

**MICROBially-MEDIATED TRANSFORMATIONS OF  
CHLORINATED SOLVENTS AND ENGINEERED  
NANOMATERIALS IN THE SUBSURFACE:  
IMPLICATIONS FOR BIOREMEDIATION, NATURAL  
ATTENUATION, FATE AND TRANSPORT**

A Dissertation

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## Abstract

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Ubiquitous subsurface microorganisms, including organohalide-respiring, iron- and sulfate-reducing, and biofilm-forming bacteria can impact the fate and transport of chlorinated organic contaminants and engineered nanoparticles released into the subsurface. Bioremediation via microbial reductive dechlorination (MRD) is a common treatment remedy for chlorinated solvents such as tetrachloroethene (PCE). The delivery of fermentable electron donor (ED) substrates to stimulate MRD in chlorinated solvent groundwater plumes and source zones is impeded by ED consumption by non-target microbes, preventing sustained MRD and leading to formation of undesirable byproducts such as sulfide. The effect of iron reduction on the MRD of PCE alone and in the presence of sulfate reduction was evaluated in 1-dimensional column studies; increased dechlorination was observed in the presence of iron-reducing bacteria (IRB), possibly because conditions were more reduced compared to IRB-free conditions. Under both iron- and sulfate-reducing conditions, MRD proceeded under higher (10.8 mM) sulfide levels compared to concentrations previously reported (5 mM) to inhibit MRD, presumably due to removal of sulfide from solution via reaction with reduced iron. In another column study, the partitioning electron donor (PED) n-butyl acetate (nBA) was evaluated against a conventional water-soluble electron donor (lactate) for its ability to partition into a mixed PCE non-aqueous-phase liquid (NAPL), sustain PCE MRD, and support bioenhanced NAPL dissolution. In abiotic systems, nBA persisted longer and exhibited higher NAPL partitioning compared to lactate. Bioenhanced dissolution of PCE-NAPL and PCE dechlorination performance by nBA were superior or comparable to that of lactate in biotic columns, suggesting that PEDs are promising EDs for use in NAPL source zones. Iron oxide nanoparticles (IONPs) are an important class of nanoparticles that may enter the subsurface intentionally or unintentionally, with potential but

unknown impacts from IRB or biofilm-forming bacteria. The susceptibility of oleic acid (OA)-stabilized IONPs to iron reduction by a model IRB was quantified in batch reactor experiments, and the impacts of *Pseudomonas fluorescens* biofilm on IONP transport were measured in column experiments. The OA coating reduced but did not eliminate reduction of the IONPs, while IONP transport was completely inhibited by *P. fluorescens* biofilm at seepage velocities of 0.4 and 4 m/d.

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---

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# List of abbreviations and acronyms

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AROC – aquagenic refractory organic matter

BDI – Bio-dechlor Inoculum

BTC – breakthrough curve

CERLA – Comprehensive Environmental Response, Compensation and Liability Act

*cis*-DCE – *cis*-1,2-dichloroethene

COOH – carboxyl group

CNTs – carbon nanotubes

DCE – dichloroethene

*Dhc* – *Dehalococcoides mccartyi*

DLVO – Derjaguin-Landau-Verwey-Overbeek

DNAPL – dense non-aqueous phase liquid

DOC – dissolved organic carbon

DOM – dissolved organic matter

e<sup>-</sup> – reducing equivalent

ED – electron donor

ENMs – engineered nanomaterials

ENPs – engineered nanoparticles

EPS – extracellular polymeric substances

ERH – electrical resistive heating

FAs – fulvic acids

FC – fulvic compounds

FeS – iron sulfide

FFO – Federal Fine Ottawa

FID – flame ionization detector

GC – gas chromatography

GC-FID – gas chromatography with flame ionization detection

*Geo* – *Geobacter* species

*GeoSZ* – *Geobacter lovleyi* strain SZ

GONPs – graphene-oxide nanoparticles

HAs – humic acids

HPLC – high pressure liquid chromatography

HD – hexadecane

IC – ion chromatography

IHSS – International Humic Substances Society

IONP – iron oxide nanoparticles

IRB – iron-reducing bacteria

ISB – *in situ* bioremediation

ISCO – *in situ* chemical oxidation

ISCR – *in situ* chemical reduction

ISTT – *in situ* thermal treatment

*K<sub>d</sub>* – Adsorption coefficient

*K<sub>NW</sub>* – aqueous phase:NAPL partitioning coefficient

LC/MS – liquid chromatography with mass spectrometry

MCL – Maximum Contaminant Levels

MNA – monitored natural attenuation

MRD – microbial reductive dechlorination

NAPL – non-aqueous phase liquid

NaBr – sodium bromide

nBA – n-butyl acetate

nAg – nano-sized silver

nAg-CIT – citrate-coated nano-silver

nC60 – nano-sized fullerene

NOM – natural organic matter

NPs – nanoparticles

NPL – National Priorities List

nZVI – nano-scale zero valent iron

OA – oleic acid

OC – organic carbon

OM – organic matter

ORP – oxidation-reduction potential

PAA – polyacrylic acid

PCE – tetrachloroethene

PED – partitioning electron donor

PEO – polyethylene oxide

pH<sub>PZC</sub> – point of zero charge equivalent pH

PITTs – Partitioning Interwell Tracer Tests

PV(s) – pore volume(s)

PVP – polyvinylpyrrolidone

PZC – point of zero charge

qPCR – quantitative polymerase chain reaction

RCRA – Resource Conservation and Recovery Act

RDase – reductive dehalogenase

$R_f$  – retardation factor

SERDP – Strategic Environmental Research and Development Program

SM – supplementary material

SRB – sulfate-reducing bacteria

SSQ – sum of squared residuals

SVE – soil vapor extraction

SWNTs – single-walled carbon nanotubes

TCE – trichloroethene

TCH – thermal conductive heating

TEAP – terminal electron-accepting processes

TOC – total organic carbon

UFPs – ultra-fine particles

USEPA – United States Environmental Protection Agency

VC – vinyl chloride

VFA(s) – volatile fatty acid(s)

