ABIOTIC, BIOTIC, AND BIO-ENHANCED REDUCTION OF HEXAVALENT CHROMIUM, CHLOROFORM AND CO-CONTAMINANTS USING NANO-SCALE ZERO VALENT IRON IN HIGHLY CONTAMINATED GROUNDWATER

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Abstract

Investigations of groundwater in a former industrial perchlorate manufacturing site have shown high contamination with perchlorate, chlorate, nitrate, hexavalent chromium (Cr (VI)), and chloroform (CF) with levels greater than 3,000, 30,000, 300, 100, and 4 mg/L, respectively. Remediation efforts using biological reduction to desired contaminant levels at this site has been challenging due to high contaminant concentrations, and high total dissolved solids (TDS). Furthermore, removal of Cr(VI) and CF in the presence of nitrate, chlorate, and perchlorate has not been examined at the contaminated site. Nano-scale Zero-Valent-Iron (NZVI) has been effective at reducing groundwater contamination both with and without bacterial augmentation. The objective of this research was to investigate the removal of CF, Cr(VI) and co-contaminants in contaminated industrial groundwater using NZVI alone or in combination with biological reduction (bio-enhancement). The effectiveness of abiotic reduction using NZVI, biotic reduction using a 1ml bacterial sludge inoculum enriched with 20 ml/L of molasses and additional nutrients, and bio-enhanced reduction using both NZVI and bacteria was evaluated in this study. Bench-scale reactors were monitored for Cr(VI), CF, nitrate, chlorate, and perchlorate removal over 8 weeks. The use of NZVI resulted in 100% reduction of Cr(VI) in only 4 hours with doses of 5,000 mg Fe^0/L. As 100% reduction of Cr(VI) occurred at a much faster rate in abiotic treatments than biotic treatments, bio-enhancement for Cr(VI reduction relies more on NZVI reduction. For CF, removal showed 15%-40% greater results under bio-enhancement conditions than abiotic treatments. However, a bio-enhanced NZVI dose of at least 8,500 mg Fe^0/L is needed to achieve higher removal than biotic treatments alone. A bio-enhanced NZVI dose of 17,000 mg Fe^0/L resulted in 100% CF removal in 7 days. Bio-enhancement also

achieved greater nitrate and chlorate removal, showing 100% removal at NZVI doses of 17,000 and 5,000 mg Fe^0/L, respectively. No abiotic perchlorate reduction was observed using NZVI. Perchlorate showed 25-50% removal only in biotic and bio-enhanced conditions. Bio-enhancement showed greater and more consistent removal for all the examined contaminants. This endorses bio-enhancement as the best treatment for groundwater from the examined site.

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Chapter 1: Problem Statement

Investigations of groundwater contamination in a former perchlorate manufacturing facility in Henderson, NV show high contamination with perchlorate, chlorate, nitrate, and hexavalent chromium (Cr(VI)). Decades of industrial activity have contributed to perchlorate, chlorate, nitrate, and Cr(VI) levels greater than 3,000 mg/L, 30,000 mg/L, 300 mg/L, and 100 mg/L, respectively. After initial investigations, chloroform (CF) contamination at 4 mg/L. was also detected. Cr(VI) at high doses is carcinogenic and can cause severe allergic reactions (Costa et al, 2003). CF can pose a serious health risk, as it is carcinogenic even at doses as low as 200 mg/kg (Boorman et al, 1999). The high levels of contamination found at this site pose a risk of seepage of industrial contaminants from this site into major potable water sources. Efficient reduction to desired contaminant levels at this site using bioremediation has been challenging due to high contaminant concentrations and the presence of high total dissolved solid (TDS) concentrations. Removal of Cr(VI) and CF in the presence of nitrate, chlorate, and perchlorate has not been examined at the contaminated site. Thus, there is a need to evaluate strategies to remediate the contamination present at this site.

Common effective removal methods for perchlorate, chlorate, nitrate, Cr(VI), and CF include reduction with zero-valent-iron (ZVI) and bioremediation (Dhal, 2013 et al; Loyaux-Lawniczak et. al; 2001 Gillham and O'Hannesin, 1994; Matlochova et al, 2013; Naffrechoux et al, 2003; Greenhalgh, 2019; Liu, et al, 2013; Miller and Logan, 2000; Nozawa-Inoue et al, 2005; Srinivasan, 2009 et al; Van Ginkel et al 1995, & Xu et al, 2004). Though bioremediation of Cr(VI) at the examined has been attempted, removal has been limited. Additionally, remediation of CF has not yet been examined at the site. This necessitates investigation of alternative remediation methods. Remediation of CF and Cr(VI) compounds is particularly pressing, as both

are listed as priority water contaminants by the Environmental Protection Agency (EPA) in Title 40, Section 131 in the Code of Federal Regulations (U.S. EPA, 1999). Many studies have proven ZVI as an effective method of treating contaminated groundwater (Mukherjee et al, 2016) ZVI has been used successfully to reduce both Cr(VI) (Gheju et al; 2011) and CF (Gillham and O'Hannesin, 1994; Matlochova et al, 2013 & Singh et al, 2011). Abiotic remediation using ZVI has also been tested for other co-contaminants present in the groundwater used in this study, such as nitrate (Liu et al, 2012), chlorate (Zarei and Ghavi, 2016), and perchlorate (Petrucci et al, 2016). Oxidation of ZVI will generate hydrogen and its electrons will be used to reduce other contaminants (Mukherjee et al, 2016). Advances in nano-particle technology have developed nano-scale ZVI (NZVI) as the most efficient form of ZVI for groundwater remediation due to its high surface area (Matlochova et al, 2013 & Mukherjee et al, 2016). Additionally, both Cr(VI) (Losi et al, 1994; Turick et al 1998, & Wang and Shen, 1995) and CF (Cappelletti et al, 2012 & Grostern et al, 2010) are biodegradable compounds. Furthermore, denitrifying conditions have shown stimulation of halogenated aliphatic compound removal (Bouwer and McCarty, 1983). Environments in which denitrifying bacteria are common also harbor perchlorate and chlorate reducing bacteria (Nozawa-Inoue et al, 2005 & Xu et al, 2004). This endorses using biotic remediation for the examined groundwater despite the limited results shown at the site in the past. The production of hydrogen gas from NZVI oxidation in both aerobic and anaerobic conditions can benefit remediation in biotic conditions (Xu et al, 2017). Augmentation of NZVI with bacterial inoculums (bio-enhancement) has also shown success for contaminants present at the site including nitrate (Liu et al, 2013), chlorate (Greenhalgh, 2019), and CF (Lee et al, 2015). However, bio-enhanced ZVI remediation is still experimental and has not been commercially applied. This endorses investigation of abiotic, biotic and bio-enhanced remediation for Cr(VI)

and CF in the presence of nitrate, chlorate, and perchlorate at the investigated site. Finally, it is important to consider potential differences between in-situ and ex-situ remediation using abiotic, biotic, and bio-enhanced removal, as treatment results using NZVI can vary when applied in-situ or ex-situ, particularly with chlorinated organics, (Stevenson and Herrera, 2018).

The Goals of the research performed for this thesis are to:

1) Investigate the efficacy of abiotic, biotic, and bio-enhanced Cr(VI) and CF removal using NZVI and/or enriched bacterial sludge in the presence of nitrate, chlorate, and perchlorate.
2) Monitor the additional contaminants (co-contaminants) present in this study, i.e. nitrate, chlorate, and perchlorate under abiotic, biotic, and bio-enhanced treatments.
3) Determine potential differences between in-situ, and ex-situ remediation through the addition of site soil to mimic in-situ conditions on abiotic, biotic, and bio-enhanced remediation of all contaminants measured in this study.
4) Study the effects of increasing NZVI doses on contaminant reduction in abiotic and bio-enhanced treatments.

Hypotheses

- Due to the effectiveness of NZVI in in-situ remediation (Matlochova et al, 2013), the presence of soil is not expected to have any negative effect on remediation using any treatment.
- For Cr (VI), previous research in this laboratory has shown rapid reduction by ZVI alone, no statistically significant difference was found between bio-enhanced reactors and biotic reactors (Greenhalgh, 2019). Therefore, Cr(VI) removal is expected to be mostly due to abiotic reactions with NZVI in abiotic and bio-enhanced removal.

- For CF, previous research has shown higher removal efficacy in bio-enhanced reactors, as opposed to abiotic and biotic treatments alone (Lee et al, 2015 & Weathers et al, 1997). Therefore, CF reduction will be the greatest in bio-enhanced reactors.
- For all contaminants, greater removal can be expected with an increasing NZVI dose in all treatments containing NZVI. This has been proven for nitrate (Liu et al, 2012), chlorate (Greenhalgh, 2019), perchlorate (Petrucci et al, 2016), and CF (Xiao et al,2014).
- Though some perchlorate reduction by ZVI is anticipated (Petrucci et al, 2016 & Schaefer et al, 2007), limited removal is expected in abiotic reactors containing NZVI and perchlorate.

Chapter 2: Literature Review

This research focuses on the remediation of Cr(VI) and (CF) from contaminated groundwater. This chapter provides information on the occurrence, health impacts, and technologies used to remove these contaminants from soil and water.

2.1 Chromium Contamination, Health Effects and Regulation

Pure metallic chromium is uncommon in the environment, as it is quick to react with atmospheric gases (Jacobs et al, 2005). The most stable oxidation states of chromium are Cr(III) and Cr(VI). Most commonly, chromium is oxidized to form Cr(III) oxide (Cr_2O_3), which is one of the most abundant compounds on the Earth's Surface (Jacobs et al, 2005). Due to its high redox potential, Cr(VI) predominates over Cr(III) in aqueous environments, with chromate (CrO_4^{2-}) as a monomer and dichromate ($Cr_2O_7^{2-}$) as the dimeric form (Loyaux-Lawniczak et al, 2001 & Li et al, 2009). The most common anthropogenic source of chromium waste is the production of chromium-containing byproducts through its use in metal hardening in the metallurgical industry (Jacobs et al, 2005 & Palmer et al, 1991). Within metallurgy, the strong oxidative potential of Cr(VI) in steel passivation is one of the most prevalent anti-corrosion practices (Berger et al, 2007), and the reason for Cr(VI) contamination in the site related to this research. However, the use of chromium is prevalent in many other industries including chemical manufacturing, photography, printing, dyeing, leather tanning, agriculture, mining, and cooling systems (Palmer et al, 1991). Currently, most chromium in the U.S. is mined offshore from chromite ($FeCr_2O_4$) (Palmer et al, 1991 & Nriagu and Nieboer, 1988). Cr(VI) can also be extracted from liquid and solid wastes using chemical solvents and adsorption (Kalidhasan and Rajesh, 2009, & Rajesh et al, 2008)

Due to its importance in industry, public exposure to chromium is mainly due to exposure to industrial byproducts and contamination. Though safety measures and protective equipment can limit exposure to chromium in industrial workplaces, leakage into groundwater reservoirs is a significant source of chromium contamination in the environment (Palmer et al, 1991). Chromium exposure can happen through a variety of pathways. Cr(VI) exposure is most commonly associated with ingestion contaminated water (Jacobs et al, 2005). Inhalation of chromium dust arising from its use in metallurgy and dermal exposure of chromium-contaminated water and soil are also common sources (Jacobs et al, 2005). Cr(VI) is highly toxic, and its ingestion even at low doses can cause cellular inhibition. Higher levels (>100 µg/L) of Cr(VI) can result in a variety of health hazards including carcinogenicity and cutaneous anaphylaxis (Costa et al, 2003).

Currently, the U.S. EPA has set the maximum contaminant level for total chromium at 100 µg/L (U.S. EPA., 2004). However, under the Clean Water Act, the U.S. EPA listed both Cr (III) and Cr(VI) as priority contaminants to be regulated in freshwater and saltwater, with respective minimum contaminant level (MCL) goals of 550 µg/L and 15 µg/L (U.S. EPA, 1999), respectively. For environments with chronic chromium contamination, MCL's for continuous exposure fall to 180 µg/L for Cr(III) and 10 µg/L for Cr(VI) in water (U.S. EPA, 1999). Though contamination is more prevalent in industrial waste, Cr(VI) can be found in various media across the U.S. Typical levels of industrial chromium contamination far exceed the water safety standards implemented by the EPA (Table 2.1).

Table 2.1: U.S. Ambient and Industrial Chromium contamination

Ambient Cr Contamination			
Medium		**Cr Concentration**	**Source**
U.S. Soil		25-85 mg/kg	Zayed and Ghavi, 2003
U.S. Air		0.1 µg/m^3	
U.S. Tap Water		0.18 µg/L	Sutton, 2010
Industrial Cr Contamination			
Contamination Source	**Medium**	**Cr Concentration**	**Source**
Ore Processing, NJ	Ore Residue	1,000-10,000 mg/kg	Li et al, 2008
Ore Processing, NJ	Ore Residue	4,575-6,530 mg/kg	Dhal et al, 2013
Metal Plating, NC	Soil	28-168 mg/kg	Nivas et al, 1996
Metal Plating, OR	Wastewater	19-1,293 mg/L	Greene, 1988
Superfund, OR	Soil	25,900 mg/kg	Zayed and Terry, 2003
Superfund, OR	Groundwater	14,600 mg/L	

2.2 Chloroform Contamination, Health Effects and Regulation

As a highly volatile compound, most CF contamination can be found atmospherically and is naturally occurring (McCulloch et al, 2003). The greatest sources of natural CF are due to terrestrial and aquatic algal activity (Laturnus et al, 2002 & McCulloch et al, 2003). Another significant source of environmental CF is release by volcanic activity (Laturnus et al, 2002), as the presence of CF among other organic gases can also be found inside the Earth's crust (Isidorov et al, 1990). Naturally burning biomass and microbiological activity in peatlands are also major sources of natural CF (Laturnus et al, 2002). While the most common anthropogenic source of CF pollution is byproduct formation due to paper products manufacturing, other current anthropogenic sources of CF include chemical manufacturing, fumigation, solid waste removal, and chlorination during water treatment (McCulloch et al, 2003).

Though the industrial production of halogenated aliphatic compounds produces noticeable levels of groundwater CF contamination (Petura et al, 1981), public exposure to CF

and other trihalomethanes (THM's) is primarily due to their ingestion as disinfection byproducts formed during potable and wastewater treatment (McCulloch et al, 2003). The formation of CF arises from chlorination during disinfection, where chlorinated oxidation of humic compounds results in the formation of THM's (McCulloch et al, 2003). Ingestion and inhalation of CF arising from THM formation in chlorinated pools and showers has also been identified as another exposure pathway (Jo et al, 1990 & Hsu et al, 2009). The carcinogenic effects of CF ingestion have been well documented by previous research in both animals and humans (Boorman, 1999, & Tardiff, 1977). Additionally, chronic exposure to CF has also been linked to a variety of health detriments including reproductive inhibition, teratogenic effects, and hepatic, kidney and bronchial damage (Kramer et al, 1992 & Hsu et al, 2009).

Like chromium, CF is listed as a priority contaminant under the Clean Water Act. As a THM, the national total THM MCL listed by the EPA is 80 µg/L (U.S. EPA, 2004). However, the EPA recommends a CF MCL goal of 60 µg/L for potable water and 2,000 µg/L for organism consumption (U.S. EPA., 2015). As a priority carcinogen, the MCL of CF for a reference dose (RfD) with a carcinogenic risk of 10^{-6} is 5.7 µg/L for potable water and 470 µg/L for organism consumption (U.S. EPA, 1999). Due to its high volatility, CF contamination is primary limited to water and air (Hoekstra et al, 1998 & McCulloch et al, 2003). The widespread variety of natural CF sources makes estimation of global CF release challenging (Laturnus et al, 2002 & McCulloch et al, 2003). Despite this, previous studies have provided several measurements of ambient CF and CF in industrial waste (Table 2.2).

Table 2.2: U.S. Ambient and Industrial Chloroform contamination

Ambient CF Contamination			
Medium		**CF Concentration**	**Source**
Air (Global)		0.09 µg/m^3	McCulloch et al, 2003
Air, NJ		0.068-8.7 µg/m^3	
Tap Water, FL		4 µg/L	Gibbons and Laha, 1999
Industrial CF contamination			
Contamination Source	**Medium**	**CF Concentration**	**Source**
US Paper Mills	Paper Products	138 µg/g	McCulloch et al, 2003
U.S. Potable Water Treatment	Chlorinated Water	13 µg/L	
U.S. Pool Treatment	Air	507-1630 µg/L	Lévesque et al, 1994

2.3 Technologies for Chromium and Chloroform Remediation

A variety of technologies have been developed to remove chromium from both soil and water (Dhal et al, 2013 & Owlad et al, 2009). In soil, chromium can be removed through traditional extraction and treatment, leaching, chemical reduction, vitrification, and biological reduction (Loyaux-Lawniczak et al, 2001). In water, methods for chromium removal include adsorption, inorganic and liquid membrane filtration, electrolysis, and biological reduction (Owlad et al, 2009). ZVI reduction of Cr(VI) has been proven effective in both soil and water (Dhal et al, 2013 & Xu et al, 2014).

Most CF contamination is airborne, while airborne CF removal through filtration has been documented (Palanisamy et al, 2016), primary exposure to CF is waterborne (McCulloch et al, 2003). Because of this, most CF remediation is focused on its removal from water. As a THM, waterborne CF remediation primarily involves hydrolysis, UV irradiation, adsorption, bioremediation, ZVI reduction, and ion exchange (Lee, 2015 et al; Matlochova et al 2013 & Naffrechoux et al, 2003). An overview of the Cr(VI) and CF reducing technologies assessed in this study is shown on Table 2.3. Overall, previous studies show greater removal of contaminants

with an increasing NZVI dose in abiotic (Li et al, 2010) and bio-enhanced (Xiao et al, 2014) treatments. Biotic treatments also show greater removal with an increased bacterial dose and increased nutrient amendment (Schaefer et al, 2007 & Wu et al, 2001).

Table 2.3: Biotic, Abiotic and Bio-enhanced Remediation Technologies to Degrade Hexavalent Chromium and Chloroform

Technology	Treatment Dose	Target Contaminant	Medium	Initial Contamination	Final Contaminant Level	Source
NZVI (60nm)	226.36 mg Fe^0/mg	Cr(VI)	Contaminated groundwater	10.9 mg/L	1-2 µg/L	Li et al, 2008
NZVI (60nm)	50 mg Fe^0/mg	Cr(VI)	Spiked DI water	100 mg/L	1-2 µg/L	
NZVI (20-100nm)	20 mg Fe^0/mg	Cr(VI)	Spiked DI water	10 mg/L	5 mg/L	Wang et al, 2010
NZVI (20-100nm)	142.86 mg Fe^0/mg	Cr(VI)	Spiked DI water	10 mg/L	3.5 mg/L	Xu et al, 2014
Bacterial Sludge	10 ml/L	Cr(VI)	Spiked LB Broth	20-600 mg/l	0-570 mg/L	Molokwane et al, 2008
Bacterial Soil Extract	10 ml/L			20-300 mg/L	0-60 mg/L	
Activated Sludge	240-2,000 mg Fe^0/mg	Cr(VI)	Municipal Wastewater	5 mg/L	0.01 mg/L	Stasinakis et al, 2003
Macro ZVI	124,192 mg Fe^0/mg	CF	Spiked Synthetic Groundwater	2,013 µg/L	2.6 µg/L	Gillham and O'Hannesin, 1994
Macro ZVI	1.5-2.5% (39 tons)	CF	Contaminated Soil	6,100 mg/kg	0 mg/kg	Ovbey et al, 2010
Nano ZVI	1,135.0 mg Fe^0/mg	CF	Spiked Bacterial Medium	1,134.11 µg/L	0 µg/L	Lee et al, 2015
Activated Carbon/ NZVI	49,619 mg Fe^0/mg	CF	Municipal Wastewater	42.19 µg/L	3.8-21.09 µg/L	Xiao et al, 2014
Bacterial Culture	100 ml Stock culture	CF	Spiked Broth Culture	102 µg/L	0 µg/L	Becker and Freedman, 1994
Bacterial Soil	200 g/L	CF	Spiked Synthetic Groundwater	4-400 µg/L	0.8-120 µg/L	Van Beelen and Van Keulen, 1990
Bacterial Sludge	250ml/l	CF	Spiked Broth Culture	2 mg/L	0.5-1 mg/l	Lu and Li, 2010
Bio-enhanced ZVI	0.03 mg Fe^0/mg 26 g/L Dry Sludge	COD	Synthetic Wastewater	6000 mg/L	900-1800 mg/L	Zhang et al, 2011
Bio-enhanced ZVI	246.15 mg Fe^0/mg 400 mg/L Dry Sludge	Perchlorate	Spiked Broth Culture	65 mg/L	0 mg/L	Son et al, 2006

Bio-enhanced ZVI	0.56 mg Fe^0/mg 628ml Bacterial Column	Nitrate	Synthetic Wastewater	177 mg/L	10.63 mg/L	Liu et al, 2013

2.3.1 Abiotic ZVI Remediation of Chromium, Chloroform, and Co-Contaminants

As stated before, ZVI has been proven effective at removing a variety of contaminants, including both Cr(VI) (Gheju et al, 2011) and CF (Garcia et al, 2020; Lee et al, 2015; Matlochova et al 2013; Singh et al, 2011, & Wang et al, 2012). The use of ZVI has achieved great success at reducing common groundwater contaminants (Fu et al, 2014, & Matlochova et al, 2013). As ZVI reactivity benefits from increased particle surface area, nano-scale ZVI (NZVI) presents the most efficient form of ZVI available for treatment (Matlochova et al, 2013 & Mukherjee et al, 2016). The capability of NZVI to reduce and adsorb a variety of contaminants makes it a viable alternative to using ion exchange and adsorption through resins (Singh et al, 2011). Furthermore, NZVI is effective in in-situ remediation, requiring only direct injection of a suspension into a contaminated water reservoir (Cundy et al, 2008 & Matlochova et al, 2013). In in-situ remediation, NZVI reduces and precipitates contaminants, immobilizing them (Cundy et al, 2008). This eliminates the production of brines, and limits exposure to harmful contaminants (Cundy et al, 2008). Methods for in-situ NZVI remediation include jet grouting, direct soil mixing, high-pressure pumping, pneumatic injection, and hydraulic fracturing (Mukherjee et al, 2016; Ovbey et al, 2010 & Thiruvenkatachari et al, 2008).

ZVI's (Fe^0) effectiveness is due to its chemical structure, which allows for easy oxidation. This oxidation results in the release of electrons, which can then reduce both organic and inorganic compounds (Mukherjee et al, 2016). This is shown in the following equation (eq. 1, Mukherjee et al, 2016).

Electron Release during ZVI Oxidation

$$Fe^0 \rightarrow Fe^{2+} + 2e^- \quad \text{(eq. 1)}$$

Under aerobic conditions, oxygen will corrode ZVI. Oxidation will occur in two steps until iron(III) (Fe^{3+}) is formed. Aerobic oxidation of ZVI will result in the production of water molecules. This is shown below (eq. 2-3, Gheju et al, 2011).

Aerobic ZVI Oxidation

$$2Fe^0 + 4H^+ + O_2 \rightarrow 2Fe^{2+} + 2H_2O \quad \text{(eq. 2)}$$

$$4Fe^{2+} + 4H^+ + O_2 \rightarrow 4Fe^{3+} + 2H_2O \quad \text{(eq. 3)}$$

Under anaerobic conditions, ZVI will oxidized by water alone. This will also proceed until iron(III) is formed. Anaerobic ZVI oxidation will produce hydrogen gas (H_2) and hydroxide ions (OH^-). Hydrogen gas will then reduce contaminants in water. Additionally, hydrogen gas can be used by bacteria as an electron donor in the reduction of various contaminants, including Cr(VI) (Thatoi et al, 2014), and CF (Cappelletti et al, 2012 & Lee et al, 2015). The production of hydrogen gas during aqueous ZVI oxidation is shown below (eq. 4-5, Gheju et al, 2011, & Reardon, 2014).

Aqueous ZVI Oxidation

$$Fe^0 + 2H_2O \rightarrow Fe^{2+} + 2OH^- + H_2 \quad \text{(eq. 4)}$$

$$2Fe^{2+} + 2H_2O \rightarrow 2Fe^{3+} + 2OH^- + H_2 \quad \text{(eq. 5)}$$

ZVI oxidation will form an oxide/hydroxide layer on the metal surface, which can serve as an attachment point for microbial biofilms (Greenhalgh, 2019). However, the formation of oxide layers will decrease ZVI reactivity, which will result in less contaminant reduction, in a process called passivation (Greenhalgh, 2019 & Zhang et al, 2016). Finally, the oxidation of ZVI in water usually results an increase in pH through production of excess hydroxide ions. This

means ZVI will react more rapidly at a decreased pH, where the concentration of hydroxide is less (Mukherjee, 2016).

The reduction of Cr(VI) into a less toxic form, Cr(III), is the principal method by which ZVI remediates chromium (Singh et al, 2011). The speciation of aqueous Cr(VI) into chromate and dichromate, makes metallic chromium species compounds the primary contaminant forms of Cr(VI) in most industrial wastewater effluent containing high amounts of metallic solutes (Li et al, 2009). Dichromate is the predominant chromium species in aquatic conditions with high Cr(VI) concentrations in acidic conditions, while chromate predominates in neutral and basic conditions (Gheju et al, 2011). ZVI can reduce both chromate species. Aqueous Cr(VI) reduction by ZVI predominantly involves reduction to Cr(III) through direct electron donation by hydrogen gas formed during anaerobic ZVI oxidation (eq. 6-9). Cr(III) will bind to hydroxide ions as chromium hydroxide ($Cr(OH)_3$) which will be adsorbed and precipitated when ionically coupled to charged iron particles (eq. 10-11). The remediation of Cr(VI) as chromate and dichromate by ZVI is shown in the equations below (Cundy et al, 2008; Gheju et al, 2011; Singh et al, 2011, & Xu et al, 2014).

Aqueous ZVI Reduction of Chromate

$$2CrO_4^{2-} + 3Fe^0 + 16H^+ \rightarrow 2Cr^{3+} + 3Fe^{2+} + 8H_2O \quad \text{(eq. 6)}$$

$$2CrO_4^{2-} + 3Fe^{2+} + 8H^+ \rightarrow 2Cr^{3+} + 3Fe^{3+} + 4H_2O \quad \text{(eq. 7)}$$

Aqueous ZVI Reduction of Dichromate

$$Cr_2O_7^{2-} + 3Fe^0 + 14H^+ \rightarrow 2Cr^{3+} + 3Fe^{2+} + 7H_2O \quad \text{(eq. 8)}$$

$$Cr_2O_7^{2-} + 6Fe^{2+} + 14H^+ \rightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O \quad \text{(eq. 9)}$$

Aqueous Precipitation of Cr(III) Hydroxide by Iron

$$Fe^{2+} + CrO_4^- + 4H_2O \rightarrow (Fe_x, Cr_{1-x})(OH)_3 + 5OH^- \quad \text{(eq. 10)}$$

$$xCr^{3+} + (1-x)Fe^{3+} + 3H_2O \rightarrow Cr_x^{3+}Fe_{1-x}^{3+}(OH)_3 + 3H_2O \quad \text{(eq. 11)}$$

The reduction of CF using ZVI is more inclusive towards other organics, which can react with several halogenated organic compounds (Gillham and O'Hannesin, 1994). This reaction involves reductive dechlorination catalyzed by electron donation by ZVI (Garcia et al, 2020; Cundy et al, 2008 & Wang et al, 2012). Under aqueous conditions, ZVI will react with hydronium ions in water and displace chlorine in chlorinated organics to form iron(II)(Fe^{2+}), water and chloride ions (Cundy et al, 2008). A lower pH will result in faster reduction of chlorinated aliphatics due to an increased hydronium concentration, as shown below (eq. 12, Cundy et al, 2008).

Aqueous ZVI Reduction of Halogenated Aliphatics.

$$Fe^0 + RCl + H_3O^+ \rightarrow Fe^{2+} + RH + Cl^- + H_2O \quad \text{(eq. 12)}$$

Reduction of CF by ZVI is usually identified by the production of dichloromethane (DCM) and methane (Lee et al, 2015). Lee's study showed the reduction of CF by ZVI alone will produce DCM and methane in equal ratios. Though the complete reduction of CF into methane is possible, ZVI reduction of DCM by ZVI is limited and requires higher ZVI doses or longer contact time (Lee et al, 2015 & Plagentz et al, 2006). The production of chloromethane (CM) is only transient, as it can be quickly reduced to methane (Lee et al, 2015 & Yu et al, 2016). Assuming complete reduction to methane, the proposed mechanism for CF reduction by ZVI is as follows (eq. 13, Yu et al, 2016).

Aqueous ZVI Reduction of Chloroform

$$CHCl_3 + 3Fe^0 + 3H_2O \rightarrow CH_4 + 3Cl^- + 3OH^- + 3Fe^{2+} \quad \text{(eq. 13)}$$

The additional contaminants measured in this study, chlorate, nitrate, and perchlorate have also shown reduction by ZVI in aqueous conditions. However, perchlorate reduction with ZVI has shown limited success (Petrucci et al, 2016 & Schaefer et al, 2007). Mechanisms for

aqueous reduction of nitrate, chlorate and perchlorate are shown in the following equations (eq. 14-16; Westerhoff, 2003 & Zarei and Ghavi, 2016).

Aqueous ZVI Reduction of Nitrate

$$NO_3^- + 4Fe^0 + 10H^+ \rightarrow 4Fe^{2+} + NH_4^+ + 4H_2O \quad \text{(eq. 14)}$$

Aqueous ZVI Reduction of Chlorate

$$ClO_3^- + 3Fe^0 + 6H^+ \rightarrow 3Fe^{2+} + Cl^- + 3H_2O \quad \text{(eq. 15)}$$

Aqueous ZVI Reduction of Perchlorate

$$ClO_4^- + 3Fe^0 + 8H^+ \rightarrow 4Fe^{2+} + Cl^- + 4H_2O \quad \text{(eq. 16)}$$

2.3.2 Biotic Remediation of Chromium and Chloroform

Many studies have recognized biological reduction as an effective method for Cr(VI) reduction both in soil and groundwater (Losi et al, 1994; Wang and Shen, 1995 & Turick et al, 1998). A wide variety of commonly abundant bacteria such as *E. coli, P. aeruginosa,* and *B. subtillis* (Wang and Shen, 1995) can reduce Cr(VI). Like ZVI degradation, the primary method for bacterial degradation is the reduction of Cr(VI) into Cr(III). This reduction can happen in both aerobic and anaerobic conditions (Wang and Shen, 1995). Cr(VI) is reduced due to enzymatic activity, most commonly by chromate reductase (Thatoi et al, 2014).

Under aerobic conditions, a common electron donor required for Cr(VI) reduction is nicotinamide adenine dinucleotide (NADH) (Fig 2.1). Aerobic Cr(VI) reduction has been identified in a variety of bacterial species in *Pseudomonas* and bacterial strains in *E. Coli* (Thatoi et al, 2014 & Wang and Shen, 1995). Aerobically, bacterial reduction of Cr(VI) involves the use of membrane-bound and/or cytoplasmic enzymes such as chromate reductase (Thatoi et al, 2014, & Turick et al, 1998). Aerobic reduction of chromium usually reduces Cr(VI) in two steps from Cr(VI) to Cr(V), then Cr(V) to Cr(III) using different enzymes and cytochromes (Malaviya and Singh, 2016, & Thatoi et al, 2014). These enzymes are usually soluble and contained within the cytoplasm (Thatoi et al, 2014). The formation of Cr(V) is short-lived and undergoes one cycle of

oxidation/reduction in which Cr(VI) is regenerated and oxygen will accept electrons (Malaviya and Singh, 2016). Ultimately, reduction will continue until Cr(III), the stable end-product, is formed (Malaviya and Singh, 2016). The overall process is shown in the equations below (eq. 17-18, Malaviya and Singh, 2016, & Thatoi et al, 2014).

Two-Step reduction of Cr(VI) in Aerobic Bioremediation

$$Cr^{6} + e^{-} \rightarrow Cr^{5+} \quad \text{(eq. 17)}$$

$$Cr^{5+} + 2e^{-} \rightarrow Cr^{3+} \quad \text{(eq. 18)}$$

Under anaerobic conditions, various substrates can be used as electron donors, these include NADH, carbohydrates, fatty acids and proteins (Fig 2.1). The anaerobic reduction of Cr(VI) can also be enzymatic, and/or can involve reduction in membranous cytochromes. Most commonly, it involves the use of membranous cytochromes in an electron transport chain, in which chromate is deposited on the cell surface and reduced in the final step (Thatoi et al, 2014). Unlike aerobic metabolism, Cr(VI) as chromate is more commonly reduced in one step to Cr(III) as the final electron acceptor under anaerobic conditions (Thatoi et al, 2014). Bacterial strains in *Pseudomonas* and *Enterobacter* found in industrial wastewater commonly employ this pathway. Though a variety of bacteria can reduce Cr(VI) into Cr(III) anaerobically, sulfate reducing bacteria are the most commonly used organisms in Cr(VI) reduction in wastewater treatment (Thatoi et al, 2014). Finally, the reduction of Cr (VI) can be catalyzed by glucose, which is followed by the precipitation of chromium hydroxide, as presented in the following equations (eq. 19-20, Thatoi et al, 2014).

Reduction of Chromate by Glucose

$$C_6H_{12}O_6 + 8CrO_4^{2-} + 14H_2O \rightarrow 8Cr(OH)_3 + 10OH^{-} + 6HCO^{-} \quad \text{(eq. 19)}$$

Reduction of Dichromate by Glucose

$$C_6H_{12}O_6 + Cr_2O_7^{2-} + H_2O \rightarrow 2Cr(OH)_3 + 2OH^{-} + 6HCO^{-} \quad \text{(eq. 20)}$$

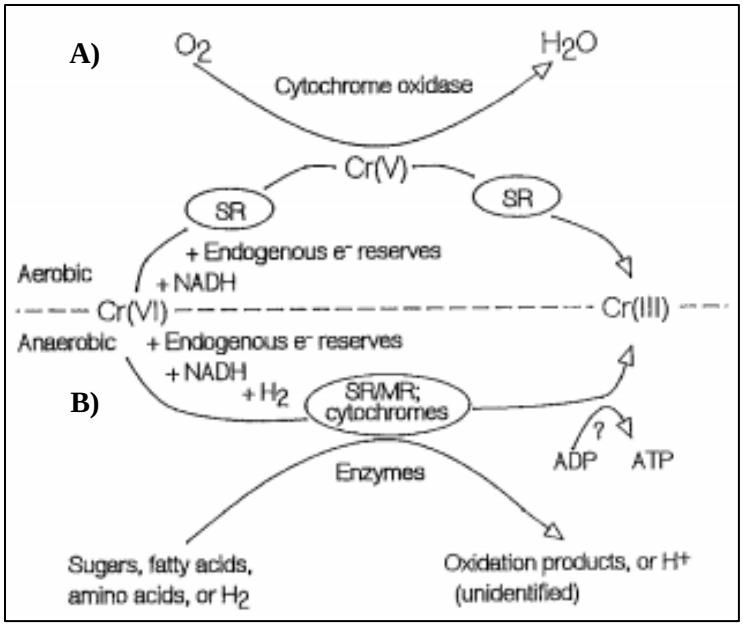


Figure 2.1: Aerobic and Anaerobic Bacterial Degradation of Hexavalent Chromium
A) Aerobic Cr(VI) metabolism showing chromium reduction by soluble reductases.
B) Anaerobic metabolism shows reduction membrane-bound cytochrome complexes. Figure credit: Wang, Y. T., & Shen, H. (1995). Bacterial reduction of hexavalent chromium. *Journal of Industrial Microbiology*, *14*(2), 160.

The biological metabolism of CF is less understood, requiring specialized bacteria with less common metabolic pathways. However, several viable bacterial strains in *Pseudomonas*, *Dehalobacter*, *N. europea* and *Rhodoccocus* (Cappelletti et al, 2012 & Grostern et al, 2010), have been demonstrated to reduce CF. CF can be degraded under aerobic and anaerobic conditions (Cappelletti et al, 2012) using readily available microbial consortia (Lu and Li, 2010). In both cases, degradation of CF is primarily cometabolic, in which CF can only be used as a non-growth substrate once a bacterial population has achieved growth using a different substrate/energy source (Cappelletti et al, 2012).

Under aerobic conditions, CF biodegradation is cometabolic (Cappelletti et al, 2012) CF is oxidized by monooxygenases (MO's). Cometabolism arises due to the unspecific nature of the substrate binding site of MO's, which can oxidize CF in addition to their targeted growth substrate (Fig 2.2). Genes that can encode for these enzymes have also identified, which include

butane MO gene clusters *bmoXYBZDC,* and *prmABCD,* and ammonia MO operons *amoC, amoA* and *amoB* (Cappelletti et al, 2012). Though other chlorinated aliphatics such as DCM and CM can be used exclusively as a growth substrate, a limited amount of bacterial strains can use CF as a main energy source (Cappelletti et al, 2012). Cappelletti also states chlorinated aliphatics and aromatic compounds are the main cometabolic growth substrates used in aerobic CF degradation, though denitrifying bacterial MO's have also shown the ability to oxidize CF. Out of these growth substrates, organisms that use methane as their main growth substrate form the largest group of aerobic CF oxidizers (Cappelletti et al, 2012). In pathways involving methane, the oxidation of methane into methanol is the catalyzing step, which is followed by oxidation into formaldehyde, formic acid and carbon dioxide (Cappelletti et al, 2012). Though the cometabolic substrates are varied, most aerobic pathways involving degradation of CF will result in the oxidation of a carbon-based growth substrate to carbon dioxide (Fig. 2.2). Aerobically, inhibition of CF oxidation due to CF concentration is possible, but toxicity inhibition in CF metabolism is more dependent on intermediate CF byproducts and growth substrate concentration (Cappelletti et al, 2012).

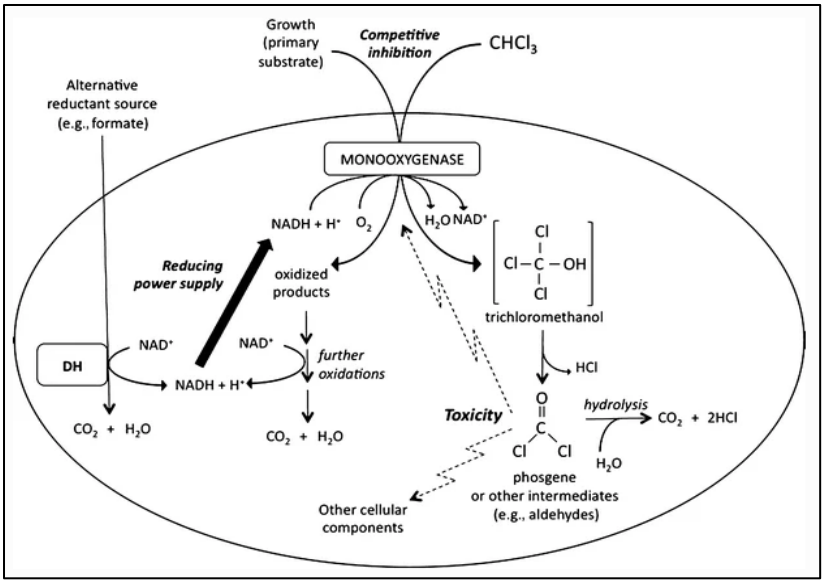


Figure 2.2: Aerobic Bacterial Degradation of Chloroform
Microbial degradation of CF under aerobic conditions. Both the growth substrate and CF are shown will compete for the MO binding. Figure credit: Cappelletti, M., Frascari, D., Zannoni, D., & Fedi, S. (2012). Microbial degradation of chloroform. *Applied microbiology and biotechnology*, *96*(6), 1397

Anaerobic degradation of CF is also cometabolic (Cappelletti et al, 2012). Cappelletti identified 3 pathways for CF biodegradation, dehalorespiration, reductive dechlorination, and hydrolysis (Fig. 2.3). Dehalorespiration involves reduction of CF as the final electron acceptor, which results in the accumulation of DCM and is catalyzed by hydrogen gas the electron donor (Cappelletti et al, 2012). In reductive dechlorination, CF is also an electron acceptor, but further reduction into methane is possible (Cappelletti et al, 2012). However, reduction of DCM into CM is not prevalent, resulting in accumulation of DCM as well. Hydrolysis will displace chloride ions with oxygen molecules, fully oxidizing CF into carbon dioxide (Cappelletti et al, 2012). This can be done by direct hydrolysis of a CF molecule or following dechlorination of CF into intermediate organic byproducts such as formaldehyde and formic acid (Cappelletti et al, 2012). Since pathways for anaerobic CF degradation are more varied, it can be performed by a variety of organisms including *Methanosarcina*, *Clostridium* and *Acetobacterium*. Increased metabolic diversity also means a greater variety of growth substrates such as fatty acids,

carbohydrates, aliphatics, and alcohols can be used in anaerobic CF degradation (Cappelletti et al, 2012). However, Cappelletti reports methanogenic bacteria are the most prevalent group of organisms known to anaerobically degrade CF. Within methanogenic bacteria, oxidation through hydrolysis is the preferred pathway of CF removal and is usually catalyzed by fatty acids and vitamin B12 (Cappelletti et al, 2012 & Shan et al 2010). Anaerobic CF degradation is more susceptible to toxicity by CF alone (Cappelletti et al, 2012), but bacterial cultures using both dehalorespiration, (Nijenhuis et al, 2016) and hydrolysis (Shan et al, 2010) have shown success in removing high levels of CF in in-situ and bench scale applications.

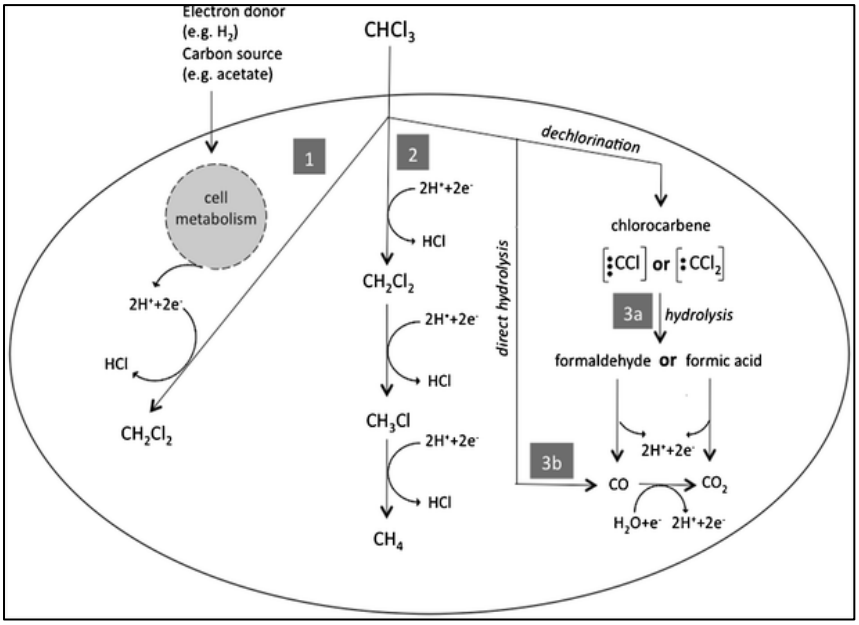


Figure 2.3: Anaerobic Bacterial Degradation of Chloroform
Three Pathways for anaerobic degradation of chloroform
1) dehalorespiration,
2) reductive dechlorination,
3a) direct hydrolysis
3b) hydrolysis of chlorocarbenes
Figure credit: Cappelletti, M., Frascari, D., Zannoni, D., & Fedi, S. (2012). Microbial degradation of chloroform. *Applied microbiology and biotechnology*, *96*(6), 1404

2.3.3 Remediation of Contaminants Using Bio-enhanced ZVI

Limited research exists on remediation using ZVI augmented by bacterial inoculation (bio-enhancement). While this technology has not been implemented at a commercial level, analyses at the experimental level show considerable success in nitrate (Liu et al, 2013), perchlorate (Miller and Logan, 2000 & Son et al, 2006), chemical oxygen demand (COD) as a measure of organic contaminants (Zhang et al, 2011), and CF (Lee et al, 2015) degradation. More specifically, production of hydrogen gas during ZVI oxidation has been proven to enhance the growth of methanogens (Xu et al, 2017), the primary bacterial group associated with CF degradation (Cappelletti et al, 2012 & Xu et al, 2017). In remediation systems using bio-enhanced ZVI, many factors can affect remediation performance. As in abiotic conditions, higher ZVI surface area, dose, and contact time can achieve more hydrogen gas production, but reactions with ambient contaminants can cause precipitation, which will encapsulate bacteria, diminishing microbial activity (You et al, 2017). Bacterial inhibition by ZVI is a primary concern in bio-enhanced reactors (Xu et al, 2017 & You et al, 2017). Previous studies have shown 2-log inactivation of *E.coli*, a prominent chromium reducer, with relatively low NZVI doses of 0.1 g/L (Li et al, 2010 & Auffan et al, 2008). Inactivation by NZVI also extends to a variety of bacteria, showing more acute effects in anaerobic microbial populations (Diao et al, 2009 & Velimirovic et al, 2015). NZVI cytotoxicity is mainly due to oxidative stress, but cell surface agglomeration and inhibition due to pH increase can also cause bacterial inhibition (Lei et al, 2016). However, studies have shown NZVI toxicity is dependent on particle size, purity and oxidative state; analyses have shown significant reduction in NZVI toxicity at particle diameters greater than 100 nm (Lei et al, 2016) and 90% reduction of inactivation when used in the presence of natural organic matter (NOM) and other ambient contaminants (Li et al, 2010).

Furthermore, the introduction of ZVI into natural aquatic environments has a noticeable but nontoxic effect on water chemistry such as increase in oxidation-reduction potential and decreased dissolved oxygen (Barnes et al, 2010). However, these changes did not adversely affect the naturally occurring biota of the environment.

