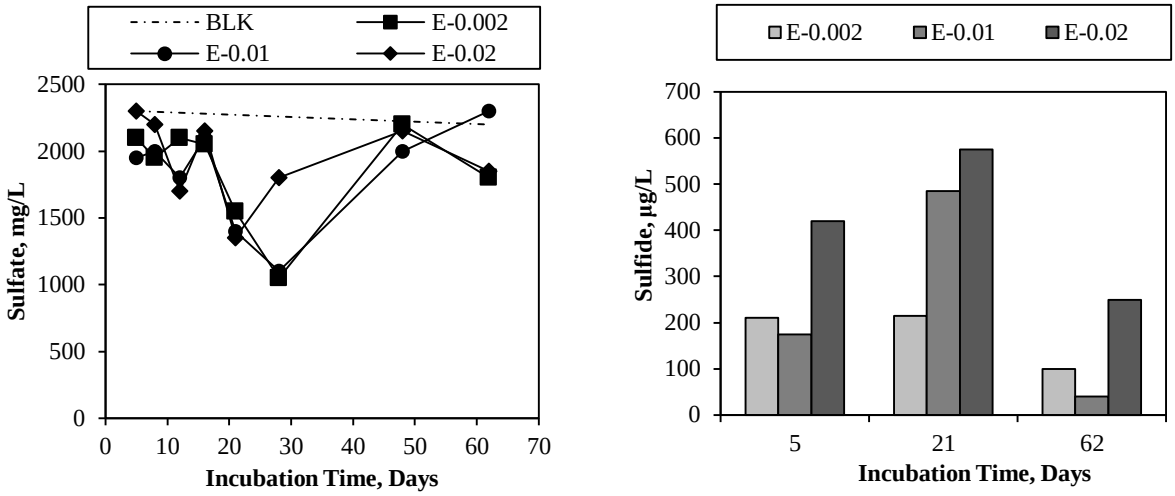

Figure 18. Sulfate Reduction in the Groundwater and Saturated Soil Microcosms - EOS-100

4. 3. 5. Abiotic Controls in Groundwater and Saturated Soil Microcosms-EOS-100

Perchlorate reduction was not observed in the abiotic controls. This result was expected since no native bacteria were expected to survive the autoclaving process. However, similar to the experiments described in Chapter 3, nitrate reduction was still observed during the incubation period as shown in Figure 19. Again, this reduction was likely attributable to incomplete sterilization of the soil, which allowed for the survival and subsequent metabolic activity of nitrate-reducing microorganisms. Indeed, based on the literature review performed during this research, there are no studies that demonstrated that perchlorate reducing bacteria are capable of supporting temperatures higher than 80 ^{o}C, thus perchlorate reducing bacteria are unable of forming spores (Thrash, 2009; Thrash et al., 2010). Conversely, there are studies that demonstrated the existence of nitrate reducing spore-forming bacteria (L'Haridon et al., 2006; Vekhoeven, 1954). Other research has documented abiotic reduction of nitrate and nitrite during

autoclaving (Dail et al., 2001). However, based on the results of the two different microcosms experiments (i.e., vadose zone and saturated soil) and the fact that no perchlorate reductions were observed in either of the experiments, it can be assumed that nitrate reductions may have resulted by the formation of spores that survive to the autoclaving process.

Table 21. Nitrate Reduction in Abiotic vs. Biotic Microcosms - EOS-100

Electron Donor	Nitrate Abiotic Control Microcosms[1]		Nitrate Biotic Microcosms[2]	
	Day 5	Day 62	Day 5	Day 62
EOS-100	12.8 mg-N/L	2.9 mg-N/L	10.15 mg/L	0.2 mg/L

[1]Nitrate Concentration in autoclaved microcosms with 0.7 mL of EOS-100
[2]Nitrate Concentration in 140X (0.02 g oil/ g soil or 0.7 mL) microcosms

Based on the results shown in Table 21, the biodegradation rates of nitrate for both biotic and abiotic microcosms were determined. Surprisingly both experiment resulted with same rate of reduction of ~0.17 mg/L/d. However, comparing the rate of nitrate reductions in the abiotic controls presented in chapter 3 (Table 11), the nitrate biodegradation rate was higher in the vadose zone soil than in the saturated soil (i.e., ~0.85 mg/L/d). This faster rate is most likely due to the higher initial nitrate concentration in the vadose zone soil.