VOCs – volatile organic compounds

ZVI – zero-valent iron

## 1. Introduction

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Subsurface microbial communities directly and indirectly influence the fate, transport, and transformations of numerous naturally occurring materials, including iron oxides, sulfate, colloids (e.g., inorganic colloids, fulvic compounds, rigid biopolymers, etc.; Buffle et al., 1998), and chlorinated organic compounds, among other compounds and materials. The widespread natural occurrence of iron-bearing ores and dissolved sulfate has given rise to ubiquitous subsurface bacteria that derive energy from sulfur- and iron-reduction. Though less abundant, geogenic chlorinated organic compounds have also resulted in the evolution of bacteria capable of deriving energy from the organohalide respiration of these chemical species. Additionally, many species of bacteria produce biofilms, which can impact transport of colloids. Iron-, sulfur- and chlorinated ethene-reducing microbial communities and biofilm-producing bacteria can also impact the fate, transformation, and transport of anthropogenic materials with properties similar to naturally occurring substances, such as chlorinated solvents and iron oxide nanoparticles (IONPs). The work presented herein addresses key challenges and uncertainties associated with bioremediation of chlorinated solvents and impacts of subsurface iron-reducing and biofilm-forming bacteria on the fate and transport on IONPs.

Chlorinated solvent bioremediation is principally accomplished by bacteria capable of deriving energy from the microbial reductive dechlorination (MRD) of compounds such as tetrachloroethene (PCE) and trichloroethene (TCE), among others (Löffler et al., 2003; Adrian and Löffler, 2016). Few species can reduce PCE and TCE beyond *cis*-dichloroethene (*cis*-DCE) to vinyl chloride (VC) and ultimately to ethene, the non-toxic end product. The most-studied PCE-to-ethene dechlorinating microbe is *Dehalococcoides mccartyi* (*Dhc*) (DiStefano et al., 1991; Freedman and Gossett, 1989; Löffler et al., 2013; Maymó-Gatell et al., 2001). The

addition of subsurface amendments to stimulate MRD (aka biostimulation) frequently includes electron donors (Henry, 2010). However, due to the abundance of many different types of subsurface bacteria, electron donors injected into the subsurface to stimulate dechlorination by organohalide-respiring microbes can be consumed in competing terminal electron-accepting processes (TEAPs), increasing remediation costs and reducing the effectiveness of remedial actions (Aulenta et al., 2006; 2007; Fennell et al., 1997).

Biostimulation of MRD in PCE and/or TCE-contaminated aquifers often causes unintended stimulation of competing microbial metabolic processes including iron and sulfate reduction (Aulenta et al., 2007; Fennell et al., 1997; Mao et al., 2017). Sulfide produced during sulfate reduction has been shown to inhibit (5 mM) the MRD of PCE and TCE by *Dhc* in batch reactor and chemostat studies (Berggren et al., 2013; Mao et al., 2017; Hoelen and Reinhard, 2004). However, the performance of MRD in the presence of concurrent iron- and sulfate-reducing conditions and in dynamic (flow-through) systems representative of groundwater flow has not been addressed in previous studies. Since reduced (ferrous) iron removes sulfide from solution through the production of iron(II) sulfide (FeS) minerals, it is hypothesized that under both iron- and sulfate-reducing conditions, the inhibitory effects of sulfide on MRD will be mitigated. The effects of reduced iron on MRD of PCE alone and on MRD in the presence of PCE and reduced sulfate products were studied in column experiments, as presented in Chapter 3.

Consumption of electron donors by non-target bacteria during delivery of electron donor to chlorinated solvent source zones is an obstacle for the successful implementation of *in situ* bioremediation (ISB) in these zones. Bioremediation has emerged as a remedy in chlorinated solvent source zones because of enhanced contaminant mass removal via bioenhanced

dissolution of dense non-aqueous-phase liquid (DNAPL) contaminants (by up factors of 2-4 over expected abiotic dissolution; Amos et al., 2008; Sleep et al., 2006; Cápiro et al. 2015). However, delivering and maintaining electron donor in source zones at sufficient concentrations to support bioenhanced dissolution of chlorinated solvents, and targeting delivery to DNAPL-water interfaces, remains a challenge to the successful implementation of biostimulation in these areas (Chu et al., 2004; Da Silva et al., 2006; Yang and McCarty, 2000; 2002). Partitioning electron donors (PEDs) favorably partition into DNAPL (Cápiro et al., 2011) and could therefore selectively maintain electron donor concentrations at levels sufficient to maintain MRD in and near aqueous phase:DNAPL interfaces in source-zone regions. At the same time, electron donor competition by non-dechlorinating bacteria would theoretically be reduced, because high concentrations of dissolved chlorinated solvents located in these regions can be toxic to some competing microorganisms (McCarty et al., 2007; Aulenta et al., 2006), but tolerated by dechlorinating microbes (Adamson et al., 2003; Isalou et al., 1998; Nielsen and Keasling, 1999; Yang and McCarty, 2000). A one-year field-scale PED implementation test using n-butyl acetate (nBA) was performed in a TCE-DNAPL source zone. During the pilot test, mass removal of TCE DNAPL, ethene production (indicating microbial reductive dechlorination), and an increase in *Dhc* biomass were measured (Bartlett, 2016). However, a parallel, quantitative comparison between the aqueous phase:DNAPL partitioning behavior, rates of bioenhanced NAPL dissolution, and extent of dechlorination achieved when using a PED versus using a water-soluble electron donor is needed to determine whether PEDs are as effective as conventional electron donors. Addressing this knowledge gap is the focus of Chapter 4.