2.4 Contamination, Health Effects and Regulation of Co-Contaminants

Though this study focuses on Cr(VI) and CF removal, groundwater from this study showed a prevalence of other contaminants common in industrial wastewater. These contaminants include nitrate, chlorate, and perchlorate (co-contaminants), which were found at extremely high concentrations. As a result, complications from exposure from these contaminants are likely, and must also be considered in remediation. Concentrations of more than 10 mg/L (as nitrogen, 44 mg/L as nitrate) nitrate can cause methemoglobinemia (Fewtrell, 2004). Chlorate concentrations as low as 200 µg/L can cause congenital defects (Righi et al, 2012). High levels of perchlorate can cause hormonal imbalances (Srinivasan et al, 2009). The U.S. EPA has set an MCL for nitrate 10 mg/L (as nitrogen, 44 mg/L as nitrate) (U.S. EPA. 2004). Though no MCL has been established for chlorate and perchlorate, the EPA sets recommended MCL goals of 210 µg/L and 56 µg/L, respectively (Greenhalgh, 2019, & U.S. EPA, 2019). Like Cr(VI) and CF, remediation of nitrate, chlorate, and perchlorate also involves ion exchange, adsorption, and bioremediation (Greenhalgh, 2019; Liu et al, 2013; Miller and Logan, 2000; Nozawa-Inoue et al, 2005; Srinivasan et al, 2009; Van Ginkel et al, 1995, & Xu et al, 2004). ZVI has also been effective at removing nitrate (Westerhoff, 2003), chlorate (Zarei and Ghavi, 2016), and shown limited removal of perchlorate (Petrucci et al, 2016).

Chapter 3: Methodology

Experiments for this study tested Cr(VI), CF, CF, nitrate, chlorate, and perchlorate, removal using microcosm batch reactors under abiotic, biotic, bio-enhanced conditions. Additionally, the potential differences between in-situ and ex-situ abiotic, biotic, and bio-enhanced removal were tested through the addition/exclusion of soil from the contaminated site. The experiments used contaminated groundwater and soil from a former industrial perchlorate manufacturing facility. The objectives of the experiments and contaminants monitored for each phase are shown on Table 3.1. To examine the efficacy of abiotic, biotic, and bio-enhanced removal of Cr(VI), CF, and co-contaminants, this study was conducted in 3 phases. Abiotic treatments were those in which NZVI was used with no bacterial component, except for bacteria that may naturally occur in the groundwater and soil. Biotic treatments consisted of the addition bacterial sludge, along with enrichment with bacterial nutrients in the absence of NZVI. Bio-enhanced treatments used NZVI supplemented with a bacterial seed and nutrients. For this research, the bacterial seed was taken from on-site fluidized bed reactors (FBR) that currently treat the groundwater biologically, using ethanol as a carbon substrate.

Table 3.1: Summary of Batch Reactor Tests Performed to Investigate Abiotic, Biotic, and Bio-enhanced Removal of Chromium(VI). Chloroform, Nitrate, Chlorate, and Perchlorate

Experiment	Contaminants Tested	Objective	No. Batch Reactors
Phase 1: Impact of NZVI on hexavalent chromium removal under biotic, abiotic, and bio-enhanced conditions.	Hexavalent Chromium	Testing efficacy of abiotic, biotic, and bio-enhanced hexavalent chromium reduction using NZVI in the presence and absence of soil.	28
Phase 2: Impact of NZVI on chloroform removal under biotic, abiotic, and bio-enhanced conditions in the presence co-contaminants	Chloroform, Nitrate Chlorate & Perchlorate	Testing efficacy abiotic, biotic, and bio-enhanced chloroform removal and removal of nitrate, chlorate, and perchlorate in the presence and absence of soil.	20
Phase 3: Effects of increasing NZVI dose under bio-enhanced conditions for the removal of chloroform in the presence of co-contaminants	Chloroform, Nitrate Chlorate & Perchlorate	Testing the effects of increasing NZVI on contaminant removal in bio-enhanced reactors in the Presence of Soil.	30

3.1 Phase 1: Impact of NZVI on Hexavalent Chromium Reduction under Abiotic, Biotic, and Bio-enhanced conditions.

Phase 1 tested reduction of Cr(VI) under abiotic, biotic, and bio-enhanced conditions. The presence of soil was also varied within treatments. This was performed to identify potential differences between in-situ and ex-situ remediation, as encapsulation by accumulating sediment decreases reactivity of abiotic in-situ reduction using NZVI (Thiruvenkatachari et al, 2008), and treatment results using NZVI can vary when applied in-situ or ex-situ (Stevenson and Herrera, 2018). Abiotic, biotic, and bio-enhanced reactors were tested periodically for Cr(VI). Microcosm batch reactors consisted of borosilicate glass bottles containing a 100ml mixture of diluted groundwater, NZVI, soil, bacterial sludge, sodium bicarbonate and bacterial nutrients. To mimic the low oxygen conditions, all reactors were sealed with a butyl rubber stopper and aluminum rings. Reactors were incubated in a rotational shaker at room temperature at 25 rpm for up to 3 weeks. Cr(VI) was monitored at predetermined times. Two replicates were taken at measurement

times. Samples were collected using syringes via the butyl rubber septum to limit the introduction of oxygen into each microcosm. The abiotic components used in this study consisted of NZVI, and contaminated groundwater. The biotic component used in this study consisted of bacterial sludge. A molasses solution was added in biotic reactors as a carbon source. Reactors containing the molasses solution were buffered with sodium bicarbonate to maintain a neutral pH. Additionally, vitamin B12, and a urea and diammonium phosphate solution (UDAP) were used as nutrients to stimulate biodegradation (Cappelletti et al, 2012 & Appenzeller et al, 2001). Bio-enhanced microcosm batch reactors contained both the abiotic and biotic components. Table 3.2 depicts the components and doses used in this study.

3.2 Phase 2: Impact of NZVI on Chloroform Removal under Abiotic, Biotic, and Bio-enhanced Conditions in the Presence Co-Contaminants

In the second phase, the removal efficacy of abiotic, biotic, and bio-enhanced treatments for CF remediation was investigated. This was also subjected to investigation of potential in-situ and ex-situ differences through presence and absence of soil. Batch reactors prepared in the same manner as Phase 1 were sent to be tested offsite in a certified environmental testing laboratory located in Irvine, CA for CF, co-contaminants, and CF degradation byproducts. Testing lasted 4-8 weeks. This extended period of testing was due to the high amount and variety of contaminants in the sample groundwater, which was speculated to add a considerable delay to the time needed for remediation. Abiotic remediation using NZVI has been shown to cause reduction of nitrate (Zhang et al, 2010), chlorate (Westerhoff, 2003), and limited reduction of perchlorate (Petrucci et al, 2016). Additionally, denitrifying conditions can stimulate the removal of halogenated aliphatic compounds (Bouwer and McCarty, 1983). Environments in which denitrifying bacteria are common also harbor perchlorate and chlorate-reducing bacteria (Nozawa-Inoue et al, 2005 &

Xu et al, 2004). Consequently, nitrate, chlorate, and perchlorate were also monitored in this study. It has been shown that formaldehyde, formic acid, dichloromethane (DCM), and chloromethane (CM) are intermediate byproducts of anaerobic CF metabolism (Cappelletti, et al 2012). Therefore, these were also tested when measuring CF removal. Lactic acid was also monitored to test the presence of anaerobic metabolism using carbohydrates (Luedeking et al, 1959, & Reddy et al, 2008) present in molasses. Due to the limited volume of groundwater in the bottles, the amount of sample needed to perform the various analysis, and cost of analysis, replicate measurements could not be tested for all the contaminants.

3.3 Phase 3: Effects of Increasing NZVI dose under Bio-enhanced Conditions for the Removal of Chloroform in the presence of Co-Contaminants

The third phase evaluated the efficacy of increasing bio-enhanced NZVI doses on CF removal and co-contaminants in the presence of soil. Increasing NZVI doses were added to bio-enhanced batch reactors prepared as in Phase 1. These were monitored for the same contaminants as in Phase 2. Due to sample volume limitations and the high cost of offsite testing, sample analysis in Phase 3 was not as frequent as in other phases.

3.4 Experimental Components

All components stock solutions used in the microcosm batch reactors in this study and their doses are shown on Table 3.2.

Table 3.2: Batch Reactor Amendment Overview

Component	Stock solution	Dose in Microcosm	Purpose
Groundwater	4X Diluted (1 part GW, 3 parts Lake Mead Water), Collected at 75-115m depth	Equalize to 100ml	Source of Contaminants
NZVI	17% 25S NZVI, 78% Propylene Glycol, 5% Iron Oxide Stock solution	30-100 g/L	Abiotic Reduction of Contaminants
Bacterial Sludge	Collected from Fluidized Bed Reactor	1ml/100ml	Biotic Removal of Contaminants
Soil	Mixed from soil from borehole at 75-115m depth	15g/100ml	Simulation of Ambient Conditions and possible Biotic Removal of Contaminants
Blackstrapp Molasses (Unsulfured, Golden Barrel, North Georgia Still Co.)	400 ml/L DI Stock Solution	5ml/100ml	Carbon Source
Vitamin B12	0.48 g/L Stock Solution	1ml/100ml	Bacterial Nutrients
39% Urea/DAP Blend	39% UDAP/L DI Stock solution U/DAP Containing: 0.43 kg Urea 0.22 kg DAP In one liter DI	1ml	Bacterial Nutrients
Sodium Bicarbonate	0.55 M Stock Solution	3ml/100ml	Buffer for Initial Neutral pH

3.4.1 Nano Zero-Valent-Iron

The NZVI used in this study was a 25S NZVI solution provided by NanoIron Future Technology in Židlochovice, Czech Republic. The NZVI solution had an approximate density of 1.2 g/ml and surface area of 25 m^2/g. The solution consisted of 17% NZVI (as Fe^0), 78% propylene glycol, and 5% iron oxide by weight. NZVI particles were sized using 632.8 nm absorbance using a ZSU5800 Malvern Zetasizer, for an average particle diameter of 420-894nm. Detailed specifications for the NZVI used in this study are provided in APPENDIX A.

3.4.2 Groundwater

The groundwater in this experiment was collected from a well at a depth of 75-115 meters and was diluted by a factor of 4 using Lake Mead water. As groundwater contaminants and solutes were high, dilution was necessary to mitigate the toxicity towards bacteria, (Park and Marchland, 2002 & Thatoi et al, 2014) and to help reduce TDS interference with biodegradation and ion chromatography analysis (Pfaff, 1993). Additionally, in actual applications, well injection of both iron particles (Zhang, 2003), and microbial organisms and nutrients (Anderson et al, 1997) will inevitably result in diluted groundwater conditions, which further promote the use of diluted groundwater in this study. Additionally, dilution is useful to attenuate used bacteria for groundwater bioremediation (Küster et al, 2004). To ensure the exclusion of bacteria in groundwater, the groundwater was filtered through a 0.22 μm filter prior to batch reactor preparation. Filtration did not affect initial contaminant levels. Additional measured parameters for the groundwater used in study are shown in APPENDIX B.

Table 3.3: Experimental Components Present in Diluted Groundwater

Groundwater (4X Diluted: 1 volume of groundwater and 3 volumes of dilution water) Component Concentration	**Unit**	**Value**
TDS	mg/L	5,286
COD	mg/L	1.25
Phosphate	mg/L	3.06E-4
Cr(VI)	μg/L	22.5
CF	mg/L	1125
Nitrate	mg/L	88.5
Chlorate	mg/L	6825
Perchlorate	mg/L	910
pH	6.8	

3.4.3 Soil

Doses of 15 g used in the microcosm batch reactors consisted of mixed soil collected from boreholes drilled in the contaminated site at a 75-115 meter depth from the same well as the groundwater. Contaminants measured in the soil were measured using extraction, precipitation, and decantation of 15 g of soil in 0.3 L distilled (DI) water. Additional measured parameters for the soil used in this study are shown in APPENDIX B.

Table 3.4: Experimental Components Present in Soil

Soil Component Concentration	Unit	Value
TDS	mg/g	14.43
COD	mg/g	0.14
Phosphate	mg/g	4.50E-5
Cr(VI)	mg/g	0.04
CF	µg/g	0.01
Nitrate	mg/g	0.13
Chlorate	mg/g	11.6
Perchlorate	mg/g	1.33

3.4.4 Bacterial Sludge

The biotic components in this study consisted of a 1ml bacterial sludge inoculation into batch reactors. Due to availability, a different batch of bacterial sludge was used during Phase 3. Due to this, the phosphate, nitrate, and the COD were measured in both batches of bacterial sludge prior to inoculation as a measure of bacterial growth-promoting conditions (Appenzeller et al, 2001). Both batches of bacterial sludge were collected on-site from the same fluidized bed reactor (FBR).

Table 3.5: Experimental Components Present in Seed Bacterial Sludge

Phase 1-2 Bacterial Sludge	Unit	Value
Phosphate	mg/L	55
COD	mg/L	33500

Phase 3 Bacterial Sludge	Unit	Value
Phosphate	mg/L	243
COD	mg/L	63000

3.4.5 Bacterial Nutrients

Enrichment with bacterial nutrients in this study consisted of an inoculation of 5 ml of a blackstrap molasses solution (400 ml/L DI stock solution), 1 ml of a 39% Urea/DAP blend (0.43 kg Urea and 0.22 kg DAP in one liter DI) and 1ml of a cobalamin (Vitamin B12) solution (0.48 g Vitamin B12/L DI stock solution). Blackstrapp molasses procured from the Golden Barrel, North Georgia Still Co was used as a carbon source for bacterial reduction, as anaerobic Cr(VI) and CF removal usually requires a carbon substrate (Cappelletti et al, 2012; Thatoi et al, 2014, & Wang and Shen, 1995). The chemical composition of the blackstrap molasses solution can be found in APPENDIX B. The addition of vitamin B12 was for the stimulation of bacterial CF reduction (Becker and Freedman, 1994 & Cappelletti et al, 2012). The addition of U/DAP was to provide a nitrogen and phosphate source to promote denitrifying bacterial growth in a contaminated environment (Appenzeller et al, 2001). Buffering with 3 ml of a 0.55M sodium bicarbonate solution was used to maintain a neutral pH, as the addition of the molasses solution decreased pH, and a neutral pH is optimal for bacterial chromium reduction (Wang and Shen, 1995).

3.5 Analyses

Analytical testing for Cr(VI), CF, nitrate, chlorate, perchlorate, and byproducts of CF metabolism was performed in this study. Testing for CF, nitrate, chlorate, perchlorate, and byproducts of CF metabolism were sent to be tested off-site to a certified environmental laboratory (TestAmerica Labs) in Irvine, California. Due to the high cost of off-site testing,

contaminant testing in Phase 3 was not as frequent as in Phase 2. The analytical procedures used for each contaminant are listed on Table 3.6.

Table 3.6: Analytical Procedures, Detection Limits and Equipment Used in the Analyses of the Components of Interest.

Parameter	Method	Limits	Equipment
Cr(VI)	Hach 8023	0.01-0.60 mg/L	Colorimeter DR5000
CF	EPA 8260B	0.5-10 µg/L 35-270 m/z	GC/MS System
Chloromethane		0.5-10 µg/L 35-270 m/z	
Dichloromethane		0.5-10 µg/L 35-270 m/z	
Nitrate	EPA 300.0	1.86-62 mg/L	Ion Chromatograph DIONEX (ICS-2000)
Chlorate	EPA 300.1	1.31-500 µg/L	
Perchlorate	EPA 314.0	0.53-2 µg/L	
Chemical Oxygen Demand	HACH 8000	20-1500 mg/L (HR)	Spectrophotometer DR 5000
Formaldehyde	EPA 8315A	0.39-2.45 mg/L	High Performance Liquid Chromatograph
Lactic, Formic, Acetic Acid	Proprietary	Proprietary	Ion Chromatograph DIONEX (ICS-2000)

3.5.1 Chromium

The Cr(VI) concentration was tested using HACH method 8023 (Hach Company), in which 0.1 g of 1, 5-diphenylcarbohydrazide (ChromaVer 3 Chromium Reagent Powder, Hach Co) was added to collected samples. The 543 nm absorbance of each sample was then measured on a Hach DR 900 colorimeter. Due to the fine grain nature of NZVI, each sample was clarified prior to testing through centrifugation at 3500 rpm for 1 hour. Finally, all samples were filtered through a 0.22 µm membrane filter prior to analysis.

3.5.2 Chloroform

The concentration of CF was tested off-site using EPA Test Method 8260B (Techniquea, 1996): Volatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS). The presence of chloromethane (CM) and dichloromethane (DCM), byproducts of abiotic and biotic CF removal (Cappelletti 2012 et al, & Lee et al, 2015), was also recorded using this method.

Purge and trapping of CF was performed in a GC with a 60-meter x 0.75 mm VOCOL capillary column and a retention time of 9 minutes. The mass spectrometry detection was performed at 83 m/z for CF, 50 m/z for CM, and 83 & 127 m/z for DCM. Quantification was measured by evaluating the response of major ions relative to calibration standards (Techniquea, 1996). Samples were preserved using 0.2 ml of HCL.

3.5.3 Nitrate, Chlorate, and Perchlorate

Nitrate and chlorate were tested off-site using EPA method 300.0 Anions and 300.1 Disinfection By-Products with Ion Chromatography (Pfaff, 1993). Testing perchlorate used EPA method 314.0 LL Perchlorate (IC). These methods subject an aqueous sample to ion chromatography (IC) using a DIONEX (ICS-2000). As with chromium testing, each sample was subjected to the clarification and filtering process as when measuring Cr(VI).

3.5.4 Bacterial Sludge Chemical Analysis

The nutrient analysis for the groundwater, bacterial sludge, and soil involved colorimetry for chemical oxygen demand (COD) and phosphate. COD was measured using HACH method 8000 (Hach Company), heat digestion and reaction with potassium dichromate and was used as a surrogate method to determine the organic composition of sludge. The phosphate concentration was measured using HACH method 10210 (Hach Company), heat digestion and reaction with ammonium molybdate and antimony potassium tartrate. Absorbance for these contaminants was measured using a HACH DR 5000 spectrophotometer (Hach Company). The COD measurement required 880 nm absorbance. Absorbance for phosphate was measured at 543 nm.

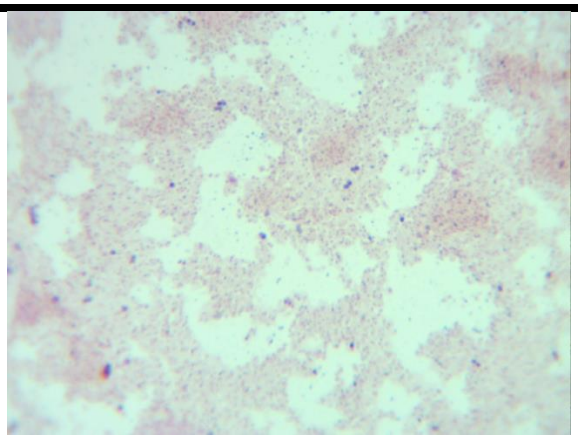
3.5.5 Anaerobic Byproduct Analysis

Formaldehyde was measured using EPA Method 8315A (U.S. EPA, 1996), high performance liquid chromatography (HPLC) with a 250 mm x 4.6 mm column. A 5 µm particle

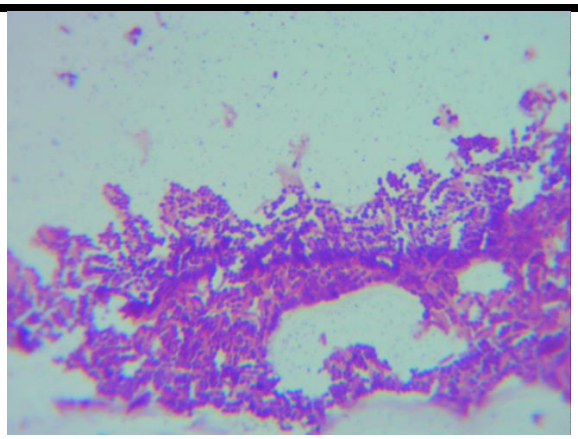
size and a retention time of 5.3 minutes was also used. Ultraviolet absorption at 360 nm was used for formaldehyde determination. A proprietary method using ion chromatography was used for the determination of lactic, acetic and formic acid.

3.6 Bacterial Microscopy

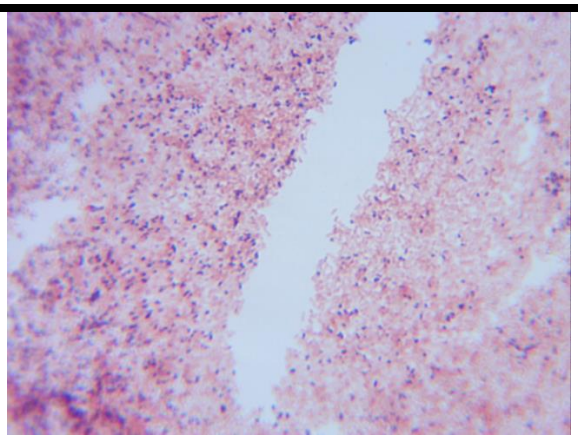
Bacterial samples taken from bacterial sludge, biotic reactors, and soil were grown aerobically and anaerobically on tryptic soy agar (TSA) plates. One ml bacterial samples were taken from the sludge, soil, and microcosms. The bacterial soil samples were taken from a 1g soil and 10ml 1:9 mixture of 4-1,1,3,3-phenyl-polyethylene glycol (Triton X-100) and DI water after 1 hour of incubation. These samples were inoculated onto the TSA plates and incubated at 33C^{o} for 5 days. Samples were grown in both aerobic and anaerobic conditions. Gram staining was then performed on each microbial sample. Wet mounts of stained samples were viewed under a compound microscope. This was to discern any morphological differences in the bacterial populations in the bacterial sludge, soil, and microcosms.



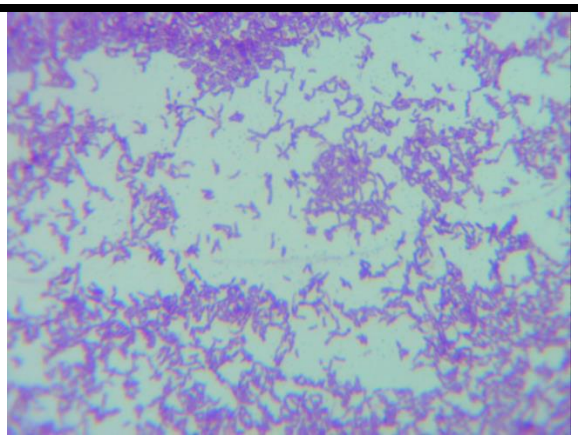
A) Biotic Reactor Containing Soil Grown Aerobically, 1000X Magnification



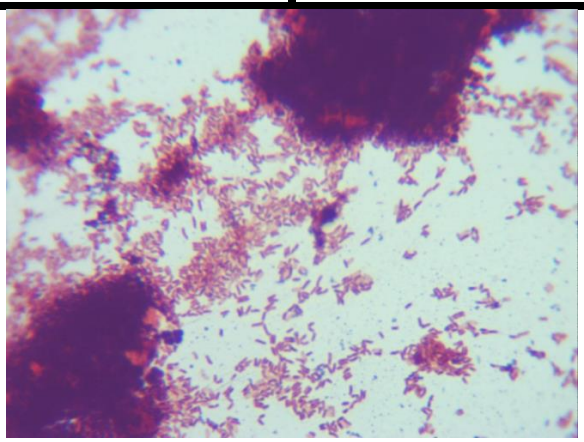
B) Biotic Reactor Containing Soil Grown Anaerobically, 1000X Magnification



C) 1ml Sludge Sample Grown Aerobically, 1000X Magnification



D) 1ml Sludge Sample Grown Anaerobically, 1000X Magnification



E) 1ml Soil Sample Grown Aerobically, 1000X Magnification

Fig 3.1: Bacterial Survey of Soil, Bacterial Sludge and Biotic Reactors

In aerobic conditions, both in the sludge and biotic reactors, bacterial populations showed a prevalence for gram-negative bacteria (Fig. 3.1 A & C), with a minor presence of gram-positive bacteria. Aerobically, the bacterial population of biotic reactors (Fig 3.1 A) morphologically resembles the bacterial population of the sludge (Fig. 3.1 C), though a higher presence of gram-positive was found in the sludge. When culturing soil under aerobic conditions (Fig. 3.1 E), the bacterial morphology still resembled the morphology found in both the bacterial sludge and biotic reactors. Anaerobic growth caused a considerable shift towards gram-positive dominance (Fig. 3.1 B & D). When comparing anaerobic bacterial populations, reactor bacterial populations show more gram-positive cocci (Fig 3.1 B), while FBR sludge bacteria are dominated by gram-positive rods (Fig 3.1 D). The shift in bacterial morphology in the absence of oxygen may indicate treating groundwater with bacterial sludge under aerobic conditions may rely on different bacterial populations. After three different attempts, soil bacteria were not able to be grown in TSA media under anaerobic conditions. This could mean the bacterial population in soil requires more specific conditions. Overall, these results are speculative, as the media used in this study may not contain the nutritional requirements for all bacteria in the bacterial sludge and soil. Molecular detection tools, particularly for organic-reducing bacteria in contaminated groundwater using DNA heat extraction and 16s rRNA sequencing of bacterial primers f27, f518, r800, and r1492 is recommended to better understand the bacterial populations of these environments (Santos et al, 2017). Additionally, a novel method using mass spectra of known proteins using a matrix-assisted laser desorption/ionization and time-of-flight analysis (MALDI-TOF MS) system has been proposed for identifying unknown groundwater bacteria (Santos et al, 2017).

3.7 NZVI Stoichiometric Calculations

Previous studies have shown complete Cr(VI) reduction by NZVI (as Fe^0) at mass ratios from 2.5-50 mg Fe^0/mg (Selvarani et al, 2012 & Xu et al, 2014). For CF, much higher mass ratios of 1,135 mg Fe^0/mg have shown to be sufficient for complete reduction (Lee et al, 2015). NZVI:contaminant mass ratios in this study were calculated based on the stoichiometric molar ratios shown in equations 6-9, and 13-16 on Section 2.3.1 and demonstrated in Table 3.7. Assuming complete reduction of CF into methane by NZVI, the mass ratios are shown on Table 3.7. The computation of these mass ratios for specific experiments is detailed in APPENDIX C. As seen in Table 3.7, the amount of NZVI needed to reduce chlorate is much higher when compared to the amounts required by the other examined contaminants, at 245.21 mmol/L. CF and Cr require the least amount of iron, at 0.03 an 0.65 mmol/L, respectively.

Table 3.7: Calculated Mass Ratios for Reduction of Contaminants by NZVI

Contaminant	Molar Mass	Molar Ratio (Section 2.3.1)	Groundwater (4X) Contaminant Concentration		Molar Ratio Fe^0: Contaminant		Mass Ratio Fe^0: Contaminant
	g/mol	Fe^0: Contaminant	mg/L	mmol/L	mmol Fe^0/L	mmol Fe^0/mmol	mg Fe^0 /mg
Cr(VI)	52.0	1.5	22.5	0.43	0.65	1.5	1.63
Nitrate	62.0	4.0	88.50	1.36	5.42	3.8	3.43
Chlorate	83.5	3.0	6,825.00	81.74	245.21	3.0	2.02
Perchlorate	99.5	4.0	910.00	9.15	37.11	4.1	2.31
CF	119.5	3.0	1.24	0.01	0.03	3.2	1.59

3.8 Data Analysis

The average removal rates of all experiments were computed for each treatment. In addition, reaction rate constants for zero, first, and second order kinetics were calculated by establishing linear correlation between time and contaminant concentration, using the reaction

rate equation relationships shown in eq.21-23. When performing linear regression to determine rate constants, the intercept was not assumed to be zero. The linear forms of rate equations used in this study are shown below (eq. 21-23), where C is concentration at time t, C_0 is the initial concentration, and k is the reaction rate constant.

Linear form of 0 Order Rate Kinetics

$$C_0 - C = -kt \quad \text{(eq. 21)}$$

Linear form of 1st Order Rate Kinetics

$$\ln\left(\frac{C}{C_0}\right) = kt \quad \text{(eq. 22)}$$

Linear form of 2nd Order Rate Kinetics

$$\left(\frac{1}{C^2} - \frac{1}{C_0^2}\right) = -kt \quad \text{(eq. 23)}$$

Statistical significance testing was performed to determine whether there was a significant difference in the removal of the contaminants using different treatments. Two factor ANOVA testing between treatments was performed in Excel software. Due to the low number of replicates, testing for significance based on time was not considered. Assuming a normal distribution, critical values for a 95% confidence interval were considered significant.

Chapter 4: Results and Discussion

To understand the results of Chapter 4, it is important to note that the NZVI doses added were used to reduce all contaminants present in each reactor. Therefore, the mass ratios reported in this study reflect the amount added rather than the actual NZVI amount that was used to reduce individual contaminants. Although stoichiometric mass ratios of NZVI to contaminants were computed using theoretical reduction reactions, it is not possible from the experiments performed for this research to determine NZVI consumption for individual contaminants. Based on the mass ratios calculated in this study, the total NZVI needed to reduce all contaminants in the groundwater from this study is presented on Table 4.1. Depending on reactor amendments, the total NZVI dose needed to reduce all the tested contaminants also changed and ranged from 16,138.10-20,168 mg Fe^0/L (Table 4.1). The calculation process is presented in APPENDIX C.

Table 4.1: Total NZVI Needed based on Mass Ratio

Contaminant	**Molecular Weight**	**Molar Ratio**	**Groundwater (4X) Contaminant Concentration**		**Mass Ratio Fe^0: Contaminant**	**NZVI Needed for Contaminant**
	g/mol	**Fe^0: Contaminant**	**mg /L**	**mmol /L**	**mg Fe^0 /mg**	**mg Fe^0 /L**
Cr(VI)	52	1.5	22.5	0.43	1.63	36.35
Nitrate	62	4.0	88.5	1.36	3.43	319.74
Chlorate	83.5	3.0	6,825.0	81.74	2.02	13,731.74
Perchlorate	99.5	4.0	910.0	9.15	2.31	2,048.64
CF	119.5	3	1.13	0.01	1.59	1.59
Total						**16,138.21 *20,168.16 **18,393.15**

*Total NZVI needed for groundwater mixed with soil.
**Total NZVI needed for groundwater mixed with soil with molasses and nutrient amendment.

4.1 Chromium Removal

Reactors containing 500 mg Fe^0/L of NZVI, at mass ratios of 17.54-22.22 mg Fe^0/mg Cr(VI), achieved complete Cr(VI) reduction in 4-5 days (Fig. 4.1.1), with 95% reduction in 24 hours regardless of soil presence. Even at 500 mg Fe^0/L, the stoichiometric mass ratios were high, at 10.86-13.71X greater than the theoretical stoichiometric dose required (Table 4.1.1). In the presence of soil, abiotic reactors with 5,000 mg Fe^0/L of NZVI, at a mass ratio of 175.43 mg Fe^0/mg resulted in complete reduction of Cr(VI) in only 4 hours, with an average reduction rate of 171.0 $mg/l * d$. At 5,000 mg Fe^0/L, the stoichiometric mass ratio was 108.6X (Table 4.1.1). Results were similar to previous research on industrial contaminated wastewater, where NZVI achieved 90% reduction within 4 hours with a higher mass ratio of 50 mg Fe^0/mg under ambient conditions (Li et al, 2008). Li's study also shows near-complete reduction in batch experiments with contaminated industrial wastewater at a mass ratio of 230 mg Fe^0/mg within 6 hours. This is consistent with results in this study, which show near complete reduction in 4 hours at a similar mass ratio of 175.43 mg Fe^0/mg (Fig. 4.1.1). Finally, Table 4.1.4 shows a statistically significant increase in Cr(VI) reduction when increasing NZVI from 500-5,000 mg Fe^0/L which is consistent with previous studies showing higher reduction levels with a greater NZVI dose (Li et al, 2008, Xu et al, 2014, & Wang et al, 2010).

In Fig. 4.1.1, bio-enhanced Cr(VI) reduction with 5,000 mg Fe^0/L of NZVI at a mass ratio of 192.12 mg Fe^0/mg showed similar average reduction to abiotic Cr(VI) reduction, at 150.9 $mg/l * d$. The stoichiometric mass ratio for bio-enhanced reactors was 118.90X in the presence of soil (Table 4.1.1). Table 4.1.4 shows no statistically significant difference in reduction between abiotic and bio-enhanced reactors with 5,000 mg Fe^0/L of NZVI. A previous study using sequential bio-enhancement after abiotic Cr(VI) reduction using NZVI shows a

lower average reduction rate to this study at $93.88 \mathrm{mg/L} * \mathrm{d}$ under ambient conditions (Ravikumar et al, 2018). This occurred even at a much higher mass ratio of 1,666.7 mg Fe^0/mg (Ravikumar et al, 2018). Additional research on bio-enhanced Cr(VI) reduction performed in this laboratory has shown a slightly lower average reduction rate of $117.60 \mathrm{mg/l} * \mathrm{d}$ for a similar mass ratio of 211.86 mg Fe^0/mg using macro-scale ZVI (Greenhalgh, 2019).

Previous studies have shown first order abiotic Cr(VI) reduction using NZVI, where a first order rate constant of k = -3.9 d^{-1} was observed for a mass ratio of 50 mg Fe^0/mg (Xu et al, 2014). At lower mass ratios of 2.5 mg Fe^0/mg, rate constants can increase to k = -82.08 d^{-1} (Selvarani et al, 2012). First order kinetics in reactors using bio-enhancement through sequential NZVI reduction and biodegradation of Cr(VI) show a rate constant of k = -3.6 d^{-1} (Ravikumar et al, 2018). Additionally, previous studies in this laboratory have shown first-order kinetics for Cr(VI) reduction with a rate constant of k = -26.4 d^{-1} in bio-enhanced reactors with a mass ratio of 211.8 mg Fe^0/mg using macro-scale ZVI (Greenhalgh, 2019). In this study, Fig. 4.1.2 shows an average to high correlation ($R^2 = 0.6 - 0.9$) for first order kinetics in abiotic reactors with NZVI doses of 500 mg Fe^0/L at mass ratios of 17.54-22.22 mg Fe^0/mg. Average rate constants at 500 mg Fe^0/L were k = -1.44 d^{-1} to -2.44 d^{-1}. At 5,000 mg Fe^0/L, with a mass ratio of 175.43 mg Fe^0/mg, high correlation for first order kinetics in abiotic and bio-enhanced reactors was found ($R^2 = 0.9$), with rate constants of k = -32.4 d^{-1} to -34.8 d^{-1} respectively. Overall, abiotic and bio-enhanced reduction of Cr(VI) showed the highest correlation for first order kinetics ($R^2 = 0.9$, Table 4.1.5). Additionally, the rate constants found at 5,000 mg Fe^0/L of NZVI resembled rate constants for macro-scale ZVI.

In Fig. 4.1.3, biotic reactors achieved complete Cr(VI) reduction in 6-7 days, with 80-90% reduction occurring within 3 days, showing an average reduction rate of $5.63 \mathrm{mg/L} * \mathrm{d}$.

The addition of soil to biotic reactors (Fig. 4.1.5) showed similar results, with an average reduction rate of 5.25 $mg/L * d$. Biotic reactors containing soil alone in Fig. 4.1.5 also achieved total Cr(VI) reduction without the addition of bacterial sludge, albeit at a slower rate of 2.63 $mg/L * d$, reaching completion at 10 days. Complete Cr(VI) reduction was still achieved even under undiluted groundwater conditions. However, biotic reduction in undiluted conditions was slower, reaching completion in 14 days. Undiluted biotic reactors containing soil alone also totally reduced Cr(VI), reaching completion in 21 days. The average reduction rates for sludge and soil where similar to their undiluted counterparts, at 5.03 $mg/L * d$ and 3.87 $mg/L * d$ respectively. This does not suggest a toxic effect of high Cr(VI) on the bacterial flora. This supports existing research on Cr(VI) toxicity, which only shows Cr(VI) toxicity at 400-600 mg/L (Molokwane et al, 2008). Additionally, Molokwane's study achieved complete anerobic reduction of Cr(VI) using 1ml inoculation of bacterial sludge at a similar initial Cr(VI) of 20 mg/L, albeit using a spiked bacterial medium. In this study, 1ml sludge inoculations also showed complete anaerobic reduction of Cr(VI). However, other studies have shown average Cr(VI) reduction rates of 17.2 $mg/l * d$ or higher by activated sludge (Stasinakis et al, 2003), which are substantially higher than the biotic reduction rates shown in this study.

High levels of Cr(VI) reduction were also seen in controls containing only molasses and bacterial nutrients, with 80-90% reduction within 3 days and an average rate of 5.63 $mg/l * d$. This is likely due to Cr(VI) reduction by phenolic hydroxides in molasses (Chen et al, 2015). No statistically significant difference was found between biotic reactors in the absence of soil, biotic reactors in the presence of soil, and controls only containing molasses and bacterial nutrients (Table 4.1.4). Because of this, it is unknown if reduction in biotic reactors was caused by microbial activity, or reduction by phenolic epoxides. In Figs 4.1.4 and 4.1.6, first order kinetics

in these reactors achieved high correlation ($R^2 = 0.9$), with rate constants of k = -1.03 d^{-1} to -1.13 d^{-1}. These are similar to Chen's study, which shows a rate constants of, k = -1.03 d^{-1} to -2.4 d^{-1} for Cr(VI) reduction using a molasses dose 1-4 ml/L at initial Cr(VI) concentrations of 25 mg/L at neutral pH and 20 C^o. A statistically significant decrease in reduction rates was found when comparing biotic reactors only containing soil to other biotic reactors. Furthermore, a statistically significant difference was found between biotic reactors only containing sludge and biotic reactors only containing soil under undiluted conditions, with soil being significantly slower at reducing Cr(VI). This suggests nutrient uptake in the soil might not be used for Cr(VI)reduction. Adsorption of molasses by the soil is also a possibility for the reduced reduction rate. However, contaminant concentrations in the soil were substantially high, limiting the possibility of adsorption. Overall, biotic reactors showed the highest correlation for first order kinetics (Table 4.1.5), but reactors under undiluted conditions showed better correlation at zero order kinetics ($R^2 = 0.9$). Finally, no reduction occurred in controls with no bacterial nutrients. While the addition molasses and nutrients will result in Cr(VI) reduction, it is unclear whether reduction in reactors with a microbial component will be biotic or abiotic.

Regarding Cr(VI) removal, the main findings of this study are:

- The addition of soil showed no statistically significant difference in abiotic Cr(VI) removal at 500 mg Fe^0/L, thus soil does not affect the performance of NZVI in Cr(VI) reduction at higher doses.
- The rate of Cr(VI) removal by NZVI in abiotic and bio-enhanced reactors is substantially faster than the rate of reduction of biotic reactors. Additionally, no statistically significant difference in Cr(VI) reduction between abiotic and bio-enhanced reactors was seen. Thus, bio-enhanced reduction is mostly abiotic and dependent on NZVI activity.

- Total biotic Cr(VI) reduction is possible, even under undiluted conditions.
- The lack of a statistically significant difference between biotic reactors and controls only containing molasses/nutrients cannot determine if reduction was due to bacterial activity or reaction with molasses. However, the absence of Cr(VI) reduction in controls without bacterial nutrients gives strong evidence for the need of a carbon source for Cr(VI) reduction in reactors lacking NZVI.

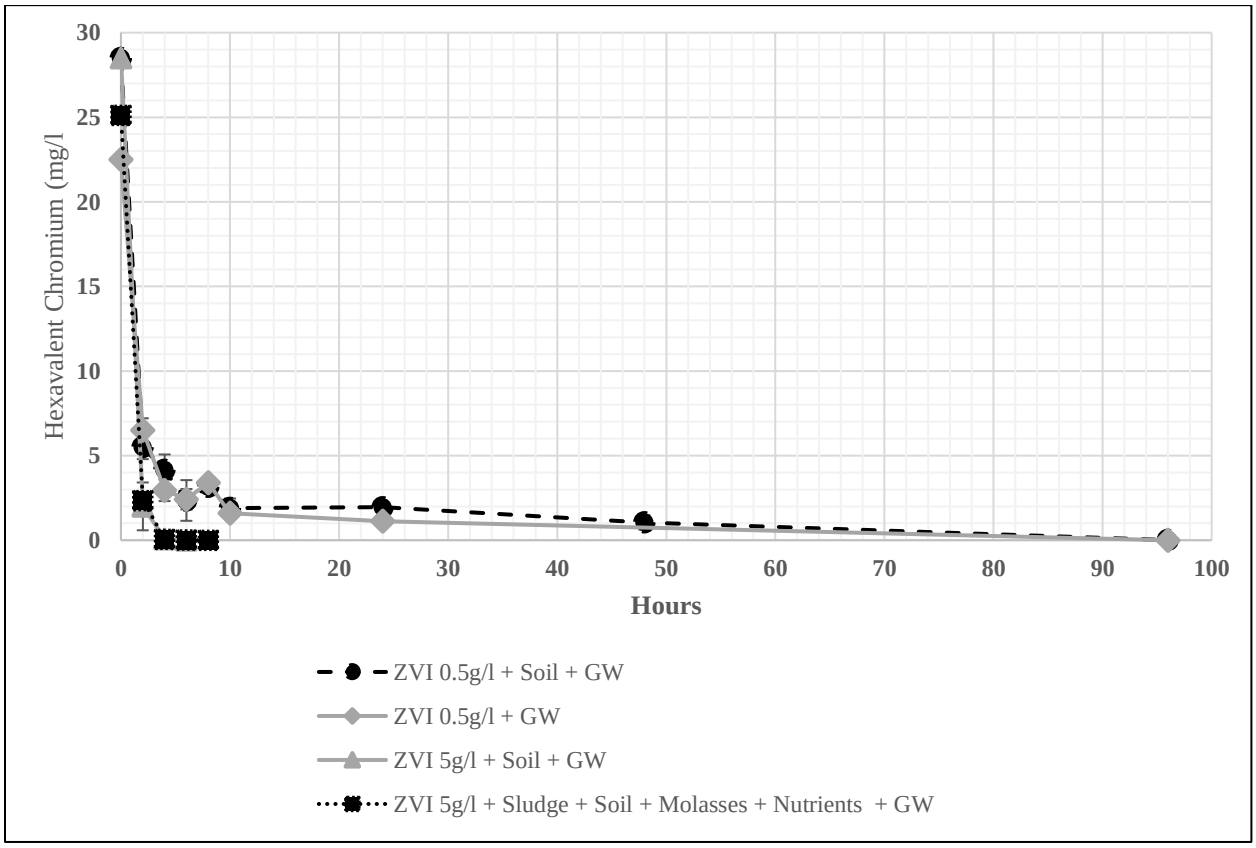


Figure 4.1.1: Abiotic and Bio-enhanced Chromium(VI) Reduction using NZVI in the Presence and Absence of Soil at Stoichiometric Mass Ratios of 13.71X (0.5 g/L), 10.86X (0.5 g/L + Soil), 108.60X (5 g/L + Soil), and 118.93X (5 g/L + Sludge + Soil + Molasses Nutrients)

Table 4.1.1: Chromium(VI) Reduction Rates for Abiotic and Bio-enhanced Reactors using NZVI in the Presence and Absence of Soil

Treatment	Average Rate (mg/l*d)	Cr(VI) Mass Ratio mg Fe^0/mg	Cr(VI) Stoichiometric Mass Ratio
ZVI 0.5 g/L + Soil + GW	1.11	17.54	10.86X
ZVI 0.5 g/L + GW	1.14	22.22	13.71X
ZVI 5 g/L + Soil + GW	171.0	175.43	108.60X
ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	150.9	192.21	118.93X

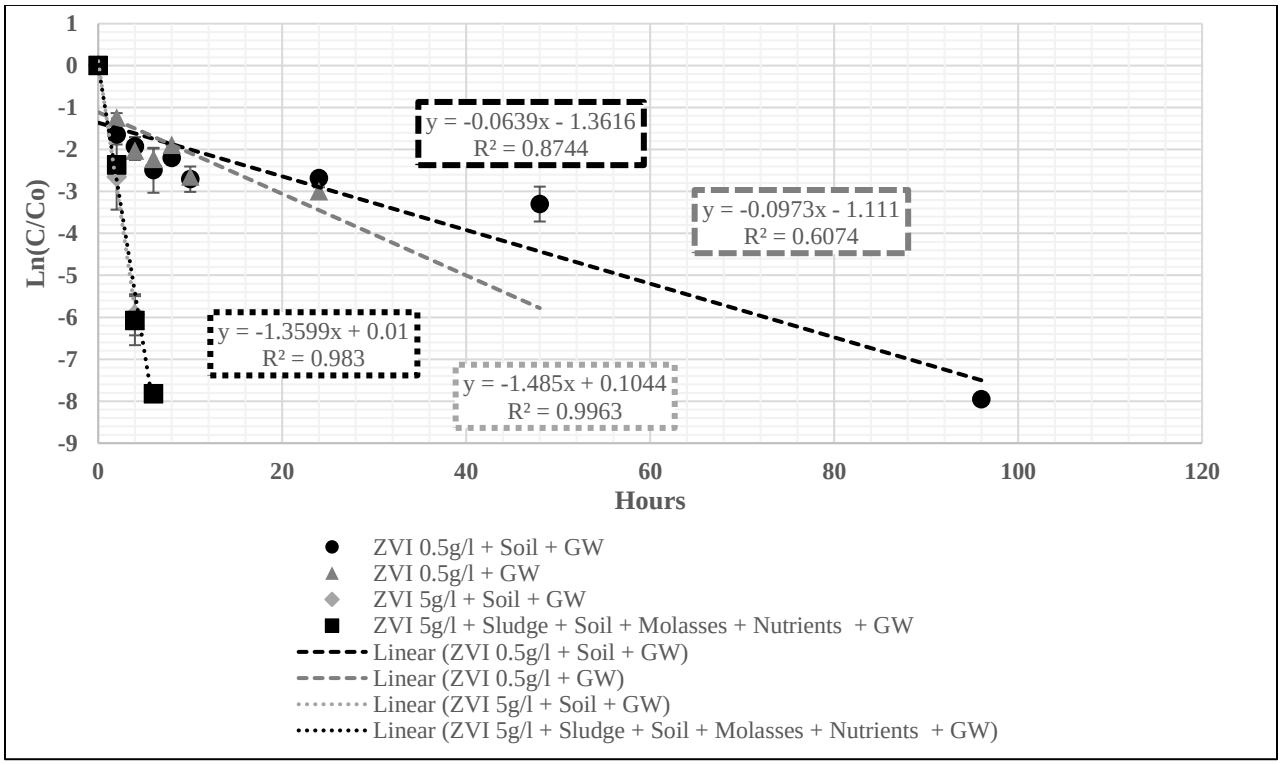


Figure 4.1.2: First Order Kinetics for Abiotic and Bio-enhanced Chromium(VI) Reduction using NZVI in the Presence and Absence of Soil at Stoichiometric Mass Ratios of 13.71X (0.5 g/L), 10.86X (0.5 g/L + Soil), 108.60X (5 g/L + Soil), and 118.93X (5 g/L + Sludge + Nutrients + Soil)