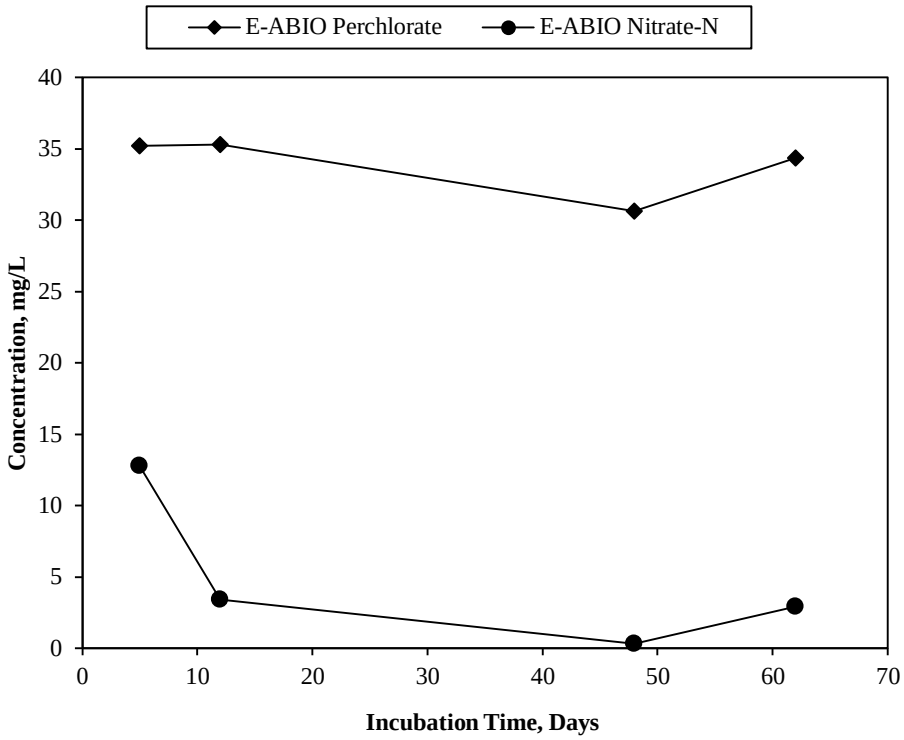

Figure 19. Nitrate and Perchlorate Concentrations in Abiotic Control Microcosms with EOS-100 at 140X Stoichiometric COD.

4. 3. 6. Nitrate Reduction Kinetics in Groundwater and Saturated Soil Microcosms-EOS-100

The rate constants describing the reduction of nitrate and perchlorate were determined based on linear regression over defined incubation periods. The rate constants for nitrate reduction were evaluated between days 0 and 28, at which point nitrate had been reduced to the method detection limit of the assay. Recall the initial nitrate concentration (i.e., nitrate concentration at day 0) was assumed as the same found in the blank controls microcosms (~19 mg-N/L). Perchlorate reduction kinetics were not characterized for these experiments due to insufficient data. The linearized data for pseudo first order degradation of nitrate with EOS-100 are shown in Figure 20. The corresponding rate constants were determined to be 0.06 d^{-1}, 0.10 d^{-1}, and 0.14 d^{-1} for EOS-100 doses of 14X, 70X, and 140X stoichiometric COD. Compared with

the experiments presented in chapter 3, the nitrate biodegradation rate constants in the saturated soil were considerably lower than in the vadose zone soil (i.e., 0.60 d^{-1}, for microcosms with 100X stoichiometric COD). As mention earlier, these results are likely due to lower nitrate concentrations in the in the saturated soil (0.0039mg-N/g soil) as compared with the higher nitrate contents in the vadose zone soil (~0.76 mg-N/g soil) or to lower activity of nitrate reducing bacteria due to high salinity contests characteristic of saturated soils (Holden & Fierer, 2005) or just to the limited data collected during the nitrate reduction period in the vadose zone soil and Lake Mead water microcosms.

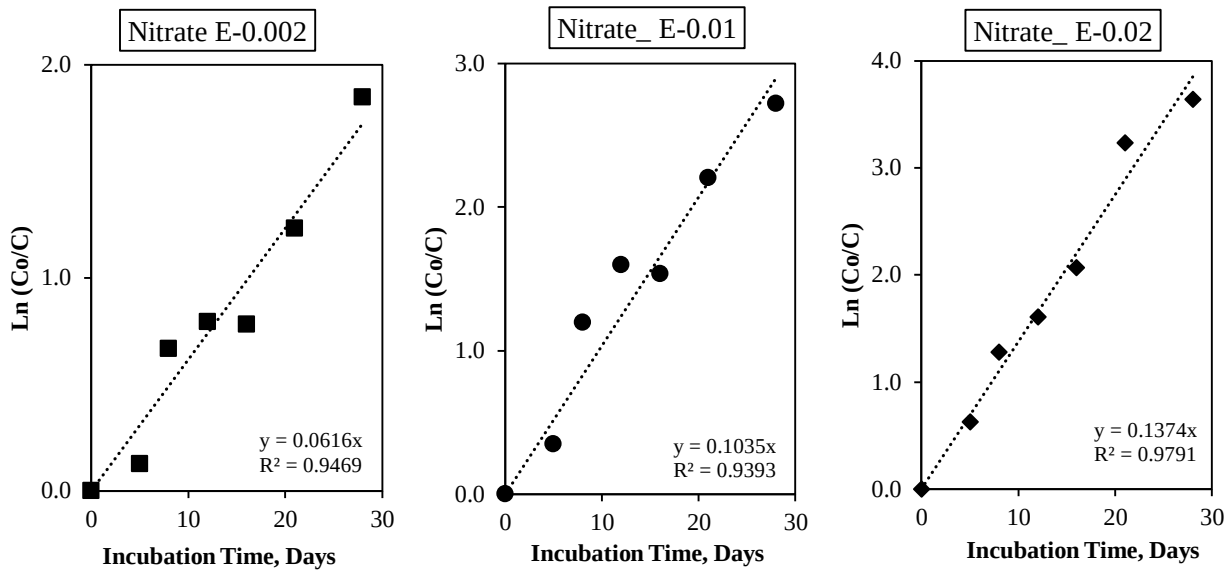

Figure 20. Nitrate Reaction Kinetics in Saturated Soil and Groundwater Microcosms - EOS-100 at 21±2°C. a) Rate constant of nitrate for microcosms amended with 0.070 mL of EOS-100 (E-0.002). b) Rate constant of nitrate for microcosms amended with 0.35 mL of EOS-100 (E-0.01). c) Rate constant of nitrate for microcosms amended with 0.7 mL of EOS-100 (E-0.02).