Engineered nanoparticles (ENPs) are manufactured and used in an increasing number of industrial applications, consumer products, and environmental technologies; therefore, their entry

into the environment through intentional or accidental pathways is inevitable (Klaine et al., 2008; Lin et al., 2010; Park et al., 2016). Iron oxide nanoparticles (IONPs) are an important class of ENPs with a potential for both unintentional and intentional release into the subsurface due to their use in consumer products (Ali et al., 2016; Campos et al., 2015; Moahapatra and Anand, 2010; Zhang et al., 2015), wastewater treatment (Lei et al., 2014; Li and Zhang, 2006; Liang et al., 2013; Lu et al., 2016; Ngomsik et al., 2012; Shipley et al., 2013), and environmental remediation (Araújo et al., 2015; Gutierrez et al., 2017; Jiang et al., 2018; Mohammed et al., 2017). They are also being considered as subsurface contrast agents, owing to their superparamagnetic properties, and may be intentionally released to the environment (Bagaria et al., 2013; Li et al., 2014; Park et al., 2012). However, the impacts of subsurface microorganisms on the fate and transport of IONPs are not well understood.

Surface coatings are commonly applied to ENPs to increase stability and enhance transport; particle surface characteristics are likely to control the susceptibility of ENPs to microbial transformations (Zhang et al., 2014). Production of oil-soluble IONPs is an effective way to obtain electrostatically-stabilized, monodisperse IONPs. Oleic acid (OA) is one of the most common organic compounds used to produce oil-soluble IONPs due to its ability to produce highly uniform IONPs with high saturation magnetization values (Wu et al., 2015). Some evidence (Bose et al., 2009; Yan et al., 2008) suggests that IONPs can be transformed by iron-reducing bacteria (IRB); the size and morphology of IONPs, along with species-specific biological reduction mechanisms (such as modes of electron transfer) are likely to influence the observed reduction rate. However, the potential for iron reduction of surface-coated IONPs by IRB is unknown. In Chapter 5, potential transformations of OA-coated IONPs by iron-reducing bacteria are investigated.

While there have been previous investigations of the impacts of biofilm on the transport of several types of electrostatically-stabilized ENPs through saturated porous media (Mitzel et al., 2016; Tripathi et al., 2012; Xiao and Wiesner, 2013), reports of impacts on the transport of OA-coated IONPs are lacking and there have been no studies of the potential impacts of biofilm on the transport of any ENP type at realistic groundwater velocities ( $< 1 \text{ m d}^{-1}$ ). The commonly-occurring biofilm-forming bacteria *Pseudomonas fluorescens* has a range of known habitats, including soil/rhizosphere, plant tissue, and various aquatic environments including wastewaters (Moore et al., 2006; Vasileva-Tonkova et al., 2006) and is adapted to survive in low-oxygen environments (Pitcher and Watmough, 2004; Moore et al., 2006). *P. fluorescens* is, therefore, an environmentally-relevant microbe with which to study potential interactions between biofilm-producing microbes and IONPs released into the environment through wastewater and other pathways, yet column transport studies employing *P. fluorescens* biofilms and ENPs have not been reported to date. Chapter 6 examines impacts of biofilm on OA-coated IONP transport through saturated media under groundwater flow rates of ca.  $0.4$  and  $4 \text{ m d}^{-1}$ .

The research presented in this dissertation aims to address the following identified research gaps:

- Considering that competition for electron donor by alternate TEAPs during *in situ* biostimulation of MRD at chlorinated organic solvent-contaminated sites is common, few studies have characterized potential impacts of these processes on MRD under dynamic (flow-through) conditions representative of groundwater flow. In particular, reports on the effects of Fe-reduction on MRD alone, or in the presence of sulfate reduction, are lacking, despite the potential for microbially-mediated iron reduction to offset MRD inhibition by sulfide.

- The use of a PED (e.g. nBA) for source zone bioremediation offers potential advantages over conventional water-soluble electron donors, including the ability to selectively maintain high electron donor concentrations at DNAPL:water interfaces, thereby possibly reducing electron donor consumption by non-target microbes and enhancing contaminant mass removal via bioenhanced dissolution. Although nBA has been successfully implemented at the field scale for remediation of chlorinated solvent DNAPL in a pilot study, a side-by-side comparison of the performance of nBA relative to a conventional electron donor is needed to quantify the advantages of nBA.
- While iron reduction of IONPs by IRB has been previously reported, the potential for iron reduction of surface-stabilized IONPs by IRB has not been studied, despite the fact that surface coatings are commonly applied to confer stability to particles, prevent surface passivation, and preserve intrinsic properties such as superparamagnetism. Since IONPs are being considered for applications that would require subsurface transport and recalcitrance to degradation and/or surface passivation, investigating the susceptibility of surface-coated IONPs to microbial iron reduction is critical.
- There have been no studies of the potential impacts of biofilm on the transport of IONPs, nor on any class of ENPs under condition representative of realistic groundwater flow rates ( $< 1 \text{ m d}^{-1}$ ). Additionally, no prior studies have investigated ENP transport through *P. fluorescens*-derived biofilms, despite the fact that this species is commonly detected in numerous subsurface environments and is therefore, an environmentally relevant biofilm-forming species.

The work presented in this dissertation addresses knowledge gaps related to bioremediation of chlorinated organic solvents in the presence of alternate TEAPs and in source-zones. This work

also increases our understanding of subsurface microbial impacts on the environmental fate and transport of OA-coated IONPs. Specifically, the experiments described below were designed to address the following objectives:

- *Determine the effect of iron reduction on MRD of PCE in a flow-through system and the performance of PCE MRD under iron- and sulfate-reducing conditions.*
- *Compare aqueous phase:non-aqueous-phase liquid (NAPL) partitioning behavior and residence time, MRD, and bioenhanced dissolution performance achieved using the PED nBA versus a conventional water-soluble electron donor substrate (sodium lactate) in a residual mixed PCE- NAPL.*
- *Quantify the susceptibility of OA-coated IONPs to microbially-mediated iron reduction by the dissimilatory IRB, *Geobacter sulfurreducens*.*
- *Characterize the impacts of *P. fluorescens* biofilm on the transport of OA-coated IONPs at environmentally-relevant pore water velocities and in transport fluids of differing electrolyte composition and ionic strength.*

Overall, the four studies completed and detailed below aim to improve bioremediation performance in chlorinated solvent source-zones, enhance our understanding of MRD under conditions of iron- and sulfate-reduction, and advance our understanding of the impacts of iron-reducing and biofilm-forming bacteria on OA-IONPs fate and transport. The results of this research can assist ISB practitioners in making decisions regarding electron donor substrate selection when chlorinated solvent DNAPL is present at a site and predict ISB performance at sites with high iron and/or sulfate concentrations. This research will also be of use to industries and decision makers interested in the subsurface fate and transport of IONPs, including (but not limited to) regulators considering the potential impacts of IONPs released unintentionally into

the subsurface or enterprises exploring the viability of utilizing IONPs as subsurface contrast agents.