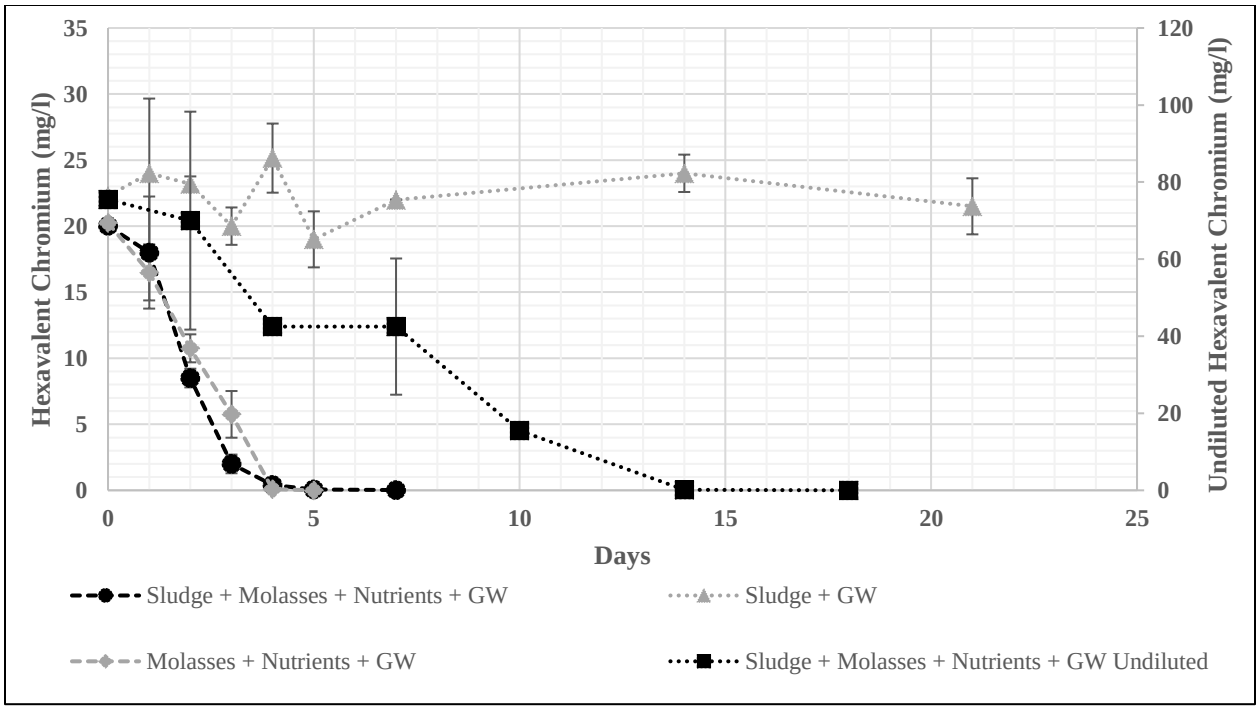


Figure 4.1.3: Biotic Chromium(VI) Reduction using Bacterial Sludge in the Absence of Soil

Table 4.1.2: Chromium(VI) Reduction Rates for Biotic Reactors using Bacterial Sludge in the Absence of Soil

Treatment	Average Rate (mg/L*d)
Sludge + Molasses + Nutrients + GW	5.63
Sludge + GW	No Change
Molasses + Nutrients + GW	5.63
Sludge + Molasses + Nutrients + GW (undil)	5.03

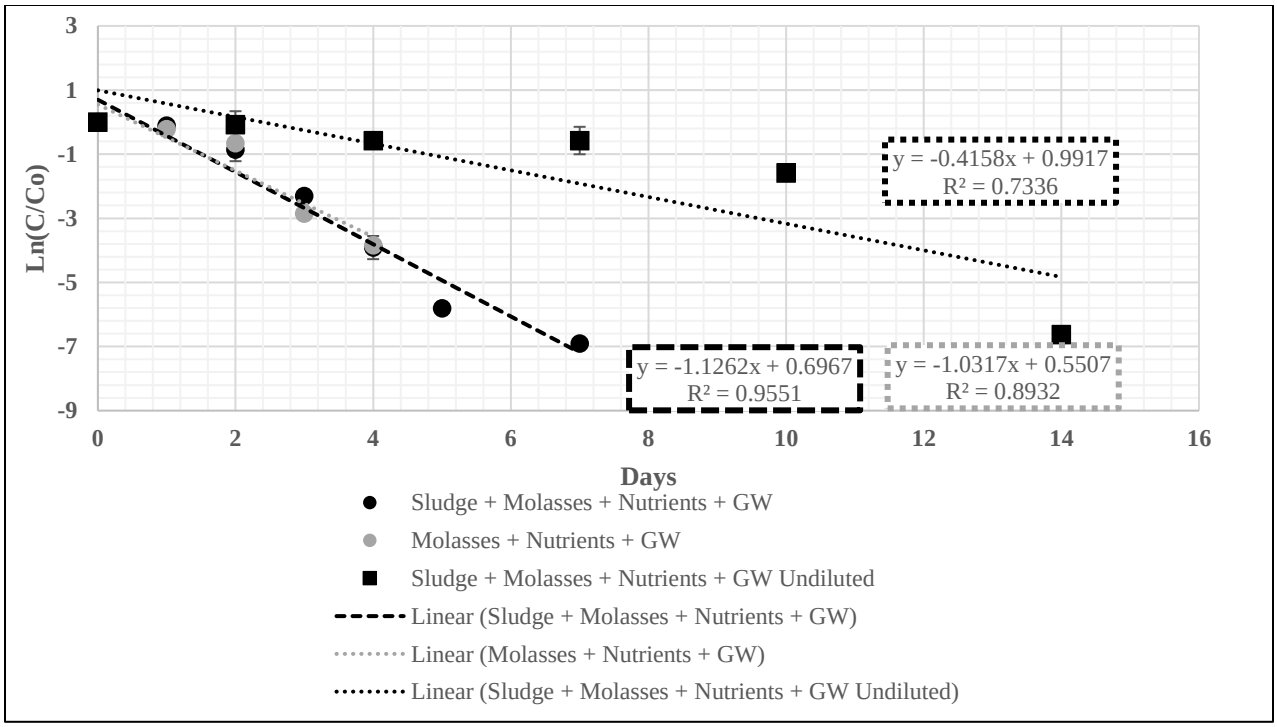


Figure 4.1.4: First Order Kinetics for Biotic Chromium(VI) Reduction using Bacterial Sludge in the Absence of Soil

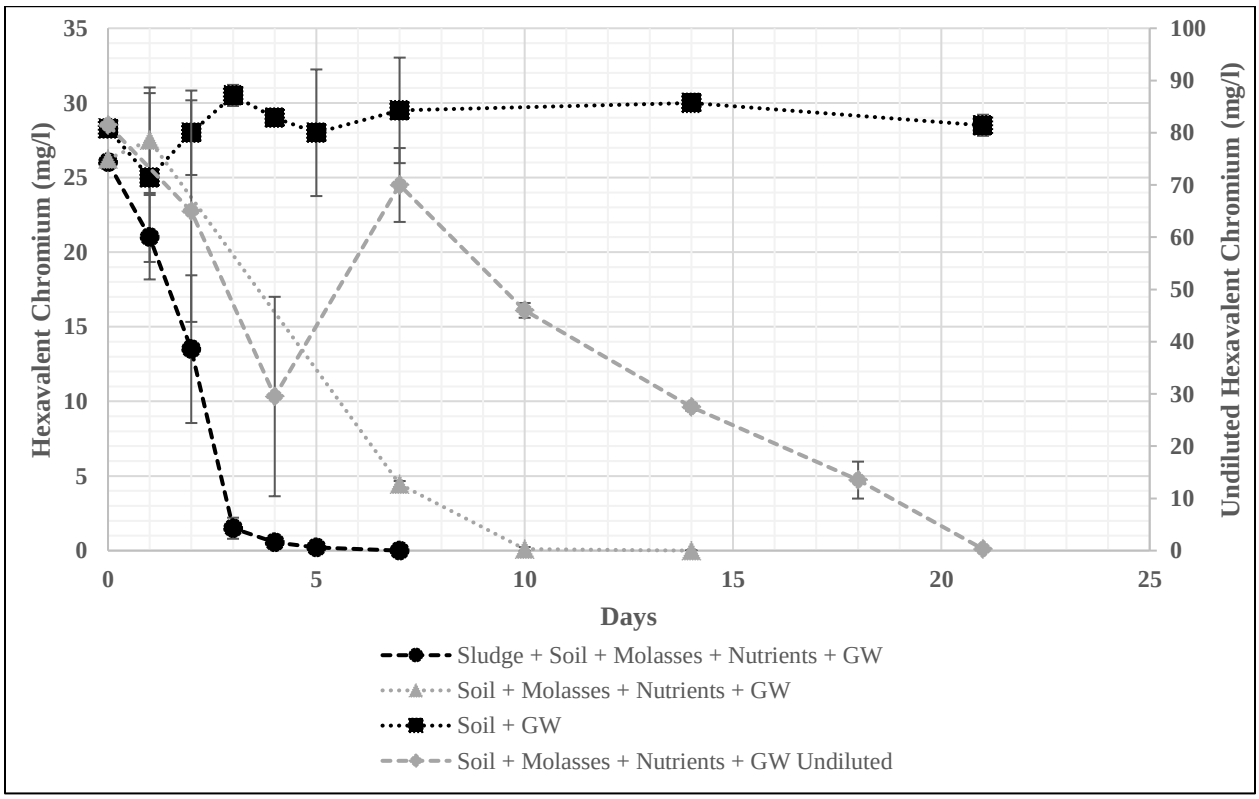


Figure 4.1.5: Biotic Chromium(VI) Reduction using Bacterial Sludge in the Presence of Soil

Table 4.1.3: Chromium(VI) Reduction Rates for Biotic Reactors using Bacterial Sludge in the Presence of Soil

Treatment	Average Rate (mg/L*d)
Sludge + Soil + Molasses + Nutrients + GW	5.25
Soil + GW	No Change
Soil + Molasses + Nutrients + GW	2.63
Soil + Molasses + Nutrients + GW (undil)	3.87

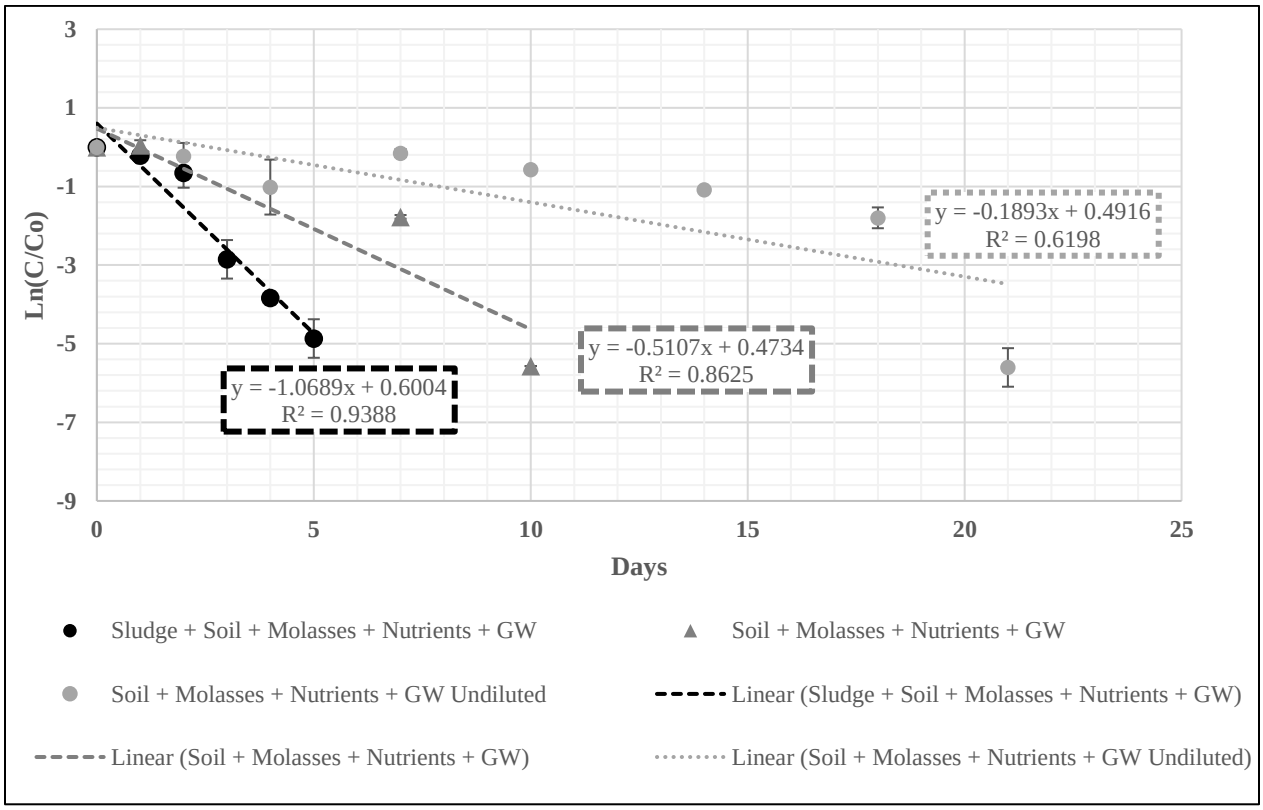


Figure 4.1.6: First Order Kinetics for Biotic Chromium(VI) Reduction using Bacterial Sludge in the Presence of Soil

Table 4.1.4: Summary of Two Factor ANOVA for Significant Difference in Chromium(VI) Reduction between Different Biotic, Abiotic, Bio-enhanced Treatments.

Description	ANOVA Analysis Between:	P-Value
Determination of significant change in NZVI reduction due to soil.	ZVI 0.5 g/L + Soil + GW	0.57
	ZVI 0.5 g/L + GW	
Determination of significant change in NZVI reduction due to bio-enhancement.	ZVI 5 g/L + Soil + GW	0.76
	ZVI 5 g/L + Sludge + Nutrients + Soil GW	
Determination of significant change due increase in NZVI.	ZVI 0.5 g/L + Soil + GW	2.50E-04
	ZVI 5 g/L + Soil + GW	
Determination of significant change between biotic treatments in the absence of NZVI	Sludge + Molasses + Nutrients + GW	0.70
	Sludge + Molasses + Nutrients + Soil + GW	
	Molasses + Nutrients + GW	
	Sludge + Molasses + Nutrients + GW	9.50E-03
	Sludge + Molasses + Nutrients + Soil + GW	
	Molasses + Nutrients + GW	
	Soil + Molasses + Nutrients + GW	
Determination of significant change in biotic reduction due to the absence of nutrients	Sludge + Molasses + Nutrients + GW	1.10E-06
	Sludge + Molasses + Nutrients + Soil + GW	
	Molasses + Nutrients + GW	
	Sludge + GW	
	Sludge + Molasses + Nutrients + GW	1.50E-05
	Sludge + Molasses + Nutrients + Soil + GW	
	Molasses + Nutrients + GW	
	Soil + GW	
	Soil + Molasses + Nutrients + GW	9.30E-05
	Sludge + GW	
	Soil + Molasses + Nutrients + GW	6.60E-04
	Soil + GW	
Determination of significant change in reduction between enriched soil and sludge in undiluted conditions	Sludge + Molasses + Nutrients + GW(undil)	9.80E-04
	Soil + Molasses + Nutrients + GW(undil)	

Table 4.1.5: Summary of Kinetics for Chromium(VI) Reduction in Abiotic, Biotic, and Bio-enhanced Treatments.

Treatment	Reaction Rate Constant			Reaction Order & Highest R^2
	0 Order	1st Order	2nd Order	
	$k = \left(\frac{mg}{L}\right) d^{-1}$	$k = d^{-1}$	$k = \left(\frac{mg}{L}\right)^{-1} d^{-1}$	
ZVI 0.5 g/L + Soil + GW	-2.4	-2.4	-4.8	**1st R^2 =0.9**
ZVI 0.5 g/L + GW	-2.4	-1.4	-0.2	**1st R^2 = 0.6**
ZVI 5 g/L + Soil + GW	-62.4	-30.0	-86.4	**1st R^2 = 0.9**
ZVI 5 g/L + Sludge + Nutrients + Soil + GW	-62.4	-31.2	-74.3	**1s R^2 = 0.9**
Sludge + Molasses + Nutrients + GW	-3.2	-1.1	-1.3	**1st R^2 = 0.9**
Molasses + Nutrients + GW	-4.4	-1.0	-1.1	**1st R^2 = 0.9**
Sludge + Molasses + Nutrients + GW (undil)	-4.6	-0.4	-0.4	**0 R^2 = 0.9**
Sludge + Soil + Molasses + Nutrients + GW	-4.1	-1.1	-0.2	**1st R^2 = 0.9**
Soil + Molasses + Nutrients + GW	-2.2	-0.5	-0.3	**1st R^2 = 0.9**
Soil + Molasses + Nutrients + GW (undil)	-3.2	-0.2	-0.1	**0 R^2 = 0.9**

4.2 Nitrate Removal

In Phase 2, abiotic, biotic, and bio-enhanced nitrate removal was compared in the absence and presence of soil (Fig. 4.2.1). In the absence of soil, nitrate reduction plateaued after Day 6 for all treatments. The effects of pH on denitrification has been recently summarized by Šimek and Cooper, 2002, suggesting denitrification is slower in acidic environments. Šimek's study concludes an optimal pH for denitrification is possible, but this has little meaning without reference to specific attributes of the experiment performed. In this study, the low pH seems to have slowed down, but not completely inhibited nitrate reduction. Final nitrate reduction for biotic and bio-enhanced reactors ranged from 50%-60%. Biotic and bio-enhanced reactors showed statistically significant removal when compared to groundwater controls (Table 4.2.3). However, no statistically significant difference in removal was found between bio-enhanced

reactors and biotic reactors. In abiotic reactors, 20%-25% reduction was seen using 5,000 mg Fe^0/L of NZVI. However, final measurements in abiotic reactors showed no change in nitrate concentration. Table 4.2.3 shows abiotic reduction was statistically significant when compared to groundwater controls despite the elevated nitrate shown at the last day of measurement. It is likely the final elevated level is due to variation of results between reactors, as each measurement was taken from a separate reactor. A statistically significant difference between abiotic and biotic reactors was found. Additionally, a statistically significant difference in removal was also found between abiotic and bio-enhanced treatments, with abiotic reduction showing the slowest average rate in both cases. This suggests abiotic nitrate reduction was slower than biotic and bio-enhanced removal.

With the addition of soil (Fig. 4.2.2), nitrate removal seemed to increase in all treatments (Table 4.2.1). However, the addition of soil increased the variability of results. This can be seen for measurements on Day 6, where nitrate readings were substantially higher for all treatments. This increased reading could have resulted from reactor variability, as different reactors were used every instance of measurement. The high variation of measurements made the determination of significant differences unreliable, where no statistically significant difference in nitrate removal between all treatments was found (Table 4.2.5). However, statistically significant removal was found when comparing all treatments to groundwater controls. Though the addition of soil resulted in higher average removal rates, no statistically significant difference between treatments with soil and treatments without soil was found (Table 4.2.6). Despite this, biotic and bio-enhanced reactors show complete nitrate removal by Day 13 and Day 28, respectively. Even though nitrate measurements in reactors containing soil are more variable, complete nitrate

reduction was not seen in the absence of soil. Overall, reactors showing the lowest final levels of nitrate contained bacterial sludge, regardless of NZVI presence.

In abiotic reactors with 5,000, 8,500, and 17,000 mg Fe^0/L, had mass ratios of 56.50, 96.06, and 191.94 mg Fe^0/mg, respectively (Table 4.2.7). Stoichiometric mass ratios for 5,000, 8,500, and 17,000 mg Fe^0/L were 15.65X, 26.61X, and 53.17X greater than the theoretical stoichiometric dose, respectively. Total nitrate reduction was achieved only at 17,000 mg Fe^0/L at Day 28 (Fig. 4.2.3). However, a resurgence in nitrate after total reduction was seen in abiotic reactors containing 17,000 mg Fe^0/L of NZVI at Day 56. It is likely this resurgence is due to variation, as each measurement was performed in separate reactors. Increasing the NZVI dose increased the average reduction rate. A moderate correlation ($R^2 = 0.7$) between increasing ZVI and nitrate reduction was in seen (Fig. 4.2.3A). A statistically significant increase in abiotic reduction was seen only when increasing the NZVI dose from 5,000-17,000 mg Fe^0/L (Table 4.2.8). Despite doses of 5,000 mg Fe^0/L of NZVI showing no change at Day 56, statistically significant reduction was found when comparing abiotic reactors to groundwater controls (Table 4.2.8). However, no statistically significant reduction was found when NZVI the dose was 8,500 mg Fe^0/L despite showing 65% reduction at Day 56. These results indicate unreliable reduction at NZVI doses of 5,000-8,500 mg Fe^0/L. Thus, higher doses of NZVI are needed to promote nitrate reduction at statistically significant levels. Previous studies have shown effective abiotic nitrate reduction using NZVI, showing first order kinetics with a rate constant as a high as, k = -123.55 d^{-1} (Zhang et al, 2010). Zhang's study yielded average reduction rates as a high as 1,800 $mg/L * d$ for a mass ratio of 20.0 mg Fe^0/ mg in spiked DI water under ambient conditions (Zhang et al, 2010). Other studies using synthetic groundwater have shown slower rates of 489.6-61.4 $mg/L * d$ with no first order kinetics and only 90% reduction achieved at a

higher mass ratio of 100.0 mg Fe^0/mg under ambient conditions (Liu et al, 2012). In this study, average rates for abiotic NZVI reduction were much lower, even at higher mass ratios. Additionally, first order kinetics showed low correlation ($R^2 = 0.1 - 0.3$) for abiotic reduction (Fig. 4.2.3B). Abiotic reactors showed the highest correlation with zero order kinetics ($R^2 = 0.4 - 0.8$, Table 4.2.15), with rate constants of k = -1.0 to -17.8 $mg/l * d$. Overall, doses of 5,000-8,000 mg Fe^0/L, at stoichiometric mass ratios of 15.65X-26.61X, are not sufficient to reduce nitrate completely. Passivation of NZVI by nitrate is a possible reason for the limited reduction at 5,000-8,000 mg Fe^0/L, as nitrate will adhere to the surface of NZVI, decreasing its reactivity (Chen et al, 2013, & Luo et al 2010). This suggests depletion of reactivity of NZVI by nitrate passivation or reaction with other contaminants at the lower doses used in this study, particularly chlorate which requires the largest fraction of the NZVI needed (Table C3).

As mentioned before, a different bacterial sludge was used in Phase 3. Phase 3 sludge contained both a higher COD and higher phosphate than the sludge used in Phases 1-2. Fig. 4.2.4 shows that biotic reactors containing bacterial sludge in Phase 3 removed 97% of nitrate in 7 days, with an average removal rate of 1.40 $mg/L * d$. In the presence of soil, the average removal rate in biotic reactors increased to 3.75 $mg/L * d$, removing 95% of nitrate in 3 days. However, a large increase in nitrate was seen at Day 35 in biotic reactors containing soil. Though this was considered and outlier for analysis, this increase was probably due to variation, as denitrifying likely did not thrive in the reactor the measurement was taken from. Statistically significant nitrate removal was found when comparing all biotic reactors to groundwater controls (Table 4.2.11). No statistically significant difference was found in biotic reactors due to the addition of soil in Phase 3. When comparing biotic reactors using Phase 2-3 sludge, a statistically significant difference was found when using a different sludge (Table 4.2.11), where higher

nitrate removal was apparent in Phase 3. This persisted even in the presence of soil, with Phase 3 sludge again showing higher levels of nitrate removal. This could be attributed to the richer sludge conditions used in Phase 3. Therefore, the effectiveness of nitrate removal using sludge is dependent on varying microbial conditions. Biotic nitrate removal in undiluted conditions showed 50% removal after 8 weeks, with an average rate of 2.68 $mg/L * d$ (4.2.4A). When compared to groundwater controls, this removal was statistically significant (Table 4.2.11). Previous research using denitrifying bacteria from bacterial sludge to remove nitrate in groundwater has shown average rates of 200 $mg/l * d$ for an initial nitrate of 500 mg/L under ambient conditions (Ayyasamy et al, 2007). Additionally, first order kinetics for anaerobic denitrification in previous studies under ambient conditions has shown rate constants of k = -1.41 d^{-1} to -2.61 d^{-1} (Leverenz et al, 2010). Biotic nitrate removal showed a high first order correlation ($R^2 = 0.8 - 0.9$) in diluted and undiluted conditions (Fig. 4.2.4B), with rate constants of k = -0.01 d^{-1} to -0.29 d^{-1}. Overall, biotic reactors showed the highest correlation with first order kinetics (Table 4.2.15). Though first order kinetics were shown, average biotic nitrate removal rates and rate constants in this study were substantially lower than previous research.

All bio-enhanced reactors showed at least 95% removal by Day 35. However, a resurgence in nitrate was seen at Day 35 in reactors containing 5,000. This again is likely due to variation between individual reactors. Doses of 5,000-8,500 mg Fe^0/L, with mass ratios of 50.89, and 86.51 mg Fe^0/mg, showed nitrate removal rates of 1.27 $mg/L * d$, and 1.66 $mg/L * d$, respectively (Fig. 4.2.5). At 17,000 mg Fe^0/L with a mass ratio of 173.01 mg Fe^0/mg, total nitrate removal was seen in just 1 day, with an average rate of 98.27 $mg/L * d$. In bio-enhanced reactors, the stoichiometric mass ratios for 5,000, 8,500, and 17,000 mg Fe^0/L were 14.09,

23.96, and 47.92X, respectively (Table 4.2.12). Bio-enhanced reactors in Phase 3 (Fig. 4.2.5A), showed a strong correlation ($R^2 = 0.8$) between increasing NZVI and nitrate removal. When comparing differences in bio-enhanced reactors, no statistically significant difference was observed when increasing the NZVI dose from 5,000-8,500 mg Fe^0/L. A statistically significant increase was shown only when increasing the NZVI dose from 5,000-17,000 mg Fe^0/L (Table 4.2.13). All bio-enhanced treatments showed statistically significant removal when compared to groundwater controls. Previous studies on bio-enhanced NZVI nitrate removal using enriched sediment has shown average rates of 3.75-8.18 $mg/l * d$ for spiked groundwater with a lower mass ratio of 6.10 mg Fe^0/mg, with first order kinetics showing a large range of substrate-dependent rate constants of k = -0.15 d^{-1} to -94.23 d^{-1} under ambient conditions (Hu et al, 2018). Other studies have shown much lower range of rate constants of k = -0.07 d^{-1} to -0.29 d^{-1} for synthetic groundwater with mass ratios of 5.26-21.00 mg Fe^0/mg under ambient temperature and a pH of 8.5-9.0 (An et al, 2010). In this study, first order kinetics in bio-enhanced reactors showed moderate correlation ($R^2 = 0.7$) only at a NZVI dose of 8,500 mg Fe^0/L, with a rate constant of -0.05 d^{-1} (Fig. 4.2.5B). While the first order rate constant in bio-enhanced reactors shown in this study is similar to the low-end range of An's research, higher mass ratios were used. Except for a 5,000 mg Fe^0/L dose, which showed the highest correlation with zero order kinetics ($R^2 = 0.6, k = -12.8\ mg/L * d$), most bio-enhanced reactors showed the highest correlation at second order kinetics ($R^2 = 0.4 - 0.9$, Table 4.2.15), with rate constants of k = -4.0E-3 to -3.7 $\left(\frac{mg}{L}\right)^{-1} * d^{-1}$.

The comparison of abiotic and bio-enhanced nitrate reactors shows a statistically significant difference between abiotic and bio-enhanced treatments at all NZVI doses (Table

4.2.14), with bio-enhanced reactors showing considerably higher removal rates. Additionally, higher removal levels were seen at lower stoichiometric mass ratios in bio-enhanced reactors than abiotic reactors. Comparing biotic and bio-enhanced reactors, there was no statistically significant difference between biotic and bio-enhanced treatments. However, at 17,000 mg Fe^0/L, total nitrate removal was achieved at Day 1 in bio-enhanced reactors, where biotic reactors show only 23% (Fig. 4.2.4). Finally, comparing abiotic and biotic treatments, abiotic nitrate reduction achieved statistically similar results to biotic reactors at NZVI doses of 8,500 mg Fe^0/L or greater, though abiotic reactors with 8,500 mg Fe^0/L did not show statistically significant difference when compared to groundwater controls.

Results from this study show:

- No difference in nitrate removal was seen due to the addition of soil in any treatment.
- Abiotic nitrate reduction using NZVI was possible, but only achieved reliable effectiveness at least 17,000 mg Fe^0/L.
- Biotic and bio-enhanced nitrate treatments generally showed higher removal than abiotic reduction.
- Considerable biotic nitrate removal is possible even under undiluted conditions.
- Due to the lack of a statistically significant difference between biotic and bio-enhanced reactors, no additional reduction due to NZVI can be expected.
- No statistically significant difference between biotic and bio-enhanced nitrate reactors was found. However, the variable results achieved in biotic reactors endorses augmentation with NZVI, which showed more consistent nitrate removal.

Table 4.2.1: Final pH measurements for Abiotic Biotic & Bio-enhanced Reactors

Treatment + GW	PH (56 Days)
ZVI 5 g/L + GW	7.0
ZVI 8.5 g/L + GW	7.0
ZVI 17 g/L + GW	7.0
Sludge + Molasses + Nutrients + GW	3.6
Sludge + Soil + Molasses + Nutrients + GW	3.8
ZVI 5 g/L Sludge + Soil + Molasses + Nutrients + GW	6.5
ZVI 8.5 g/L Sludge + Soil + Molasses + Nutrients + GW	3.8
ZVI 17 g/L Sludge + Soil + Molasses + Nutrients + GW	8.3

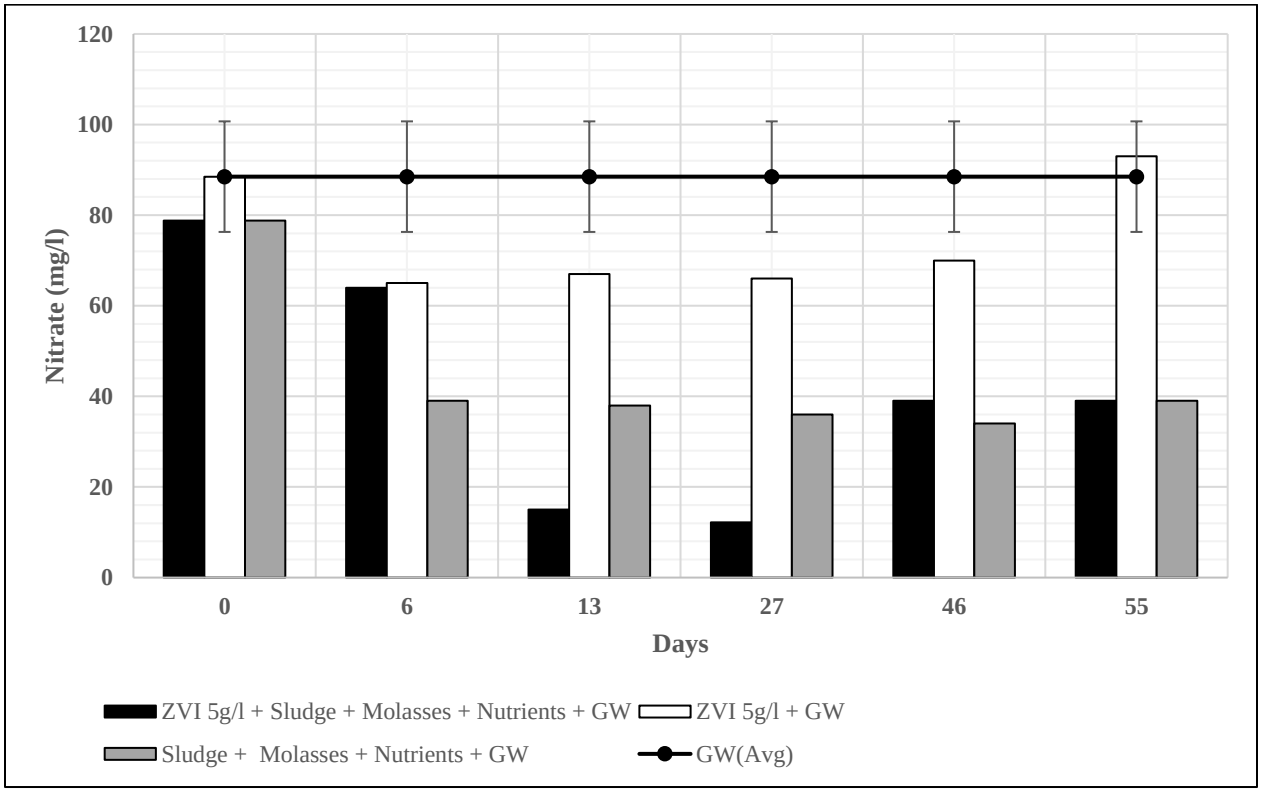


Figure 4.2.1: Phase 2: Abiotic, Biotic, and Bio-enhanced Nitrate Removal in the Absence of Soil at Stoichiometric Mass Ratios of 17.58X (5 g/L), and 15.65X (5 g/L + Sludge + Nutrients)

Table 4.2.2: Nitrate Removal Rates for Abiotic, Biotic, and Bio-enhanced Reactors in the Absence of Soil

Treatment	Average Rate (mg/l*d)	Nitrate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	1.65	17.58X
ZVI 5 g/L + GW	0.18	15.65X
Sludge + Molasses + Nutrients + GW	1.29	0X

Table 4.2.3: Summary of Two Factor ANOVA for Phase 2: Abiotic, Biotic, and Bio-enhanced Nitrate Removal in the Absence of Soil

Description	ANOVA Analysis Between:	P-Value
Determination of significant change in NZVI reduction due to bio-enhancement.	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.017
	ZVI 5 g/L + GW	
Determination of significant change in biotic treatment due to the absence of NZVI	ZVI 5 g/l + Sludge + Molasses + Nutrients + GW	0.720
	Sludge + Molasses + Nutrients + GW	
Determination of significant change between biotic and abiotic reactors with NZVI	Sludge + Molasses + Nutrients + GW	0.003
	ZVI 5 g/L + GW	
Determination of significant removal due to bio-enhanced NZVI	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.001
	GW	
Determination of significant biotic removal	Sludge + Molasses + Nutrients + GW	0.007
	GW	
Determination of significant abiotic reduction by NZVI	ZVI 5 g/L + GW	0.020
	GW	

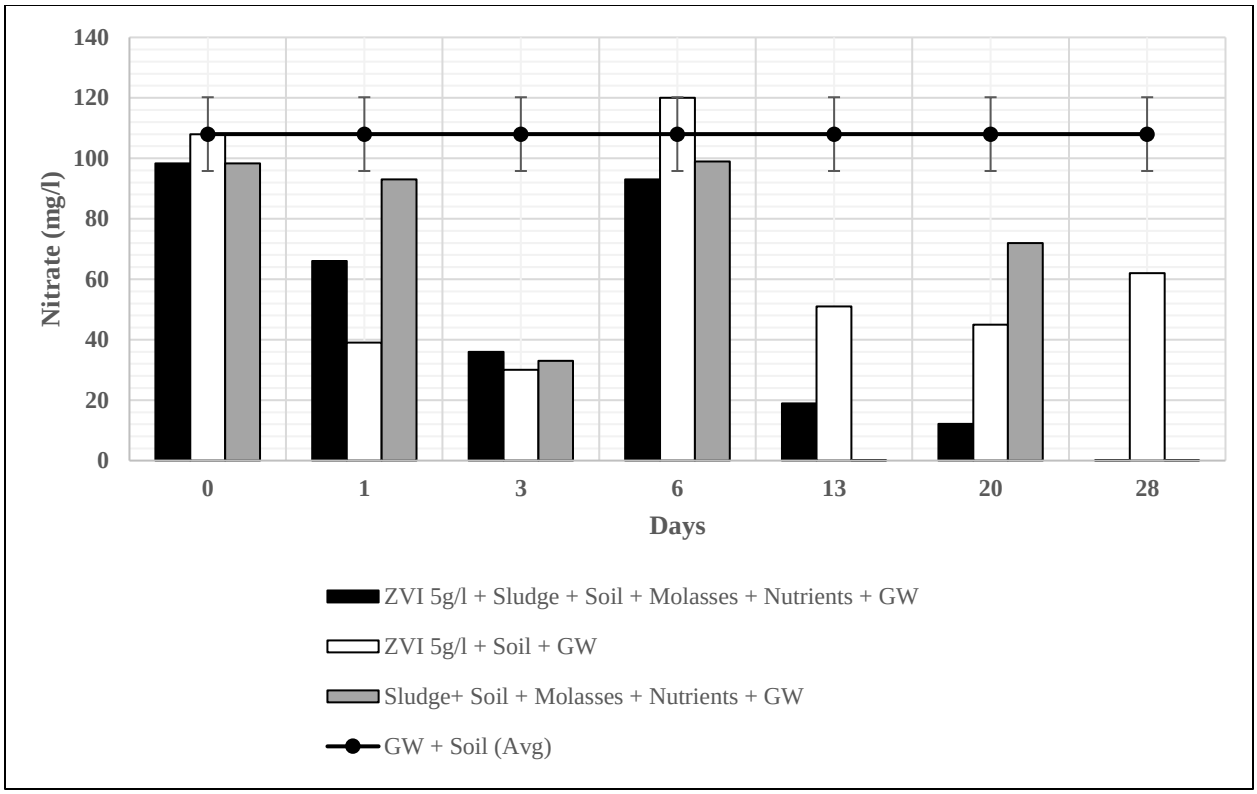


Figure 4.2.2: Phase 2: Abiotic, Biotic and Bio-enhanced Nitrate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 14.09X (5 g/L + Soil), and 12.82X (5 g/L + Sludge + Soil + Molasses + Nutrients)

Table 4.2.4: Nitrate Removal Rates for Abiotic, Biotic and Bio-enhanced Reactors in the Presence of Soil

Treatment	Average Rate (mg/l*d)	Nitrate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	7.56	14.09X
ZVI 5 g/L + Soil + GW	1.64	12.82X
Sludge+ Soil + Molasses + Nutrients + GW	3.51	0X

Table 4.2.5: Summary of Two Factor ANOVA for Phase 2: Abiotic, Biotic, and Bio-enhanced Nitrate Removal in the Presence of Soil

Description	ANOVA Analysis Between	P-Value
Determination of significant change in NZVI Removal due to bio-enhancement.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.280
	ZVI 5g/L + Soil + GW	
Determination of significant change in biotic treatment due to the absence of NZVI.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.338
	Sludge+ Soil + Molasses + Nutrients + GW	
Determination of significant change between biotic and abiotic reactors with NZVI.	Sludge+ Soil + Molasses + Nutrients + GW	0.867
	ZVI 5 g/L + Soil + GW	
Determination of significant change due to bio-enhanced NZVI removal.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.018
	GW + Soil	
Determination of significant biotic removal.	Sludge+ Soil + Molasses + Nutrients + GW	0.019
	GW + Soil	
Determination of significant abiotic reduction by NZVI.	ZVI 5 g/L + Soil + GW	0.048
	GW + Soil	

Table 4.2.6: Summary of Two Factor ANOVA in Phase 2 to Determine Significant Difference in Nitrate Removal due to the Addition of Soil

Description	ANOVA Analysis Between	P-Value
Determination of significant change due to addition of soil in bio-enhanced treatments.	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.94
	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change due to addition of soil in abiotic NZVI reduction.	ZVI 5 g/L + GW	0.524
	ZVI 5 g/L + Soil + GW	
Determination of significant change due to addition of soil in biotic treatments.	Sludge + Molasses + Nutrients + GW	0.712
	Sludge+ Soil + Molasses + Nutrients + GW	
Determination of significant change due to addition of soil in groundwater controls	GW	0.343
	GW + Soil	

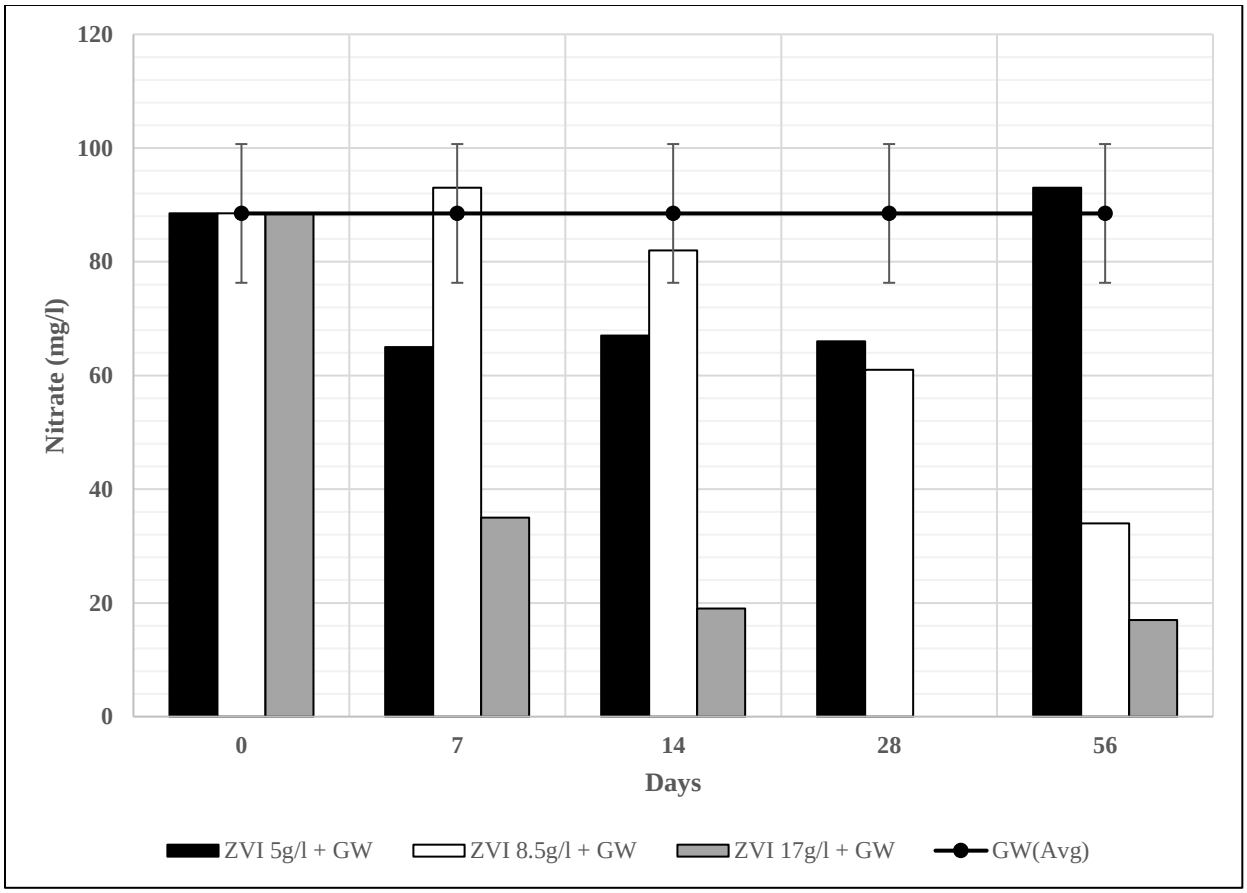


Figure 4.2.3: Abiotic NZVI Nitrate Reduction in the Absence of Soil at Stoichiometric Mass Ratios of 15.65X (5 g/), 26.61X (8.5 g/L), and 53.17X (17 g/L)

Table 4.2.7: Nitrate Reduction Rates for Abiotic Reactors in the Absence of Soil

Treatment	Average Rate(mg/l*d)	Nitrate Mass Ratio mg Fe^0/mg	Nitrate Stoichiometric Mass Ratio
ZVI 5 g/L + GW	0.80	56.49	15.65X
ZVI 8.5 g/L + GW	No Change	96.06	26.61X
ZVI 17 g/L + GW	3.16	191.94	53.17X