4. 3. 7. Phosphate Amendment in Groundwater and Saturated Soil Microcosms-EOS-100

To evaluate the effect of phosphate on the biodegradation kinetics of nitrate and perchlorate, microcosms were amended with ~21 mg-P/L of phosphate and EOS-100 (140X stoichiometric COD) and incubated for up to 21 days. Recall that the initial nitrate and perchlorate concentration were previously determined to be ~19 mg-N/L and ~35 mg/L, respectively (based on blank samples). By the first sampling day (i.e., day 5), nitrate had already been reduced by 90%, but perchlorate reduction had not yet started (Figure 21b). As shown earlier, the addition of EOS-100 at 140X stoichiometric COD without phosphate addition only achieved ~47% nitrate reduction over the same incubation period (Figure 16).

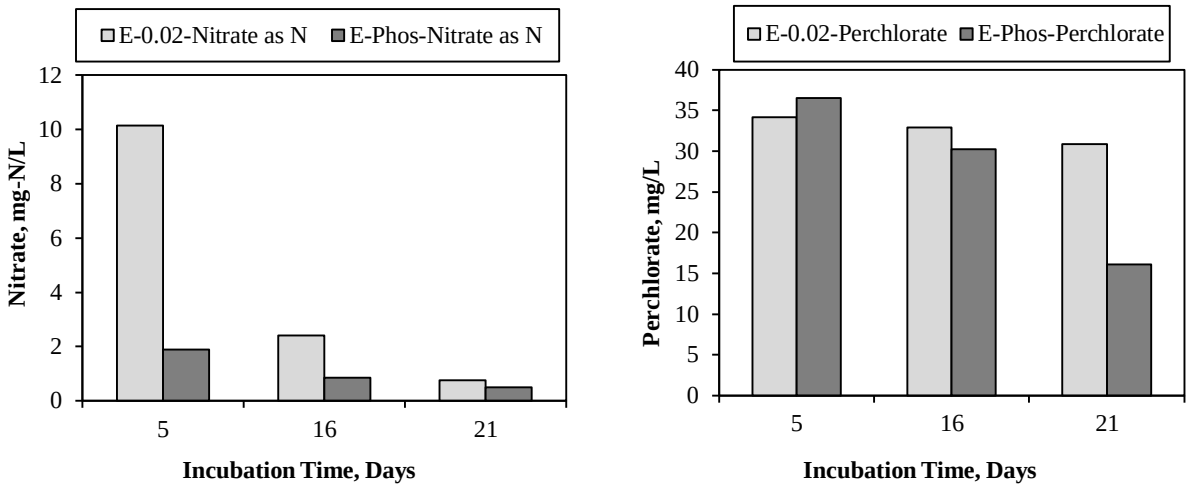

Figure 21. Nitrate and Perchlorate Concentrations in Microcosms with EOS-100 (140X Stoichiometric COD) and 65 mg- PO_4^{-3}/L of Phosphate. E-0.02: microcosms with 0.70 mL of EOS-100-No phosphate added; E-Phos: microcosms with 0.70 mL of EOS-100-Phosphate added

Once the nitrate was removed (day 21), the perchlorate concentration decreased for microcosm with EOS-100 but not phosphate addition by ~9% (E-0.02-Perchlorate, Figure 21b), and the perchlorate concentration decreased by 56% in microcosms with EOS-100 and phosphate

addition (i.e., E-Phos-Perchlorate, Figure 21b). These results are consistent with the nitrate and perchlorate biodegradation rates shown in Table 22. Nitrate biodegradation rates increased from 1.77 mg/L/d to 3.42 mg/L/d during the first five days of incubation in microcosms with and without phosphate addition, but as the concentration of nitrate decreased the biodegradation rates were also reduced (i.e., 0.33 mg/L/d vs 0.07 mg/L/d, by day 21 of incubation). As mention before, perchlorate reductions did not start until the concentration of nitrate was reduced. But, in contrast with the biodegradation rates of nitrate, the perchlorate biodegradation rates did not decrease by the end of the incubation period in the microcosms with phosphate addition (i.e., 2.83 mg/L/d). This may have resulted by the highest concentrations of perchlorate present during the entire incubation period.

Table 22. Nitrate and Perchlorate Biodegradation Rates in Microcosms with EOS-100 (140X Stoichiometric COD) and 65 mg- PO_4^{-3}/L of Phosphate.

Days	Nitrate Biodegradation Rate (mg/L/d)		Perchlorate Biodegradation Rate (mg/L/d)	
	E-0.02*	E-Phos**	E-0.02*	E-Phos**
5	1.77	3.42	1.52	0.90
16	0.70	0.10	0.12	0.57
21	0.33	0.07	0.40	2.83

* Microcosms with 0.70 mL of EOS-100- No phosphate added
** Microcosm with 0.70 mL of EOS-100- Phosphate added

These results suggest that phosphate amendment increases the biodegradation rates of perchlorate and nitrate. Alternatively, phosphate addition increases the biodegradation rate of nitrate, thereby, eliminating this competing species allowing perchlorate reduction to commence sooner. In addition, comparing these results with the obtained in the vadose zone soil and Lake Mead water microcosms (Chapter 3), ~20 mg-P/L of phosphate is an adequate concentration to enhance nitrate and perchlorate biodegradations, compare with the ~6.5 mg-P/L of phosphate used in the vadose zone soil microcosms, in which any improvement in nitrate and perchlorate degradation were observed.