## Chapter 1 References

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## 2. Background: Chlorinated solvents and engineered nanoparticles in the subsurface and their interactions with microbial communities

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This chapter provides a literature review with background information on chlorinated solvent contamination occurrence and remediation and on the environmental fate and transport of engineered nanoparticles (ENPs). Section 2.1 provides a general introduction to the problem of subsurface chlorinated solvent contamination and a history and summary of remediation technologies with emphasis on chlorinated ethene bioremediation via microbial reductive dechlorination (MRD), and remaining key challenges and uncertainties related to the application of MRD under iron- and sulfate-reducing conditions and in chlorinated solvent source zones. Section 2.2 provides a broad overview of ENPs and factors that influencing their transport in subsurface environments. Uncertainties pertaining to the impact of metal-reducing and biofilm-forming bacteria on the subsurface transformation and transport, respectively, of iron oxide nanoparticles (IONPs), an important class of ENPs, are highlighted.

### 2.1. Chlorinated solvents

#### **2.1.1. History of use, extent of contamination**

Chlorinated organic solvents such as tetrachloroethene (PCE) and trichloroethene (TCE) have been produced in large quantities and used in a broad variety of industrial, commercial, and even medical applications (Doherty, 2000). Their attractiveness is a result of their unique chemical properties and strong solvent characteristics. The use of PCE and TCE as degreasing agents became widespread in the U.S. during the 1940's (Grindstaff, 1998) and continued to increase into the 1970's when production peaked at ~800 and 600 million pounds, respectively.

Their use declined thereafter as public awareness of adverse ecosystem and human health impacts grew (Doherty, 2000; McCarty, 2010).

Regulations on the production and use of chlorinated solvents were passed, starting in California in 1966, as public knowledge of the extent of environmental contamination by chlorinated solvents expanded. In parallel the scientific understanding of the carcinogenicity of these chemicals increased (Doherty, 2000; McCarty, 2010). These occurrences also led in part to the creation of the U.S. Environmental Protection Agency (USEPA) and the passage of legislation including the Clean Air and Water Pollution Control Acts in the 1970's (Doherty, 2000; McCarty, 2010). However, the damage had already been done: Improper disposal techniques, historical ignorance about environmental fate and health impacts, and accidental releases have made chlorinated solvent contamination of groundwater a widespread problem (Grindstaff, 1998; McCarty, 2010). Chlorinated solvents are some of the most frequently occurring and persistent soil and groundwater contaminants; they have been detected at ca. 80% of uncontrolled hazardous waste (Superfund) sites and at thousands of U.S. Department of Defense sites (USEPA, 2004a; Kueper et al. 2014). Estimates on the extent of chlorinated solvent contamination vary but suggest that there are >100,000 and up to 400,000 contaminated sites in the US alone (Grindstaff, 1998; Kueper et al. 2014).

### 2.1.2. Chemical properties of chlorinated solvents and behavior in the subsurface

Chlorinated solvents such as PCE and TCE belong to a class of soil and groundwater contaminants known as Dense Non-Aqueous Phase Liquids (DNAPLs), owing to the fact that they exist as a separate phase in the presence of water and are denser than water (Cwiertny and Scherer, 2010). Both PCE and TCE are 2-carbon molecules containing one double bond (Fig 2.1) with properties including low flammability and aqueous solubility, and high volatility. A

summary of the relevant physical and chemical properties of PCE, TCE, and their chlorinated dechlorination daughter products is presented in Table 2.1. In addition to degreasing/dry cleaning applications, the unique properties of chlorinated solvents spurred their use as chemical intermediates, heat-transfer fluids, refrigerants, and metal cleaning agents, among other uses (Doherty et al., 2000; McCarty, 2010).

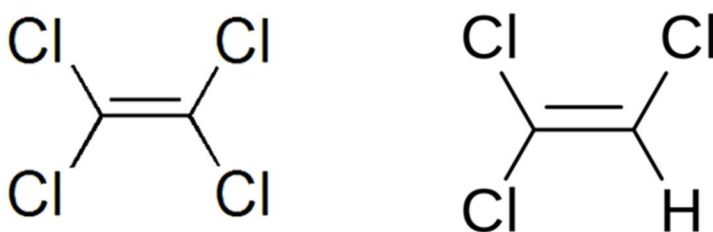


**Figure 2.1.** Chemical structures of PCE (left) and TCE (right)

**Table 2.1.** Nomenclature, chemical and physical properties of tetrachloroethene (PCE) and its chlorinated dechlorination daughter products. Adapted from Cwiertny and Scherer (2010)

| IUPAC Name                     | Formula                                       | Molar Weight | Density (g mL <sup>-1</sup> ) | Aqueous Solubility (mg L <sup>-1</sup> ) | Vapor Pressure (kPa) | K <sub>H</sub> <sup>a</sup> | Log K <sub>ow</sub> <sup>b</sup> | MCL <sup>c</sup> (mg L <sup>-1</sup> ) |
|--------------------------------|-----------------------------------------------|--------------|-------------------------------|------------------------------------------|----------------------|-----------------------------|----------------------------------|----------------------------------------|
| tetrachloroethene              | C <sub>2</sub> Cl <sub>4</sub>                | 165.8        | 1.63                          | 150                                      | 2.4                  | 26.3                        | 2.88                             | 0.005                                  |
| trichloroethene                | C <sub>2</sub> HCl <sub>3</sub>               | 131.4        | 1.46                          | 1,100                                    | 9.9                  | 11.7                        | 2.53                             | 0.005                                  |
| <i>cis</i> -1,2-dichloroethene | C <sub>2</sub> H <sub>2</sub> Cl <sub>2</sub> | 96.9         | 1.28                          | 3,500                                    | 27.1                 | 7.4                         | 1.86                             | 0.07                                   |
| vinyl chloride                 | C <sub>2</sub> H <sub>3</sub> Cl              | 62.5         | 0.91                          | 2,763                                    | 355                  | 79.2                        | 1.38                             | 0.002                                  |