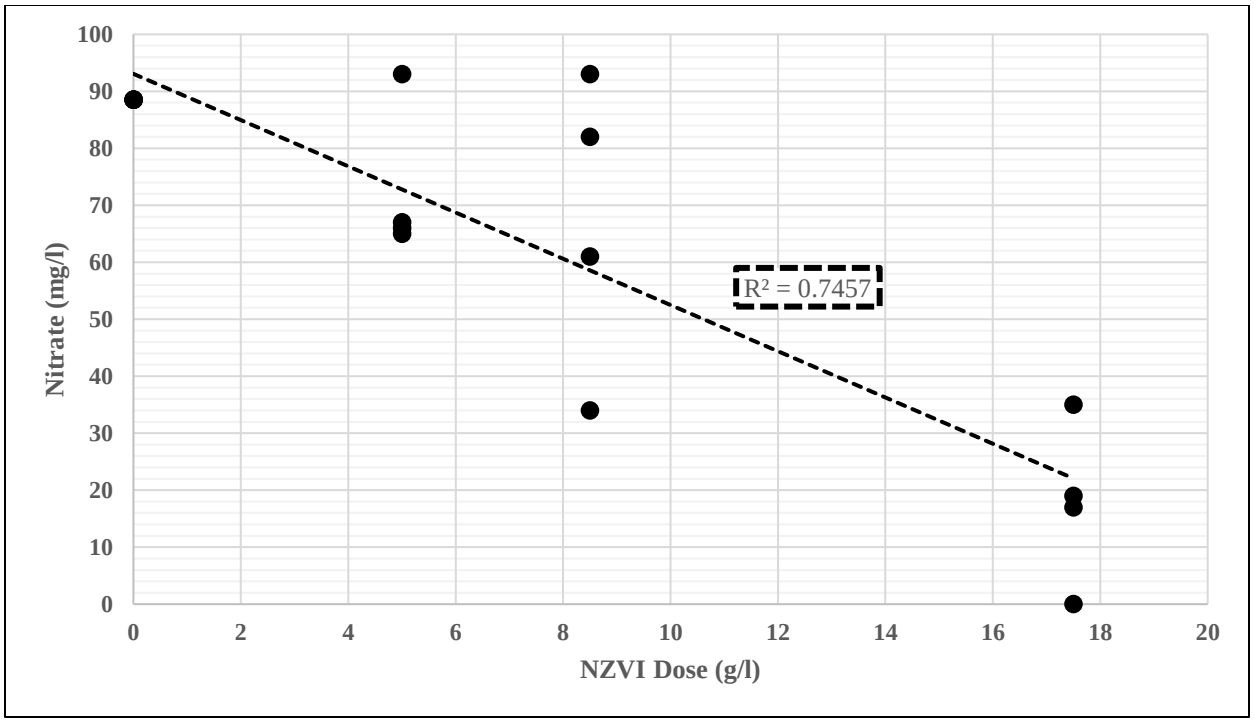


Figure 4.2.3A: Correlation between Nitrate Reduction and NZVI Concentration

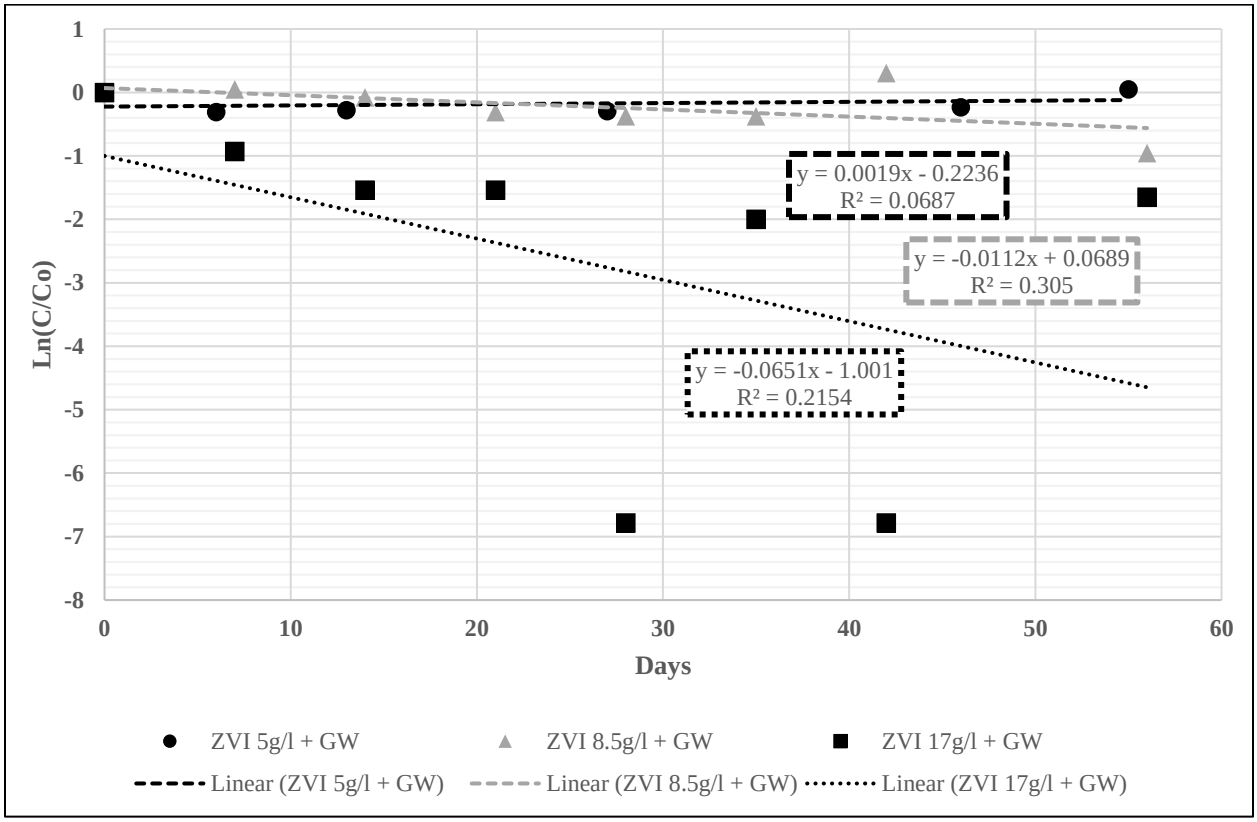


Figure 4.2.3B: First Order Kinetics for Abiotic Nitrate Reduction in the Absence of Soil at Stoichiometric Mass Ratios of 15.65X (5 g/L), 26.61X (8.5 g/L), and 53.17X (17 g/L)

Table 4.2.8: Summary of Two Factor ANOVA to Determine Significant Difference in Abiotic Nitrate Reduction due to increase in NZVI

Description	ANOVA Analysis Between	P-Value
Determination of significant change in abiotic reduction due to 1.7X increase in NZVI	ZVI 5g/L + GW	0.762
	ZVI 8.5g/L + GW	
Determination of significant change in abiotic reduction due to 3.3X increase in NZVI	ZVI 5g/L + GW	0.010
	ZVI 17g/L + GW	
Determination of significant abiotic reduction by NZVI	ZVI 5g/L + GW	0.020
	GW	
	ZVI 8.5g/L + GW	0.32
	GW	
	ZVI 17g/L + GW	0.007
	GW	

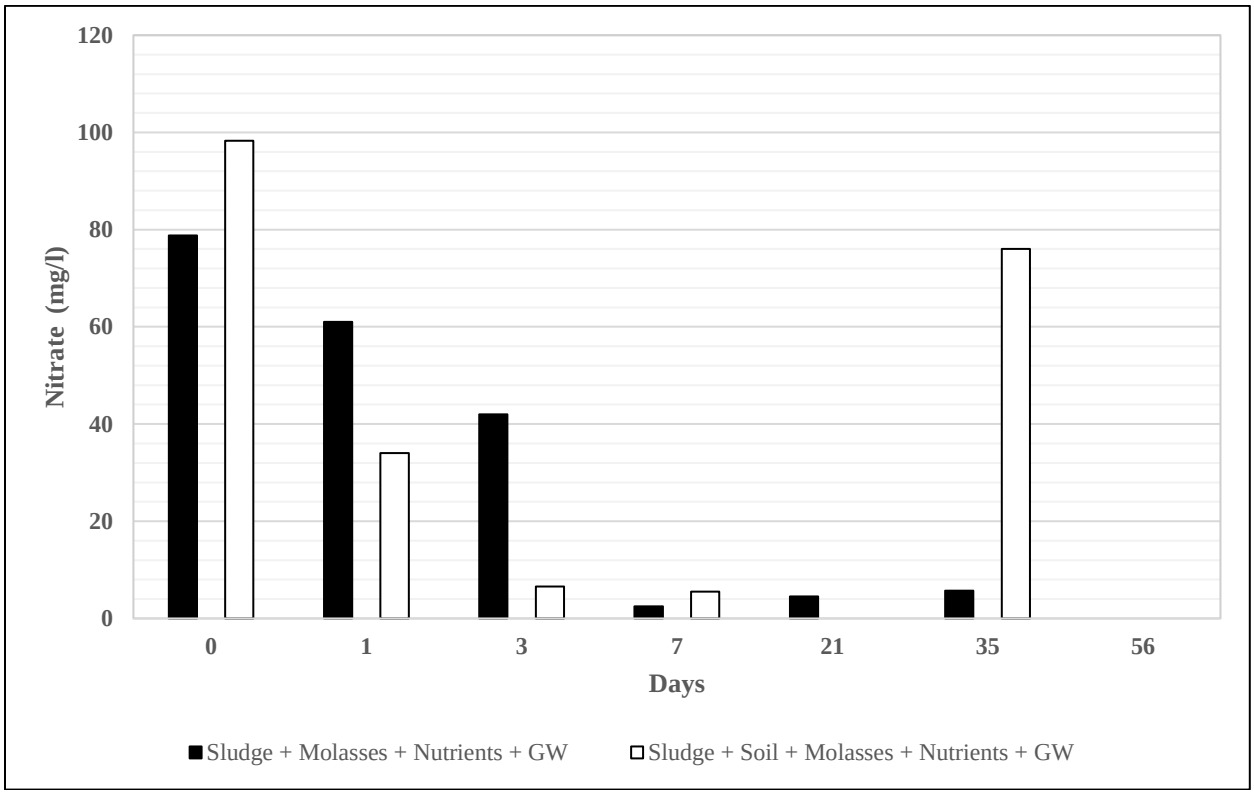


Figure 4.2.4: Diluted Biotic Nitrate Removal in the Presence and Absence of Soil Using Phase 3 Sludge

Table 4.2.9: Nitrate Removal Rates for Biotic Reactors in the Presence and Absence of Soil Using Phase 3 Sludge

Treatment	Average Rate (mg/L*d)
Sludge + Molasses + Nutrients + GW	1.40
Sludge + Soil + Molasses + Nutrients + GW	3.75

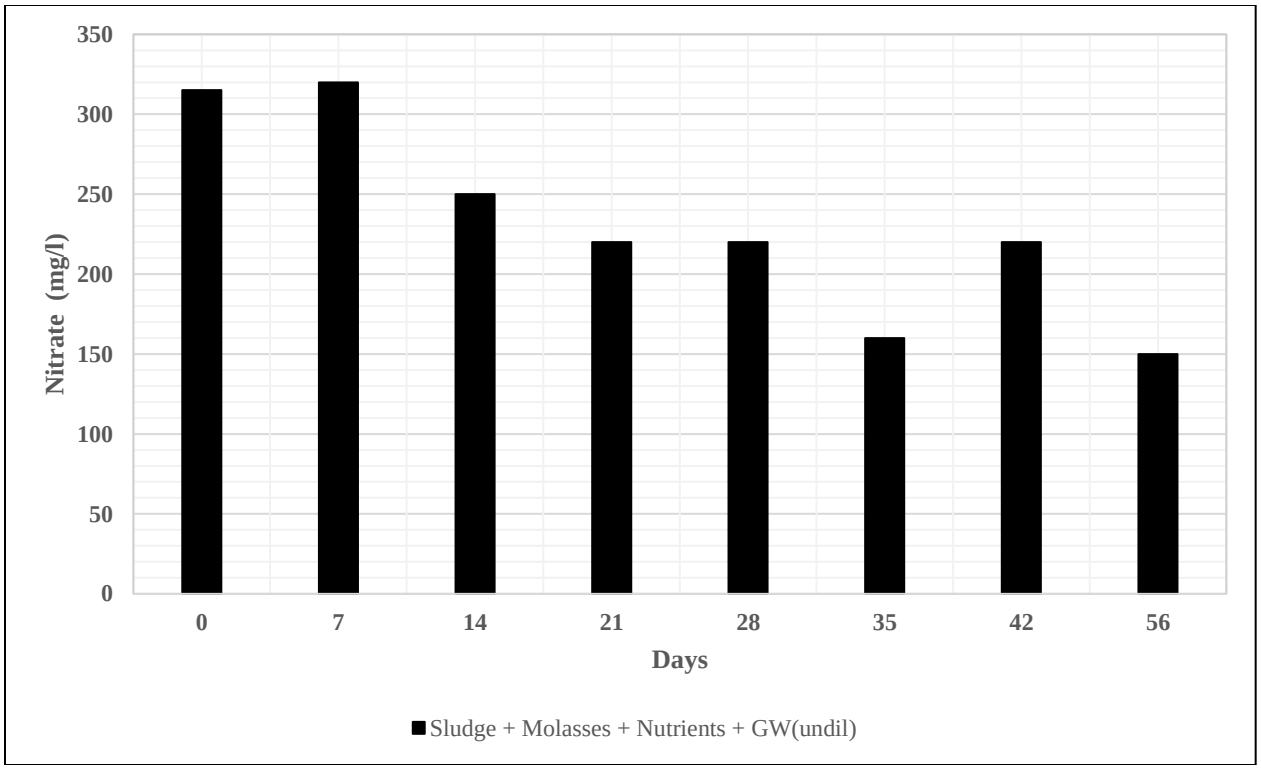


Figure 4.2.4A: Undiluted Biotic Nitrate Removal in the Absence of Soil using Phase 3 Sludge

Table 4.2.10: Nitrate Removal Rates for Undiluted Biotic Reactors in the Absence of Soil using Phase 3 Sludge

Treatment	Average Rate (mg/L*d)
Sludge + Molasses + Nutrients + GW(undil)	2.68

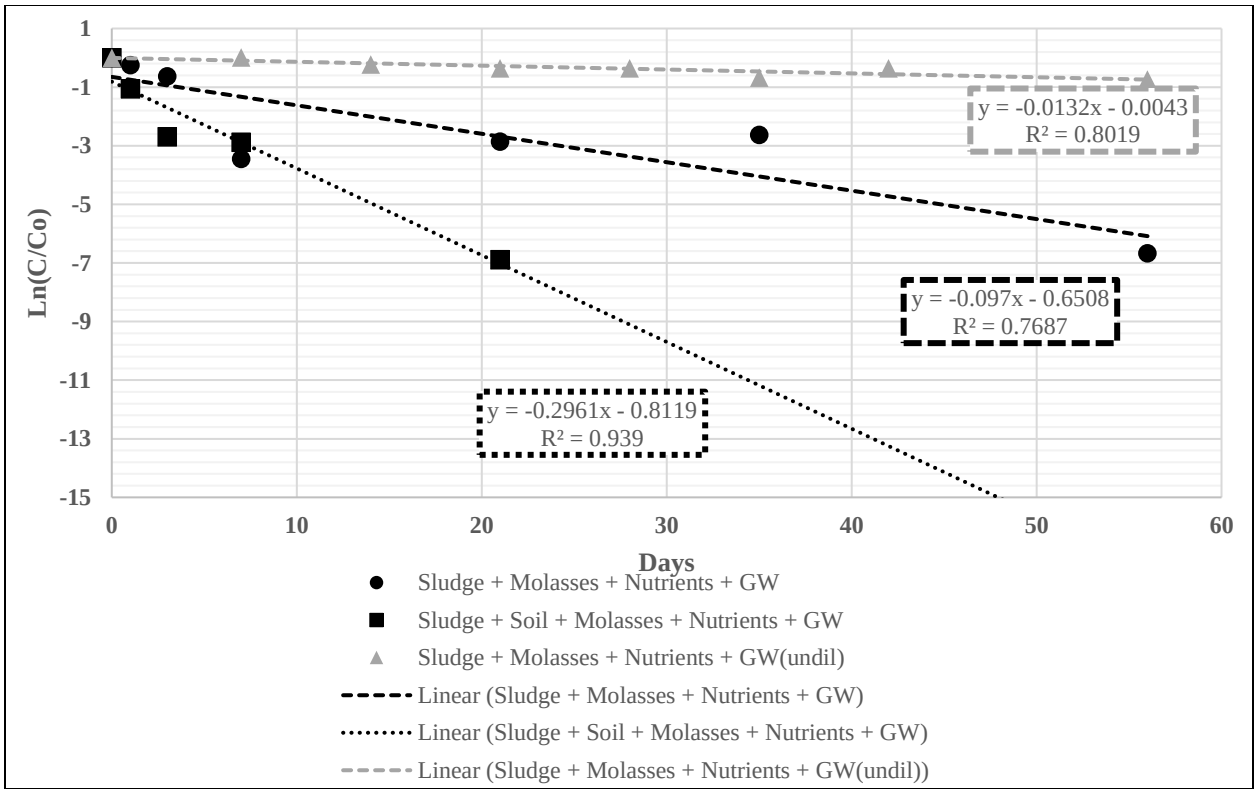


Figure 4.2.4B: First Order Kinetics for Biotic Nitrate Removal in the Presence and Absence of Soil using Phase 3 Sludge

Table 4.2.11: Summary of Two Factor ANOVA in to Determine Significant Removal in Biotic Nitrate Reactors in Phase 3

Description	ANOVA Analysis Between	P-Value
Determination of significant change in biotic reactors between Phase 2 and Phase 3 enriched sludge.	Sludge (Phase 2) + Molasses + Nutrients + GW	0.018
	Sludge (Phase 3) + Molasses + Nutrients + GW	
Determination of significant change in biotic reactors between Phase 2 and Phase 3 enriched sludge in the presence of soil.	Sludge (Phase 2) + Soil + Molasses + Nutrients + GW	0.032
	Sludge (Phase 3) + Soil + Molasses + Nutrients + GW	
Determination of significant change in biotic reactors due the addition of soil.	Sludge (Phase 3) + Molasses + Nutrients + GW	0.831
	Sludge +(Phase 3) Soil + Molasses + Nutrients + GW	
Determination of significant biotic removal using Phase 3 sludge in diluted and undiluted conditions.	Sludge (Phase 3) + Molasses + Nutrients + GW	0.007
	GW	
	Sludge (Phase 3) + Soil + Molasses + Nutrients + GW	0.005
	GW + Soil	
	Sludge (Phase 3) + Molasses + Nutrients + GW(undil)	0.012
	GW	

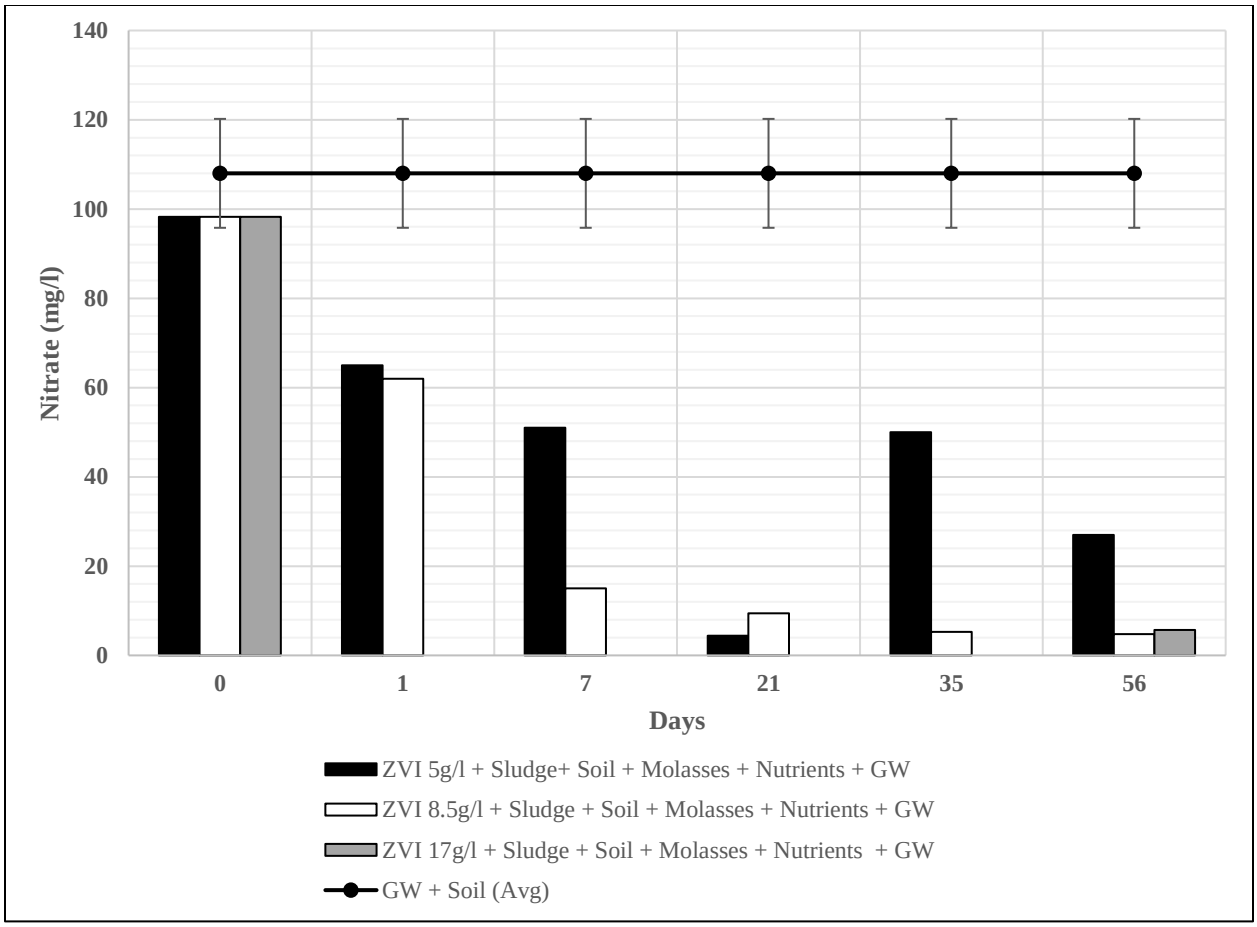


Figure 4.2.5: Phase 3: Effects of Increasing NZVI on Bio-enhanced Nitrate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 14.09X (5 g/L), 23.96X (8.5 g/L), and 47.92X (17 g/L)

Table 4.2.12: Nitrate Removal Rates for Bio-enhanced Reactors in the Presence of Soil

Treatment	Average Rate (mg/l*d)	Nitrate Mass Ratio $mgFe^0/mg$	Nitrate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	1.27	50.89	14.09X
ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	1.66	86.50	23.96X
ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	98.27	173.01	47.92X

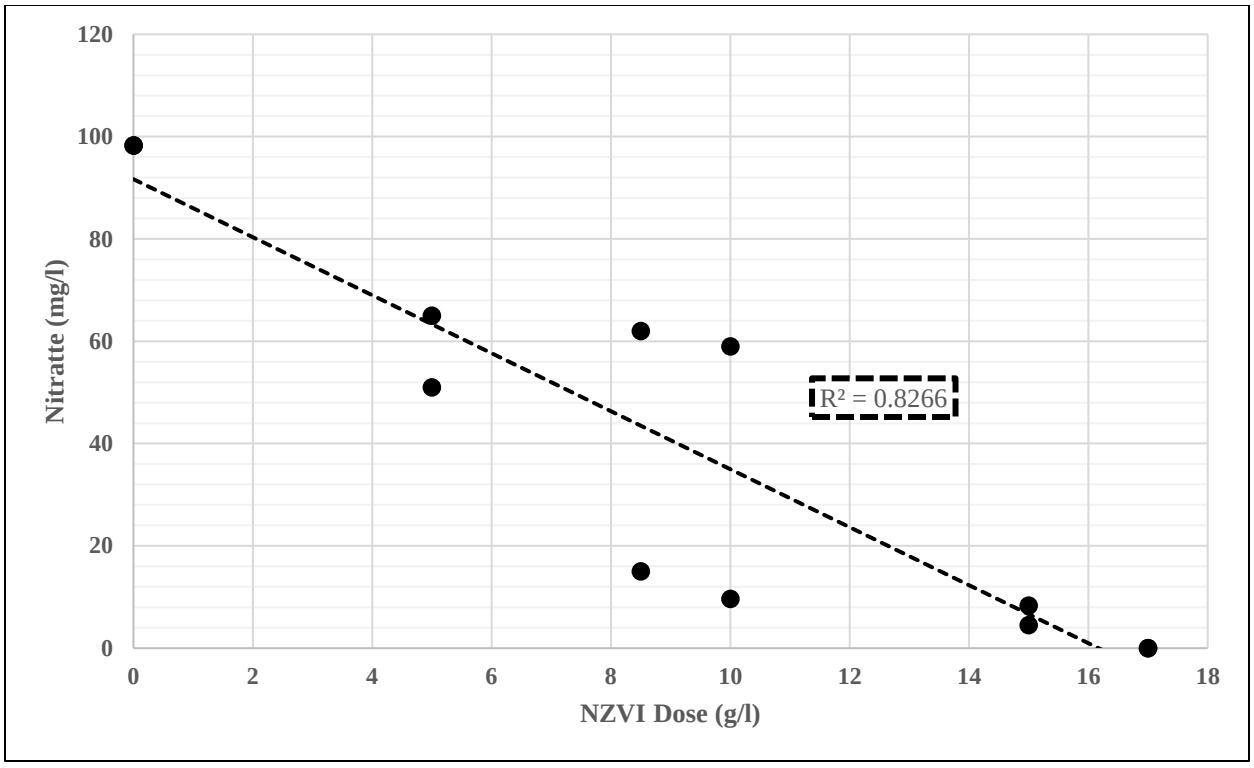


Figure 4.2.5A: Correlation between Nitrate Removal and NZVI concentration under Bio-enhanced conditions in the presence of soil

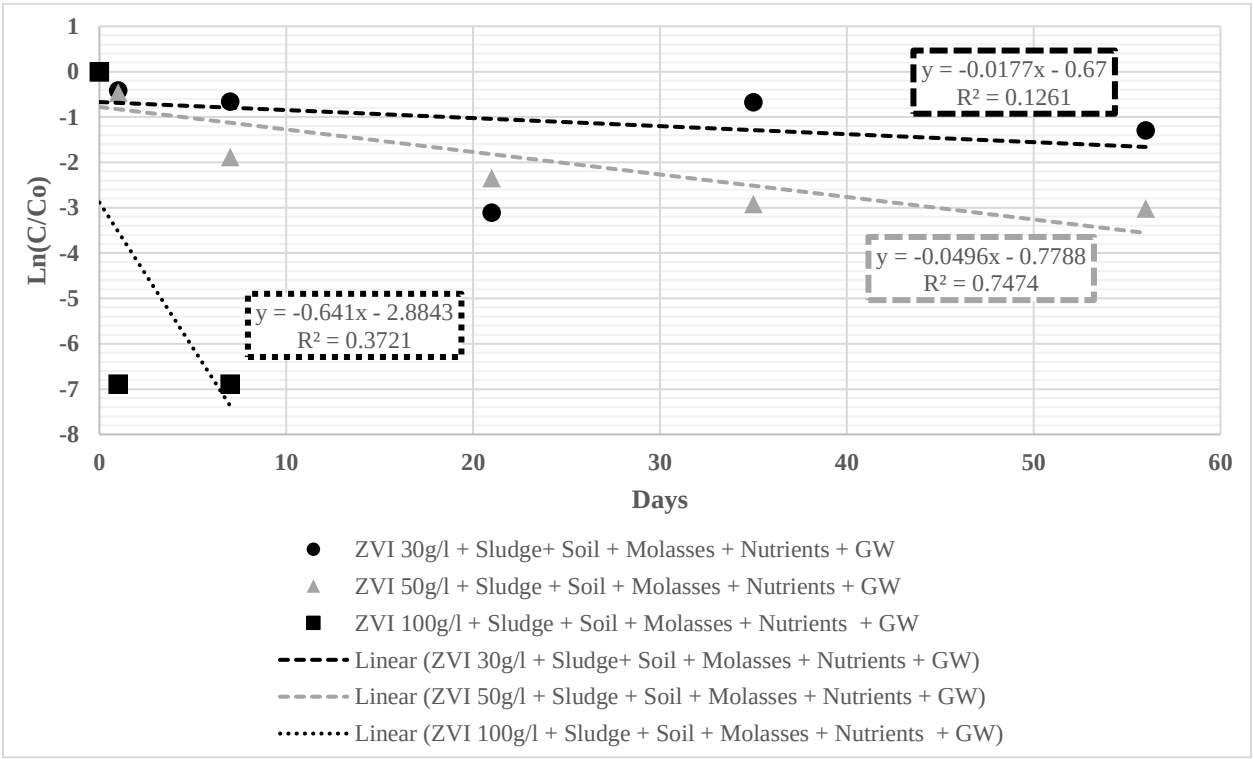


Figure 4.2.5B: First Order Kinetics for Phase 3: Effects of Increasing NZVI on Bio-enhanced Nitrate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 14.09X (5 g/L), 23.96X (8.5 g/L), and 47.92X (17 g/L)

Table 4.2.13: Summary of Two Factor ANOVA to Determine Significant Difference in Bio-enhanced Nitrate Removal due to increase in NZVI in Phase 3

Description	ANOVA Analysis Between	P-Value
Determination of significant change in bio-enhanced removal due to 1.7X increase in NZVI	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.100
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change in bio-enhanced removal due to 3.3X increase in NZVI	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.030
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant removal between bio-enhanced treatments and groundwater controls.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.009
	GW + Soil	
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.006
	GW + Soil	
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.003
	GW + Soil	

Table 4.2.14: Summary of Two Factor ANOVA Comparing Abiotic, Biotic, and Bio-enhanced Nitrate Removal in Phase 3

Description	ANOVA Analysis Between	P-Value
Determination of significant change between bio-enhanced and abiotic reactors.	ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	0.056
	ZVI 5 g/L + GW	
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.031
	ZVI 8.5 g/L + GW	
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.053
	ZVI 17 g/L + GW	
Determination of significant change between bio-enhanced and biotic reactors.	ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	0.252
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.831
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.215
	Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change between abiotic and biotic reactors.	ZVI 5 g/L + GW	0.044
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 8.5 g/L + GW	0.121
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 17 g/L + GW	0.907
	Sludge + Soil + Molasses + Nutrients + GW	

Table 4.2.15: Summary of Kinetics for Nitrate Removal in Abiotic, Biotic, and Bio-enhanced Treatments

Treatment	Reaction Rate Constant			Reaction Order & Highest R^2
	0 Order	1st Order	2nd Order	
	$k = \left(\frac{mg}{L}\right) d^{-1}$	$k = d^{-1}$	$k = \left(\frac{mg}{L}\right)^{-1} d^{-1}$	
ZVI 5 g/L + GW	-1.0	-2.0E-3	-2.0E-5	**0** $R^2 = 0.4$
ZVI 8.5 g/L + GW	-14.1	-0.01	-2.0E-4	**0** $R^2 = 0.8$
ZVI 17 g/L + GW	-17.8	-0.06	-0.31	**0** $R^2 = 0.6$
Sludge + Molasses + Nutrients + GW	-13.7	-0.10	-0.56	**1st** $R^2 = 0.8$
Sludge + Soil + Molasses + Nutrients + GW	-17.5	-0.29	-0.03	**1st** $R^2 = 0.9$
Sludge + Molasses + Nutrients + GW(undil)	-22.9	-0.01	-6.0E-5	**1st** $R^2 = 0.8$
ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	-12.8	-0.02	-3.0E-5	**0** $R^2 = 0.6$
ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	-18.4	-0.05	-4.0E-3	**2nd** $R^2 = 0.9$
ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	-2.2	-0.64	-3.71	**2nd** $R^2 = 0.4$

4.3 Chlorate Removal

In Phase 2, abiotic, biotic, and bio-enhanced chlorate removal was compared in the absence and presence of soil (Table 4.3.2). Chlorate removal plateaued for most treatments after 6 days in the absence of soil. Though this could be attributed to low pH in biotic reactors, further investigation did reveal total chlorate removal in biotic reactors with the addition of soil. Removal in biotic and bio-enhanced treatments ranged from 30-35% to 50-55%, respectively. Analysis showed a statistically significant difference between biotic and bio-enhanced reactors, with bio-enhanced reactors showing greater removal rates. Additionally, statistically significant removal was seen in both biotic and bio-enhanced reactors when compared to groundwater controls. A large chlorate increase was seen in abiotic reactors at the end of 8 weeks (Fig. 4.3.1). Current research within the same lab has shown chlorate precipitation by metal ions. The precipitation of chlorate and perchlorate salts by metal ions such as potassium and sodium in

aqueous conditions when solute concentrations are high has been documented during chlorate production for the wood and pulp industry (Wanngard, 1992). However, no chlorate removal was detected in groundwater controls. It is likely this increase was due to variation, as each measurement was taken from individual reactors. However, the extent of this increase should be noted, as it is nearly 200% over the initial chlorate. This increased final reading resulted in no statistically significant difference between abiotic NZVI reduction at 5,000 mg Fe^0/L and groundwater controls (Table 4.3.2).

In the presence of soil (Fig. 4.3.2), complete reduction of chlorate in biotic and bio-enhanced reactors occurred within 13 days. Average chlorate removal rates in the presence of soil under biotic and bio-enhanced conditions only differed slightly (Table 4.3.3). No statistically significant difference was seen between biotic and bio-enhanced reactors in the presence of soil. Additionally, both biotic and bio-enhanced reactors showed statistically significant removal when compared to groundwater controls (Table 4.3.4). Abiotic chlorate reduction with 5,000 mg Fe^0/L also showed statistically significant removal when compared to groundwater controls, with a plateau after 6 days at 40-55% (Fig. 4.3.2). A statistically significant difference was seen between all treatments with soil and all treatments without soil (Table 4.3.5). All treatments showed statistically significant increased removal in the presence of soil. This suggests the presence of chlorate reducing bacteria in the soil. Greater chlorate removal was seen even in abiotic reactors, where no additional nutrients were introduced. Thus, anaerobic chlorate reduction using substrates provided by the NZVI solution is possible. The production of hydrogen gas by NZVI (Gheju et al, 2011, & Reardon, 2014) and chlorate microbial reduction using propylene glycol (Adrian et al, 2007 & Van Ginkel et al, 1995), which is present in the

NZVI solution, might explain the increased removal. Therefore, chlorate reducing bacteria in the soil might not be limited to carbohydrate substrates.

Fig. 4.3.3 shows high variability of results in abiotic NZVI chlorate reduction at doses of 5,000-8,500 mg Fe^0/L with respective mass ratios of 0.73-1.25 mg Fe^0/mg chlorate. The stoichiometric mass ratios for 5,000-8,500 mg Fe^0/L were only 0.36X and 0.62X, respectively (Table 4.3.6). Which means these doses were less than the theoretical stoichiometric dose. Comparing abiotic reduction to groundwater controls, no significant statistically removal by NZVI doses lower than 17,000 mg Fe^0/L was observed (Table 4.3.7). At 17,000 mg Fe^0/L, with a mass ratio of 2.49 mg Fe^0/mg, total chlorate reduction was achieved within 7 days. The stoichiometric mass ratio for 17,000 mg Fe^0/L was 1.24X (Table 4.3.6). At 17,000 mg Fe^0/L, the average reduction rate was 242.46 $mg/L * d$. However, chlorate increased from the detection limit to 2,100 mg/L in abiotic reactors with 17,000 mg Fe^0/L of NZVI at Day 56. This again might be due to variation between reactors. An average correlation between increasing NZVI and chlorate reduction was seen ($R^2 = 0.5$, Fig. 4.3.3A). Previous research on abiotic chlorate reduction by NZVI has shown first order rate constants of k = -75.02 d^{-1}, with average removal rates as high as 8,258 $mg/l * d$ for spiked DI water with a mass ratio of 2.50 mg Fe^0/mg in ambient conditions (Petrucci et al, 2016). Another study performed in this laboratory showed lower rate constants of k = -0.81 d^{-1} to -1.24 d^{-1} and an average rate of 2,730.24 $mg/L * d$ for a mass ratio of 130.03 mg Fe^0/mg for synthetic groundwater using macro-scale ZVI under ambient conditions (Greenhalgh, 2019). Furthermore, another study using macro-scale ZVI showed an average rate of 3,674.0 $mg/L * d$ and a first order rate constant of k = -25.92d^{-1} for spiked DI water with a mass ratio of 21.78 mg Fe^0/mg under ambient conditions (Westerhoff, 2003). In reactors showing statistically significant removal in this study, first order kinetics for abiotic

reactors in Fig. 4.3.3B showed low correlation ($R^2 = 0.2$). Additionally, averages rates were lower than previous studies. Abiotic reactors showing significant reduction showed the highest, albeit low correlation for zero order kinetics ($R^2 = 0.4$, Table 4.3.14), with a rate constant of k = -2,046.7 $mg/l * d$. Overall, doses of 5,000-8,500 mg Fe^0/L were not sufficient to reliably reduce chlorate. It is likely partial reaction of NZVI with chlorate and other contaminants impeded significant removal at 5,000-8,500 mg Fe^0/L. This is due to these doses accounting for low stoichiometric mass ratios (Table 4.3.6). Therefore, chlorate is likely the contaminant that impedes total reduction at NZVI doses of 5,000-8,500 mg Fe^0/L. Passivation of NZVI due to nitrate before reaction with chlorate could also be a significant source of the decrease in NZVI reactivity (Chen et al, 2015 & Luo et al 2010), resulting in low reduction at 5,000-8,500 mg Fe^0/L. Finally, the total abiotic chlorate reduction of chlorate at 17,000 mg Fe^0/L suggests a stoichiometric mass ratio of at least 1.24X is sufficient to remediate chlorate.

In Phase 3, where a richer bacterial sludge was used, biotic reactors containing bacterial sludge alone showed 35% removal after 1 week of treatment (Fig. 4.3.4). However, chlorate increased over the subsequent 8-week testing period. A statistically significant difference was seen between biotic reactors only containing sludge and groundwater controls was observed (Table 4.3.10). However, biotic containing sludge showed increasing chlorate in Phase 3. This unlikely the case of variation between reactors, as this happened consistently in subsequent measurements. The reason for this increase is unknown, but chlorate formation is usually an intermediate byproduct of bacterial perchlorate metabolism under anoxic conditions (Xu et al, 2004), where decreased perchlorate was detected within the first week (Fig 4.4.4). Precipitation and subsequent dissolution of chlorate salts is also a possible reason for this (Wanngard, 1992), as the addition of the molasses solution increased aqueous potassium and sodium, and

subsequent bacterial activity likely dissolved precipitated chlorate salts. A statistically significant difference between biotic chlorate reactors using Phase 2 -3 sludge was found, where moderate biotic removal was achieved in Phase 2, and increasing chlorate was seen in Phase 3 (Table 4.3.10). In the presence of soil, biotic reactors achieved complete chlorate removal within 7 days, with an average rate of 1,116.18 $mg/L * d$. No statistically significant difference was seen between biotic reactors in Phase 2-3 when soil was present (Table 4.3.10). Despite low chlorate detection at Day 21, biotic reactors using sludge alone showed little to no change in chlorate under undiluted conditions (Fig. 4.3.4A). It is likely a chlorate reducing bacterial population from the sludge was able to be established in the undiluted biotic reactor at Day 21, which would result in this decreased reading. Despite the low detection at Day 21, no statistically significant removal was seen between undiluted biotic reactors containing sludge alone and groundwater controls (Table 4.3.10). Previous studies have shown first order kinetics using bacterial sediment from the same FBR reactor, with rate constants of k = -0.031 d^{-1} to -0.110 d^{-1} and an average rate of only 0.221 $mg/l * d$ using synthetic groundwater under ambient conditions (Greenhalgh, 2019). In this, study, first order kinetics showed high correlation ($R^2 = 0.9$) in biotic reactors showing statistically significant removal (Fig. 4.3.4B), with a substantially higher rate constant of k = -1.31 d^{-1}. First order kinetics showed the highest correlation in biotic reactors with significant removal (Table 4.3.14). However, rate constants were not similar to previous research within the same laboratory. Fatty acid substrates were used in Greenhalgh's research, whereas this study used a carbohydrate substrate. It is likely this difference is due to the difference in substrates, as chlorate metabolism is substrate dependent (Van Ginkel et al, 1995). Finally, no chlorate removal in biotic reactors using phase 3 sludge alone presents evidence for bacterial toxicity for chlorate-reducing bacteria by the groundwater

in this study. One cause for this toxicity is the high TDS concentration in the groundwater used in this study of 30,000-50,000 mg/L.

Under bio-enhanced conditions, all doses of NZVI resulted in total chlorate removal within 7 days (Fig. 4.3.5). Average rates for bio-enhanced NZVI at 5,000-8,500 mg Fe^0/L were both 1,116.18 $mg/l * d$. The mass ratios for 5,000-8500 mg Fe^0/L were 0.64 and 1.09 mg Fe^0/mg, respectively. At 17,000 mg Fe^0/L, with a mass ratio of 2.18 mg Fe^0/mg, total chlorate removal was achieved in only 1 day and showed an average rate of 7,813.25 $mg/l * d$. In bio-enhanced reactors, the stoichiometric mass ratios for 5,000, 8,500, and 17,000 mg Fe^0/L were 0.32, 0.54, and 1.08X, respectively (Table 4.3.11). Bio-enhanced reactors in Phase 3 showed a strong correlation ($R^2 = 0.9$) between NZVI and chlorate removal (Fig. 4.3.5A). Though average rates greatly increased with a dose increase of 5,000-17,000 mg Fe^0/L, no statistically significant difference between bio-enhanced treatments was found (Table 4.3.12). Statistically significant removal was found between bio-enhanced treatments and groundwater controls at all doses of NZVI (Table 4.3.12). Previous research has shown first order kinetics in bio-enhanced treatments using macro-scale ZVI and bacterial sediment from the same site, with a rate constant of k = -1.17 d^{-1} and an average rate of 18.8 $mg/l * d$ for a mass ratio of 198.02 mg Fe^0/mg for synthetic groundwater using macro-scale ZVI (Greenhalgh, 2019). In this study, first order kinetics for bio-enhanced reactors in Fig. 4.3.5B show a moderate to high correlation ($R^2 = 0.6 - 0.8$). Though the average rate was much higher, a similar rate constant range of k = -0.7 d^{-1} to -1.00 d^{-1} to Greenhalgh's research was found. However, Greenhalgh's research used macro-scale ZVI, which has lower surface area. Overall, bio-enhanced reactors showed the highest correlation for second order kinetics ($R^2 = 0.7 - 0.9$, Table 4.3.14), with rate constants of k = -0.1 to -0.2 $\left(\frac{mg}{L}\right)^{-1} d^{-1}$.

Comparing abiotic and bio-enhanced chlorate removal, previous results showed no chlorate removal at 5,000-8,500 mg Fe^0/L of NZVI in abiotic reactors. Bio-enhanced reactors showed a statistically significant difference when compared to abiotic reactors, in which only bio-enhanced treatments showed statistically significant removal (Table 4.3.13). At 17,000 mg Fe^0/L, abiotic and bio-enhanced chlorate reactors showed no statistically significant difference. Comparing biotic and bio-enhanced reactors, doses of 8,500-17,000 mg Fe^0/L were statistically different, with bio-enhanced treatments at these doses showing greater rates. Comparing abiotic and biotic reactors, abiotic chlorate reduction only achieved statistically similar results to biotic removal at NZVI doses of 17,000 mg Fe^0/L, with lower doses showing no statistically significant removal.

Overall, results from this study show:

- The addition of soil will result in higher chlorate removal across all treatments.
- Abiotic reduction of chlorate using NZVI only showed statistically significant reduction at 17,000 mg Fe^0/L.
- Bio-enhanced treatments achieved the highest removal efficiency. However, at least 8,500 mg Fe^0/L of NZVI was needed to achieve higher efficiency than biotic treatments alone.
- Biotic chlorate removal by bacterial sludge alone showed dubious results. Only biotic treatments in the presence of soil achieved consistent chlorate removal, which suggests using soil in biotic treatments.