4. 3. 8. Nitrate and Perchlorate Biodegradation in Groundwater and Saturated Soil Microcosms-EOS-Pro

Additional microcosms were prepared to evaluate nitrate and perchlorate biodegradation in groundwater and saturated soil with an alternative slow-release electron donor—EOS-Pro. Microcosm preparation was similar to the EOS-100 experiments in that the ratio of saturated soil to groundwater was held constant at 40 g to 100 mL, but EOS-Pro was dosed at 0.01 g oil per g of soil and 0.005 g oil per gram of soil (i.e., 77X and 39X stoichiometric COD, respectively). The experimental matrix was summarized previously in Table 18.

Based on the EOS-Pro dosing conditions, the theoretical COD concentrations in the microcosms were expected as ~8,000 mg/L and ~4,000 mg/L for 77X and 39X stoichiometric COD, respectively. But according to Figure 22, the COD was considerably lower with average values of ~217 mg/L for 77X stoichiometric COD and ~122 mg/L for 39X stoichiometric COD microcosms, thereby suggesting adsorption of the slow-release oil onto the soil.

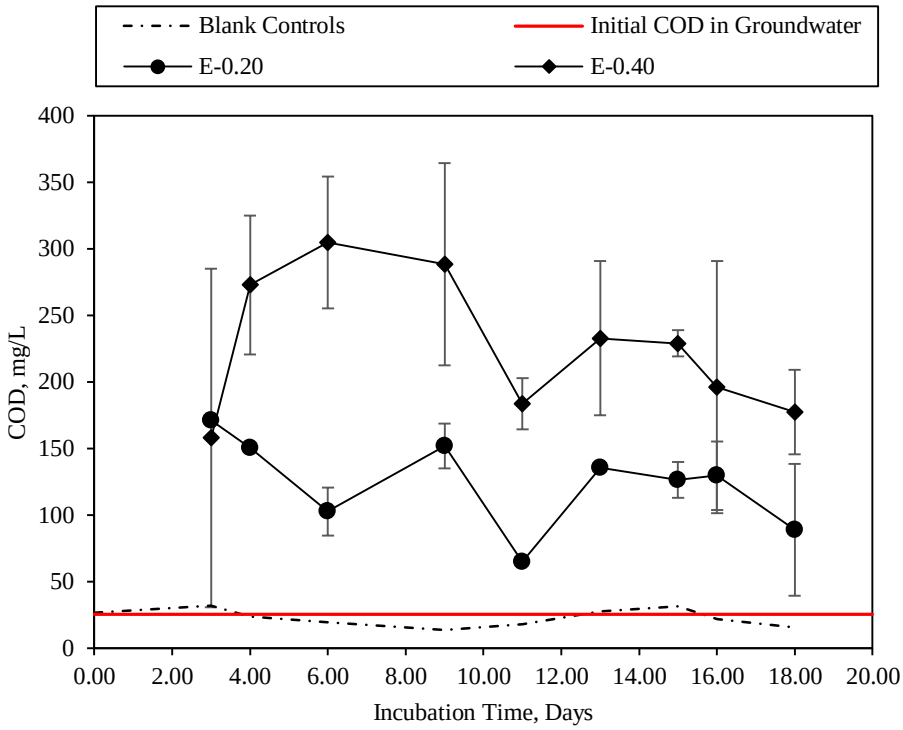

Figure 22. Chemical Oxygen Demand in Groundwater and Saturated Soil - EOS-Pro. E-0.20: 0.005 g oil/ g soil; E-0.40: 0.01 g oil/g soil; BLK: no EOS-Pro added. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

As shown earlier in Table 14, the initial nitrate concentration in the saturated soil and groundwater were 1.6mg-N/L and 16 mg-N/L, respectively. But, the nitrate concentration in these experimental microcosms was unusually low (<1 mg-N/L), even for the first sample analyzed on day 3 (Figure 23). The nitrate concentration in the blank microcosm was higher (~6.4 mg-N/L) but still considerably lower than the groundwater itself. Coupled with the fact that the initial perchlorate concentration was consistent with previous experiments, these data suggest rapid nitrate degradation even in the absence of electron donor amendment.

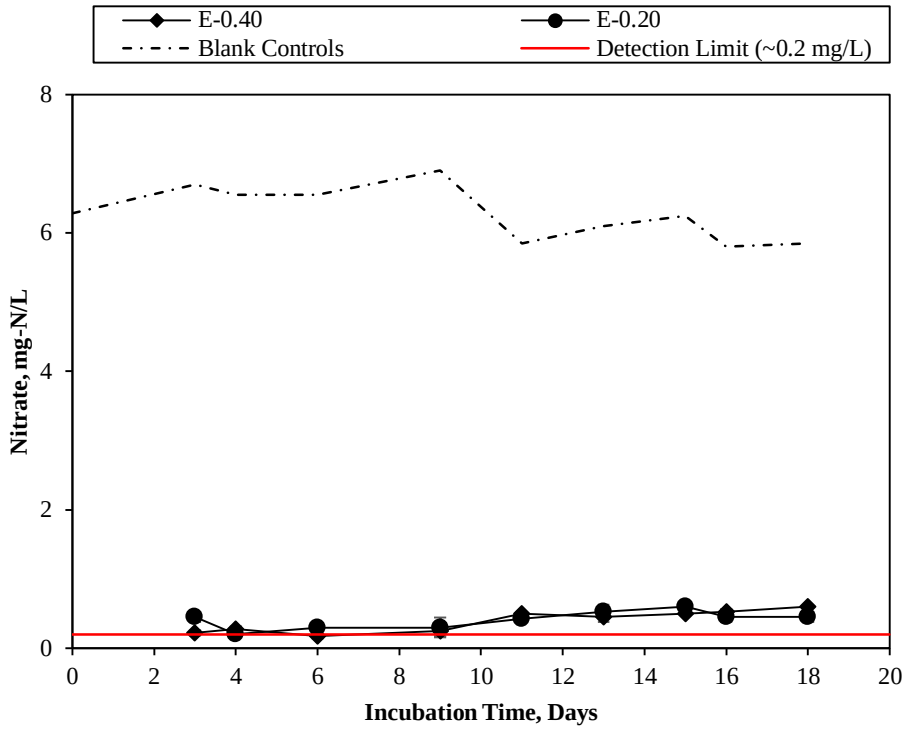

Figure 23. Nitrate Reduction in Groundwater and Saturated Soil Microcosms - EOS-Pro. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