Notes: <sup>a</sup> K<sub>H</sub> (Henry's Law constant) given in units of (x10<sup>-3</sup> atm • m<sup>3</sup> mol<sup>-1</sup>)

<sup>b</sup>K<sub>ow</sub> = octanol-water partitioning coefficient

<sup>c</sup>MCL = Maximum Contaminant Levels, source:

<https://www.epa.gov/sites/production/files/201803/documents/dwtable2018.pdf>

When released into the environment, PCE and TCE DNAPLs seep to the bottom of the water table, settling on impermeable or semi-permeable surfaces such as clay or bedrock (Kueper

et al., 2014; Sale and Newell, 2010). A number of factors influence the architecture of the resultant DNAPL source zones, including the duration, rate, and number of initial releases at a site, and the composition of the DNAPL (i.e., whether it is a mixed or pure substance). Site characteristics such as permeability and heterogeneity of aquifer materials, organic carbon content, and groundwater flow characteristics also play a large role in the evolution and distribution of source zone DNAPL (Kueper et al., 2014; Sale and Newell, 2010). The result is the occurrence of potentially mobile DNAPL “pools” and/or DNAPLs distributed in the form of discrete blobs and ganglia referred to as residual DNAPL (Figure 2.2). Owing to their low aqueous solubilities, PCE and TCE DNAPLs dissolve slowly into the groundwater, sustaining plumes of contaminated water that can persist for long periods of time at concentrations that are often orders of magnitude higher than drinking water standards (Cwiertny and Scherer, 2010; Kueper et al., 2014; McCarty, 2010). The remediation of sites contaminated with PCE and/or TCE therefore often involves treatment of both source-zone contaminants and groundwater plumes (Kueper et al., 2014; Sale and Newell, 2010).

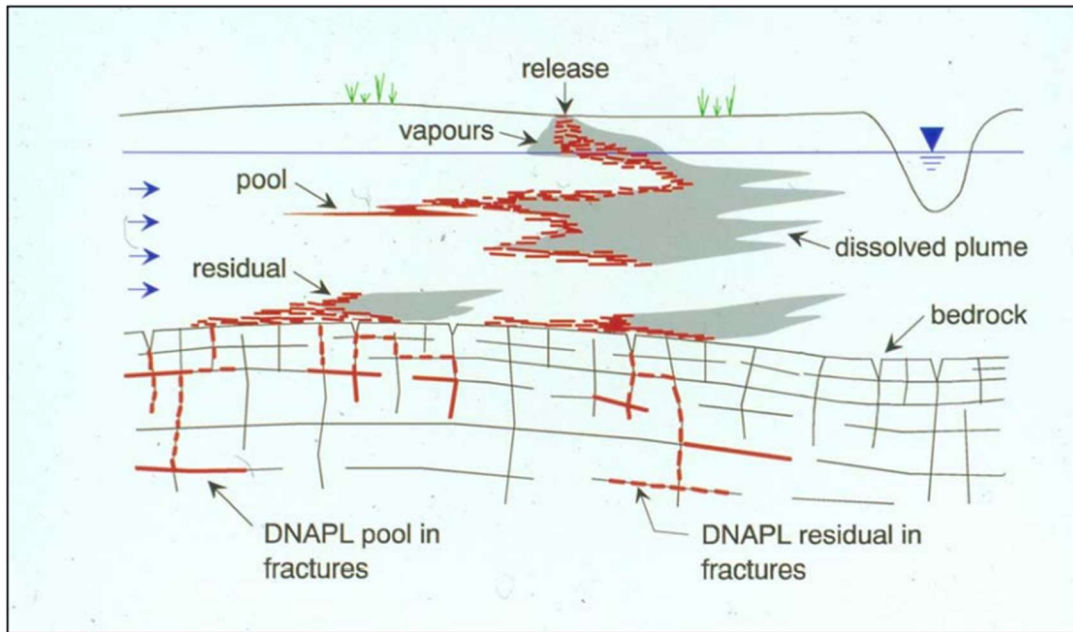


**Figure 2.2. Schematic showing development of DNAPL source zone. Following initial release of DNAPL (shown in red) in a heterogeneous aquifer system in which pooling occurs on low-permeability lenses, residual DNAPL is present in numerous locations including in fractured bedrock, and the flow of groundwater over the source material (blue arrows) creates plumes (shown in grey) of dissolved contaminant that migrate away from the source zone. Image: ITRC (2008).**

### **2.1.3. Overview of approaches for remediation of chlorinated organic solvents**

In 1980, the U.S. congress passed the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), which established a federal program for the cleanup of Superfund sites. Early technologies applied to the remediation of chlorinated solvents included primarily above-ground pump and treat processes, contaminated soil removal and *in situ* containment strategies including slurry walls. An increasing realization of the difficulty and expense associated with *ex situ* clean-up approaches, and a growing understanding of the movement and fate (i.e., advection, diffusion, sorption and degradation) of chlorinated organic contaminants in the subsurface during the 1980's led to the realization that residual and pooled DNAPL in subsurface source zones sustained long-term groundwater plumes (McCarty, 2010; Kueper et al., 2014). The passage of CERCLA spurred significant research and the development of numerous *in situ* technologies including air injection, thermal treatment technologies,

chemical reduction and oxidation, and co-solvent and surfactant flushing. The understanding of natural biotic and abiotic degradation pathways for many chlorinated organic contaminants including PCE and TCE also grew at this time (Kueper et al., 2014).