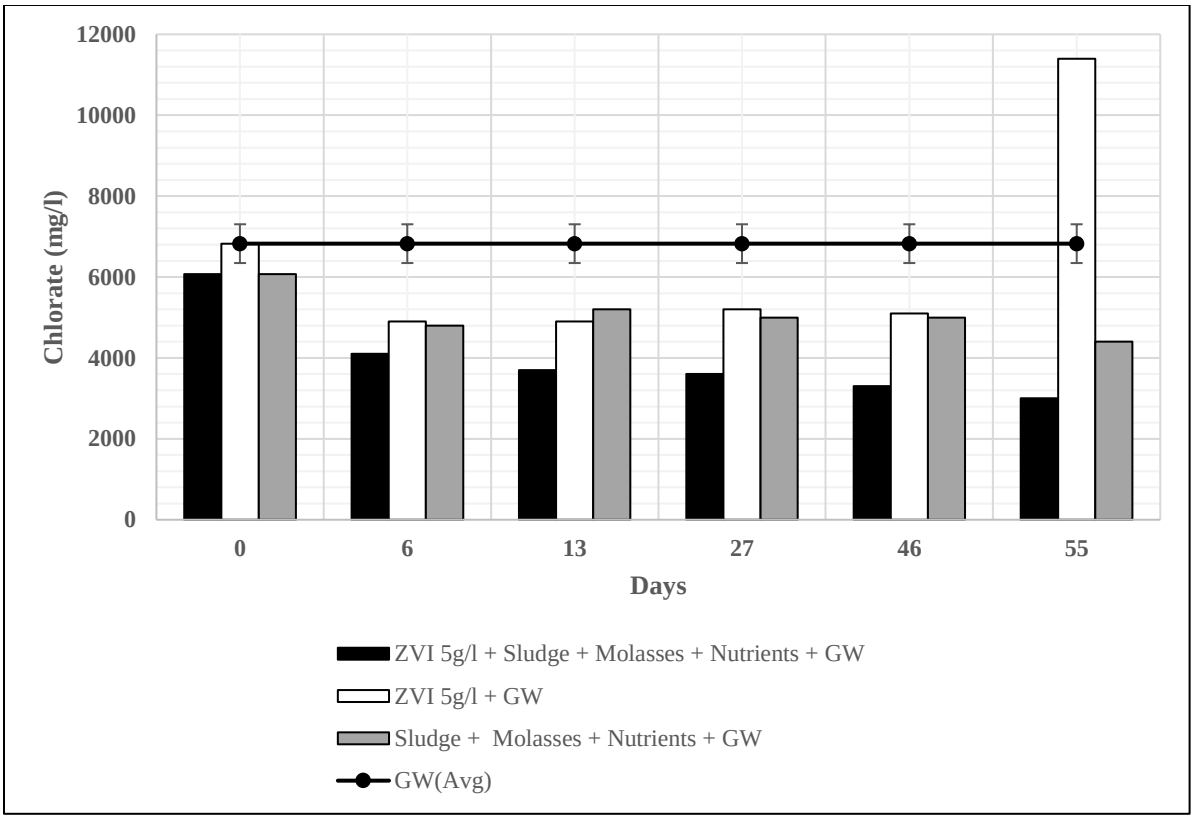


Figure 4.3.1: Phase 2: Abiotic, Biotic, and Bio-enhanced Chlorate Removal in the Absence of Soil at Stoichiometric Mass Ratios of 0.41X (5 g/L), and 0.35X (5 g/L + Sludge + Nutrients)

Table 4.3.1: Chlorate Removal Rates for Abiotic, Biotic, and Bio-enhanced Reactors in the Absence of Soil

Treatment	Average Rate (mg/L*d)	Chlorate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	65.54	0.35X
ZVI 5 g/L + GW	No Change	0.41X
Sludge + Molasses + Nutrients + GW	44.10	0X

Table 4.3.2: Summary of Two Factor ANOVA for Phase 2: Abiotic, Biotic, and Bio-enhanced Chlorate Removal in the Absence of Soil

Description	ANOVA Analysis Between:	P-Value
Determination of significant change in NZVI reduction due to bio-enhancement.	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.018
	ZVI 5 g/L + GW	
Determination of significant change in biotic treatment due to the absence of NZVI	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.008
	Sludge + Molasses + Nutrients + GW	
Determination of significant change between biotic and abiotic reactors with NZVI	Sludge + Molasses + Nutrients + GW	0.003
	ZVI 5g/L + GW	
Determination of significant removal due to bio-enhanced NZVI	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.007
	GW	
Determination of significant biotic removal	Sludge + Molasses + Nutrients + GW	0.024
	GW	
Determination of significant abiotic reduction by NZVI	ZVI 5 g/L + GW	0.861
	GW	

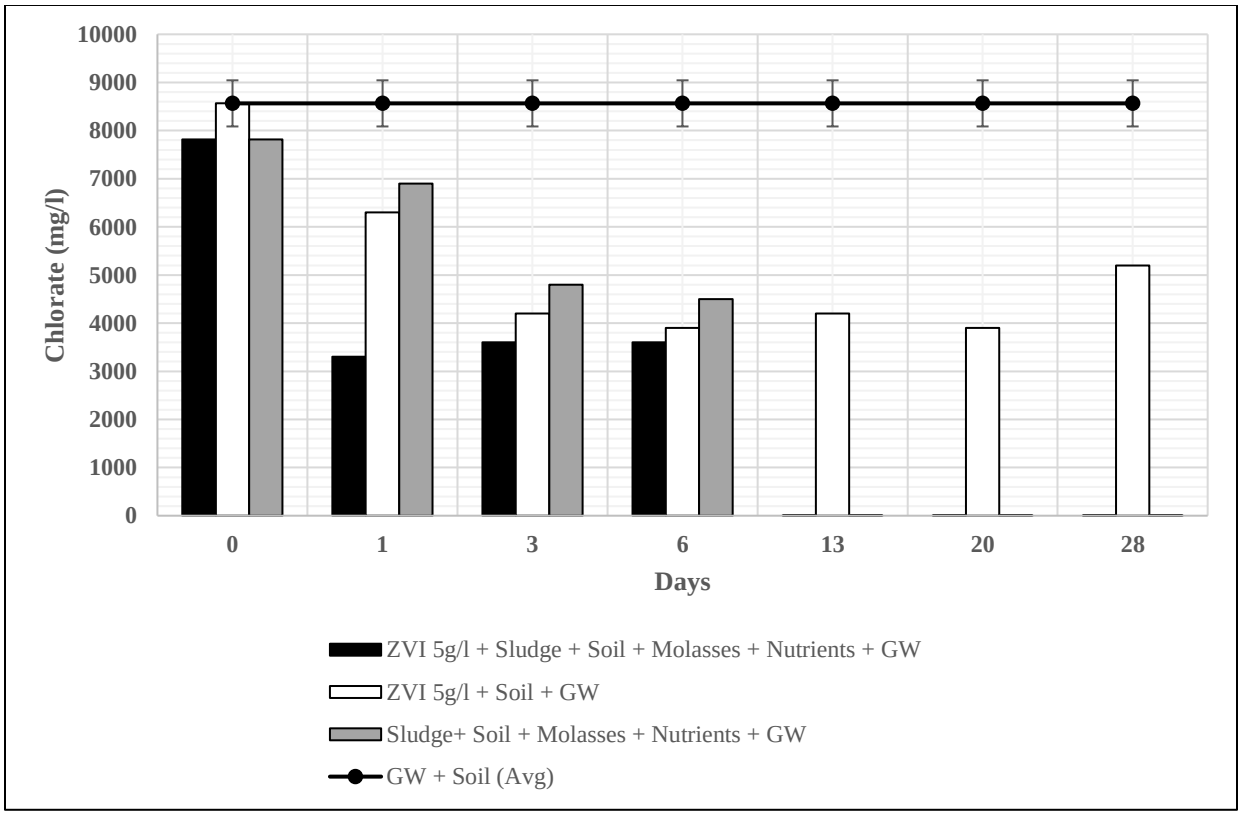


Figure 4.3.2: Phase 2: Abiotic, Biotic and Bio-enhanced Chlorate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 0.32X (5 g/L + Soil), and 0.29X (5 g/L + Sludge + Soil + Molasses + Nutrients)

Table 4.3.3: Chlorate Removal Rates for Abiotic, Biotic and Bio-enhanced Reactors in the Presence of Soil

Treatment	Average Rate (mg/L*d)	Chlorate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	601.04	0.32X
ZVI 5 g/L + Soil + GW	195.0	0.29X
Sludge+ Soil + Molasses + Nutrients + GW	601.20	0X

Table 4.3.4: Summary of Two Factor ANOVA for Phase 2: Abiotic, Biotic and Bio-enhanced Chlorate Removal in the Presence of Soil

Description	ANOVA Analysis Between	P-Value
Determination of significant change in NZVI Removal due to bio-enhancement.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.033
	ZVI 5 g/L + Soil + GW	
Determination of significant change in biotic treatment due to the absence of NZVI.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.156
	Sludge+ Soil + Molasses + Nutrients + GW	
Determination of significant change between biotic and abiotic reactors with NZVI.	Sludge+ Soil + Molasses + Nutrients + GW	0.051
	ZVI 5g/L + Soil + GW	
Determination of significant change due to bio-enhanced NZVI removal.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.003
	GW + Soil	
Determination of significant biotic removal.	Sludge+ Soil + Molasses + Nutrients + GW	0.016
	GW + Soil	
Determination of significant abiotic reduction by NZVI.	ZVI 5 g/L + Soil + GW	0.002
	GW + Soil	

Table 4.3.5: Summary of Two Factor ANOVA in Phase 2 to Determine Significant Difference in Chlorate Removal due to the Addition of Soil

Description	ANOVA Analysis Between	P-Value
Determination of significant change due to addition of soil in bio-enhanced treatments.	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.054
	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change due to addition of soil in abiotic NZVI reduction.	ZVI 5 g/L + GW	0.017
	ZVI 5 g/L + Soil + GW	
Determination of significant change due to addition of soil in biotic treatments.	Sludge + Molasses + Nutrients + GW	0.040
	Sludge+ Soil + Molasses + Nutrients + GW	
Determination of significant change due to addition of soil in groundwater controls	GW	0..201
	GW + Soil	

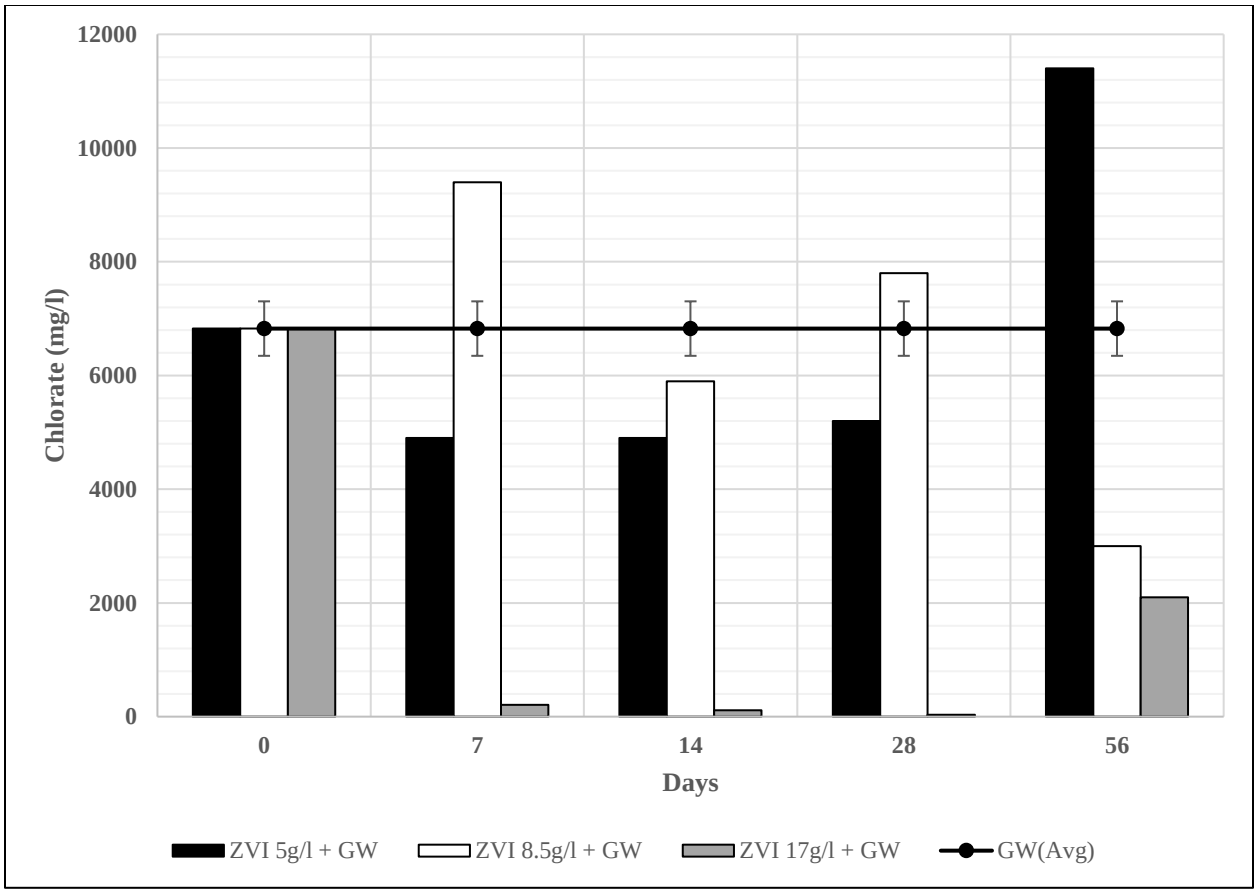


Figure 4.3.3: Abiotic NZVI Chlorate Reduction in the Absence of Soil at Stoichiometric Mass Ratios of 0.36X (5 g/L), 0.62X (8.5 g/L), and 1.24X (17 g/L)

Table 4.3.6: Chlorate Reduction Rates for Abiotic Reactors in the Absence of Soil

Treatment	Average Rate (mg/L*d)	Chlorate Mass Ratio mg Fe^0/mg	Chlorate Stoichiometric Mass Ratio
ZVI 5 g/L + GW	No Change	0.73	0.36X
ZVI 8.5 g/L + GW	No Change	1.25	0.62X
ZVI 17 g/L + GW	975.00	2.49	1.24X

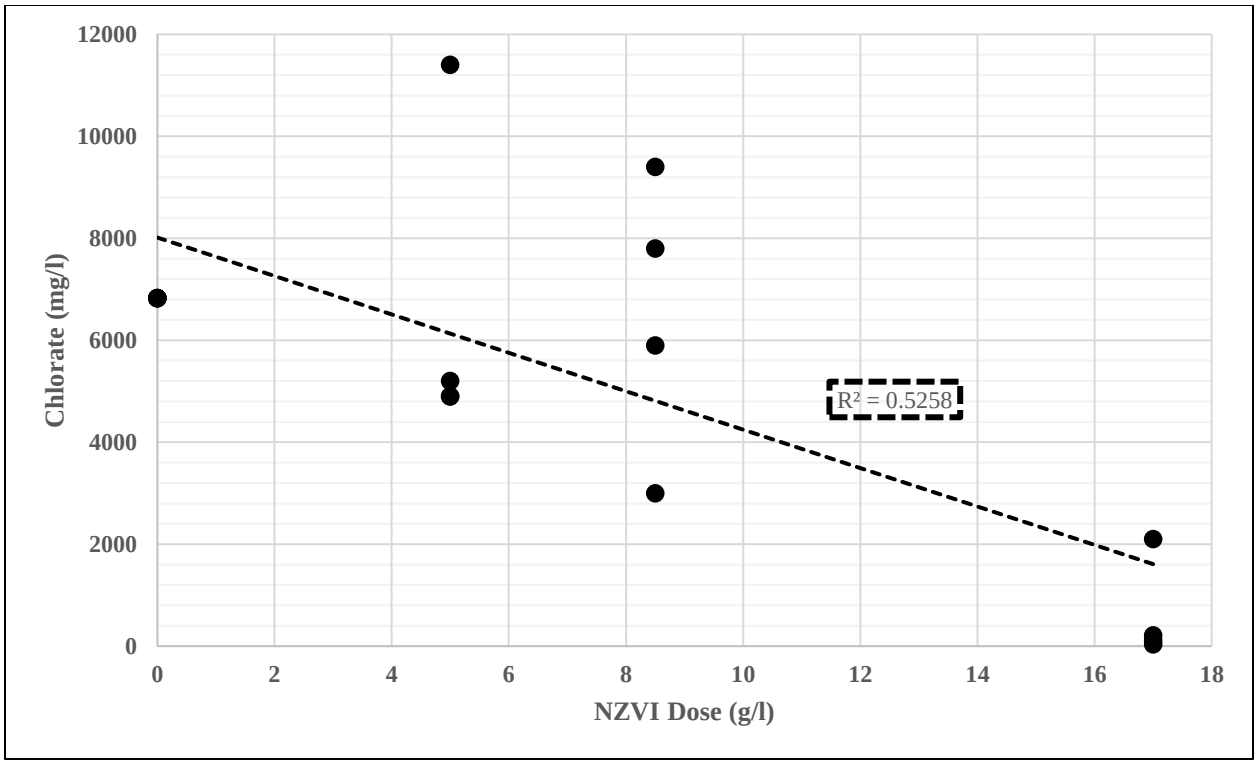


Figure 4.3.3A: Correlation between Chlorate Reduction and NZVI concentration

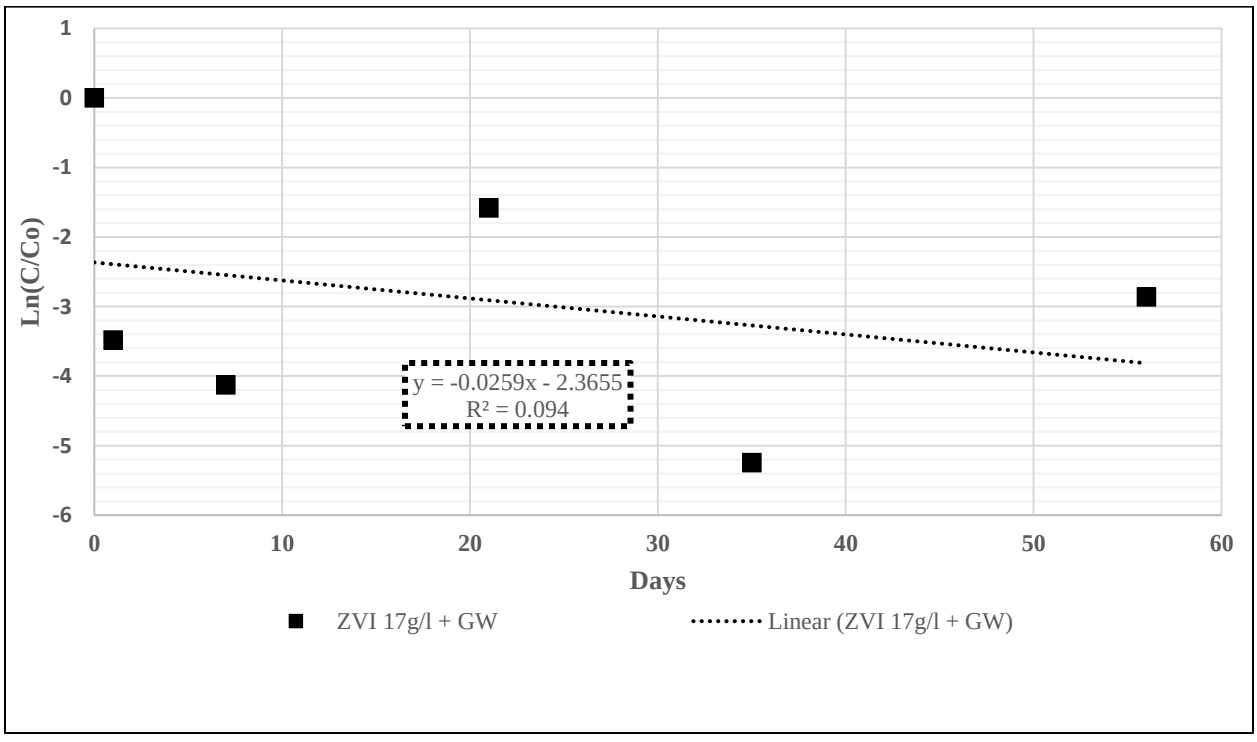


Figure 4.3.3B: First Order Kinetics for Abiotic Chlorate Reduction in the Absence of Soil at a Stoichiometric Mass Ratio of 1.24X (17 g/L)

Table 4.3.7: Summary of Two Factor ANOVA to Determine Significant Difference in Abiotic Chlorate Reduction due to increase in NZVI

Description	ANOVA Analysis Between	P-Value
Determination of significant change in abiotic reduction due to 1.7X increase in NZVI	ZVI 5 g/L + GW	0.993
	ZVI 8.5 g/L + GW	
Determination of significant change in abiotic reduction due to 3.3X increase in NZVI	ZVI 5 g/L + GW	0.011
	ZVI 17 g/L + GW	
Determination of significant abiotic reduction by NZVI	ZVI 5 g/L + GW	0.861
	GW	
	ZVI 8.5 g/L + GW	0.581
	GW	
	ZVI 17 g/L + GW	0.017
	GW	

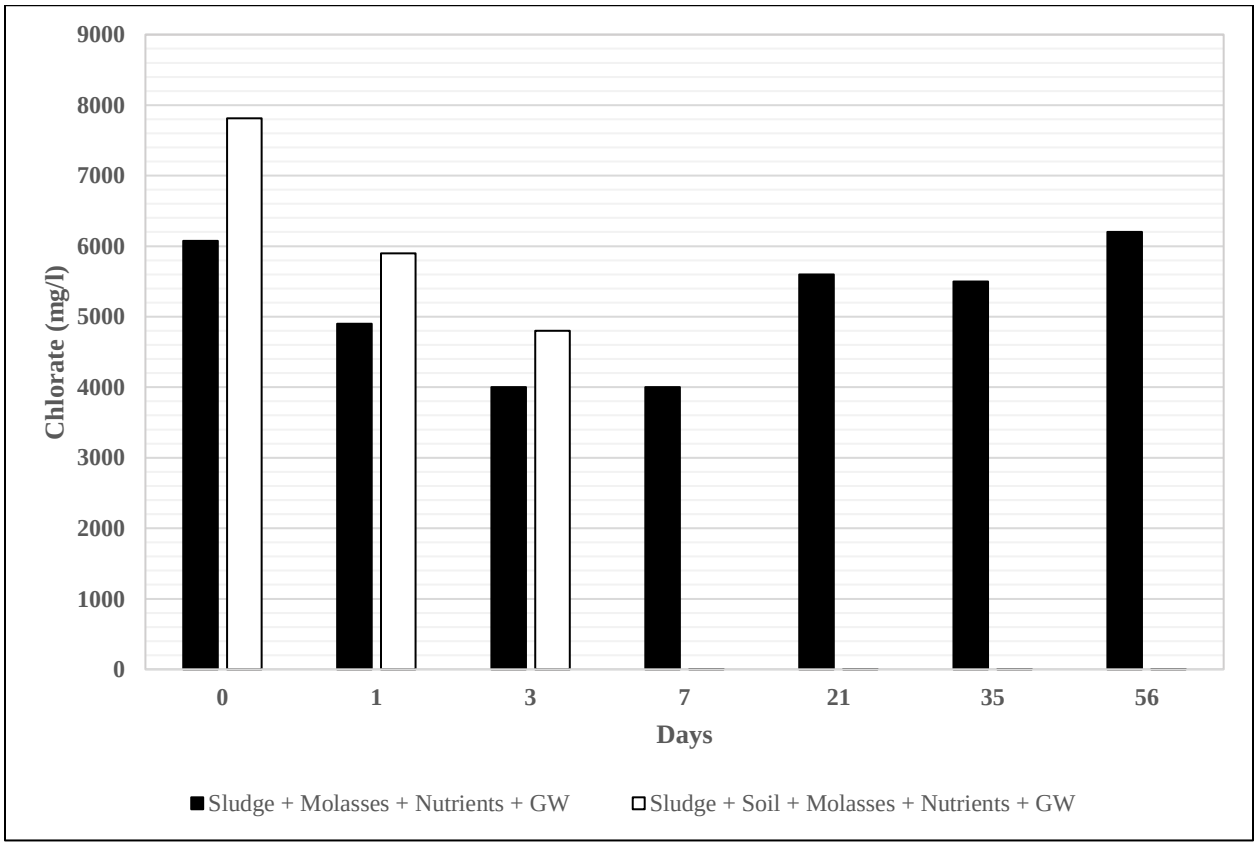


Figure 4.3.4: Diluted Biotic Chlorate Removal in the Presence and Absence of Soil Using Phase 3 Sludge

Table 4.3.8: Chlorate Removal Rates for Biotic Reactors in the Presence and Absence of Soil Using Phase 3 Sludge

Treatment	Average Rate (mg/L*d)
Sludge + Molasses + Nutrients + GW	No Change
Sludge + Soil + Molasses + Nutrients + GW	1,116.18

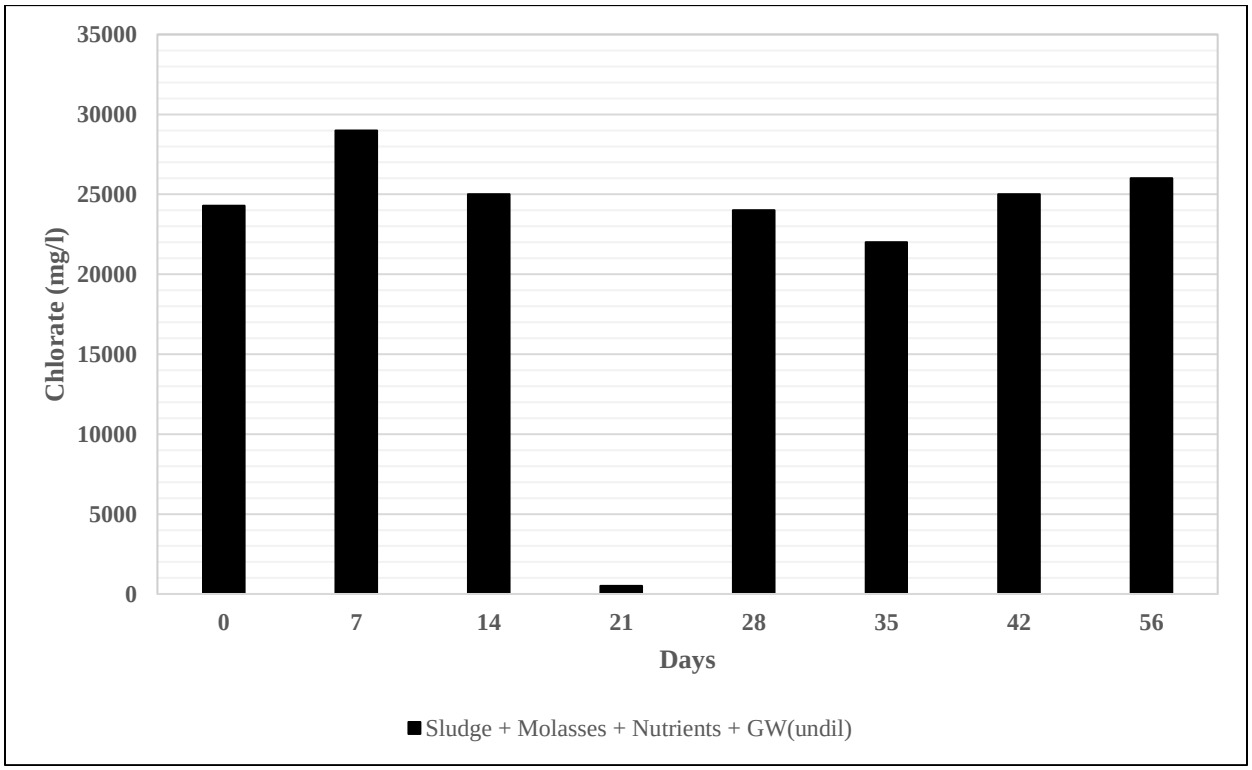


Figure 4.3.4A: Undiluted Biotic Chlorate Removal in the Absence of Soil using Phase 3 sludge

Table 4.3.9: Chlorate Removal Rates for Undiluted Biotic Reactors in the Absence of Soil using Phase 3 sludge

Treatment	Average Rate (mg/L*d)
Sludge + Molasses + Nutrients + GW(undil)	No Change

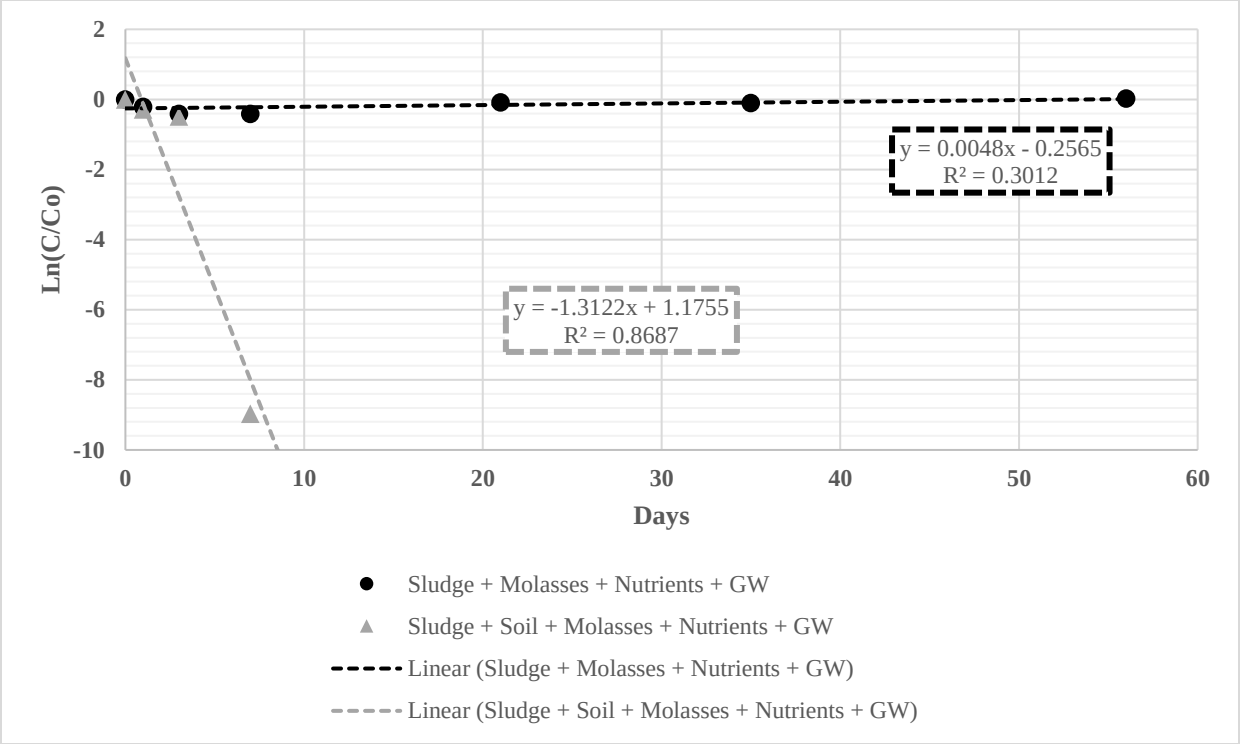


Figure 4.3.4B: First Order Kinetics for Biotic Chlorate Removal in the Presence of Soil using Phase 3 Sludge

Table 4.3.10: Summary of Two Factor ANOVA in to Determine Significant Removal in Biotic Chlorate Reactors in Phase 3

Description	ANOVA Analysis Between	P-Value
Determination of significant change in biotic reactors between Phase 2 and Phase 3 enriched sludge.	Sludge (Phase 2) + Molasses + Nutrients + GW	0.038
	Sludge (Phase 3) + Molasses + Nutrients + GW	
Determination of significant change in biotic reactors between Phase 2 and Phase 3 enriched sludge in the presence of soil.	Sludge (Phase 2) + Soil + Molasses + Nutrients + GW	0.275
	Sludge (Phase 3) + Soil + Molasses + Nutrients + GW	
Determination of significant change in biotic reactors due the addition of soil.	Sludge (Phase 3) + Molasses + Nutrients + GW	0.026
	Sludge + Soil + Molasses + Nutrients + GW (Phase 3)	
Determination of significant biotic removal using Phase 3 sludge in diluted and undiluted conditions.	Sludge (Phase 3) + Molasses + Nutrients + GW	0.028
	GW	
	Sludge (Phase 3) + Soil + Molasses + Nutrients + GW	0.008
	GW + Soil	
	Sludge (Phase 3) + Molasses + Nutrients + GW(undil)	0.477
	GW	

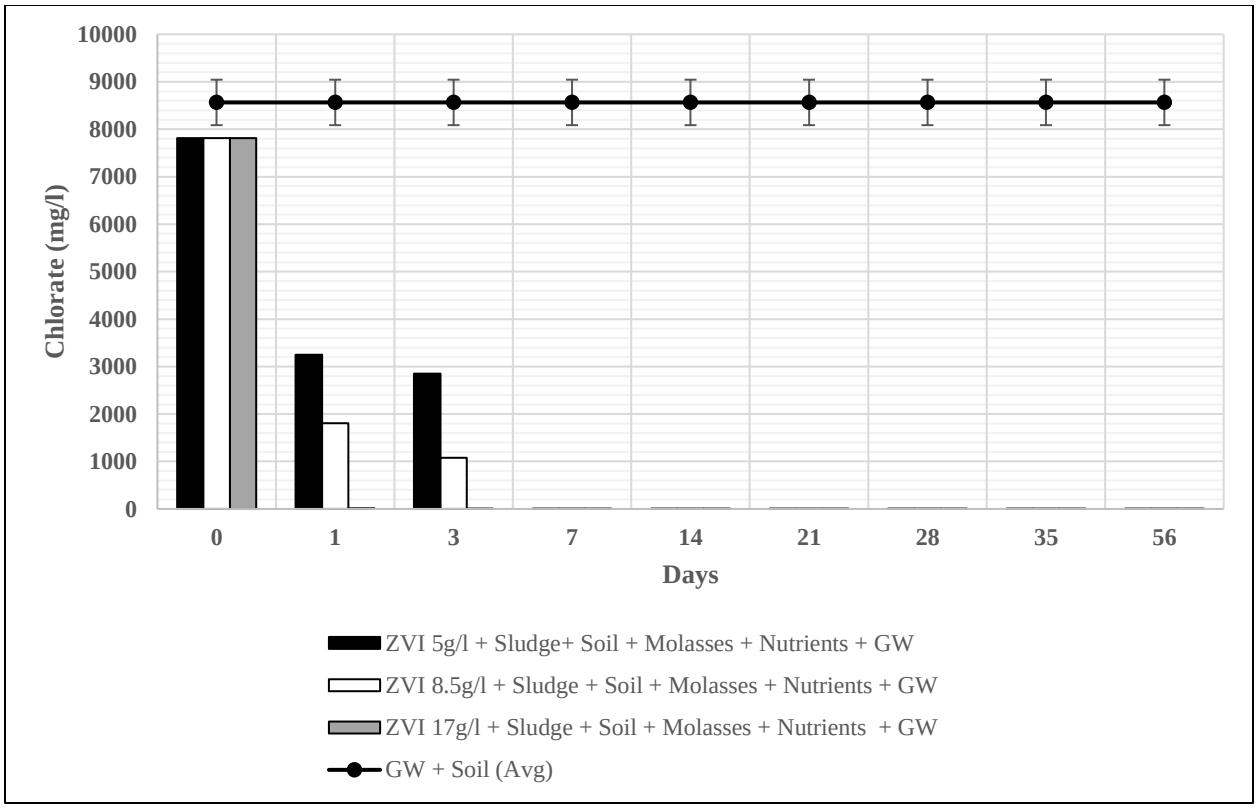


Figure 4.3.5: Phase 3: Phase 3: Effects of Increasing NZVI on Bio-enhanced Chlorate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 0.32X (5 g/L), 0.54X (8.5 g/L), and 1.08X (17 g/L)

Table 4.3.11: Chlorate Removal Rates for Bio-enhanced Reactors in the Presence of Soil

Treatment	Average Rate (mg/L*d)	Chlorate Mass Ratio mg Fe^0/mg	Chlorate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	1,116.18	0.64	0.32X
ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	1,116.18	1.09	0.54X
ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	7,813.25	2.18	1.08X

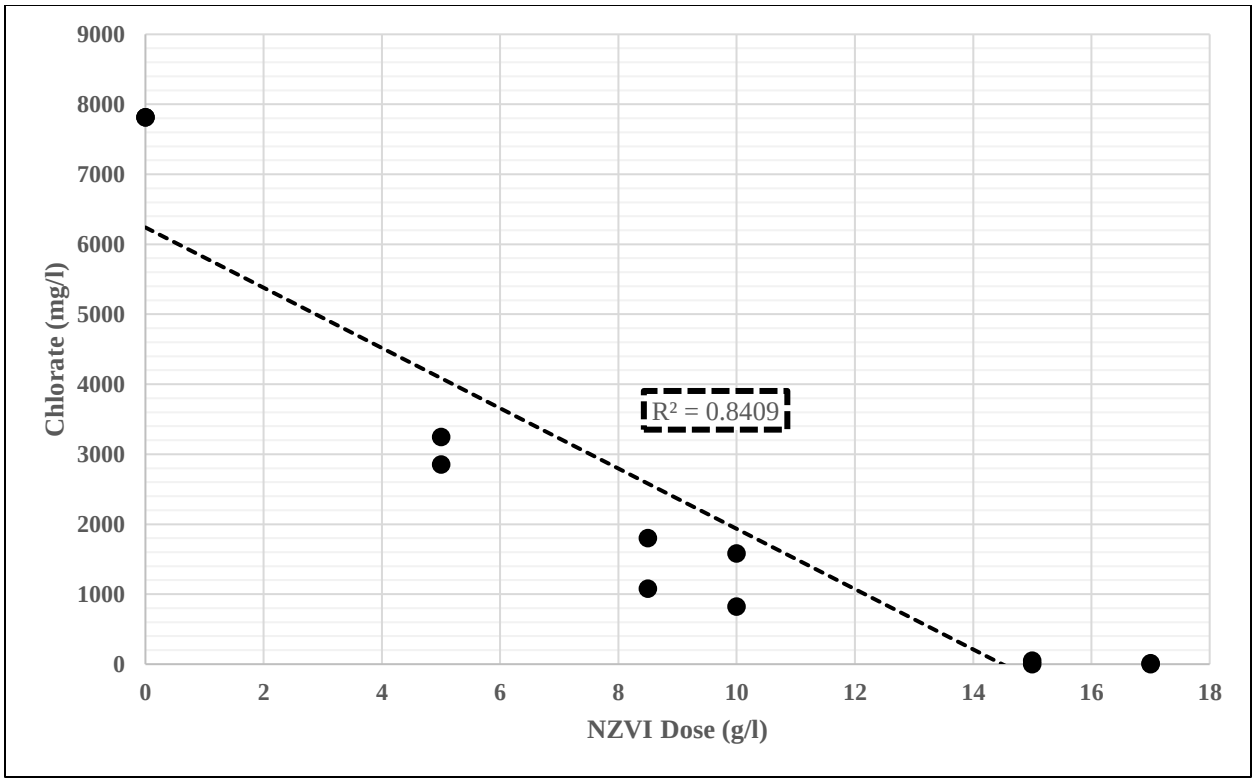


Figure 4.3.5A: Correlation between Chlorate Removal and NZVI concentration under Bio-enhanced conditions in the presence of soil

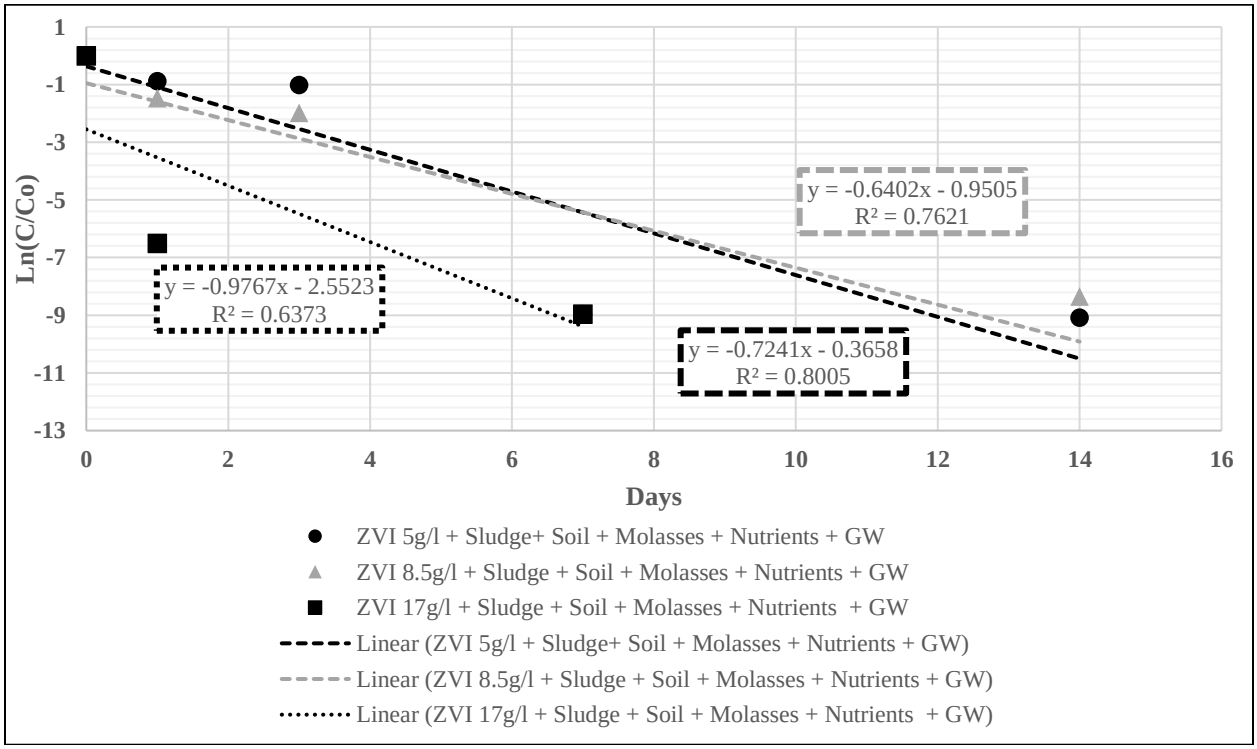


Figure 4.3.5B: First Order Kinetics for Phase 3: Effects of Increasing NZVI on Bio-enhanced Chlorate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 0.32X (5 g/L), 0.54X (8.5 g/L), and 1.08X (17 g/L)

Table 4.3.12: Summary of Two Factor ANOVA to Determine Significant Difference in Bio-enhanced Chlorate Removal due to increase in NZVI in Phase 3

Description	ANOVA Analysis Between	P-Value
Determination of significant change in bio-enhanced removal due to 1.7X increase in NZVI	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.188
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change in bio-enhanced removal due to 3.3X increase in NZVI	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.185
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant removal between bio-enhanced treatments and groundwater controls.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	1.20E-04
	GW + Soil	
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	5.75E-05
	GW + Soil	
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	4.53E-05
	GW + Soil	

Table 4.3.13: Summary of Two Factor ANOVA Comparing Abiotic, Biotic and Bio-enhanced Chlorate Removal in Phase 3

Description	ANOVA Analysis Between	P-Value
Determination of significant change between bio-enhanced and abiotic reactors.	ZVI 5g/L + Sludge+ Soil + Molasses + Nutrients + GW	0.016
	ZVI 5g/L + GW	
	ZVI 8.5g/L + Sludge + Soil + Molasses + Nutrients + GW	0.004
	ZVI 8.5g/L + GW	
	ZVI 17g/L + Sludge + Soil + Molasses + Nutrients + GW	0.097
	ZVI 17g/L + GW	
Determination of significant change between bio-enhanced and biotic reactors.	ZVI 5g/L + Sludge+ Soil + Molasses + Nutrients + GW	0.097
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 8.5g/L + Sludge + Soil + Molasses + Nutrients + GW	0.030
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 17g/L + Sludge + Soil + Molasses + Nutrients + GW	0.054
	Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change between abiotic and biotic reactors.	ZVI 5g/L + GW	0.044
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 8.5g/L + GW	0.039
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 17g/L + GW	0.110
	Sludge + Soil + Molasses + Nutrients + GW	

Table 4.3.14: Summary of Kinetics for Chlorate Removal in Abiotic, Biotic, and Bio-enhanced Treatments

Treatment	Reaction Rate Constant			Reaction Order & Highest R^2
	0 Order	1st Order	2nd Order	
	$k = \left(\frac{mg}{L}\right) d^{-1}$	$k = d^{-1}$	$k = \left(\frac{mg}{L}\right)^{-1} d^{-1}$	
ZVI 17g/L + GW	-2,046.7	-0.03	-1.0E-4	**0** $R^2 = 0.4$
Sludge + Soil + Molasses + Nutrients + GW	113.5	5.0E-3	1.0E-6	**1st** $R^2 = 0.3$
Sludge + Soil + Molasses + Nutrients + GW	-1,429.9	-1.3	-0.05	**1st** $R^2 = 0.9$
ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	-2,833.0	-0.7	-0.1	**2nd** $R^2 = 0.9$
ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	-2,416.5	-0.6	-0.1	**2nd** $R^2 = 0.8$
ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	-3,906.6	-1.0	-0.2	**2nd** $R^2 = 0.7$

4.4 Perchlorate Removal

In Phase 2, abiotic, biotic, and bio-enhanced perchlorate removal was compared in the absence and presence of soil (Fig. 4.4.1). In the absence of soil, both biotic and bio-enhanced reactors achieved 30-35% removal after 8 weeks. No statistically significant difference between biotic and bio-enhanced treatments was found. Both biotic and bio-enhanced treatments showed statistically significant removal when compared to groundwater controls. Abiotic reactors containing 5,000 mg Fe^0/L of NZVI showed increased perchlorate at Day 46. This might be due to reactor measurement variation. However, lower perchlorate concentrations observed at all other testing times. Despite this, no statistically significant removal was observed between abiotic reactors and groundwater controls (Table 4.4.2). Furthermore, no statistically significant difference between any treatments was found. As a result, the data on abiotic perchlorate reduction by NZVI are inconclusive.

With the addition of soil, removal rates were higher in biotic and bio-enhanced reactors (Fig. 4.4.2). Biotic reactors showed higher average removal levels than bio-enhanced reactors. No statistically significant difference between biotic and bio-enhanced treatments was found in

the presence of soil (Table 4.4.4). When comparing biotic and bio-enhanced reactors to groundwater controls, statistically significant removal was found. In abiotic reactors containing 5,000 mg Fe^0/L of NZVI, increased perchlorate was found at Day 13 (Fig.4.4.2). Again, this increase is likely the result of variation due to using different reactors at each measurement. Though showing reduced perchlorate at all other testing times, no statistically significant removal between abiotic reactors and groundwater controls was found. This again makes the efficacy of abiotic perchlorate reduction using 5,000 mg Fe^0/L of NZVI inconclusive (Table 4.4.4). Despite the increase in average rates in biotic and bio-enhanced reactors, similar removal at 30-35% was found by biotic and bio-enhanced treatments at the end of the testing period. This resulted in no statistically significant difference due to the addition of soil (Table 4.4.5). Though the low decrease in pH in biotic and bio-enhanced reactors might have resulted in limited removal, chlorate removal was highly successful with the addition of soil. Perchlorate and chlorate-reducing bacteria normally share common environments (Nozawa-Inoue et al, 2005 & Xu et al, 2004). As a result, environmental factors affecting chlorate-reducing are likely to affect perchlorate-reducing bacteria, which was not the case in this study.

Results from Phase 2 showed inconclusive results for perchlorate reduction by NZVI at a dose of 5,000 mg Fe^0/L at a mass ratio of 5.49 mg Fe^0/mg perchlorate. However, no reduction was seen in abiotic reactors containing higher NZVI doses of 8,500-17,000 mg Fe^0/L, with mass ratios of 9.34-18.68 mg Fe^0/mg (Fig. 4.4.3). The stoichiometric mass ratios for 5,000, 8,500, and 17,000 mg Fe^0/L were 2.44X, 4.15X, and 8.30X greater than the theoretical stoichiometric dose, respectively (Table 4.4.6). No statistically significant reduction was found between abiotic reactors and groundwater controls at any dose of NZVI (Table 4.4.7). Abiotic reactors showed no correlation ($R^2 = 2.0E-3$) between increasing NZVI and decreasing perchlorate (Fig.

4.4.3A). Therefore, perchlorate reduction using NZVI is unlikely. Previous research has shown limited reduction of perchlorate by NZVI in spiked industrial groundwater with an average rate of only 0.02 $mg/L * d$ at a mass ratio of 20 mg Fe^0/mg under ambient conditions (Schaefer et al, 2007). In another study, NZVI at a mass ratio of 100 mg Fe^0/mg showed limited reduction with average removal rates of 14 $mg/L * d$ using spiked DI water and 30^oC (Petrucci et al, 2016). First order kinetics were not performed in Schaefer's and Petrucci's studies. Though the highest dose NZVI dose used in this study (18.68 mg Fe^0/mg) is similar to Schaefer's dose in contaminated wastewater, NZVI was not successful at reducing any amount of perchlorate. This suggests passivation by nitrate (Chen et al, 2013 & Luo et al, 2010) and depletion by reaction with other contaminants, necessitating higher doses to reduce perchlorate abiotically.