Although nitrate had essentially been eliminated prior to day 3 of incubation, with maximum nitrate degradation rates of 1.96 mg-N/L/d and 1.88 mg-N/L/d for microcosms augmented with 77X and 39X stoichiometric COD, respectively. No significant perchlorate degradation was observed for the first 15 days of incubation, but nearly complete perchlorate degradation was observed between days 15 and 18 of incubation, with maximum perchlorate degradation rates of 4.86 mg/L/d for 77X stoichiometric COD and 6.00 mg/L/d for 39X stoichiometric COD microcosms. It is unclear what caused the 15-day perchlorate degradation lag period. However, the rapid degradation of nitrate and the fact that perchlorate was nearly completely degraded within 20 days—compared to >45 days for EOS-100—indicates that EOS-Pro might be a superior slow-release electron donor for the groundwater and saturated soil at the study site.

Comparing the maximum degradation rates of both EOS-100 and EOS-Pro in the biodegradation of nitrate and perchlorate, EOS-Pro generates significantly higher nitrate and perchlorate biodegradation rates than EOS-100. Although different stoichiometric COD were considered during the two microcosms batch test, based on the results of this research, EOS-Pro is considered a superior electron donor for nitrate and perchlorate contaminations in groundwater and saturated soil.

Table 23. Nitrate and Perchlorate Biodegradation Rates in Groundwater and Saturated Soil Microcosms Augmented with EOS-100 and EOS-Pro

Microcosms Batch Test	Grams of Oil to Grams of Soil Ratio	Maximum Nitrate Biodegradation Rates, mg-N/L/d	Maximum Perchlorate Biodegradation Rates, mg/L/d
EOS-100	0.002 g oil/g soil - 14X	0.45	0.10
	0.01 g oil/g soil - 70X	1.11	0.95
	0.02 g oil/g soil - 140X	1.17	0.84
EOS-Pro	0.005 g oil/g soil - 39X	1.88	6.00
	0.01 g oil/g soil - 77X	1.96	4.86

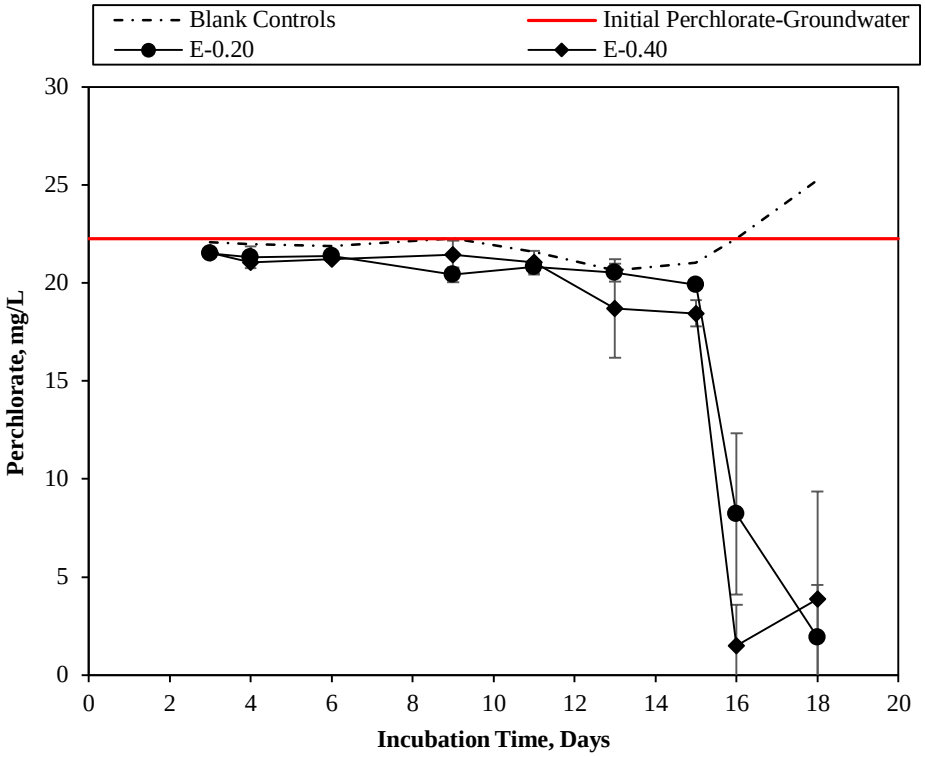

Figure 24. Perchlorate Reduction in Groundwater and Saturated Soil Microcosms - EOS-Pro. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

In contrast with previous experiments with EOS-100, EOS-Pro did not achieve sulfate degradation, and in fact, the sulfate concentration actually increased slightly in all microcosms (i.e., blank controls and those amended with EOS-Pro), as shown in Figure 25. This increase likely resulted from sulfate desorption from the saturated soil into the groundwater during the incubation period. Based on the initial extraction experiments (Table 14), the initial concentrations of sulfate in the aggregated extract obtained during the soil extraction process and in the groundwater were 140 and 1,520 mg/L, respectively. Therefore, desorption over the experimental period appeared to release additional sulfate that had not been previously measured. However, nitrate and perchlorate degradation did not appear to be affected by the high

sulfate concentration, presumably because of the thermodynamic favorability of nitrate and perchlorate over sulfate.