Air injection removes contaminants by capitalizing on their volatilities and includes technologies such as soil vapor extraction (SVE), bioventing, and air sparging (Johnson et al., 1994; McCarty, 2010). Bioventing (Wilson and Ward, 1987; Hincee and Alleman, 1997) is of limited use in the treatment of contaminants including PCE and TCE that are not readily degraded aerobically. Biosparging has also been used to stimulate the anaerobic biodegradation of TCE (Brockman et al., 1995), but this produces only co-metabolic degradation, which is a relatively slow and inefficient process (McCarty, 2010). *In situ* air stripping can also be a slow process, especially for rate-limited desorption of sorbed contaminants with low diffusion rates. *In situ* thermal treatment (ISTT) technologies were developed to increase desorption rates since these are temperature-dependent and include injection of heated fluids, electrical resistive heating (ERH) and thermal conductive heating (TCH) (McCarty, 2010), but these methods can be costly (USEPA, 2004b).

*In Situ* Chemical Oxidation (ISCO) has mainly focused on DNAPL treatment (McCarty, 2010), using oxidants such as permanganate, peroxide, Fenton's reagent, and persulfate (Kueper et al., 2014; McCarty, 2010). *In Situ* Chemical Reduction (ISCR) relies on reductive processes to reduce the organic carbon in chlorinated solvents to a lower oxidation state (e.g., ethane), but, both ISCO and ISCR result in a release of the organic chlorine in the form of chloride, and both generally utilize reactive and often hazardous materials. Cosolvent flushing and surfactant flushing are also technologies primarily used to target and remove DNAPL source mass that entail the subsurface injection of solvents or surfactants into contaminated zones to mobilize and

subsequently remove DNAPL (Abdul et al., 1990; Augustijn et al., 1994; Fountain et al., 1991; McCarty, 2010; Vigon and Rubin, 1989). Field-scale implementation of both cosolvent and surfactant flushing has shown that residual cosolvent and/or surfactant (assuming the selected surfactant is food-grade and biodegradable) remaining in the treatment zone could act as an electron donor and stimulate biodegradation of remaining contaminant (McCarty, 2010; Mravik et al., 2003; Ramsburg et al., 2004). Thus, these techniques are sometimes coupled with bioremediation as a combined DNAPL treatment remedy (McCarty, 2010). One potential hazard associated with these methods is the mobilization and subsequent migration of DNAPL (Fountain et al., 1991; Pennell et al., 1996).

### ***Bioremediation***

Bioremediation (the use of organisms to degrade contaminants) is the most commonly employed remediation technique for groundwater and soil sites contaminated by chlorinated solvents (Pandey et al., 2009; Lyon and Vogel, 2013, McGuire et al., 2016). Bioremediation compares favorably with other available physical-chemical treatment technologies because it has several advantages including relatively low capital costs, minimal infrastructure requirements, and no effluent capture requirements due to the benign nature of the dechlorination products (e.g., ethene, ethane) formed after full dehalogenation of target pollutants (Grindstaff, 1998; USEPA, 2000; Lyon and Vogel, 2013). Additionally, like ISCO and ISCR, bioremediation can result in the destruction and removal of chlorinated solvents, but unlike ISCO and ISCR, bioremediation does not entail the injection of potentially harmful or costly reductants or oxidants into the subsurface. Bioremediation frequently entails biostimulation, in which amendments used by dechlorinating microbes are supplied to a contaminated aquifer (often via

well injection) to stimulate organohalide respiration. Treatments may also include bioaugmentation; the addition of dechlorinating bacteria to a site (Lyon and Vogel, 2013).

#### **2.1.4 Microbial reductive dechlorination (MRD)**

Biodegradation pathways (e.g., reductive dechlorination, aerobic oxidation or co-metabolism, anaerobic oxidation, etc.) for chlorinated ethenes depend heavily on site-specific redox conditions and their succession (Chapelle and Bradley, 2004). Microbial reductive dechlorination (MRD) of chlorinated ethenes proceeds in a stepwise fashion via hydrogenolysis (replacement of a chlorine substituent with a hydrogen atom, Figure 2.3). The number of chlorine atoms contained within a chlorinated ethene molecule is predictive of the ease with which MRD will occur under anaerobic conditions: PCE, which is a strong oxidant, is readily reduced to TCE in any reducing environment, while the formation of cis-1,2-dichloroethene (cis-DCE) from TCE requires iron-reducing conditions; a further dechlorination step to yield vinyl chloride (VC) requires sulfate ( $\text{SO}_4^{2-}$ )-reducing conditions (Vogel et al., 1987; Chapelle, 1996). The reductive dechlorination of VC to ethene is the least thermodynamically-favorable step in anaerobic environments and requires extremely reduced (i.e. methanogenic) conditions (Vogel and McCarty, 1985; Barrio-Lage et al., 1987; 1990; Freedman and Gossett, 1989; DiStefano et al., 1991; De Bruin et al., 1992; Carter and Jewell, 1993; Maymó-Gatell et al., 1995). Chlorinated PCE daughter products with fewer chlorine atoms (e.g. DCE, VC) can also be degraded aerobically (Badin et al., 2016; Bradley, 2000; Bradley and Chapelle, 1998; Chapelle, 1996; Coleman et al., 2002; Gerritse et al., 1995), a process that can be enhanced in systems that also contain humic acids, which can act as electron acceptors (Bradley et al., 1998). Some aerobic methanotrophic bacteria can also degrade chlorinated ethenes (Yoon and Semrau, 2008; Lee et al., 2006; Hanson and Hanson, 1996).