Biotic reactors in Phase 3, where a richer bacterial sludge was used, show an initial perchlorate removal of 30% in just 1 day in reactors containing sludge alone (Fig. 4.4.4). Subsequent monitoring showed increasing perchlorate over the 8-week testing period. This unlikely the case of variation between reactors, as this happened consistently in subsequent measurements. Like chlorate, it is likely that perchlorate is precipitated as salt compounds due to increased metal ion concentration (Wanngard, 1992) resulting from the addition of molasses. If this is true, subsequent dissolution by bacterial activity would increase perchlorate concentration after the initial decrease. This trend also persisted in biotic reactors only containing sludge under undiluted conditions (Fig. 4.4.4A), showing 15% decreased perchlorate at Day 14, and increasing perchlorate over the remainder of the testing period. No statistically significant removal between biotic reactors using Phase 3 sludge alone and groundwater controls was found (Table 4.4.10). With the addition of soil, biotic reactors in Phase 3 had a similar trend, showing 50% removal at Day 21 followed by a subsequent increase in perchlorate (Fig. 4.4.4). Despite

the subsequent increase, this showed statistically significant removal when compared to groundwater controls (Table 4.4.10), with an average rate of 2.67 $mg/L * d$. Comparing Phases 2-3, a statistically significant difference between biotic reactors only containing sludge was found (Table 4.4.10). Phase 2 sludge achieved statistically significant removal, while sludge in Phase 3 did not. However, differences between Phase 2-3 sludge were mitigated with the addition of soil, where no statistically significant difference between biotic treatments was found (Table 4.4.10). This suggests bacterial activity in the soil might augment perchlorate removal. Previous research has shown bacterial perchlorate removal is substrate-dependent (Miller and Logan, 2000, & Shaefer et al, 2007). An average rate of 331.2 $mg/L * d$ was shown in a previous study using carbon dioxide and hydrogen gas are used as substrates under ambient conditions (Miller and Logan, 2000). Other studies have shown perchlorate removal in wastewater using molasses and bacterial sediment, with an average rate of 26.25 $mg/L * d$ (Wu et al, 2001). However, the molasses dose in Wu's study was much higher, at 300 ml/L. First order kinetics were not shown in Wu's study. This study only used 20 ml/L, but biotic reactors with statistically significant removal showed average rates of 2.67-12.48 $mg/L * d$. First order kinetics for biotic removal in this study did not show correlation in biotic reactors showing statistically significant removal ($R^2 = 0.01$, Fig. 4.4.4B). Zero order kinetics showed the highest correlation for biotic removal. However, this correlation was still very low ($R^2 = 0.02$, Table. 4.4.14).

Bio-enhanced reactors showed limited perchlorate removal for NZVI doses of 5,000, 8,500, and 17,000 mg Fe^0/L with mass ratios of 4.95, 8.44, and 16.87 mg Fe^0/mg. Removal was similar at all NZVI doses, ranging from 25-30% at Day 56, though little additional removal was seen after Day 1 (Fig. 4.4.5). The average removal rates for 5,000 mg Fe^0/L, 8,500 mg Fe^0/L,

and 17,000 mg Fe^0/L were 4.46, 3.39, and 7.68 $mg/L * d$, respectively. In bio-enhanced reactors, the stoichiometric mass ratios for 5,000, 8,500, and 17,000 mg Fe^0/L were 2.20X, 3.75X, and 7.50X, respectively (Table 4.4.11). However, a high correlation ($R^2 = 0.8$) between increasing NZVI and perchlorate removal was seen under bio-enhanced conditions (4.4.5A). Statistically significant removal was seen when comparing bio-enhanced reactors to groundwater controls (Table 4.4.12). No statistically significant difference was seen between any bio-enhanced reactors. Previous research has shown the generation of hydrogen gas due to ZVI oxidation resulted in complete perchlorate removal in a bio-enhanced anaerobic reactor with NZVI (Son et al, 2006). Son's study showed complete perchlorate removal in batch reactors using dry bacterial sludge and macro-scale ZVI with a higher mass ratio of 123 mg Fe^0/mg. Son's research also showed ZVI does not directly reduce perchlorate, rather ZVI provides hydrogen gas as an electron donor for bacterial perchlorate metabolism and showed a moderate first order correlation ($R^2 = 0.7$), with a rate constant of k = $18.96 d^{-1}$ and an average removal rate of 204 $mg/L * d$ at a mass ratio of 30.8 mg Fe^0/mg under ambient conditions using macro-scale NZVI. First order kinetics did not show high correlation for bio-enhanced reactors in this study ($R^2 = 0.01 - 0.20$, Fig. 4.4.5B). Though bio-enhanced removal showed the highest correlation for second order kinetics, this correlation was still low ($R^2 = 0.12 - 0.44$, Table. 4.4.14). It is likely not enough NZVI was used to stimulate bacterial perchlorate reduction, as previous studies used higher doses. Passivation by bacteria onto the surface of NZVI particles (Chen et al, 2013 & Yu et al, 2007) could have resulted in competition for hydrogen gas for reduction of bacterial perchlorate. This explain the limited removal in bio-enhanced reactors.

Comparing abiotic and bio-enhanced treatments, a statistically significant difference between bio-enhanced and abiotic reactors as seen (Table 4.4.13), with abiotic reactors showing

no statistically significant removal. Comparing biotic and bio-enhanced treatments, no statistically significant difference between biotic and bio-enhanced reactors was found. This fits previous research, where bio-enhancement of ZVI showed similar results to biotic controls (Son, 2006). Finally, no statistically significant difference between biotic and abiotic reactors with 5,000 mg Fe^0/L was found. It is likely this happened due to the decreased readings shown by abiotic reactors with 5,000 mg Fe^0/L, resulting in false positives. This is mitigated by the comparisons between biotic reactors and abiotic reactors with 8,500-17,00 mg Fe^0/L, where NZVI yielded no statistically significant removal. This means bacterial activity is main contributor in bio-enhanced perchlorate removal.

Overall, results from this study show:

- The presence of soil did not statistically influence perchlorate removal in any treatment.
- Despite initially showing statistically significant removal at 5,000 mg Fe^0/L, abiotic perchlorate reduction was not effective at higher doses. Higher doses than 17,000 mg Fe^0/L of NZVI are needed, as much of the NZVI added was likely depleted by other contaminants.
- Both biotic and bio-enhanced treatments achieved limited perchlorate removal. Due to NZVI mostly showing no perchlorate reduction, it is likely removal under bio-enhanced conditions is more dependent on microbial activity.
- Though biotic perchlorate removal was possible, using sludge alone showed inconsistent results. The presence of soil in biotic reactors yielded more consistent removal, suggesting some augmentation with soil addition is still possible.

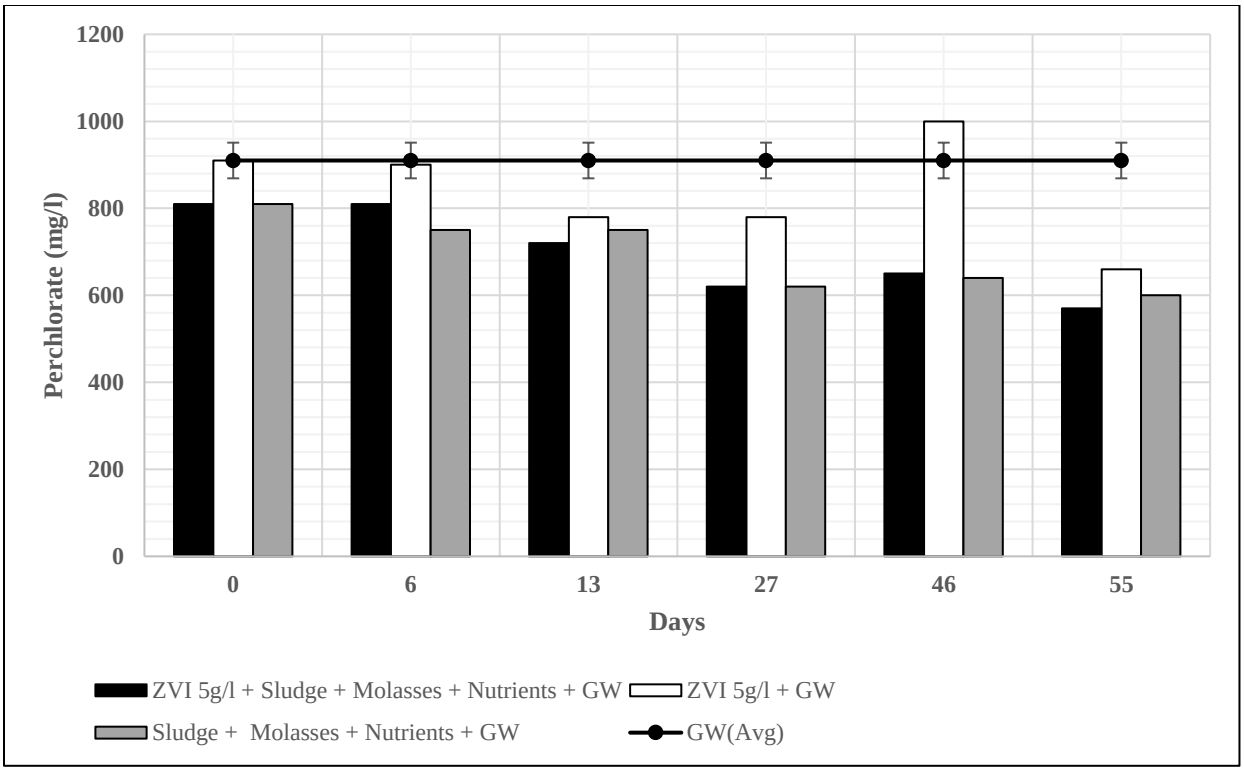


Figure 4.4.1: Phase 2: Abiotic, Biotic, and Bio-enhanced Perchlorate Removal in the Absence of Soil at Stoichiometric Mass Ratios of 2.74X (5 g/L), and 0.2.44X (5 g/L + Sludge + Nutrients)

Table 4.4.1: Perchlorate Removal Rates for Abiotic, Biotic and Bio-enhanced Reactors in the Absence of Soil

Treatment	Average Rate (mg/L*d)	Perchlorate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	4.29	2.44X
ZVI 5 g/L + GW	No Change	2.74X
Sludge + Molasses + Nutrients + GW	3.75	0X

Table 4.4.2: Summary of Two Factor ANOVA for Phase 2: Abiotic, Biotic, and Bio-enhanced Perchlorate Removal in the Absence of Soil

Description	ANOVA Analysis Between:	P-Value
Determination of significant change in NZVI reduction due to bio-enhancement.	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.278
	ZVI 5g/L + GW	
Determination of significant change in biotic treatment due to the absence of NZVI	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.907
	Sludge + Molasses + Nutrients + GW	
Determination of significant change between biotic and abiotic reactors with NZVI	Sludge + Molasses + Nutrients + GW	0.298
	ZVI 5 g/L + GW	
Determination of significant removal due to bio-enhanced NZVI	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.031
	GW	
Determination of significant biotic removal	Sludge + Molasses + Nutrients + GW	0.016
	GW	
Determination of significant abiotic reduction by NZVI	ZVI 5 g/L + GW	0.144
	GW	

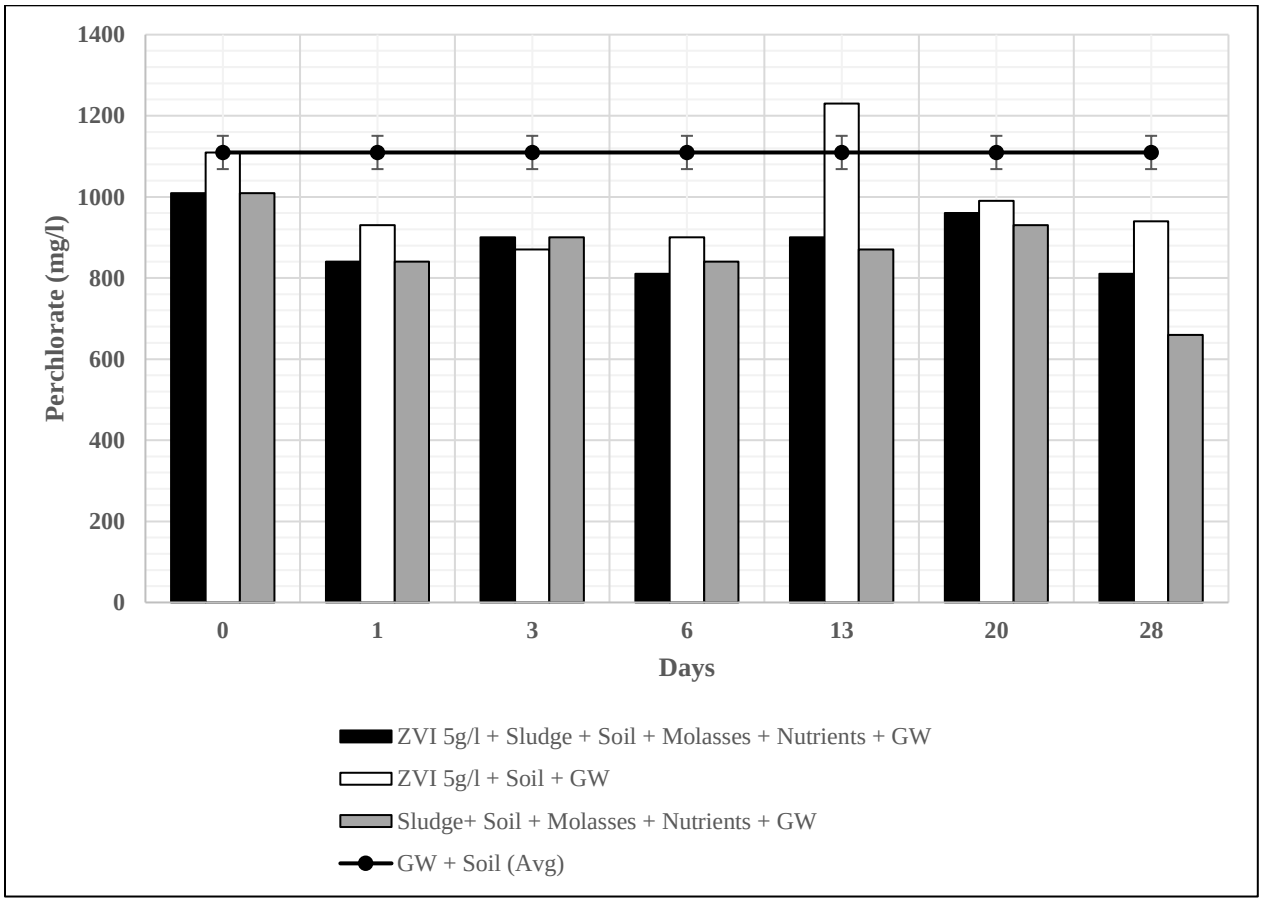


Figure 4.4.2: Phase 2: Abiotic, Biotic and Bio-enhanced Perchlorate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 2.00X (5 g/L + Soil), and 2.20X (5 g/L + Sludge + Soil + Molasses + Nutrients)

Table 4.4.3: Perchlorate Removal Rates for Abiotic, Biotic, and Bio-enhanced Reactors in the Presence of Soil

Treatment	Average Rate (mg/L*d)	Perchlorate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	7.14	2.20X
ZVI 5 g/L + Soil + GW	No Change	2.00X
Sludge+ Soil + Molasses + Nutrients + GW	12.48	0X

Table 4.4.4: Summary of Two Factor ANOVA for Phase 2: Abiotic, Biotic and Bio-enhanced Perchlorate Removal in the Presence of Soil

Description	ANOVA Analysis Between	P-Value
Determination of significant change in NZVI Removal due to bio-enhancement.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.697
	ZVI 5 g/L + Soil + GW	
Determination of significant change in biotic treatment due to the absence of NZVI.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.289
	Sludge+ Soil + Molasses + Nutrients + GW	
Determination of significant change between biotic and abiotic reactors with NZVI.	Sludge+ Soil + Molasses + Nutrients + GW	0.428
	ZVI 5 g/L + Soil + GW	
Determination of significant change due to bio-enhanced NZVI removal.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.005
	GW + Soil	
Determination of significant biotic removal.	Sludge+ Soil + Molasses + Nutrients + GW	0.007
	GW + Soil	
Determination of significant abiotic reduction by NZVI.	ZVI 5 g/L + Soil + GW	0.368
	GW + Soil	

Table 4.4.5: Summary of Two Factor ANOVA in Phase 2 to Determine Significant Difference in Perchlorate Removal due to the Addition of Soil

Description	ANOVA Analysis Between	P-Value
Determination of significant change due to addition of soil in bio-enhanced treatments.	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.513
	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change due to addition of soil in abiotic NZVI reduction.	ZVI 5 g/L + GW	0.324
	ZVI 5 g/L + Soil + GW	
Determination of significant change due to addition of soil in biotic treatments.	Sludge + Molasses + Nutrients + GW	0.071
	Sludge+ Soil + Molasses + Nutrients + GW	
Determination of significant change due to addition of soil in groundwater controls	GW	0.656
	GW + Soil	

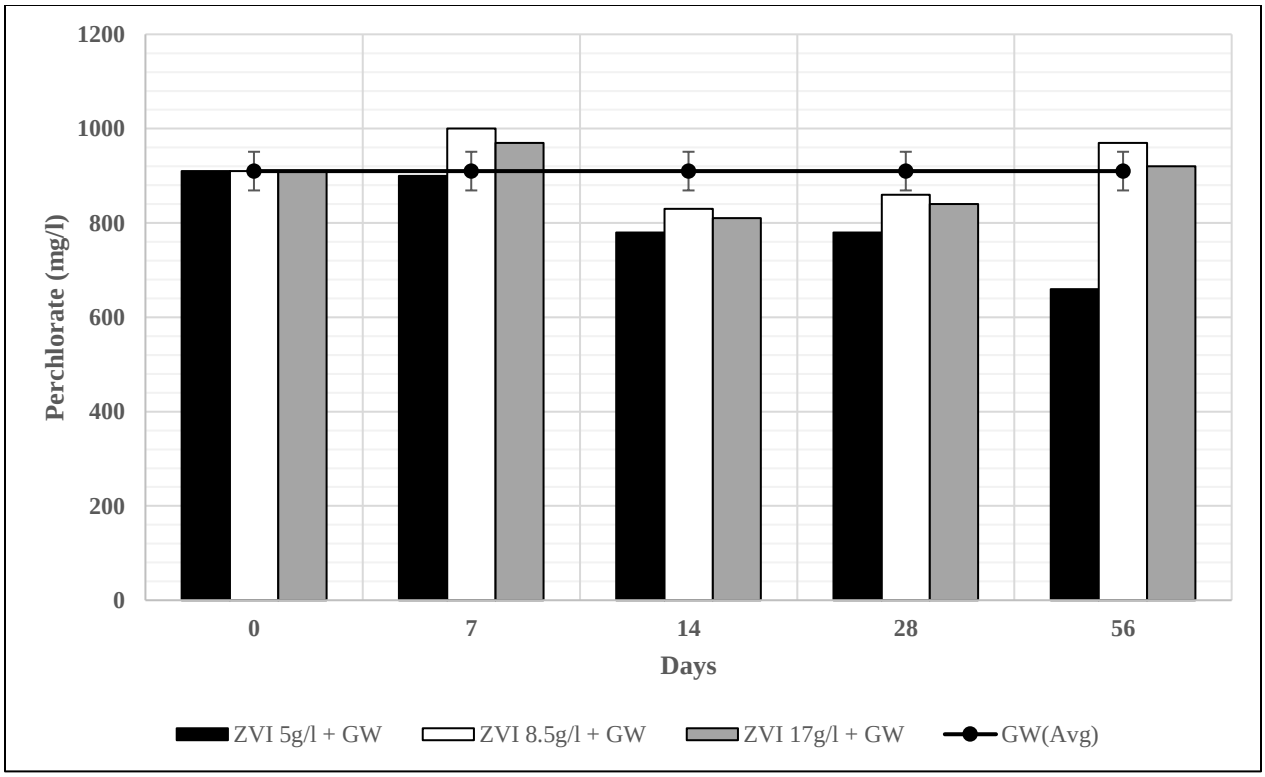


Figure 4.4.3: Abiotic NZVI Perchlorate Reduction in the Absence of Soil at Stoichiometric Mass Ratios of 2.44X (5 g/L), 4.15X (8.5 g/L), and 8.30X (17 g/L)

Table 4.4.6: Perchlorate Reduction Rates for Abiotic Reactors in the Absence of Soil

Treatment	Average Rate(mg/L*d)	Perchlorate Mass Ratio mg Fe^0/mg	Perchlorate Stoichiometric Mass Ratio
ZVI 5 g/L + GW	No Change	5.49	2.44X
ZVI 8.5 g/L + GW	No Change	9.34	4.15X
ZVI 17 g/L + GW	No Change	18.68	8.30X

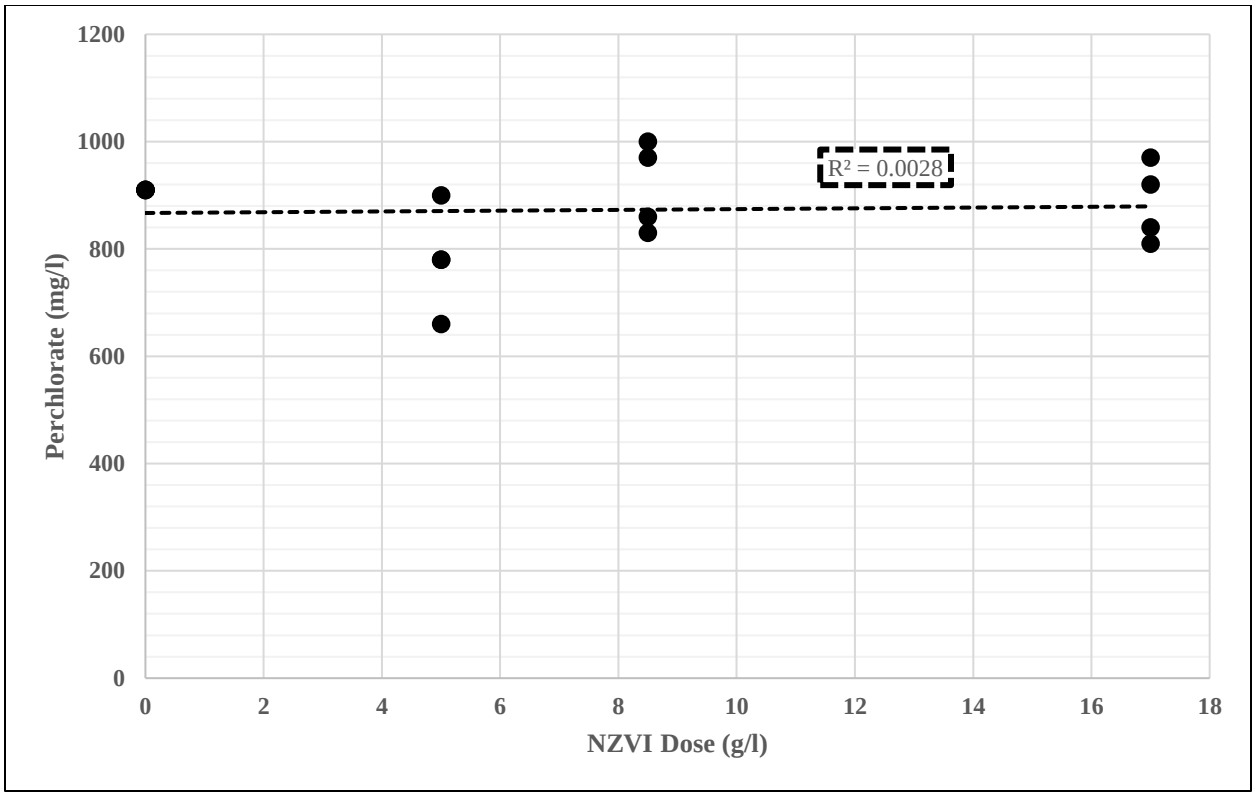


Figure 4.4.3A: Correlation between Perchlorate Reduction and NZVI concentration

Table 4.4.7: Summary of Two Factor ANOVA to Determine Significant Difference in Abiotic Perchlorate Reduction due to increase in NZVI

Description	ANOVA Analysis Between	P-Value
Determination of significant change in abiotic reduction due to 1.7X increase in NZVI	ZVI 5 g/L + GW	0.317
	ZVI 8.5 g/L + GW	
Determination of significant change in abiotic reduction due to 3.3X increase in NZVI	ZVI 5 g/L + GW	0.493
	ZVI 17 g/L + GW	
Determination of significant abiotic reduction by NZVI	ZVI 5 g/L + GW	0.144
	GW	
	ZVI 8.5 g/L + GW	0.894
	GW	
	ZVI 17g/L + GW	0.411
	GW	

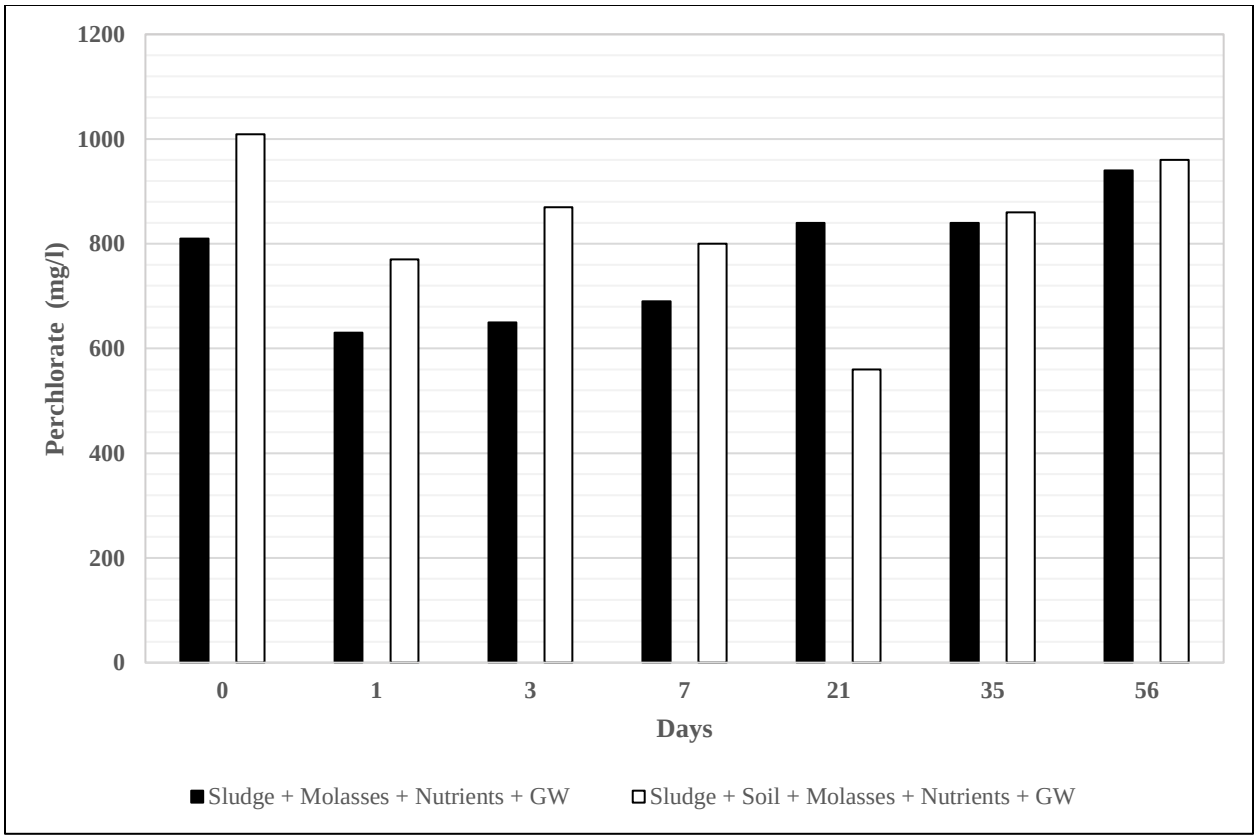


Figure 4.4.4: Diluted Biotic Perchlorate Removal in the Presence and Absence of Soil Using Phase 3 Sludge

Table 4.4.8: Perchlorate Removal Rates for Biotic Reactors in the Presence and Absence of Soil using Phase 3 Sludge

Treatment	Average Rate (mg/L*d)
Sludge + Molasses + Nutrients + GW	No Change
Sludge + Soil + Molasses + Nutrients + GW	0.88

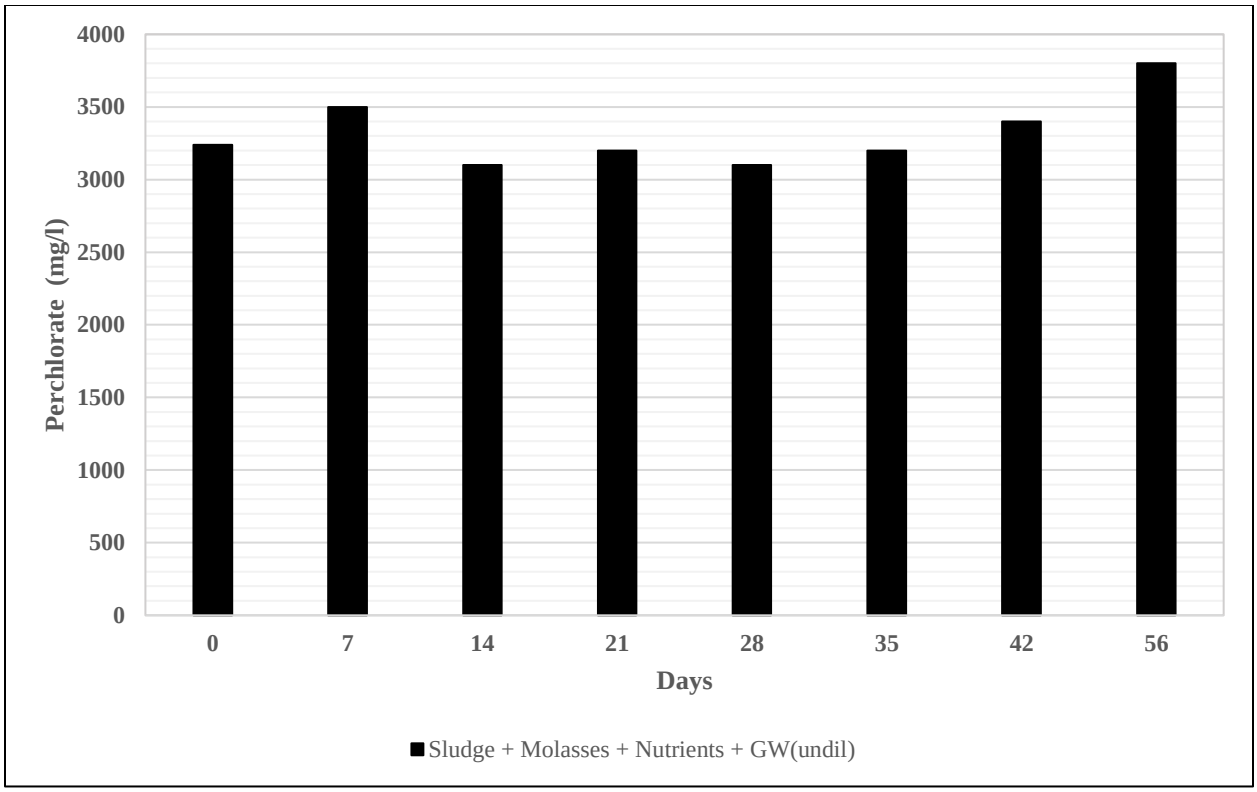


Figure 4.4.4A: Undiluted Biotic Perchlorate Removal in the Presence and Absence of Soil using Phase 3 sludge

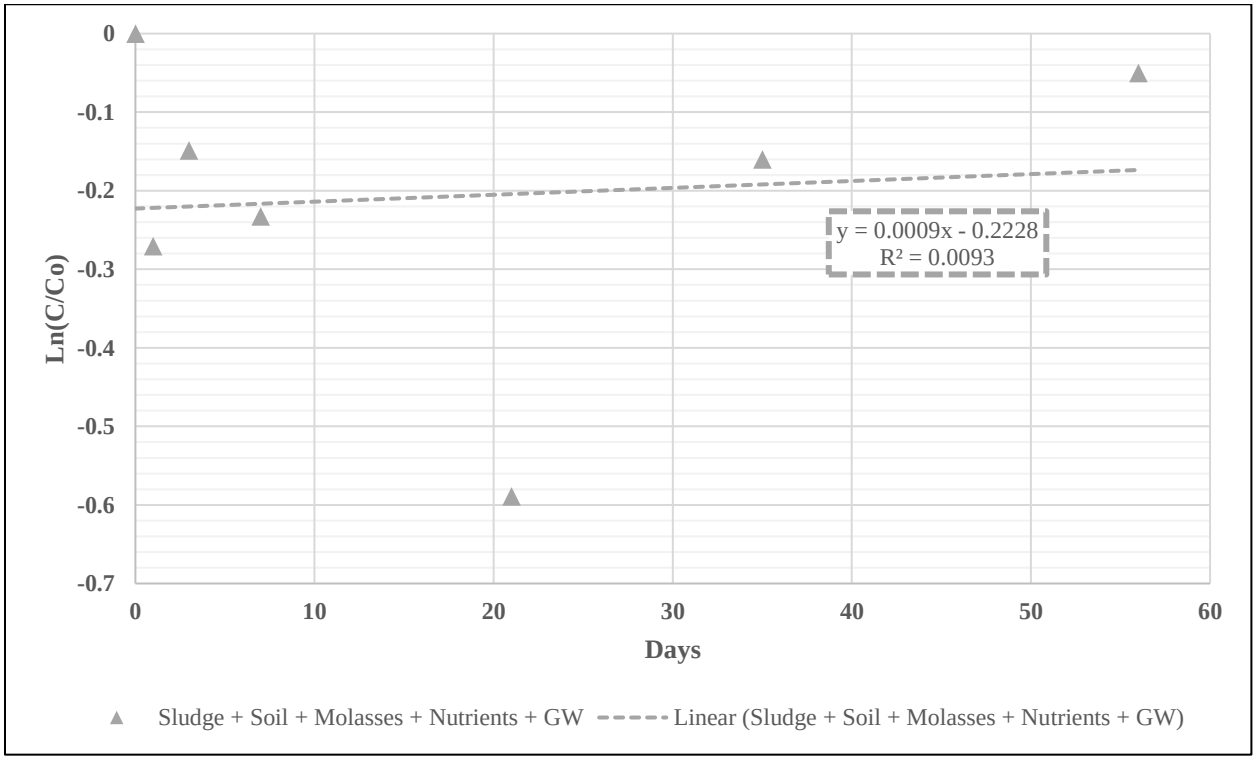


Figure 4.4.4B: First Order Kinetics for Biotic Perchlorate Removal in the Presence of Soil using Phase 3 Sludge

Table 4.4.9: Perchlorate Removal Rates for Undiluted Biotic Reactors in the Absence of Soil using Phase 3 Sludge

Treatment	Average Rate (mg/L*d)
Sludge + Molasses + Nutrients + GW(undil)	No Change

Table 4.4.10: Summary of Two Factor ANOVA in to Determine Significant Difference in Biotic Perchlorate Removal in Phase 3

Description	ANOVA Analysis Between	P-Value
Determination of significant change in biotic reactors between Phase 2 and Phase 3 enriched sludge.	Sludge (Phase 2) + Molasses + Nutrients + GW	0.051
	Sludge (Phase 3) + Molasses + Nutrients + GW	
Determination of significant change in biotic reactors between Phase 2 and Phase 3 enriched sludge in the presence of soil.	Sludge (Phase 2) + Soil + Molasses + Nutrients + GW	0.157
	Sludge (Phase 3) + Soil + Molasses + Nutrients + GW	
Determination of significant change in biotic reactors due the addition of soil.	Sludge (Phase 3) + Molasses + Nutrients + GW	0.202
	Sludge (Phase 3) + Soil + Molasses + Nutrients + GW	
Determination of significant biotic removal using Phase 3 sludge in diluted and undiluted conditions.	Sludge (Phase 3) + Molasses + Nutrients + GW	0.292
	GW	
	Sludge (Phase 3) + Soil + Molasses + Nutrients + GW	0.050
	GW + Soil	
	Sludge (Phase 3) + Molasses + Nutrients + GW(undil)	0.175
	GW	

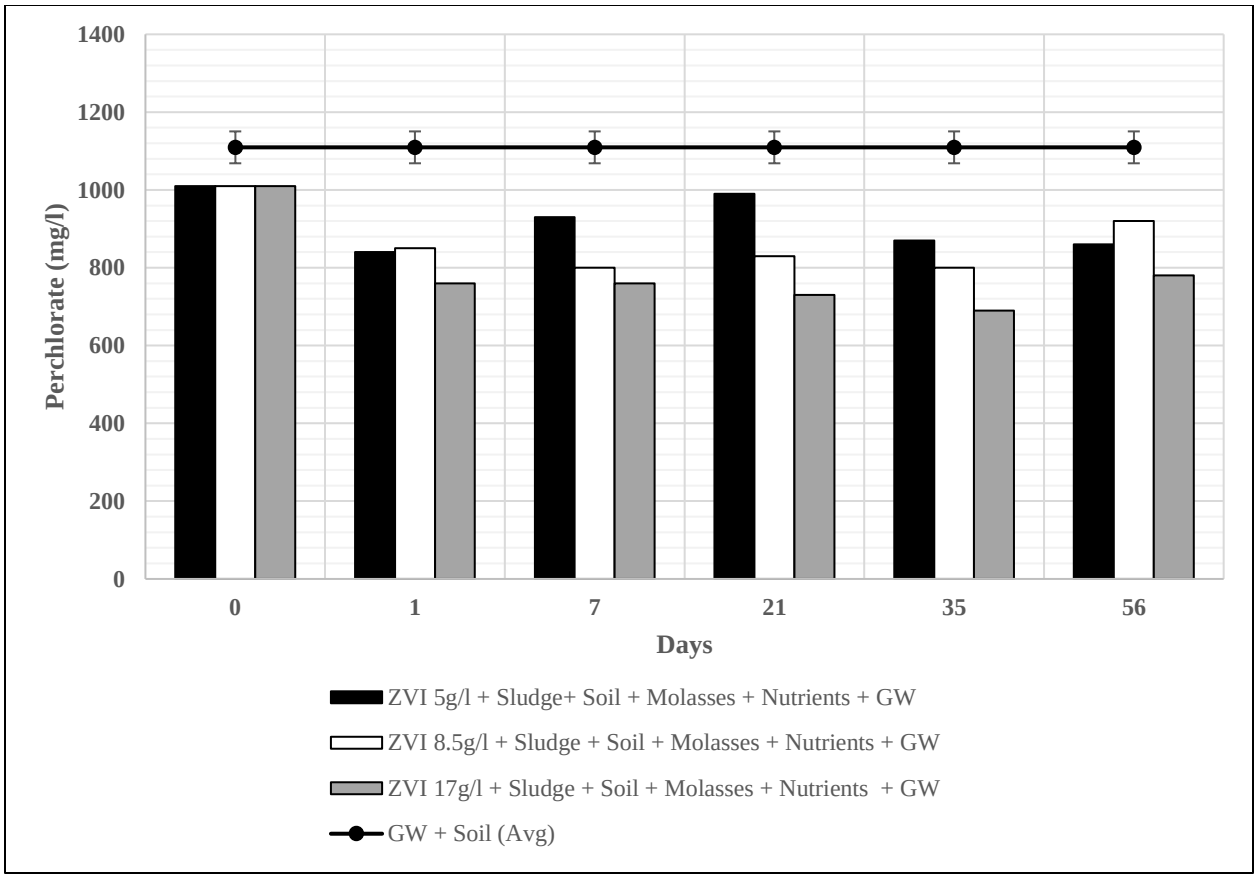


Figure 4.4.5: Phase 3: Phase 3: Phase 3: Effects of Increasing NZVI on Bio-enhanced Perchlorate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 2.20X (5 g/L), 3.75X (8.5 g/L), and 7.50X (17 g/L)

Table 4.4.11: Perchlorate Removal Rates for Bio-enhanced Reactors in the Presence of Soil

Treatment	Average Rate (mg/L*d)	Perchlorate Mass Ratio mg Fe^0/mg	Perchlorate Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	4.46	4.95	2.20X
ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	3.39	8.44	3.75X
ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	7.68	16.88	7.50X

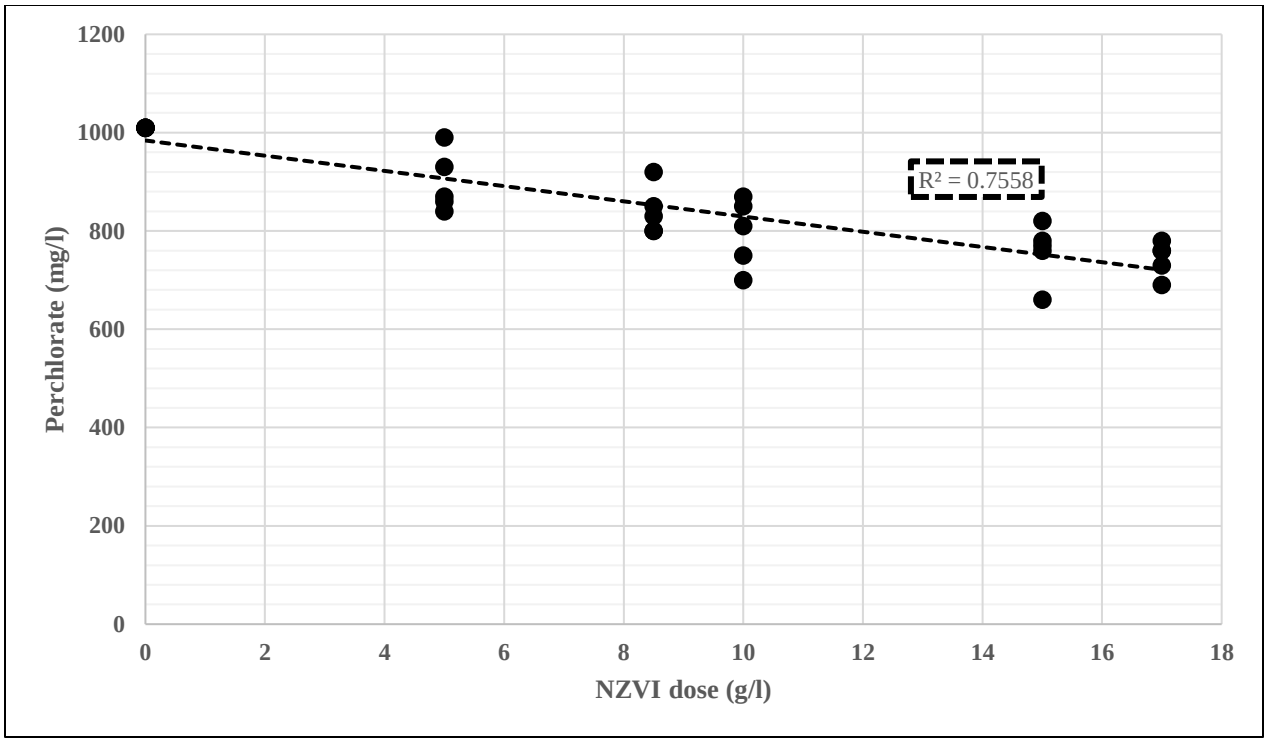


Figure 4.4.5A: Correlation between Perchlorate Removal and NZVI concentration under Bio-enhanced conditions in the presence of soil

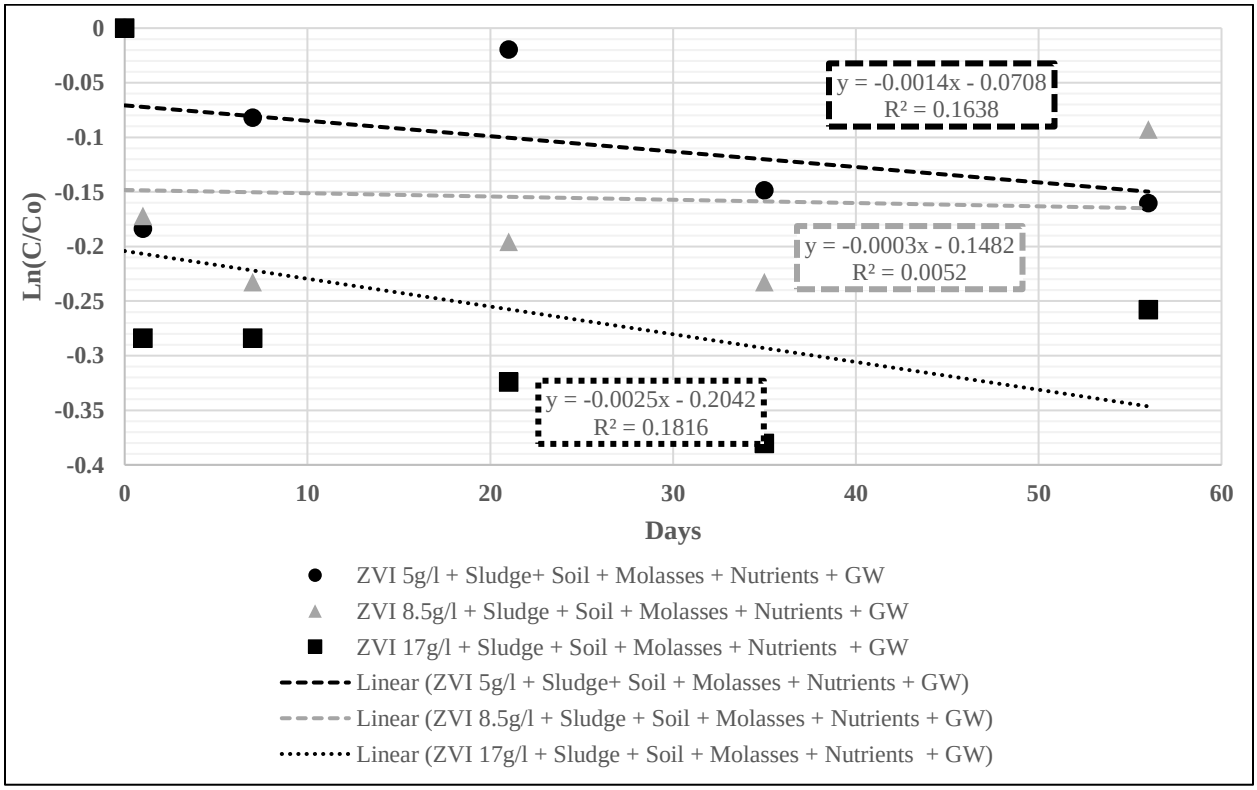


Figure 4.4.5B: First Order Kinetics for Phase 3: Effects of Increasing NZVI on Bio-enhanced Perchlorate Removal in the Presence of Soil at Stoichiometric Mass Ratios of 2.20X (5 g/L), 3.75X (8.5 g/L), and 7.50X (17 g/L)

Table 4.4.12: Summary of Two Factor ANOVA in Phase 3 to Determine Significant Difference in Bio-enhanced Perchlorate Removal due to increase in NZVI

Description	ANOVA Analysis Between	P-Value
Determination of significant change in bio-enhanced removal due to 1.7X increase in NZVI	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.227
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change in bio-enhanced removal due to 3.3X increase in NZVI	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.019
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant removal between bio-enhanced treatments and groundwater controls.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.036
	GW + Soil	
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.017
	GW + Soil	
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.006
	GW + Soil	

Table 4.4.13: Summary of Two Factor ANOVA Comparing Abiotic, Biotic and Bio-enhanced Perchlorate Removal in Phase 3

Description	ANOVA Analysis Between	P-Value
Determination of significant change between bio-enhanced and abiotic reactors.	ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	0.054
	ZVI 5g/L + GW	
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.048
	ZVI 8.5g/L + GW	
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.023
	ZVI 17g/L + GW	
Determination of significant change between bio-enhanced and biotic reactors.	ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	0.100
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.093
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.676
	Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change between abiotic and biotic reactors.	ZVI 5 g/L + GW	0.276
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 8.5 g/L + GW	0.036
	Sludge + Soil + Molasses + Nutrients + GW	
	ZVI 17 g/L + GW	0.045
	Sludge + Soil + Molasses + Nutrients + GW	

Table 4.4.14: Summary of Kinetics for Perchlorate Removal in Abiotic, Biotic, and Bio-enhanced Treatments

Treatment	Reaction Rate Constant			Reaction Order & Highest R^2
	0 Order	1st Order	2nd Order	
	$k = \left(\frac{mg}{L}\right) d^{-1}$	$k = d^{-1}$	$k = \left(\frac{mg}{L}\right)^{-1} d^{-1}$	
Sludge + Soil + Molasses + Nutrients + GW	-9.9	9.0E-4	9.0E-7	**0** $R^2 = 0.02$
ZVI 5g/L + Sludge+ Soil + Molasses + Nutrients + GW	-17.1	-1.4E-3	-2.0E-6	**0** $R^2 = 0.20$
ZVI 8.5g/L + Sludge + Soil + Molasses + Nutrients + GW	-16.2	-3.0E-4	-3.0E-7	**0** $R^2 = 0.12$
ZVI 17g/L + Sludge + Soil + Molasses + Nutrients + GW	-39.6	-2.5E-3	-3.0E-6	**0** $R^2 = 0.44$

4.5 Chloroform Removal

In Phase 2, abiotic, biotic, and bio-enhanced CF removal was compared in the absence and presence of soil (Fig. 4.5.1). In the absence of soil, CF was considerably reduced after 6 days in all treatments, but subsequent additional removal was limited. Abiotic and biotic reactors showed similar removal at 40-50% at Day 56. No statistically significant difference was seen between abiotic and biotic reactors (Table 4.5.2). Bio-enhanced reactors showed the highest level of removal, showing 50-55% at Day 55. A statistically significant difference was seen between abiotic and bio-enhanced reactors, with bio-enhanced reactors achieving a higher average rate of removal (Table 4.5.2). No statistically significant difference between biotic and bio-enhanced reactors was seen. All treatments showed statistically significant CF removal when compared to groundwater controls.