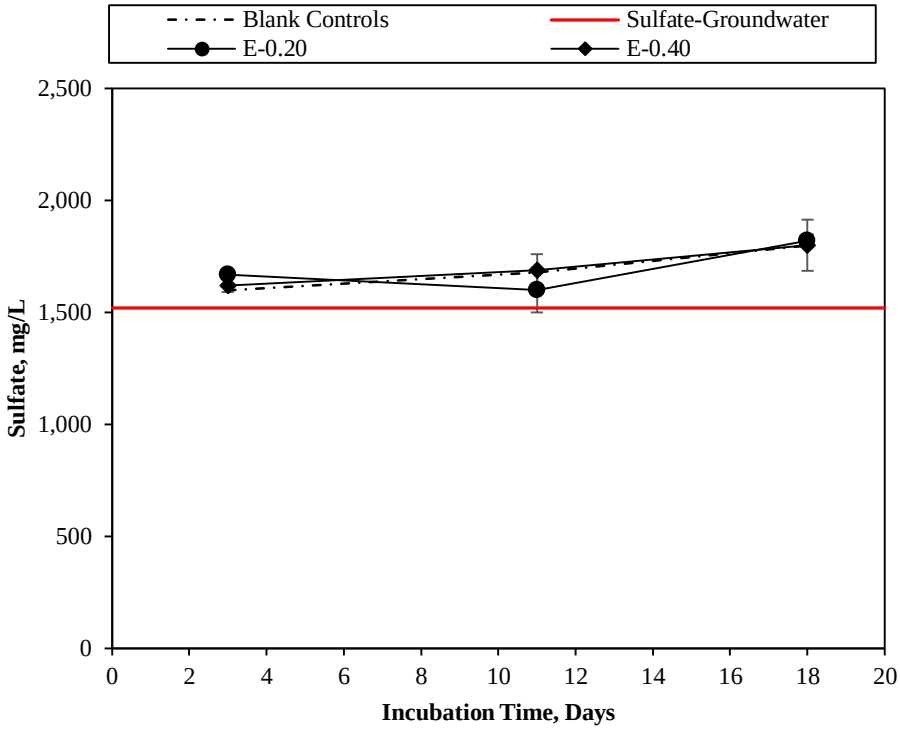

Figure 25. Sulfate Reduction in Groundwater and Saturated Soil Microcosms - EOS-Pro. The error bars indicate the standard deviation of the measurements. (Samples were tested in duplicates).

As mentioned earlier, EOS-Pro is also an additional source of phosphate (i.e., 0.116 % by weight of EOS-Pro). Thus, in this set of microcosms, no supplementary phosphate was added besides that provided by the oil. The initial phosphate concentrations in the saturated soil and groundwater were ~2.6 and 0.95 mg- PO_4^{-3}/L, respectively, but in the microcosms, the phosphate concentrations varied but were always less than 2 mg/L (Figure 26). The low concentration of phosphate in the microcosms may have resulted by the low phosphate content characteristic of saturated zone soils (Holden & Fierer, 2005; Konopka & Turco, 1991) or by phosphate

precipitations due to higher calcium content in the groundwater used in the microcosms (~1,800 mg-$CaCO_3$).

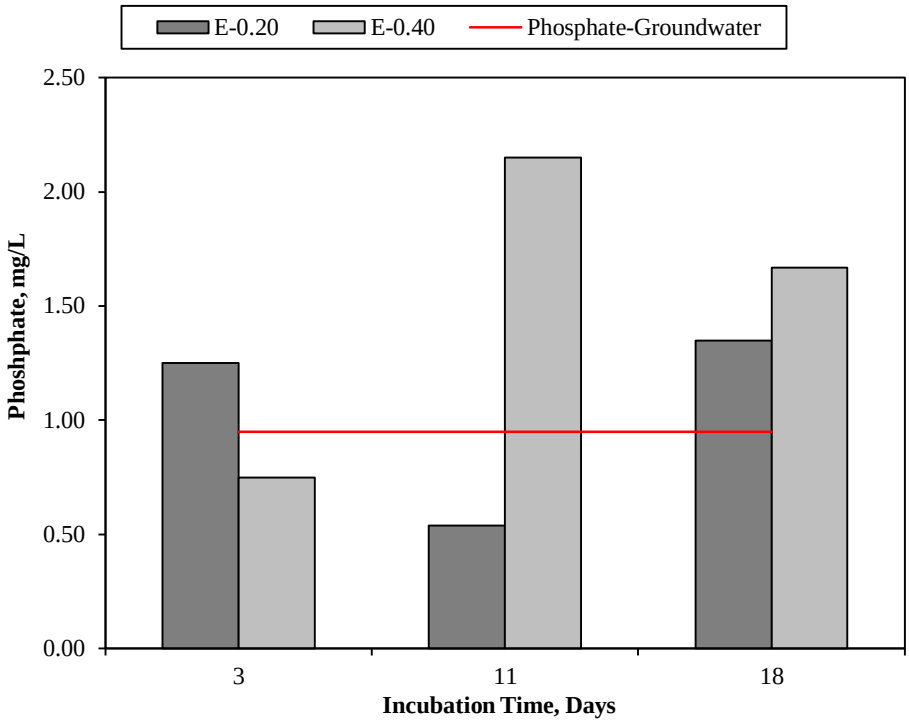

Figure 26. Phosphate Concentrations in Groundwater and Saturated Soil Microcosms - EOS-Pro