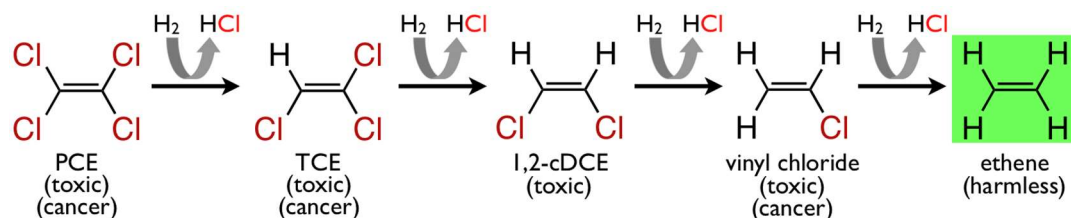


**Figure 2.3. Reductive dechlorination of PCE. Image modified from Paul Joseph McMurdie II, distributed under a CC BY-SA 3.0 license (<https://creativecommons.org/licenses/by-sa/3.0/deed.en>)**

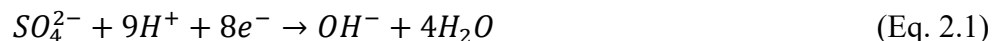
Fortuitous (cometabolic) PCE degradation has been observed across numerous anaerobic microbial populations, including fermenters, sulfate and iron-reducers, methanogens and acetogens (Fathepure et al., 1987; Futamata et al., 2001; Gerritse et al., 1995; Vogel et al., 1987; Bradley and Chapelle, 2000a). This degradation occurs at substantially lower rates compared to those observed in organohalide respiring bacteria that generate energy by utilizing chlorinated ethenes as electron acceptors (Bradley, 2000).

Numerous organohalohide-respiring bacteria can degrade PCE or TCE to cis-DCE, such as *Sulfurospirillum multivorans*, a strict anaerobe known to be able to extract energy through organohalide respiration of PCE (John et al., 2009; Buttet et al., 2013) and other *Sulfurospirillum* species (Luijten et al., 2003; Neumann et al., 1994), *Desulfuromonas michiganensis*; anaerobic bacteria which oxidize acetate and can reduce PCE (Sung et al., 2003), *Geobacter lovleyi* strain SZ (Sung et al., 2006), *Desulfitobacterium spp.* (Gerritse et al., 1996; Suyama et al., 2001), *Dehalobacter spp.* (Holliger et al., 1998; 1993), and *Clostridium bifermentans* strain DPH-1 (Chang et al., 2000). However, very few species are known to be capable of organohalide respiration of cis-DCE and VC, the best studied of which is *Dehalococcoides mccartyi* (*Dhc*, DiStefano et al., 1991; Freedman and Gossett, 1989; Löffler et al., 2013; Maymó-Gatell et al., 2001). Thermodynamic constraints on DCE and VC organohalide respiration along with

competition for electron-donor (Aulenta et al., 2007; Bennett et al., 2007; Hoelen and Reinhard, 2004) and inadequate quantities or non-uniform distributions of DCE-to-ethene respiring organisms (Bennett et al., 2007; Bradely, 2000; He et al., 2002; Lu et al., 2006; Yoshikawa et al., 2017) in the subsurface are thought to be responsible for the accumulation of cis-DCE often observed in subsurface regions of PCE and TCE contamination (Bradley, 2000; Lyon and Vogel, 2013; McCarty and Reinhard, 1993). This process is sometimes referred to as a "DCE stall" (Davis et al., 2008; McGuire et al., 2004).

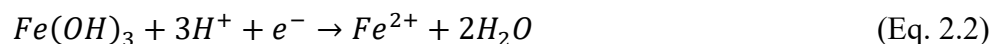
#### 2.1.5 Effects of iron- and sulfate-reduction on MRD of chlorinated solvents

Another potential obstacle to MRD of chlorinated organic solvents in the subsurface is the occurrence of sulfide, which has been shown to inhibit the activity of *Dhc* in microcosms (Hoelen and Reinhard, 2004; Mao et al., 2017) and chemostats (Berggren et al., 2013) at a concentration of 5 mM. Mao et al. (2017) investigated the effects of sulfate reduction on TCE dechlorination in batch reactors and found that sulfide inhibited dechlorination enzymes in *Dhc* strain 195 at 5 mM concentrations. The authors also hypothesized that sulfate-reducing bacteria, (SRB) which exhibit faster growth rates than *Dhc*, would out-compete *Dhc* at high H<sub>2</sub> concentrations (e- acceptor limited regimes). Sulfate is one of the most common anions found in groundwater (Liamleam and Annachhatere, 2007; Miao et al., 2012) and is readily reduced to sulfide by SRB, according to the general half-reaction:



Sulfide production is commonly measured during in situ bioremediation, however, inhibition of MRD is not always detected at sites where substantial sulfate reduction is measured (Aziz et al., 2013; Lee et al., 1998; Aulenta et al., 2006; Mao et al., 2017). The presence of iron-

reducing bacteria (IRB) and the production of reduced (ferrous) iron may be one reason why MRD inhibition is not always observed at sites co-contaminated with chlorinated solvents and  $\text{SO}_4^{2-}$ . Iron (Fe) is the fourth-most abundant crustal element, and microbially-mediated iron cycling (dominated mainly by members of the Archaea and Bacteria domains) is a critical component of biogeochemical cycles on Earth (Weber et al., 2006). Iron-oxidizing and iron-reducing microorganisms exploit the energetically-favorable redox potential between ferric [Fe(III)] and ferrous [Fe(II)] iron. At pH values above 4 in anoxic environments, Fe(III) oxides function as an electron donor and are readily-reduced to Fe(II) by IR (Weber et al., 2006). A generalized half-reaction for the reduction of ferric iron oxide is:



Under both iron- and sulfate-reducing conditions, ferrous iron rapidly reacts with sulfide, forming iron sulfide minerals; thereby removing sulfide from solution and potentially preventing sulfide-related inhibition of MRD. Competitive exclusion of SRB may also prevent sulfide inhibition of MRD in high-iron environments, where IRB have been observed to exclude sulfate-reducing activity by maintaining levels of dissolved hydrogen, formate, and acetate at lower values than minimum thresholds required SRB (Chapelle and Lovley, 1992).

The question of whether iron reduction benefits, harms, or is of no consequence to MRD is unclear. Some studies have observed or speculated that bioavailable Fe(III) in sediments within contaminated aquifers can hinder organohalide respiration of chlorinated ethenes due to competition for electron donor between iron-reducing bacteria and dechlorinators (Lu et al., 2001; McLean et al., 2006; Shani et al., 2013; Zaa et al., 2010). However, Fe(III) reduction has also been observed to have no impact (Wei and Finneran, 2011; 2013) or even to enhance MRD (Wei and Finneran, 2011). Previous studies have not reported the impacts of iron reduction on

MRD under dynamic (flow-through) conditions, or on the performance of MRD in the presence of both iron- and sulfate-reducing conditions in a flow-through system. Since *in situ* MRD in contaminated aquifers occurs under conditions of dynamic groundwater flow and in the presence of concurrent microbial processes including iron- and sulfate-reduction, it is desirable to understand the performance of MRD under these conditions. The experiments performed and evaluated in Chapter 3 are aimed at addressing these knowledge gaps.