The addition of soil increased CF removal in all treatments. Abiotic, biotic, and bio-enhanced treatments showed 55%, 60%, and 70% reduced CF after 4 weeks, respectively. (Fig. 4.5.2). Like in previous results, subsequent removal after the initial measurement was also limited. Again, the decrease in pH in biotic and bio-enhanced reactors could be the cause for the absence of subsequent removal, but following results showed more successful removal in biotic

and bio-enhanced reactors despite the decrease in pH. Despite an increase in removal, no statistically significant difference was seen in abiotic reactors due to the addition of soil (Table 4.5.5). However, a statistically significant increase in biotic reactors was found when soil was added. Additionally, bio-enhanced reactors showed a near-statistically significant (P = 0.06, Table 4.5.5) increase in removal with the addition of soil. As a result, the addition of soil could promote a higher level of CF removal in biotic, and bio-enhanced reactors due to additional CF reducing bacteria in the soil. Finally, statistically significant removal was seen in all treatments when compared to groundwater controls. However, no statistically significant difference between any treatments was found in the presence of soil (Table 4.5.4).

In abiotic reactors, NZVI doses of 5,000-8,500 mg Fe^0/L with mass ratios of 4,032.25-6,845.56 mg Fe^0/mg CF achieved 40-80% reduction, respectively (Fig. 4.5.3). Abiotic reactors containing 17,000 mg Fe^0/L with a mass ratio of 13,691.12 mg Fe^0/mg achieved total CF reduction at 21 days, with 99% reduction after 1week. However, a resurgence in CF from the detection limit to 170 µg/L was seen at Day 35-56 at 17,000 mg Fe^0/L. The formation of CF during NZVI reduction of carbon tetrachloride could explain this resurgence (Zhang et al, 2011). However, carbon tetrachloride was not monitored in this study. Measurement variation in different reactors could also explain this increase, as different reactors were used at different instances of measurement. Statistically significant reduction was seen at all NZVI doses when compared to groundwater controls (Table 4.5.7). A statistically significant increase in reduction was also observed when increasing NZVI from 5,000-8,500 mg Fe^0/L and 5,000-17,000 mg Fe^0/L. At 5,000 8,500, and 17,000 mg Fe^0/L, the average CF reduction rates were 8.41, 17.70, and 177.28 $\mu g/L * d$, respectively. Stoichiometric mass ratios for 5,000, 8,500, and 17,000 mg Fe^0/L were 2,873.56X, 4,885.05X, and 9,770.10X greater than the theoretical stoichiometric

dose, respectively (Table 4.5.6). Increasing NZVI showed a high correlation ($R^2 = 0.88$) with CF reduction (Fig. 4.5.3A). Previous research has shown limited abiotic CF reduction by macro-scale ZVI, with a first order rate constant of k = -0.50d^{-1} for a CF/NZVI surface area ratio of 28.25 $\mu g/m^2$ for spiked DI water under ambient conditions (Gillham and O'Hannesin, 1994). Another study showed a first order rate constant of k = -16.0d^{-1} at a much higher CF/NZVI surface area ratio of 8,107.15 $\mu g/m^2$ using a spiked bacterial medium under at 30°C and neutral pH (Lee et al, 2015). Using NZVI supported with activated carbon (AC) showed a first order rate constant range of k = -7.45 to -29.8d^{-1} for a CF/NZVI-AC surface area ratio of 0.48 $\mu g/m^2$ in municipal groundwater (Xiao et al, 2014). Accounting for surface area, doses of 5,000, 8,500, and 17,000 mg Fe^0/L in this study show CF/NZVI surface area ratios of 9.93, 5.84, and 2.92 $\mu g/m^2$, respectively, and are within Gillham's, Lee's, and Xiao's research. However, abiotic reactors showed low correlation ($R^2 = 1.0E-4$ to 0.3) for first order kinetics (Fig. 4.5.3B). Overall, zero order kinetics showed the highest correlation for biotic removal ($R^2 = 0.5 - 0.8$, Table 4.5.11), with rate constants of k = -135.56 to -322.11 $\mu g/L * d$. Even at 5,000,000-8,500 mg Fe^0/L NZVI dose used in this study, the stoichiometric mass ratios were substantially high, at 2,873.56X-9,770.10X (Table 4.5.6). Despite this, total CF reduction did not occur at 5,000-8,500 mg Fe^0/L. Therefore, depletion of reactivity by nitrate passivation (Chen et al, 2013) and depletion by reaction with other contaminants is likely.

Due to cost and large sample volume, CF levels were less monitored in Phase 3, where a richer bacterial sludge was used. As a result, two-factor ANOVA could not be performed due to the reduced amount of data points. Biotic reactors in Phase 3 using sludge alone reduced CF by 80% at Day 56 (Fig. 4.5.4), for an average removal rate of 16.70 $\mu g/L * d$. Comparing biotic reactors using sludge alone in Phase 2-3, CF removal in Phase 2 was 50% (Fig. 4.5.1), while

Phase 3 showed 80% removal (Fig. 4.5.4). Therefore, richer sludge conditions, such as increased COD and/or phosphate, could promote higher CF removal. With the addition of soil, biotic reactors in Phase 2 showed an increase to 60% removal (Fig. 4.5.2). Biotic removal in the presence of soil in Phase 3 again showed an increase to 85% removal (Fig. 4.5.4) with an average rate of 17.60 $\mu g/L * d$. This suggests bacteria in the soil can undergo CF metabolism and further enhance biotic removal. Biotic CF reactors showed 55% removal under undiluted conditions (Fig. 4.5.4A), with an average removal rate of 44 $\mu g/L * d$. This suggests no toxic effect on bacterial activity due to undiluted conditions. Previous studies using glucose as a main substrate in biotic anaerobic CF removal and bacterial sludge showed an average rate of 55 $\mu g/L * d$ for an initial CF concentration of 2,000 µg/L at 30^oC and neutral pH (Lu and Li, 2010), no first order kinetics were shown in Lu's study. In another study, anaerobic CF removal in river water was tested using bacterial sediment (Van Beelen and Van Keulen, 1990). Van Beelen's study showed first order kinetics, with a rate constant of k = -0.27 d^{-1} and an average removal rate of 5.69 $\mu g/L * d$ at an initial CF concentration of 400 µg/L at 10^oC and neutral pH. Though additional testing is needed to prove first order kinetics, average rates in undiluted biotic reactors using molasses were similar to Lu's study.

Bio-enhanced reactors in Phase 3 showed greatly reduced CF at all NZVI doses (Fig. 4.5.5). Again, due to the limited amount of testing, ANOVA could not be performed. With NZVI doses of 5,000, 8,500, and 17,000 mg Fe^0/L at mass ratios of 4,518.34, 7,685.21, and 15,370.43 mg Fe^0/mg, CF removal was 80%, 95%, and 99%, respectively. At 5,000, 8,500, and 17,000 mg Fe^0/L, average removal rates were 15.46, 18.57, and 39.14 $\mu g/L * d$, respectively. The total stoichiometric mass ratios for 5,000 8,500, and 17,000 mg Fe^0/L were 3,213.96X, 5,466.59X, and 10,933.19X, respectively (Table 4.5.10). A moderate inverse correlation ($R^2 =$

0.65) between increasing ZVI and CF removal in bio-enhanced reactors was seen (Fig. 4.5.5A). Previous research on bio-enhanced remediation showed complete CF removal with a CF/NZVI surface area ratio of 53.86 $\mu g/m^2$ in spiked DI water at 20^oC at neutral pH (Weathers et al, 1997). Weather's study showed a first order kinetics with a rate constant of k = -0.90 d^{-1}, and an average rate of 13.64 $\mu g/l * d$. At a much higher CF/NZVI surface area ratio of 8,107.15 $\mu g/m^2$, bio-enhanced NZVI showed a first order rate constant of k = -2.25 d^{-1} using a spiked bacterial medium at 30^oC and neutral pH (Lee et al, 2015). In this study, bio-enhanced CF/NZVI surface area ratios for 5,000 8,500, and 17,000 mg Fe^0/L were 8.85, 5.20, and 2.60 $\mu g/m^2$, respectively. First order kinetics in this study showed high correlation ($R^2 = 0.8 - 0.9$), with rate constants of k = -0.03 d^{-1} to -0.09 d^{-1} (Fig. 4.5.5B). Overall, this study showed comparable removal rates to Weather's study, but the first order rate constants were lower than those shown in Weather's and Lee's study. Overall, bio-enhanced reactors showed the highest correlation for second order kinetics ($R^2 = 0.8 - 0.9$, Table. 4.5.11), with rate constants of k = -6.0E-5 to -2.0E-3 $\left(\frac{\mu g}{L}\right)^{-1} d^{-1}$.

Comparing abiotic and bio-enhanced treatments (Fig. 4.5.3 & Fig 4.5.5), bio-enhancement resulted in a 40% and 15% increase at 5,000-8,500 mg Fe^0/L. At 17,000 mg Fe^0/L, both abiotic and bio-enhanced treatments showed near complete CF removal at similar times, though no CF resurgence is seen in bio-enhanced reactors. Comparing biotic and bio-enhanced treatments (Fig 4.5.4 & Fig 4.5.5), bio-enhanced reactors show greater removal only at NZVI doses of 8,500-17,000 mg Fe^0/L after 56 days. Comparing abiotic and biotic treatments (Fig. 4.5.3 & Fig. 4.5.4), only NZVI doses of 17,000 mg Fe^0/L achieved higher levels of removal than biotic reactors after 56 days. Overall, bio-enhancement resulted in greater removal than abiotic and biotic removal. However, only NZVI doses of 8,500 mg Fe^0/L or greater resulted in higher

levels of removal than biotic treatments. Additionally, biotic CF removal was variable, showing a dependence on sludge conditions. This endorses bio-enhancement to achieve more consistent CF removal.

At all points of measurement, only negligible amounts of CM and DCM, potential byproducts of abiotic (Weathers et al, 1997) and biotic (Cappelletti et al, 2012) CF removal, were detected throughout all instances of CF measurement. The lack of intermediate chlorinated aliphatic byproducts in abiotic reactors suggests complete reduction of CF to methane by NZVI (Weathers et al, 1997). Biotic samples also lacked any CM/DCM. This disproves the significant presence of bacterial dehalorespiration of CF, which produces DCM as a primary byproduct (Cappelletti et al, 2012). The absence of chlorinated aliphatics could result from complete reduction to methane through reductive dechlorination (Cappelletti et al, 2012, & Lee et al, 2015). However, a low amount DCM is produced in during reductive dechlorination, which could disprove the presence of reductive dechlorination (Lee et al, 2015). Anaerobic CF metabolism in this study could also be through oxidation of CF through direct hydrolysis, in which intermediate byproducts are quickly oxidized to carbon dioxide (Cappelletti et al, 2012 & Bouwer and McCarty, 1983). This supports previous research, where low DCM accumulation is seen when hydrolysis takes place (Lee et al, 2015). The detection of formaldehyde and formic acid (Fig. 4.5.6), which are intermediate products of CF hydrolysis (Cappelletti et al, 2012), in biotic and bio-enhanced samples exhibiting high levels of CF removal also supports this. Evidence for CF hydrolysis is further supported by the low pH recorded in biotic reactors after 8 weeks (Table 4.2.1), where the decrease in pH might have resulted from carbonic acid production due to aqueous carbon dioxide. However, the production of carbon dioxide could be due to anaerobic respiration using other substrates, particularly the glucose present in molasses

(Luedeking et al, 1959 & Reddy et al, 2008). This assumption is supported by the prevalence of lactic acid at higher molar levels than removed CF (Fig 4.5.6). This makes determining the presence of CF hydrolysis unreliable. As a common product of anaerobic CF metabolism (Cappelletti et al, 2012), the measurement of methane gas produced within the airspace of each reactor would mitigate this uncertainty and provide stronger evidence for the presence of bacterial CF metabolism (Weathers et al, 1997, & Lee et al, 2015).

Overall results from this study show:

- The presence of soil increased CF removal in biotic reactors. Additionally, bio-enhanced reactors with soil showed a near statistically significant increase over bio-enhanced reactors without soil. It is likely additional CF metabolizing bacteria are present in the soil, which can augment biotic and bio-enhanced removal.
- Bio-enhanced reactors achieved the highest CF removal. However, an NZVI dose of at least 8,500 mg Fe^0/L is needed to achieve higher removal than biotic treatments.
- Biotic CF removal was successful, albeit variable, showing greater CF removal when a richer sludge was used.
- The absence of chlorinated aliphatics at all points of measurement suggests total reduction of CF to methane. In biotic samples, the absence of chlorinated aliphatics and the presence of formaldehyde supports hydrolysis as the main pathway of CF metabolism in this study.

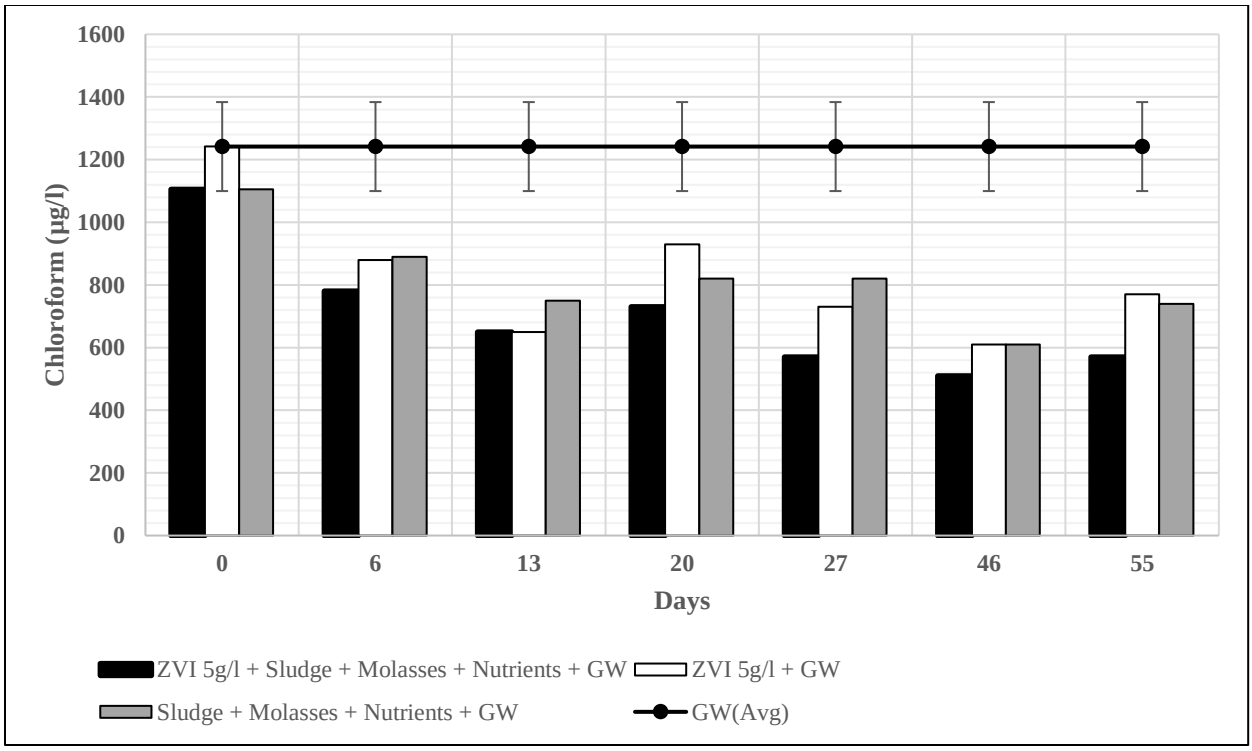


Figure 4.5.1: Phase 2: Abiotic, Biotic, and Bio-enhanced Chloroform Removal in the Absence of Soil at Stoichiometric Mass Ratios of 2,873.56X (5 g/L), and 3,205.13X (5 g/L + Sludge + Nutrients)

Table 4.5.1: Chloroform Removal Rates for Abiotic Biotic and Bio-enhanced Reactors in the Absence of Soil

Treatment	Average Rate (µg/L*d)	CF Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	9.72	3,205.13X
ZVI 5 g/L + GW	8.41	2,873.56X
Sludge + Molasses + Nutrients + GW	6.63	0X

Table 4.5.2: Summary of Two Factor ANOVA for Phase 2: Abiotic, Biotic, and Bio-enhanced Chloroform Removal in the Absence of Soil

Description	ANOVA Analysis Between:	P-Value
Determination of significant change in NZVI reduction due to bio-enhancement.	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.027
	ZVI 5 g/L + GW	
Determination of significant change in biotic treatment due to the absence of NZVI	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.069
	Sludge + Molasses + Nutrients + GW	
Determination of significant change between biotic and abiotic reactors with NZVI	Sludge + Molasses + Nutrients + GW	0.189
	ZVI 5 g/L + GW	
Determination of significant removal due to bio-enhanced NZVI	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.004
	GW	
	Sludge + Molasses + Nutrients + GW	0.004

Determination of significant biotic removal	GW	
Determination of significant abiotic reduction by NZVI	ZVI 5 g/L + GW	0.016
	GW	

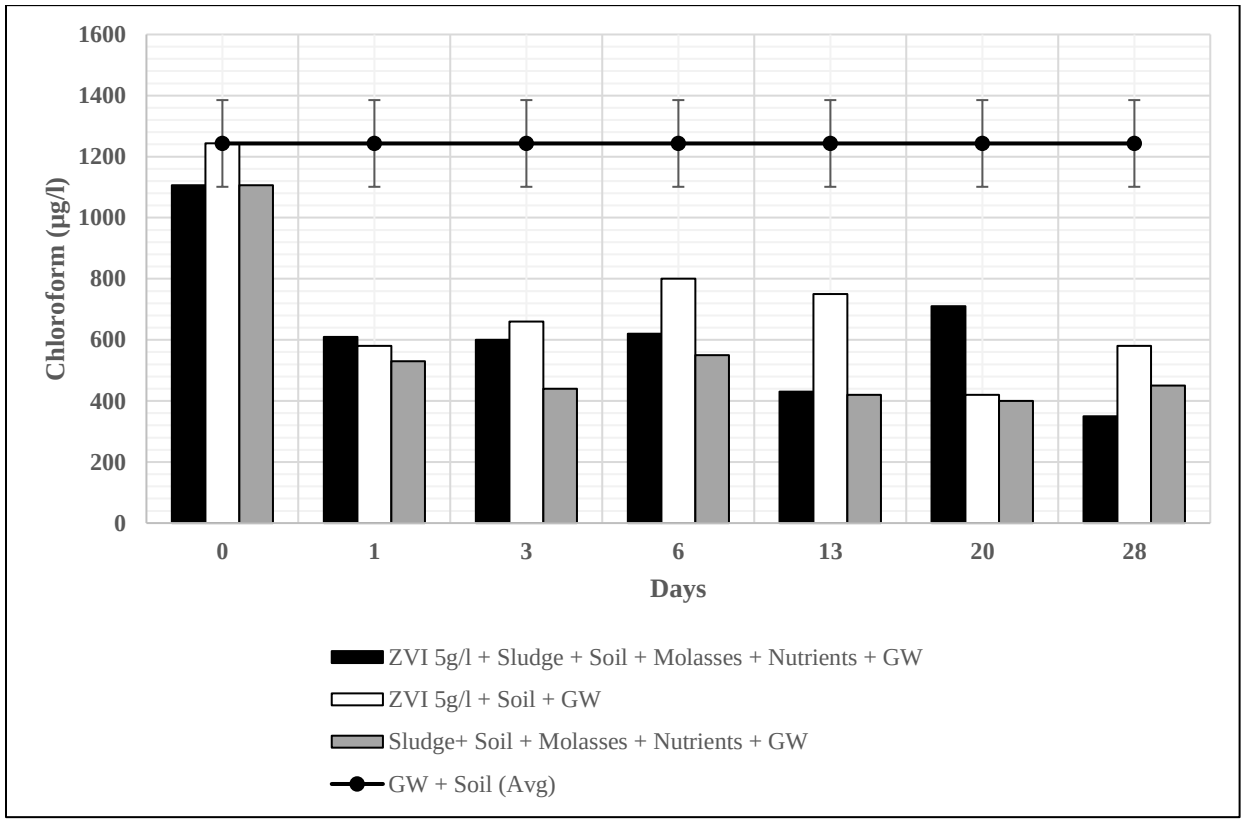


Figure 4.5.2: Phase 2: Abiotic, Biotic and Bio-enhanced Chloroform Removal in the Presence of Soil at Stoichiometric Mass Ratios of 2,873.56X (5 g/L + Soil), and 3,205.13X (5 g/L + Sludge + Soil + Nutrients)

Table 4.5.3: Chloroform Removal Rates for Abiotic, Biotic and Bio-enhanced Reactors in the Presence of Soil

Treatment	Average Rate (µg/L*d)	CF Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	27.00	3,205.13X
ZVI 5 g/L + Soil + GW	23.71	2,873.56X
Sludge+ Soil + Molasses + Nutrients + GW	23.42	0X

Table 4.5.4: Summary of Two Factor ANOVA for Phase 2: Abiotic, Biotic, and Bio-enhanced Chloroform Removal in the Presence of Soil

Description	ANOVA Analysis Between:	P-Value
Determination of significant change in NZVI Removal due to bio-enhancement.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.918
	ZVI 5 g/L + Soil + GW	
Determination of significant change in biotic treatment due to the absence of NZVI.	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	0.177
	Sludge+ Soil + Molasses + Nutrients + GW	
Determination of significant change between biotic and abiotic reactors with NZVI.	Sludge+ Soil + Molasses + Nutrients + GW	0.080
	ZVI 5 g/L + Soil + GW	
Determination of significant change due to bio-enhanced NZVI removal.	ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	0.004
	GW+ Soil	
Determination of significant biotic removal.	Sludge + Soil + Molasses + Nutrients + GW	0.002
	GW+ Soil	
Determination of significant abiotic reduction by NZVI.	ZVI 5 g/L+ Soil + GW	0.002
	GW+ Soil	

Table 4.5.5: Summary of Two Factor ANOVA in Phase 2 to Determine Significant Change in Chloroform Removal due to the Addition of Soil

Description	ANOVA Analysis Between	P-Value
Determination of significant change due to addition of soil in bio-enhanced treatments.	ZVI 5 g/L + Sludge + Molasses + Nutrients + GW	0.060
	ZVI 5 g/L + Sludge + Soil + Molasses + Nutrients + GW	
Determination of significant change due to addition of soil in abiotic NZVI reduction.	ZVI 5 g/L + GW	0.284
	ZVI 5 g/L + Soil + GW	
Determination of significant change due to addition of soil in biotic treatments.	Sludge + Molasses + Nutrients + GW	0.017
	Sludge+ Soil + Molasses + Nutrients + GW	
Determination of significant change due to addition of soil in groundwater controls	GW	0.747
	GW + Soil	

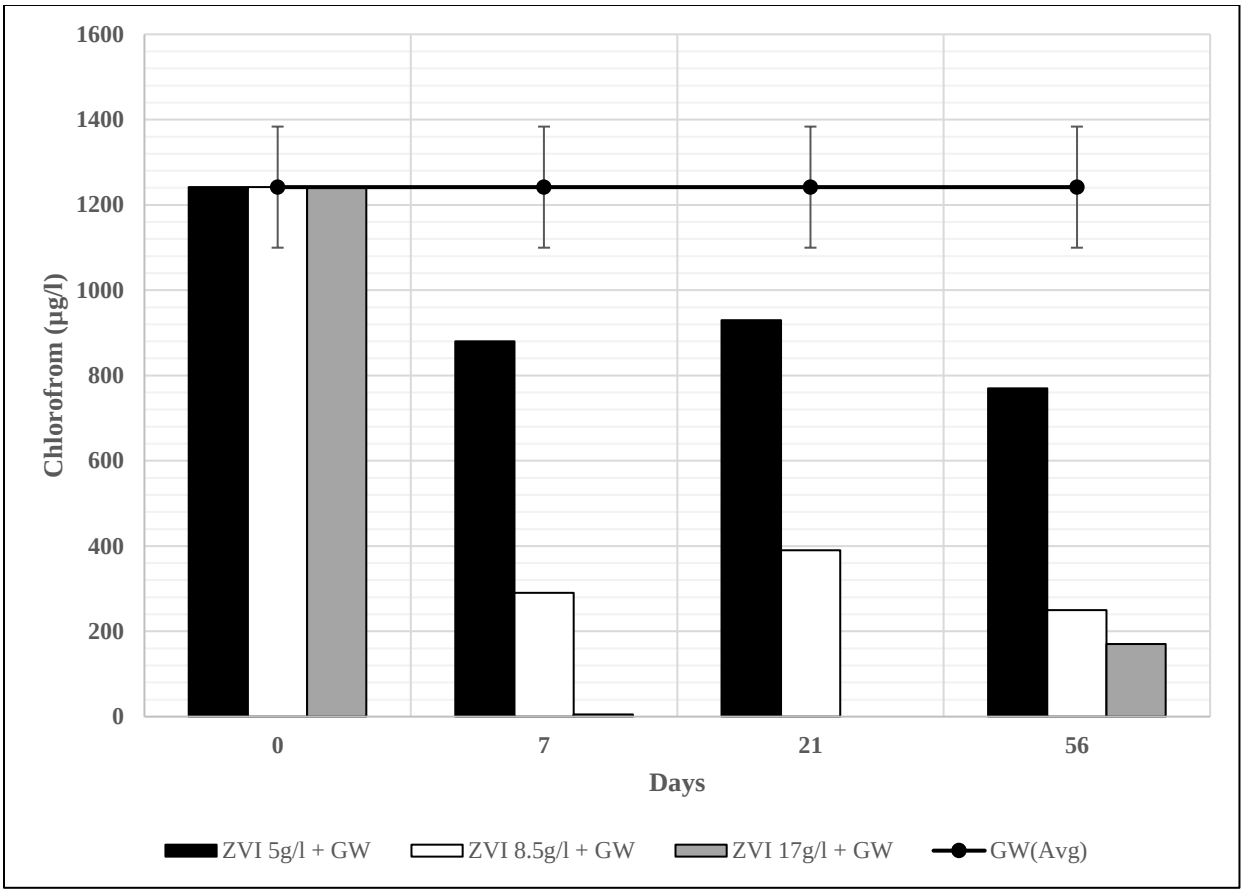


Figure 4.5.3: Abiotic NZVI Chloroform Reduction in the Absence of Soil at Stoichiometric Mass Ratios of 2,873.56X (5 g/L), 4,885.05X (8.5 g/L), and 9,770.10X (17 g/L)

Table 4.5.6: Chloroform Reduction Rates for Abiotic Reactors in the Absence of Soil

Treatment	Average Rate (µg/L*d)	CF Mass Ratio mg Fe^0/mg	CF Stoichiometric Mass Ratio
ZVI 5 g/L + GW	8.41	4,032.25	2,873.56X
ZVI 8.5 g/L + GW	17.70	6,845.46	4,885.05X
ZVI 17 g/L + GW	177.28	13,691.12	9,770.10X

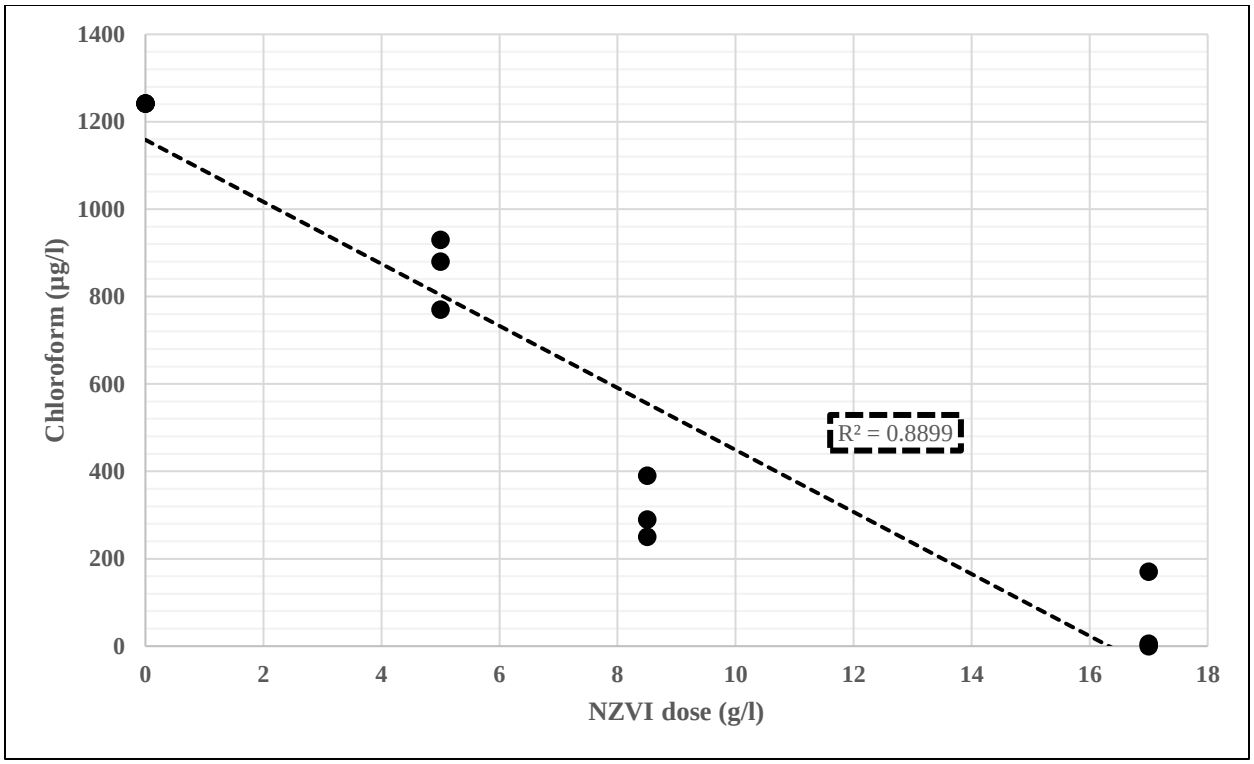


Figure 4.5.3A: Correlation between Abiotic Chloroform Reduction and NZVI concentration

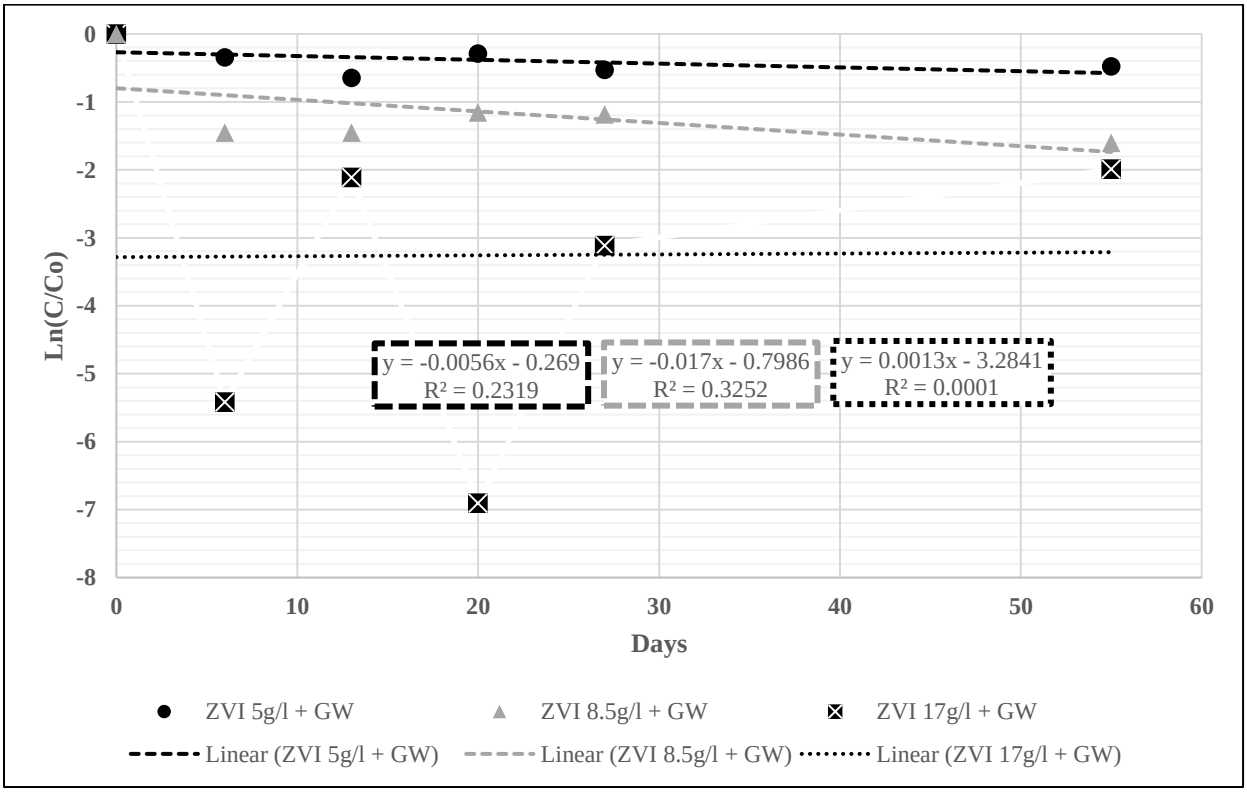


Figure 4.5.3B: First Order Kinetics for Abiotic Chloroform Reduction in the Absence of Soil at Stoichiometric Mass Ratios of 2,873.56X (5 g/L), 4,885.05X (8.5 g/L), and 9,770.10X (17 g/L)

Table 4.5.7: Summary of Two Factor ANOVA to Determine Significant Change in Abiotic Chloroform Reduction due to increase in NZVI

Description	ANOVA Analysis Between	P-Value
Determination of significant change in abiotic reduction due to 1.7X increase in NZVI	ZVI 5 g/L + GW	0.007
	ZVI 8.5 g/L + GW	
Determination of significant change in abiotic reduction due to 3.3X increase in NZVI	ZVI 5 g/L + GW	0.007
	ZVI 17 g/L + GW	
Determination of significant abiotic reduction by NZVI	ZVI 5 g/L + GW	0.016
	GW	
	ZVI 8.5 g/L + GW	0.005
	GW	
	ZVI 17 g/L + GW	0.006
	GW	

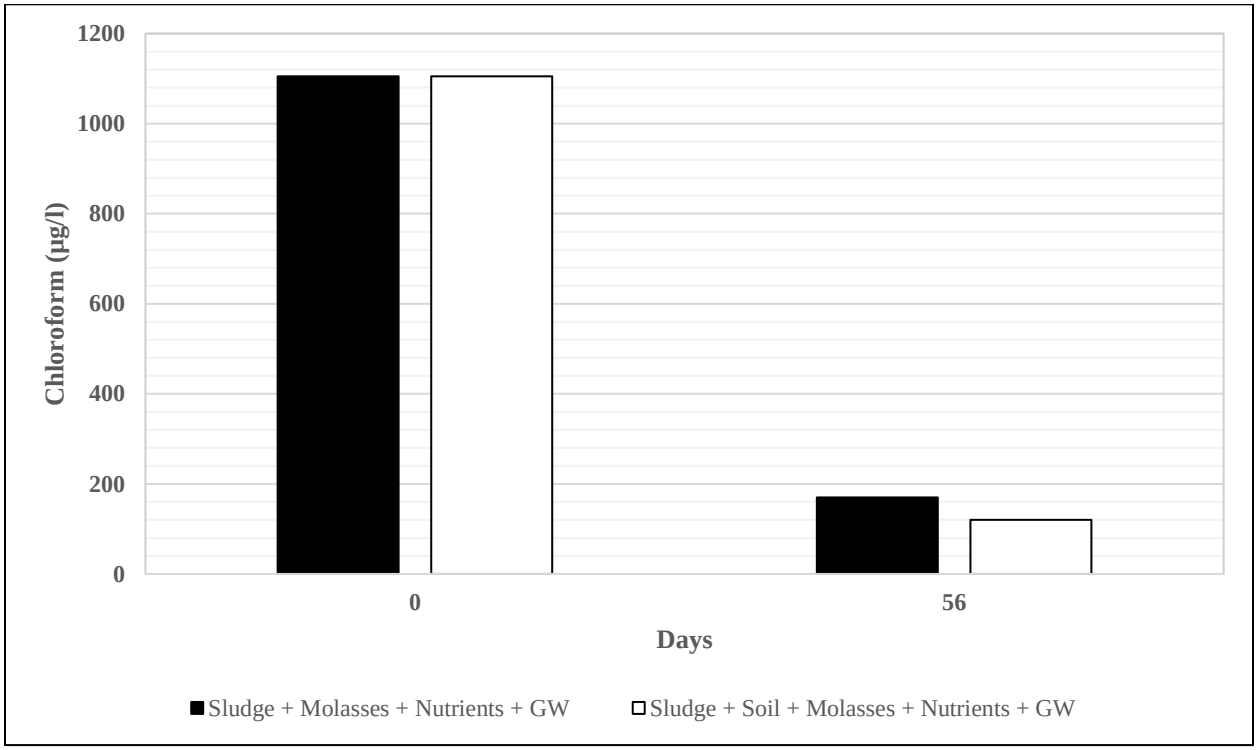


Figure 4.5.4: Diluted Biotic Chloroform Removal in the Presence and Absence of Soil using Phase 3 Sludge

Table 4.5.8: Chloroform Removal Rates for Biotic Reactors in the Presence and Absence of Soil using Phase 3 Sludge

Treatment	Average Rate (µg/L*d)
Sludge + Molasses + Nutrients + GW	16.70
Sludge + Soil + Molasses + Nutrients + GW	17.60

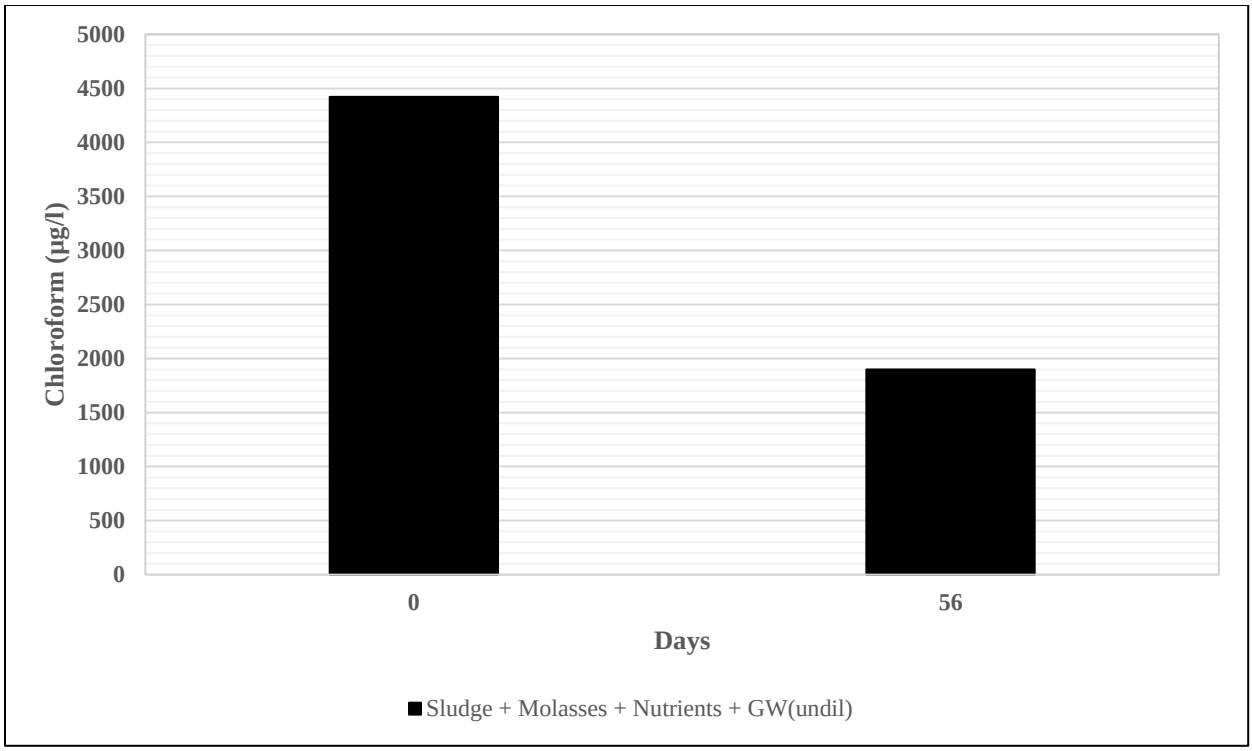


Figure 4.5.4A: Undiluted Biotic Chloroform Removal in the Absence of Soil using Phase 3 sludge

Table 4.5.9: Chloroform Removal Rates for Undiluted Biotic Reactors in the Absence of Soil using Phase 3 sludge

Treatment	Average Rate (µg/L*d)
Sludge + Molasses + Nutrients + GW(undil)	44.00

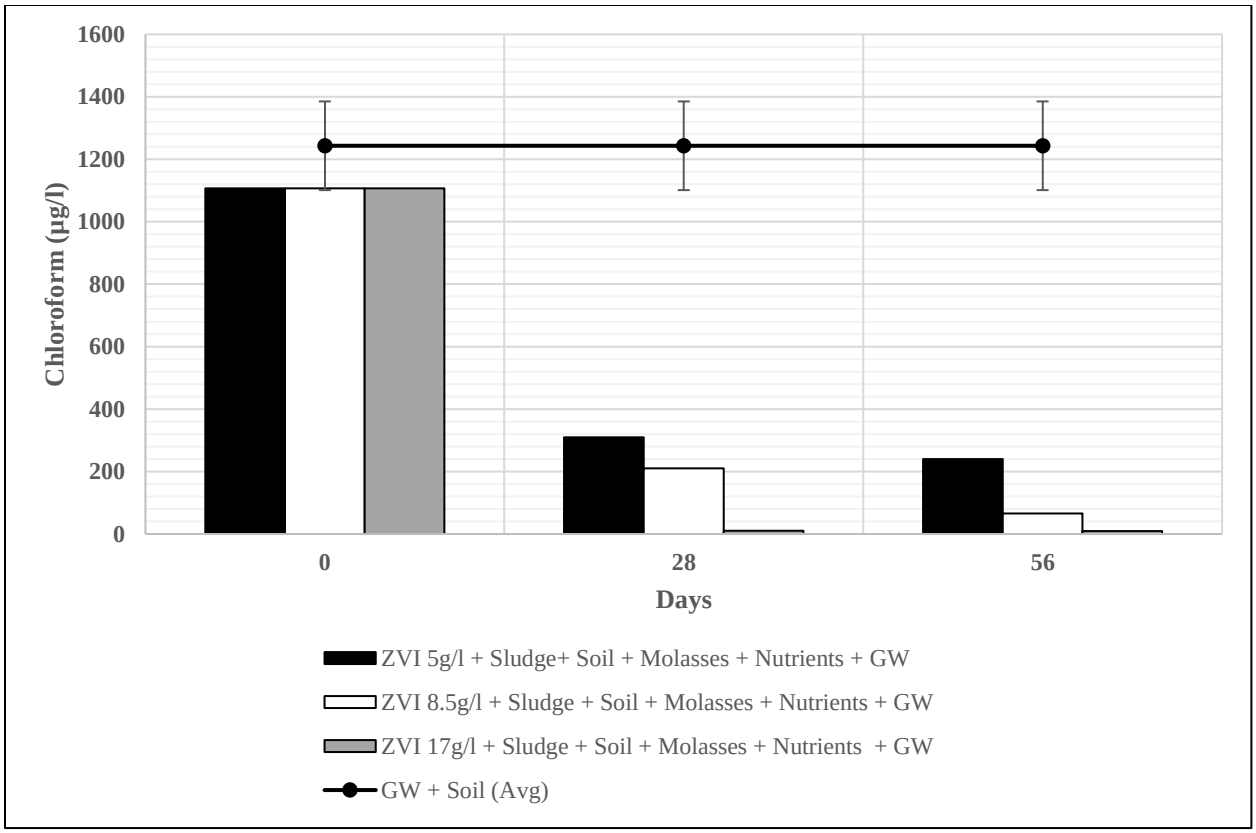


Figure 4.5.5: Phase 3: Effects of Increasing NZVI on Bio-enhanced Chloroform Removal in the Presence of Soil at Stoichiometric Ratios of 3,213.96X (5 g/L), 5,466.59X (8.5 g/L), and 10,933.19X (17 g/L)