4. 4. Summary

The goal of this part of the research was to evaluate nitrate and perchlorate biodegradation in contaminated groundwater and saturated soil when using two different electron donors (i.e., EOS-100 and ESO-Pro). Moreover, the analysis was intended to identify optimum ratios of the emulsified oils to the contaminated groundwater and saturated soil (e.g., grams electron donor/gram of soil) that stimulate higher and faster nitrate and perchlorate biodegradation.

The oil dosing ratio of 0.02 g of EOS-100 per gram of saturated soil (140X stoichiometric COD) demonstrated faster reduction of nitrate and perchlorate compared with ratios of 0.01 or 0.002 g of EOS-100 per gram of saturated soil (i.e., 70X stoichiometric COD and 40X stoichiometric COD, respectively). The 0.02 g of EOS-100 per gram of saturated soil demonstrate a nitrate degradation rate of 1.77 mg-N/L/d during the first 5 days of incubation (~47% reduction), while the 0.01 and 0.002 ratios achieved demonstrate 1.11 mg-N/L/d (~30% reduction) and 0.45 mg-N/L/d (~14% reduction), respectively, during the same incubation period. Full nitrate reduction required 28 to 48 days of incubation, at which point perchlorate reduction became thermodynamically favorable.

Perchlorate reduction lag period supports the hypothesis of sequential reduction of electron acceptors (i.e., oxygen, nitrate, perchlorate, manganese, iron, sulfate, and carbon dioxide) based on redox potentials. After the corresponding lag period, perchlorate reduction was very rapid for the higher EOS-100 dosing ratios, but only slight reductions in perchlorate were observed over the testing period for a dosing ratio of 0.002 g EOS-100 per g of saturated soil (14X stoichiometric COD) with a degradation rate of 0.09mg/L/d compared with 0.84 and 0.95 mg/L/d demonstrated in 0.02 g oil/g soil and 0.01 g of oil/g of soil, respectively. These results were presumably due to slower kinetics at the lower dosing ratio considering that soluble COD was available in the microcosm.

When using EOS-Pro, nitrate and perchlorate degradations were more rapid than with EOS-100. Nitrate degradation rates were 4.86 mg-N/L/d and 6.00 mg-N/L/d during the first 3 days of the incubation period for the two dosing ratios test (77X and 39X stoichiometric COD), whereas nitrate reduction required >40 days for EOS-100 with a similar dosing ratio. By day 16,

perchlorate had also been reduced by >80% for both dosing conditions. Therefore, EOS-Pro appears to be a superior electron donor.

CHAPTER 5. CONCLUSIONS

- Nitrate and perchlorate contamination were found to be higher in the vadose zone soil than in the saturated soil. The high nitrate and perchlorate contamination in the vadose zone soil pose significant trends to public health since these contaminants can be flushing into groundwater sources by precipitations. Once the nitrate and perchlorate contaminants reached the groundwater sources, sensitive populations such as infants and pregnant women can be potentially incurred from the consumption of nitrate and perchlorate-contaminated water and food. Nevertheless, higher nitrate and perchlorate concentrations in the vadose zone soil are advantageous for treatment purposes because kinetic reactions are benefited at higher electron acceptor concentrations increasing the biodegradation rates at contaminated zones.
- Phosphate additions demonstrated variety outlines between the different set of microcosms. In the vadose zone soil microcosms, phosphate was added at a concentration of 6.5 mg-P/L in microcosms augmented with EOS-100 and glycerol. Results indicated that nitrate and perchlorate reductions were not enhanced with phosphate addition. However, in the saturated soil the concentration of phosphate was increased to ~21 mg-P/L and the results demonstrated an improvement in nitrate and perchlorate reductions. In fact, nitrate biodegradation rates in microcosms increased from ~1.77 mg/L/d to ~3.42 mg/L/d during the first 5 days of incubation. This increment allows the elimination of this competing specie faster allowing perchlorate reduction to commence sooner with rates of biodegradation of 2.83 mg/L/d compare with 0.40 mg/L/d without phosphate addition when using the same oil to soil ratio of 0.02 g oil/g soil.

- The main intention of evaluating the compost /mulch extract as a source of electron donor for nitrate and perchlorate biodegradation was to test an economic and replicable electron donor. Unfortunately, the compost/mulch extract generated during this research did not demonstrate any effects in nitrate and perchlorate reductions. These results are due to the lower chemical oxygen demand (~253 mg/L) that could be obtained in the laboratory.
- The slow release emulsified oil EOS-100 and the highly soluble glycerol were evaluated in the vadose zone soil and Lake Mead water microcosms. Both, electron donors achieved similar overall nitrate and perchlorate reductions, but EOS-100 exhibited faster kinetic reductions. The nitrate rate constants were estimated to be 0.60 d^{-1} and 0.42 d^{-1} for EOS-100 and glycerol, respectively. In the absence of nitrate, the pseudo first order rate constants for perchlorate reduction were determined to be 0.36 d^{-1} for EOS-100 and 0.31 d^{-1} for glycerol. Based on these results and in the fact that EOS-100 is adsorbed easily into the soil, this electron donor is recommended over glycerol because it can provide long-term soil remediation in full-scale applications.
- In the vadose zone soil the maximum degradation rates for nitrate and perchlorate reduction were achieved with EOS-100, with maximum degradation rates of 3.42 mg-N/L/d and 3.21 mg/L/d for nitrate and perchlorate, respectively. Compared with the maximum degradation rates of glycerol of 2.75 mg-N/L/d and 2.85 mg/L/d for nitrate and perchlorate respectively. In addition, EOS-100 promoted nitrate and perchlorate biodegradations to levels below the detection limit of the analytical methods within 6 days for nitrate and 14 days for perchlorate reductions.
- The soil to water ratios indicated that the amount of electron donor is a limiting factor in nitrate and perchlorate biodegradation rather than water volume. Both soil to water ratios

(i.e., 30 g of soil to 60 mL of water and 30 g of soil to 100 mL water) were effective in mobilizing the adsorbed nitrate and perchlorate, but there were minor impacts on bioremediation. The two soil to water ratios achieved similar reduction of nitrate and perchlorate for 11X stoichiometric COD, but the lower water volume (i.e., 60-mL) hindered nitrate and perchlorate reduction for 2.5X stoichiometric COD. The 11X stoichiometric COD was adequate for nitrate reduction, but perchlorate reduction required significantly longer incubation times based on the observed kinetics. Furthermore, the preliminary glycerol dosing with 0.5X and 1.0X stoichiometric COD was entirely inadequate to initiate biological reduction. Therefore, more comprehensive cost analyses are warranted to determine if higher electron donor concentrations (e.g., 100X) are justifiable when considering the more rapid kinetics of nitrate and perchlorate reduction.

- Nitrate and perchlorate biodegradation in the saturated soil and groundwater was evaluated using two emulsified oils, EOS-100 and EOS-Pro. EOS-Pro contains additional nutrients, and vitamins such as phosphate (~1,000 mg-P/L) and vitamin B12, while EOS-100 does not contain either. Thus, EOS-Pro rapidly improves the availability of microbial community, thereby enhancing nitrate and perchlorate biodegradation in contaminated zones. Based on the results of this research EOS-Pro is recommended for substrates with low nitrate and perchlorate contamination contents, low nutrients availability, and low microbial content.
- Between the three EOS-100 dosing ratios used in the saturated zone soil, 0.01 g oil/g soil and 0.02 g oil/g soil resulted in a complete nitrate and perchlorate biodegradation. However, 0.02 g oil/g soil demonstrated a slightly maximum degradation rates for nitrate

(1.17 mg/L/d) compared with the 0.01 g oil/g soil (1.10 mg-N/L). However, 0.01 g oil/g soil ratio demonstrated faster perchlorate degradation rates (0.95 mg/L/d) compare with 0.02 g oil/g soil ratio (0.84 mg/L/d). Nonetheless, the data in which the maximum perchlorate degradation rates were calculated for 0.02 g oil/g soil were low than for 0.01 g oil/g soil generating uncertainty. Therefore, based on the results of this research the highest COD stoichiometric ratio (0.02 g oil/g soil) is recommended for a faster and complete perchlorate biodegradation overcoming the presence of other contaminants such as oxygen, nitrate, manganese, iron, sulfate, and carbon dioxide.

- Based on the results observed between the different set of experiments, the abiotic control microcosms suggested the presence of nitrate reducing spore-forming bacteria in the vadose zone soil and the saturated soil with rates of nitrate biodegradation of ~0.017 mg/d and ~0.085 mg/d, respectively. However, additional research is needed in order to confirm these results.

5. 1. Implications of Perchlorate Bioremediation

Using the result of this research and data from other referenced authors, the implication of in-situ biodegradation treatments, especially soil flushing techniques are explained as reference for full-scale applications.

- The vadose zone soil samples used in this research were collected from four different locations and two different profile depths (0-12 feet and 14-26 feet) at a perchlorate-contaminated site. Assuming a maximum sample depth collection of 26 ft (~8 m) and a hydraulic velocity of an upper layer of soil (vadose zone soil) reported by R. C. Border (2007) of 2.1 m/d, the approximate time of flushing the Lake Mead water into the vadose

zone soil is determined as 3.2 days. In saturated soil, the typical vertical velocity of the groundwater has been reported as 0.04 m/d (Gal et al., 2009), thereby for the total depth of saturated soil used in this research of 40 ft (~12.2 m), the time to effectively distribute the mixed water and electron donor into de saturated zone is 30.5 days.

- The total mass of perchlorate in a contaminated vadose zone soil can be calculated by using the depth and the area of a contaminated vadose zone soil, the amount of perchlorate per unit of soil, and the bulk density of the soil. For example, the total mass of perchlorate at the site of study of this research can be determined by using the calculated perchlorate concentration (0.18 mg ClO_4^-/ g of soil) and the thickness of the vadose zone soil (26 ft). Moreover, assuming a contaminated area of 1 ft^2 and typical vadose zone soil bulk density of 81.1 lb/ft^3 (Border, Robert C. 2007), the total mass of perchlorate of the contaminated vadose zone at the site of this study is determined as 0.17 kg of perchlorate.
- The bulk density of the saturated soils at the site of study has been reported as 135.895 lb/ft^3 (Shrestha, Sichu, 2016). Thereby, the total mass of perchlorate assuming a 1 ft^2 area and using the calculated perchlorate concentration in the saturated soil of 0.0041 mg ClO_4^-/ g of soil for a 40 feet thickness saturated soil, can be calculated as 0.010 kg of perchlorate.

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