Table 4.5.10: Chloroform Removal Rates for Bio-enhanced Reactors in the Presence of Soil

Treatment	Average Rate (µg/L*d)	CF Mass Ratio mg Fe^0/mg	CF Stoichiometric Mass Ratio
ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	15.46	4,518.34	3,213.96X
ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	18.57	7,685.21	5,466.59X
ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	39.14	15,370.43	10,933.19X

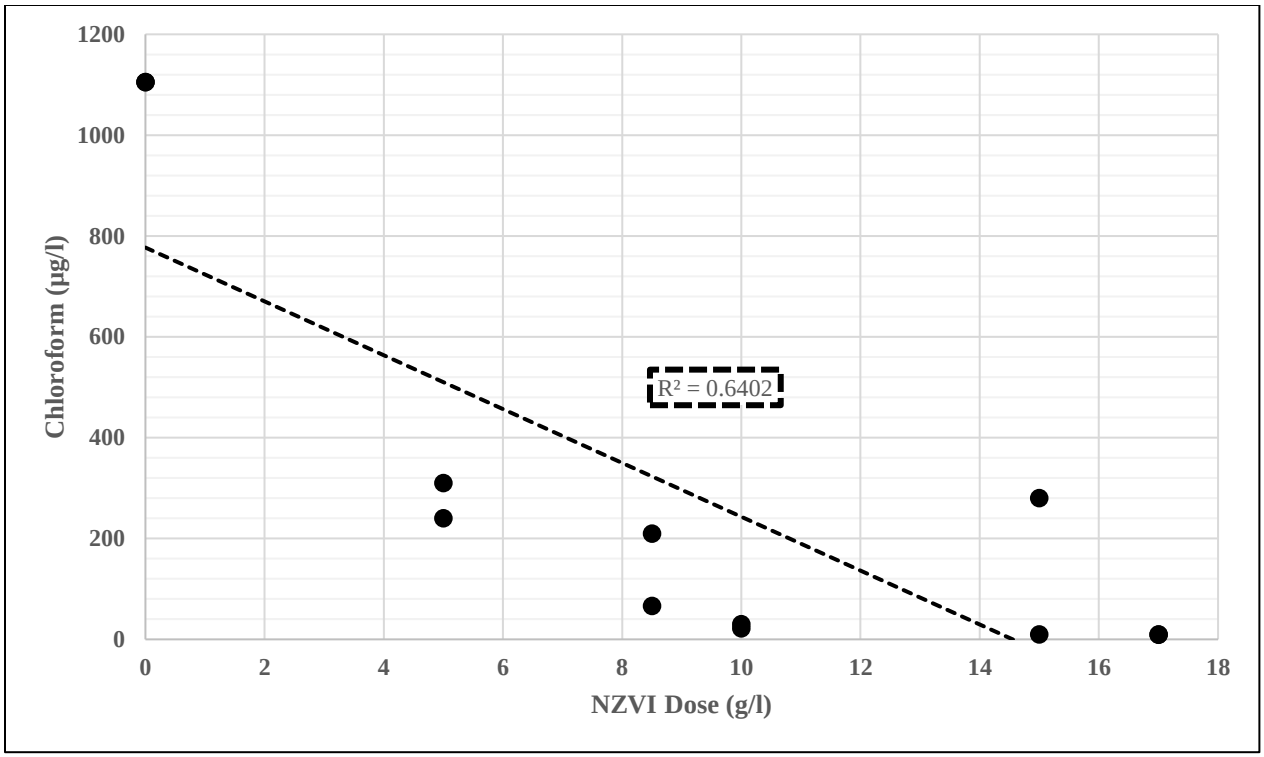


Figure 4.5.5A: Correlation between Bio-enhanced Chloroform Removal and NZVI

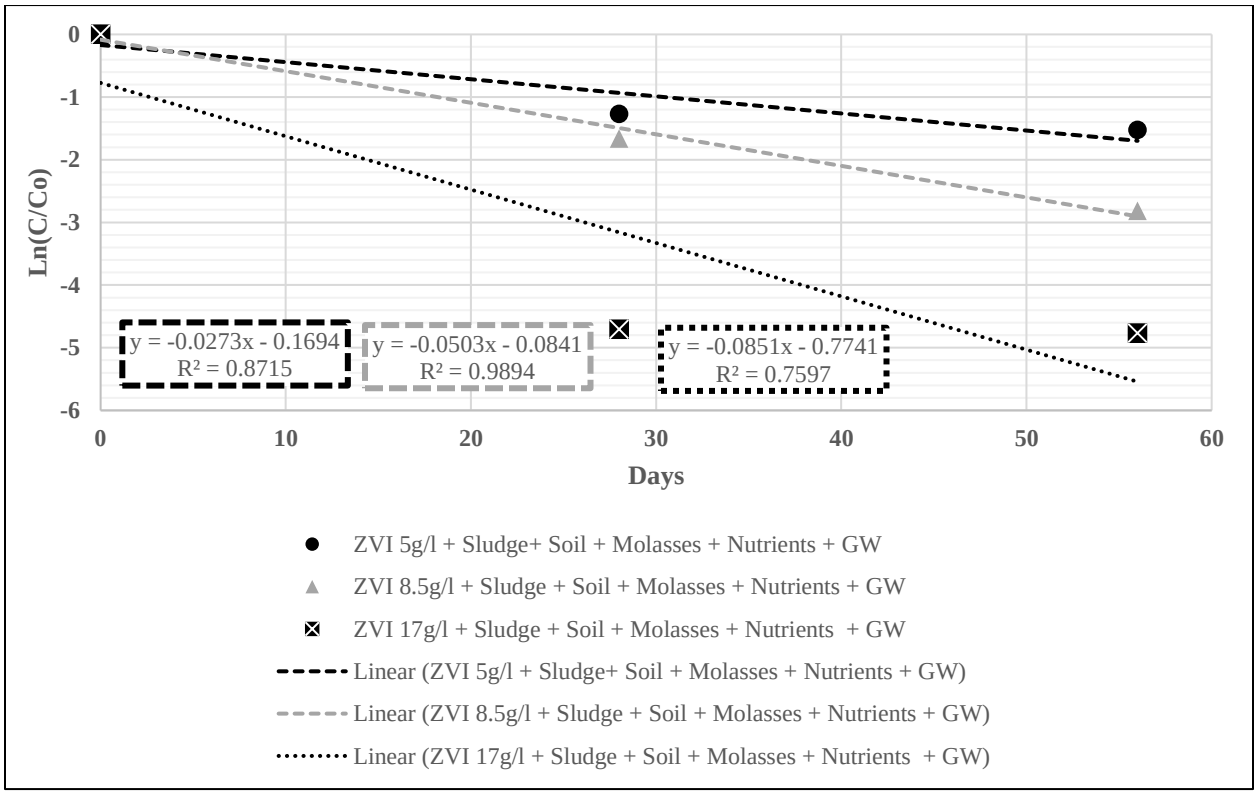


Figure 4.5.5B: First Order Kinetics for Phase 3: Effects of Increasing NZVI on Bio-enhanced Chloroform Removal in the Presence of Soil at Stoichiometric Ratios of 3,213.96X (5 g/L), 5,466.59X (8.5 g/L), and 10,933.19X (17 g/L)

Table 4.5.11: Summary of Kinetics for Chloroform Removal in Abiotic, Biotic, and Bio-enhanced Treatments

Treatment	Reaction Rate Constant			Reaction Order & Highest R^2
	0 Order	1st Order	2nd Order	
	$k = \left(\frac{\mu g}{L}\right) d^{-1}$	$k = d^{-1}$	$k = \left(\frac{\mu g}{L}\right)^{-1} d^{-1}$	
ZVI 5 g/L + GW	-136.5	-6.0E-3	-8.0E-6	**0** $R^2 = 0.8$
ZVI 8.5 g/L + GW	-287.5	-0.02	-3.0E-5	**0** $R^2 = 0.6$
ZVI 17 g/L + GW	-322.1	-1.0E-3	-7.0E-3	**0** $R^2 = 0.5$
ZVI 5 g/L + Sludge+ Soil + Molasses + Nutrients + GW	-433.3	-0.03	-6.0E-5	**2nd** $R^2 = 0.9$
ZVI 8.5 g/L + Sludge + Soil + Molasses + Nutrients + GW	-520.3	-0.05	-2.0E-3	**2nd** $R^2 = 0.9$
ZVI 17 g/L + Sludge + Soil + Molasses + Nutrients + GW	-548.6	-0.09	-2.0E-3	**2nd** $R^2 = 0.8$

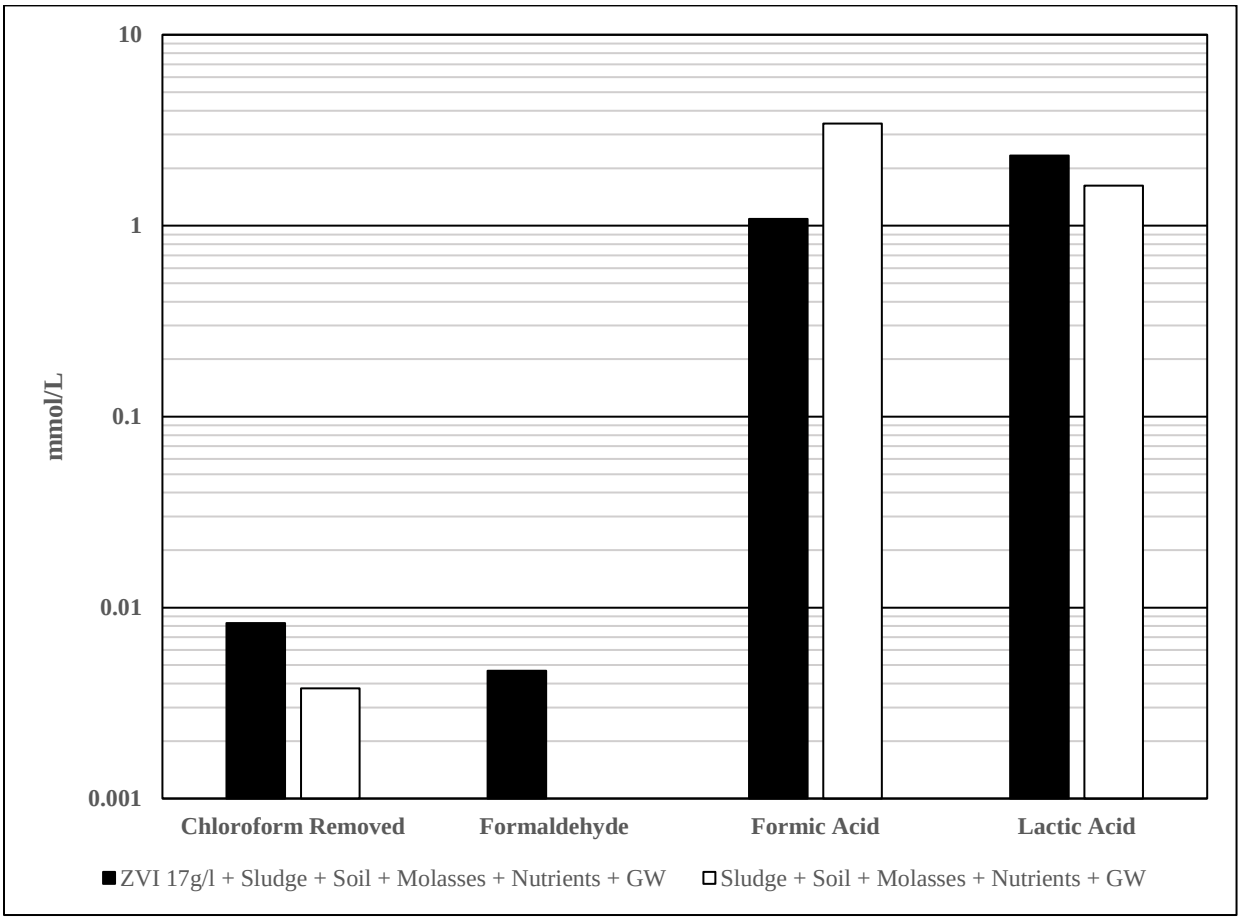


Figure 4.5.6: Biotic and Bio-enhanced Anaerobic Byproduct Formation in the Presence of Soil

4.6 Removal Summary

Rapid Cr(VI) reduction was possible with NZVI doses as low as 500 mg Fe^0/L. Substantial CF reduction was also achieved by NZVI at doses as low as 5,000 mg Fe^0/L. Despite this, abiotic reactors showed the least success, showing no perchlorate reduction, and only achieving substantial reduction for nitrate and chlorate at doses of 17,000 mg Fe^0/L. The limited removal of co-contaminants by NZVI at 5,000-8,500 mg Fe^0/L is likely due to insufficiency. Stoichiometrically, these doses are not sufficient to the contaminant concentrations present in the groundwater. At these insufficient doses, competition for NZVI by all the different contaminants in the groundwater. This could account for measurement variability in abiotic reactors. Different levels of nitrate passivation across different reactors would also result in variable results in abiotic and bio-enhanced reactors. While biotic and bio-enhanced reactors showed substantial removal for most contaminants, perchlorate removal was limited for both treatments. Additionally, more variable results were shown by biotic treatments, where different sludge characteristics produced different levels of removal for nitrate, chlorate, perchlorate, and CF. Furthermore, since sludge inoculation was separate for each reactor, differential microbial growth in individual reactors might have also contributed to measurement variability. Bio-enhanced reactors showed the highest level of removal for all treatments. However, a substantial amount of NZVI is needed to remove most contaminants at the same level as biotic treatments, particularly in nitrate, chlorate, and CF. Despite this, bio-enhanced reactors showed faster removal rates, more consistent removal than biotic treatments, and total removal of nitrate, chlorate, and CF (Fig 4.6.1). The greater and more consistent results in bio-enhanced reactors suggests a synergistic relationship between NZVI and the bacterial flora available in this study. This endorses bio-enhancement of NZVI as the preferred method of

remediation for contaminants in groundwater from the industrial complex monitored in this study Furthermore, the addition of soil had a positive effect on chlorate and CF removal under biotic and bio-enhanced conditions and did not adversely affect the performance of NZVI. Thus, in-situ conditions might benefit from the amendment of NZVI and nutrients. Finally, while bio-enhancement was the most successful treatment, total abiotic removal of all contaminants except perchlorate was possible with NZVI, albeit at the maximum dose of 17,000, 17,000 mg Fe^0/L. The most successful treatments showing complete removal of Cr(VI), nitrate, chlorate, and CF are shown in Fig. 4.6.1.

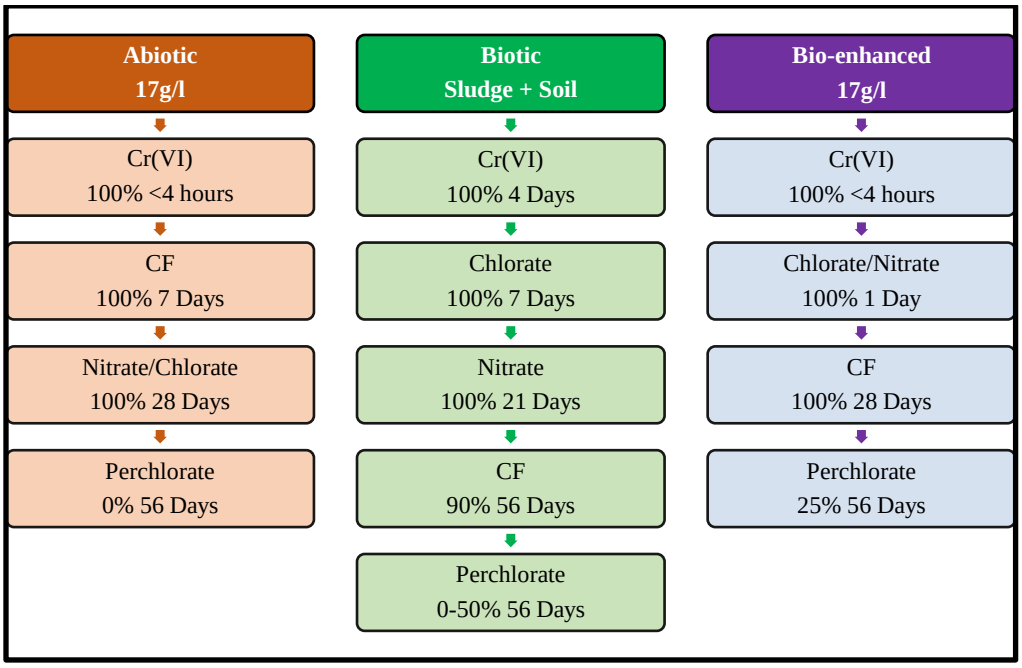


Figure 4.6.1: Treatment Timeline Showing Complete Removal

Chapter 5: Conclusion, Implications and Future Recommendations

The high and diverse contamination of soil and groundwater from the industrial site monitored in this study presents a challenge for conventional remediation methods in treating groundwater pollution. This necessitates investigation of alternative removal methods to remediate highly contaminated groundwater, both in in-situ and ex-situ remediation. As NZVI and bioremediation have been shown to be effective for the groundwater contaminants in this study, contaminant reduction using NZVI with and without biological reduction were tested in highly contaminated groundwater.

5.1 Chromium Removal

The presence of soil did not affect Cr(VI) degradation in any of the treatments. Abiotic reduction of Cr(VI) in diluted groundwater was readily apparent at concentrations as low as 500 mg Fe^0/L of NZVI. Complete Cr(VI) reduction was also shown in biotic reactors even under undiluted conditions, albeit at a considerably slower rate than abiotic reactors. However, no significant difference was found between biotic reactors and control reactors containing only molasses/nutrients. Thus, it is uncertain if Cr(VI) reduction in biotic reactors was due to bacterial activity or abiotic reaction with molasses. While total Cr(VI) reduction requires only enrichment with bacterial nutrients, abiotic reduction using NZVI was considerably faster. Additionally, no statistically significant change in reduction was seen between abiotic and bio-enhanced reactors. Therefore, Cr(VI) reduction in bio-enhanced reactors is more dependent on NZVI activity.

5.2 Nitrate Removal

No statistically significant difference in nitrate removal was seen due to the addition of soil in any treatment. Abiotic nitrate reduction using NZVI was possible, but only achieved

reliable effectiveness with at least 17,000 mg Fe^0/L. Biotic and bio-enhanced reactors showed higher levels of removal than abiotic reduction. The lack of a statistically significant difference between biotic and bio-enhanced suggests no additional reduction due to NZVI can be expected. Significant biotic nitrate significant was possible even under undiluted conditions, albeit variable.

5.3 Chlorate Removal

The presence of soil resulted in higher chlorate removal across all treatments. Abiotic reactors only showed significant chlorate reduction at 17,000 mg Fe^0/L. Bio-enhanced treatments achieved the highest levels of removal. However, at least 8,500 mg Fe^0/L of NZVI was needed to achieve higher removal efficiency than biotic treatments. Biotic chlorate removal using bacterial sludge alone showed dubious results. Only biotic treatments in the presence of soil degradation achieved consistent chlorate removal, which suggests chlorate reducing bacteria in soil as the main contributor in biotic chlorate removal.

5.4 Perchlorate Removal

The presence of soil did not influence perchlorate removal in any treatment. Despite initially showing statistically significant reduction at 5,000 mg Fe^0/L, abiotic reactors with higher doses were not effective at reducing perchlorate. Effective perchlorate reduction by NZVI likely requires higher doses than 17,000 mg Fe^0/L, as much of the NZVI was likely depleted by other contaminants. Both biotic and bio-enhanced treatments achieved limited perchlorate removal. Due to NZVI showing ineffective removal, it is likely perchlorate removal under bio-enhanced conditions is more dependent on microbial activity. Though biotic perchlorate removal was possible, it showed inconsistent results.

5.5 Chloroform Removal

Abiotic NZVI CF reduction showed statistically significant removal with doses as low as 5,000 mg Fe^0/L. The presence of soil did not reduction in abiotic reactors, but soil increased CF removal in biotic reactors. Additionally, bio-enhanced removal in the presence of soil showed a near significant increase over bio-enhanced reactors without soil. Bio-enhanced reactors achieved the highest levels of CF removal. However, at least 17,000 mg Fe^0/L of NZVI is needed to achieve higher removal than biotic treatments. Biotic CF removal was successful, albeit variable, showing greater levels when a richer sludge was used. The absence of chlorinated aliphatics in all treatments at all points of measurement suggests total reduction of CF to methane. However, the absence of chlorinated aliphatics and the presence of formaldehyde also supports oxidation through hydrolysis as the main pathway of CF removal by the bacteria used in this study.

5.6 Implications

Though abiotic and biotic removal of Cr(VI), CF, nitrate, and chlorate were possible. The faster and more consistent removal of groundwater contaminants shown by bio-enhanced NZVI endorses this method for the remediation of the groundwater from this site. However, it should be noted that even though some perchlorate removal was seen, final perchlorate readings for bio-enhanced reactors did not remove perchlorate below non-toxic levels. Therefore, more research is needed on fully remediating the contaminated water from this site.

5.7 Future Recommendations

Measurement variation between individual batch reactors was a significant problem in this research. This can be addressed by using a single batch reactor for each treatment instead of multiple small reactors. Using a single batch reactor would also address differential microbial

growth and ensure a consistent microbial colony to treat every contaminant. As most removal in this study did not fit first order kinetics, it is recommended that more frequent testing is performed, particularly within the first week of preparation, where most removal seemed to plateau. To more conclusively determine the presence of CF removal, the detection of methane is a more deterministic byproduct of biotic and abiotic reduction. Finally, a better characterization of the bacterial flora present in this study is needed, which promotes the use of molecular analysis of the sludge, soil, and biotic reactors. Inoculation with pure bacterial cultures would also help determine which bacterial species would help remediate the groundwater in this study

APPENDIX A: Specifications for 25S Nano-Scale Zero Valent Iron

NANOIRON
FUTURE TECHNOLOGY

NANOFER 25S

Safety Data Sheet

according to Regulation (EC) No. 1907/2006 (REACH) with its amendment Regulation (EU) 2015/830
Date of issue: 1/1/2009 Revision date: 12/12/2018 Supersedes: 6/26/2018 Version: 0.2

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1. Product identifier

Product form	: Mixture
Trade name	: NANOFER 25S
Chemical name	: Iron - suspension
EC-No.	: 231-096-4
CAS-No.	: 7439-89-6
REACH registration No	: A registration number is not available for this substance as the substance or its uses are exempted from registration, the annual tonnage does not require a registration or the registration is envisaged for a later registration deadline.

1.2. Relevant identified uses of the substance or mixture and uses advised against

1.2.1. Relevant identified uses

Main use category	: Industrial use
Industrial/Professional use spec	: For laboratory usage, for industrial usage, it is highly applicable in the reduction technologies of ground water remediation and waste water treatment.

1.2.2. Uses advised against

No additional information available

1.3. Details of the supplier of the safety data sheet

NANO IRON, s.r.o.
Topolová 933
667 01 Židlochovice - Czech Republic
T +420 513 033 633 - F +420 547 230 212
info@nanoiron.cz

1.4. Emergency telephone number

No additional information available

Country	Organization/company	Address	Emergency telephone number	Comment
USA	American Association of Poison Control Centers	515 King Street Suite 510 | Alexandria, VA 22314	1-800-222-1222	web site: PoisonHelp.org

SECTION 2: Hazards identification

2.1. Classification of the substance or mixture

Classification according to Regulation (EC) No. 1272/2008 [CLP]

Not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008.

Adverse physicochemical, human health and environmental effects

No additional information available ,

2.2. Label elements

Labelling according to Regulation (EC) No. 1272/2008 [CLP]

The product does not need to be labelled in accordance with EC directives or respective national laws.

Precautionary statements (CLP) : P280 - Wear Protective gloves, protective clothing, eye protection, face protection.

2.3. Other hazards

Other hazards not contributing to the classification : Upon contact with water, a small amount of explosive hydrogen gas may be released (less than 1 l / 1 kg per hour).

SECTION 3: Composition/information on ingredients

3.1. Substances

Not applicable

3.2. Mixtures

Name : NANOFER 25S

Name	Product identifier	%	Classification according to Regulation (EC) No. 1272/2008 [CLP]
Propylene glycol	(CAS-No.) 57-55-6 (EC-No.) 200-338-0	77	Not classified
Iron	(CAS-No.) 7439-89-6 (EC-No.) 231-096-4	14 - 18	Not classified

Iron oxide	(CAS-No.) 1345-25-1 (EC-No.) 215-721-8	2- 6	Not classified
Surfactant	- -	3	Not classified
Carbon	(CAS-No.) 7440-44-0 (EC-No.) 931-328-0	0 - 1	Not classified

SECTION 4: First aid measures

4.1. Description of first aid measures

First-aid measures general : Remove all contaminated clothing and footwear. If you feel unwell, seek medical advice (show the label where possible). In case of burns, it is necessary to proceed in accordance with first aid for burns. Never give anything by mouth to an unconscious person.

First-aid measures after inhalation : Assure fresh air breathing. Allow the victim to rest. If symptoms persist call a doctor.

First-aid measures after skin contact : Remove affected clothing and wash all exposed skin area with mild soap and water, followed by warm water rinse. Wash skin thoroughly with mild soap and water. Apply emollient cream. NEVER use solvents or thinners. If skin irritation or rash occurs: Get medical advice/attention.

First-aid measures after eye contact : IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.

First-aid measures after ingestion : Drink plenty of water and induce vomiting, get immediate medical attention.

4.2. Most important symptoms and effects, both acute and delayed

Symptoms/effects : Not expected to present a significant hazard under anticipated conditions of normal use. More detailed information: See section 11.

4.3. Indication of any immediate medical attention and special treatment needed

No additional information available

SECTION 5: Firefighting measures

5.1. Extinguishing media

Suitable extinguishing media : The product itself is not flammable.

Unsuitable extinguishing media : No restrictions.

5.2. Special hazards arising from the substance or mixture

Fire hazard : When burning, the combustion gases and steam can be produced. The inhalation of decomposition combustion products may result in health damage.

5.3. Advice for firefighters

Firefighting instructions : Extinguish using standard precautions from a safe distance.

Protection during firefighting : Use self-contained breathing apparatus.

SECTION 6: Accidental release measures

6.1. Personal precautions, protective equipment and emergency procedures

General measures : Respect the guidelines specified within points 7 and 8. Prohibition of entry by unauthorized persons. Remove ignition sources. Proper grounding procedures to avoid static electricity should be followed. No open flames, no sparks, and no smoking.

6.1.1. For non-emergency personnel

Emergency procedures : Evacuate unnecessary personnel.

6.1.2. For emergency responders

Protective equipment : Avoid contact with eyes, skin and clothing. In case of possible negative impact due vapors use breathing apparatus. Equip cleanup crew with proper protection.

Emergency procedures : Ensure adequate ventilation. Ventilate area.

6.2. Environmental precautions

Avoid increasing leakage. Do not allow product to spread into the environment. In case of greater leaks into the environment, to proceed according to local regulations and to contact the relevant departments of local authorities.

6.3. Methods and material for containment and cleaning up

Methods for cleaning up : Flush with plenty of water. Spill area may be slippery.

6.4. Reference to other sections

See Section 8 and 13 of this safety data sheet.

SECTION 7: Handling and storage

7.1. Precautions for safe handling

Precautions for safe handling	: Excess pressure to be let off by carefully opening the container before use. Never open the container at the temperature over 35°C. Ensure sufficient ventilation. Avoid sparks and other sources of ignition. Homogenize material and use the whole volume best. If unused material left, keep it sealed with inert gas (nitrogen, argon. Do not mix with oxidizing agents, acids, acetylene, ammonia. Do not allow the product conduction into drains, surface and ground water without permission from local authority.
Hygiene measures	: Do not eat, drink or smoke in areas where product is used. Wash hands and other exposed areas with mild soap and water before eating, drinking or smoking and when leaving work. Before break and after work take off contaminated clothing. Store this clothing separately.

7.2. Conditions for safe storage, including any incompatibilities

Technical measures	: Keep container tightly closed. Prohibit smoking. Prevent access by unauthorized persons.
Storage conditions	: Store in a cool place. Keep container tightly closed in a dry and well-ventilated place. Store under inert gas. Sensitive to moisture. Store in a tightly sealed container in a cool (the most suitable temperatures are 1-5°C), dry and well-ventilated area. Material may degrade on storage unless refrigerated. Do not freeze!
Incompatible materials	: Store separately from oxidizing agents, acids, alkalis, acetylene, ammonia.
Storage temperature	: 1 - 5 °C
Storage area	: Store in a cool place
Packaging materials	: Store in original packaging.

7.3. Specific end use(s)

Product usage is specified by its manufacturer within the instruction manual, which is on the package label or in attached documentation.

SECTION 8: Exposure controls/personal protection

8.1. Control parameters

No additional information available

8.2. Exposure controls

Appropriate engineering controls:

Avoid eye and skin contact. Before breaks and after work wash your hands.

Personal protective equipment:

Avoid all unnecessary exposure.

Materials for protective clothing:

Long sleeved protective clothing. Safety shoes. EN 344

Hand protection:

Nitrile-rubber protective gloves. EN 374. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices.

Type	Material	Permeation	Thickness (mm)	Penetration	Standard
	Nitrile rubber (NBR)	6 (> 480 minutes)	0,11		EN 374

Eye protection:

Tightly fitting safety goggles. EN 166. During a fire, use black glasses in addition (due risk of retina damage).

Skin and body protection:

Wash and dry hands. Use protective hand cream.

Respiratory protection:

Not required for normal conditions of use.

Personal protective equipment symbol(s):

Other information:

Do not eat, drink or smoke during use.

SECTION 9: Physical and chemical properties

9.1. Information on basic physical and chemical properties

Physical state	: Suspension.
Appearance	: Liquid. Nanomaterial.
Colour	: Black.
Odour	: None.
Odour threshold	: No data available
pH	: 9,5 – 11,5
Relative evaporation rate (butylacetate=1)	: No data available
Melting point	: No data available
Freezing point	: No data available
Boiling point	: No data available
Flash point	: No data available
Auto-ignition temperature	: No data available
Decomposition temperature	: No data available
Flammability (solid, gas)	: Flammable solid.
Vapour pressure	: No data available
Relative vapour density at 20 °C	: No data available
Relative density	: No data available
Density	: 1,15 - 1,25 g/cm^3 (20°C)
Solubility	: Miscible with water.
Log Pow	: No data available
Viscosity, kinematic	: No data available
Viscosity, dynamic	: No data available
Explosive properties	: No data available
It does not have oxidising properties	: No data available
Explosive limits	: No data available

9.2. Other information

Other properties	: Granulometry: d50 < 50nm Specific surface: > 25m^2/g Surface charge: zero (0)

SECTION 10: Stability and reactivity

10.1. Reactivity
With water - a small volume of hydrogen is generated in water reaction (less than 1l/1kg.hr).

10.2. Chemical stability
Stable in use and storage conditions as recommended in item 7.

10.3. Possibility of hazardous reactions
Not specified.

10.4. Conditions to avoid
Avoid air and high temperatures.

10.5. Incompatible materials
Oxidizing agent. Acids. Acetylene. Ammonia.

10.6. Hazardous decomposition products
At high temperatures, the product can produce hazardous decomposition products. See section 5.

SECTION 11: Toxicological information

11.1. Information on toxicological effects

Acute toxicity (oral)	: Not classified
Acute toxicity (dermal)	: Not classified
Acute toxicity (inhalation)	: Not classified

NANOFER 25 (values are based on pure nanoparticles whose content in NANOFER 25S is 20 wt.%)	
LD50 oral rat	30000 mg/kg bw/day

Skin corrosion/irritation	: Not classified
Additional information	: Based on available data, the classification criteria are not met
Serious eye damage/irritation	: Not classified
Additional information	: Based on available data, the classification criteria are not met
Respiratory or skin sensitisation	: Not classified
Additional information	: Based on available data, the classification criteria are not met

Germ cell mutagenicity	: Not classified
Additional information	: Based on available data, the classification criteria are not met
Carcinogenicity	: Not classified
Additional information	: Based on available data, the classification criteria are not met
Reproductive toxicity	: Not classified
Additional information	: Based on available data, the classification criteria are not met
STOT-single exposure	: Not classified
Additional information	: Based on available data, the classification criteria are not met
STOT-repeated exposure	: Not classified
Additional information	: Based on available data, the classification criteria are not met
Aspiration hazard	: Not classified
Additional information	: Based on available data, the classification criteria are not met
Potential adverse human health effects and symptoms	: Based on available data, the classification criteria are not met.
Other information	: Inhalation of dust can irritate the respiratory system.

SECTION 12: Ecological information

12.1. Toxicity

Acute aquatic toxicity	: Not classified
Chronic aquatic toxicity	: Not classified

NANOFER 25 (values are based on pure nanoparticles whose content in NANOFER 25S is 20 wt.%)	
LC50 fish 1	2976 mg/l
EC50 Daphnia 1	13248 mg/l
EC50 72h algae (1)	1080 mg/l

12.2. Persistence and degradability

NANOFER 25	
Persistence and degradability	Not established.

12.3. Bioaccumulative potential

NANOFER 25	
Bioaccumulative potential	Not established.

12.4. Mobility in soil

No additional information available

12.5. Results of PBT and vPvB assessment

PBT, vPvB: not relevant - inorganic substance

12.6. Other adverse effects

Additional information : Avoid release to the environment.

SECTION 13: Disposal considerations

13.1. Waste treatment methods

Regional legislation (waste)	: European waste catalogue. Directive 2008/98/EC of the European Parliament and of the Council on waste and repealing certain Directives, in the valid wording.
Waste treatment methods	: The substance and its surpluses must be put only to specified area for waste and must be dispose together with the sorted waste, e.g. in waste incinerators.
Sewage disposal recommendations	: See article 5.3.
Product/Packaging disposal recommendations	: Dispose in a safe manner in accordance with local/national regulations. Empty containers completely. Hand empty containers over to authorized company that has permissions for their removal. Dispose waste in accordance with relevant local regulations with suitable devices. Sort and put other waste according to type of material into containers for recycling or to places specified by local authorities.
Ecology - waste materials	: Avoid release to the environment.
European List of Waste (LoW) code	: Product: 06 03 99 - wastes not otherwise specified Burned product: 06 03 16 - metallic oxides other than those mentioned in 06 03 15 Containers and packaging: 15 01 04 - metallic packaging Determined waste catalogue numbers are recommended based on probable usage of this product. Based on special usage and its real disposal by user, the other waste catalogue numbers can be used as well.Determined waste catalogue numbers are recommended based on probable usage of this product. Based on special usage and its real disposal by user, the other waste catalogue numbers can be used as well.

SECTION 14: Transport information

The product is not dangerous for transport in accordance with ADR / RID / IMDG / IATA / ADN. Transport away from oxidising materials and heat / fire sources. Follow all regulations in your country.

SECTION 15: Regulatory information

15.1. Safety, health and environmental regulations/legislation specific for the substance or mixture

15.1.1. EU-Regulations

No REACH Annex XVII restrictions
NANOFER STAR is not on the REACH Candidate List
NANOFER STAR is not on the REACH Annex XIV List

15.1.2. National regulations

No additional information available , follow the national US regulations

15.2. Chemical safety assessment

A chemical safety assessment has been carried out

SECTION 16: Other information

Data sources	: REGULATION (EC) No 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on classification, labelling and packaging of substances and mixtures (CLP). Manufacturer/Supplier information. Registration dossier.
Training advice	: Familiarize workers with recommended use, mandatory protective equipment, first aid, and forbidden product manipulation.
Other information	: This information is based on our current knowledge and is intended to describe the product for the purposes of health, safety and environmental requirements only. It should not therefore be construed as guaranteeing any specific property of the product. They cannot be considered as a guarantee for appropriateness and usability for particular application.

APPENDIX B: Additional Measured Parameters

Table B1: Additional Measured Parameters in Groundwater

Parameter	Unit	Results
Aluminum	mg/L	0.00
Arsenic	mg/L	0.012
Boron	mg/L	1.10
Calcium	mg/L	2,263.00
Cadmium	mg/L	0.00
Cobalt	mg/L	0.00
Copper	mg/L	0.00
Iron	mg/L	0.00
Potassium	mg/L	78.90
Magnesium	mg/L	1171
Manganese	mg/L	0.01
Molybdenum	mg/L	0.01
Sodium	mg/L	7,619
Nickel	mg/L	0.00
Phosphorus	mg/L	0.10
Lead	mg/L	0.00
Sulfur	mg/L	408.4
Selenium	mg/L	0.00
Silica	mg/L	17.08
Strontium	mg/L	65.43
Zinc	mg/L	0.01
TDS	mg/L	23,700.00
Hardness	mgCaCO3/L	10,473.00
Nitrate	mg/L	338.00
Chloride	mg/L	5,440.00

Table B2: Additional Measured Parameters in Soil

Parameter	Unit	Results
Aluminum	mg/g	0.03
Arsenic	mg/g	0.00
Boron	mg/g	0.00
Calcium	mg/g	0.75
Cadmium	mg/g	0.00
Cobalt	mg/g	0.00
Copper	mg/g	0.00
Iron	mg/g	0.01
Potassium	mg/g	0.11
Magnesium	mg/g	0.44
Manganese	mg/g	0.00
Molybdenum	mg/g	0.00
Sodium	mg/g	2.96
Nickel	mg/g	0.00
Phosphorus	mg/g	0.00
Lead	mg/g	0.00
Sulfur	mg/g	0.50
Selenium	mg/g	0.00
Silica	mg/g	0.54
Strontium	mg/g	0.02
Zinc	mg/g	0.00
TDS	mg/g	14.43
Hardness	mgCaCO3/g	3.65
Nitrate	mg/g	0.07
Chloride	mg/g	1.70

Table B3: Additional Measured Parameters in Molasses Solution

Parameter	Unit	Results
Aluminum	mg/L	10.10
Arsenic	mg/L	0.00
Boron	mg/L	0.00
Calcium	mg/L	3,838.00
Cadmium	mg/L	0.00
Cobalt	mg/L	0.00
Copper	mg/L	3.03
Iron	mg/L	63.63
Potassium	mg/L	5,282.30
Magnesium	mg/L	515.10
Manganese	mg/L	2.02
Molybdenum	mg/L	0.00
Sodium	mg/L	838.30
Nickel	mg/L	0.00
Phosphorus	mg/L	90.90
Lead	mg/L	0.00
Sulfur	mg/L	1,676.60
Selenium	mg/L	0.00
Silica	mg/L	490.15
Strontium	mg/L	14.14
Zinc	mg/L	2.02
TDS	mg/L	48,682.00
Hardness	mgCaCO3/L	11,716.00
Nitrate	mg/L	0.00
Chloride	mg/L	2,383.60

APPENDIX C: NZVI Stoichiometric Ratio Calculator

Table C1: Calculator for NZVI Mass Ratios

C1	C2	C3	C4	C5	C6	C7	C6
			Input Water	C4/C2	C3*C5	C6/C5	(C6*56) /(C4*C2)
Contaminant	**Molar Mass**	**Molar Ratio**	**Groundwater (4X) Contaminant Concentration**		**Molar Ratio Fe^0: Contaminant**		**Mass Ratio Fe^0: Contaminant**
	g/mol	**Fe^0: Contaminant**	**mg/L**	**mmol/L**	**mmol Fe^0/L**	**mmol Fe^0/mmol**	**mg Fe^0 /mg**
Cr(VI)	52.0	1.5	22.5	0.43	0.65	1.51	1.63
Nitrate	62.0	4.0	88.50	1.36	5.42	3.80	3.43
Chlorate	83.5	3.0	6,825.00	81.74	245.21	3.01	2.02
Perchlorate	99.5	4.0	910.00	9.15	37.11	4.12	2.31
CF	119.5	3.0	1.25	0.01	0.03	3.21	1.59

Table C2: Calculator for Total NZVI Needed

C1	C2	C3	C4	C5	C6	C7
			Input Water	C5 /C2	Input Table C1	C4*C6
Contaminant	**Molar Mass**	**Molar Ratio**	**Groundwater (4X) Contaminant Concentration**		**Mass Ratio Fe^0: Contaminant**	**NZVI Needed for Contaminant**
	g/mol	**Fe^0: Contaminant**	**mg /L**	**mmol /L**	**mg Fe^0 /mg**	**mg Fe^0 /L**
Cr(VI)	52	1.5	22.5	0.43	1.63	36.35
Nitrate	62	4.0	88.5	1.36	3.43	319.74
Chlorate	83.5	3.0	6,825.0	81.74	2.02	13,731.74
Perchlorate	99.5	4.0	910.0	9.15	2.31	2,048.64
CF	119.5	3	1.13	0.01	1.59	1.59
Total						**16,138.1**

Table C3: Calculator for Fraction of Total NZVI Needed at Various NZVI Doses

C1	C2	C3	C4	C5
Input Stock Dose	**Input Stock Fraction**	**(C2*C3)/100**	**Input Total (Table C2)**	**C3/C4**
NZVI Stock Dose	**NZVI Stock Fraction**	**NZVI Dose**	**Total ZVI needed**	**Fraction of Total NZVI needed**
mg/L	**Percent**	**mg Fe^0/L**	**mg Fe^0/L**	
3,000	17	510	16,138.1	**0.03**
30,000	17	5,100	16,138.1	**0.32**
50,000	17	8,500	16,138.1	**0.53**
100,000	17	17,000	16,138.1	**1.06**

Table C4: Calculator for Fraction of NZVI Required by each Contaminant

C1	C2	C3	C4
	Input Total Table C2	**Input (Table C2)**	**(C3/C2)*100**
Contaminant	**Total ZVI needed**	**NZVI Needed for Contaminant**	**Percent of Total NZVI Needed for Contaminant**
	mg Fe^0/L	**mg Fe^0/L**	**Percent**
Cr(VI)	16,138.1	36.4	0.019
Nitrate	16,138.1	319.7	2.00
Chlorate	16,138.1	13,731.7	85.10
Perchlorate	16,138.1	2,048.6	12.70
CF	16,138.1	1.6	0.01

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Education

B.S. in Biological Sciences, University of Nevada, Las Vegas, Graduated 2014

M.S. in Civil and Environmental Engineering, University of Nevada, Las Vegas, Graduated 2020

SKILLS
Computer software: Microsoft Word, Excel, PowerPoint, AutoCAD.
Writing Skills: Competent in report writing and editing.
Languages: Fluent in Spanish and English.
Chemistry: Proficient in general and water chemistry.
Microscopy: Compound Light Microscope, Scanning Electron Microscope.

RELEVANT EXPERIENCE
June 2016-May2020: Graduate Assistant. UNLV Environmental Engineering Laboratory
20 hours per week, Supervisor: Dr. Jacimaria Batista
Treating water in contaminant reduction. Analyzing samples under an Electron microscope. Conducting research on chloroform reduction using bacteria and Zero-Valent-Iron.

June 2018-September 2018: Kitchen Staff. Ken's Foods
45 hours per week, Supervisor: Stephanie Adams
Performing quality control procedures on food products such as pH testing and viscosity testing.

May 2017-September 2017: General Compounder.
Genesis Pharmaceuticals, 40 hours per week, Supervisor: Miguel Chinchilla
Manufacturing and cataloguing various pharmaceutical products. Working with heavy machinery such as pumps, industrial mixers and boilers.

March 2, 2016-May 19, 2016: Anatomy Lab Technician.
Medcure Las Vegas, 40 hours per week, Supervisor: Karim Muradian
Harvesting and suturing human tissue. Using and sterilizing medical equipment for operating on cadavers.

August 25, 2014-December 8, 2015: Part-time Instructor University of Nevada, Las Vegas. 20-30 hours per week, Supervisor: Nicole Espinoza
Instructing students in introductory biology. Performing dissections on various organisms ranging from plants to animals. Prepared assignments and exams relating to the material presented.

June 20, 2015-August 20, 2015: Field Technician. University of Nevada, Reno.
40 hours per week, Supervisor: Beth Newingham
Identifying and cataloguing grassland vegetation. Performed data collection by Geotagging locations in Oregon and California with native grass species.

June 21, 2013-August 21, 2013: Undergraduate Researcher. Northern Illinois
40 hours per week. University, Supervisor: Nicholas A Barber PhD.

Performing research on population dynamics of beetles as a measure of grassland health. Using R statistical software. Writing scientific reports detailing local animal and plant relations.

November 1, 2010-June 1, 2011 Laboratory Assistant. Desert Research Institute of Las Vegas, NV. 20 hours per week, Supervisor: Kumud Acharya PhD.
Performing hazardous waste disposal. Maintaining mussel and cell colonies. Gathering field data pertaining to root samples of various plants and soil samples.

VOLUNTEER EXPERIENCE:

August 25, 2009- August 25, 2013: Member of Alpha Epsilon Delta, regularly attended humanitarian events such as Get Outdoors, Best Buddies, Habitat for Humanity, and Ragnar Relay.

AWARDS:

June 2013: REU summer research program award at Northern Illinois University.
May 2008: Honors Diploma, Silverado High school.
June 2008-May 2013: Millennium Scholarship.