### **2.1.6 Bioremediation of chlorinated solvent source zones**

As mentioned above, the presence of DNAPL in contaminant source zones can create long-lasting groundwater plumes (Cwiertny and Scherer, 2010; Kueper et al., 2014; McCarty, 2010; Stroo et al., 2012). Bioremediation of chlorinated DNAPLs such as PCE in source-zone regions was once considered infeasible due to high levels of dissolved solvents in these locations, which were thought to be toxic to dechlorinating organisms such as *Dhc*. However, subsequent studies (e.g. Amos et al., 2008; 2009; Glover et al., 2007) showed that organohalide respiration can occur in DNAPL-water interfaces near source zones and can result in enhanced contaminant mass removal via bioenhanced dissolution (relative to rates of dissolution expected under abiotic conditions) of DNAPL (by up factors of to 4.6; Amos et al., 2008; Sleep et al., 2006) and reduced source zone longevity. Bioenhanced dissolution is accomplished by increasing mass transfer of chlorinated solvent between dense DNAPL and dissolved phases species relative to what is expected under abiotic conditions. This is possible because the reductive dechlorination of parent compounds such as PCE increases the parent compound concentration gradient at DNAPL:water interfaces by lowering its concentration in solution. Additionally, the daughter compounds generated, such as cis-DCE, and vinyl chloride (VC),

have substantially higher aqueous solubilities compared to their precursor compounds (Table 2.1).

Although bioenhanced DNAPL dissolution is feasible and desirable to decrease source zone remediation times, delivering electron donor to source zones (Yang and McCarty, 2000; Löffler and Edwards, 2006) and maintaining sufficient concentrations (Yang and McCarty, 2002; Chu et al., 2004; Da Silva et al., 2006) to stimulate organohalide respiration is a major challenge to the successful implementation of source zone bioremediation. With respect to DNAPLs, electron donors can be classified according to their solubility in the DNAPL phase. Insoluble donors such as sodium lactate (water-soluble) do not partition into DNAPLs and presumably have relatively short source zone lifetimes. DNAPL-soluble electron donors include short-chain alcohols, edible oils such as emulsified vegetable oil (EVO), and partitioning electron donors (PEDs). Alcohols such as methanol, ethanol, and pentanol increased PCE DNAPL dissolution rates by factors of 2 to 3 in flow-through column (Yang and McCarty, 2002) and aquifer cell studies (Sleep et al., 2006), however in cases where competing terminal electron-accepting processes were present (Sleep et al., 2006), electron donors were quickly consumed by competing microbes. In this scenario, continuous and/or repeated injections of electron donor would be required, driving up remediation costs. Yang and McCarty (2002) also evaluated dechlorination performance of viscous olive oil premixed with PCE, and noted enhanced microbial dechlorination activity and DNAPL dissolution, presumably due to injection of a viscous electron donor capable of forming an immobile phase in proximity to the DNAPL:water interface. In practice, it would be difficult to ensure mixing of a fermentable oil-based electron donor with the DNAPL phase *in situ*. In a column study comparing MRD of PCE in various EVO treatments as well as a water-soluble electron donor, Long and Borden (2006) observed

lower biodegradation rates in emulsion treated columns and attributed these lower rates to emulsified oil barriers, suggesting that the use of EVO as an electron donor may extend the time required for remediation.

### **2.1.7 Partitioning electron donors for bioremediation of chlorinated solvent source zones**

One potentially advantageous strategy to overcome the limitations of both soluble and insoluble electron donor substrates is through targeted delivery of electron donor to DNAPL:water interfaces through the use of partitioning electron donors (PEDs), which are soluble in water but favorably partition into DNAPL phases. The concept of PEDs originated in Partitioning Interwell Tracer Tests (PITTs), which were originally developed in the fields of groundwater hydrology and petroleum reservoir engineering, but later used to estimate the volume and spatially averaged saturations of subsurface NAPL contaminants (Annable et al., 1998a, b; Brooks et al., 2002; Jin et al., 1995). The basic idea behind PITTs is that a pulse of tracers with different abilities to partition into NAPLs is injected into an injection well upstream from a zone of suspected NAPL contamination, and the timing of breakthrough of the various tracers is then monitored by collecting groundwater samples in a sampling well located downstream of the injection site. In the absence of NAPL, the tracers would all be expected to arrive at the sampling well at the same time and concentrations. However, if NAPL is present in the area swept by the tracers, those tracers which partition favorably into NAPL would be expected to arrive at the sampling well at later times and at lower concentrations compared to the tracers with unfavorable NAPL partitioning characteristics (Jin et al., 1995). Once it was confirmed that MRD DNAPL source zones was feasible (e.g., Carr et al., 2000; Yang and McCarty, 2000), the notion of an electron donor that could preferentially partition into NAPL phases and re-dissolve back into the aqueous phase concurrently with NAPL dissolution became

an attractive one, as this process could theoretically increase rates of DNAPL dissolution and, thus, reduce source zone longevity. Additionally, while consumption of electron donor by competing organisms is still a concern in DNAPL source-zone regions, the ability to selectively maintain high electron donor concentrations at DNAPL:water interfaces through the use of PEDs may give dechlorinating bacteria a competitive edge. The rationale being that high concentrations of dissolved chlorinated solvents located in these regions can be toxic to some competing microorganisms such as methanogens (McCarty et al., 2007; Aulenta et al., 2006), but tolerated by dechlorinating microbes.

One promising PED is n-butyl acetate (nBA). In an abiotic column study involving sand containing a uniform distribution of residual TCE-DNAPL, a pulse injection of 5g/L nBA resulted in strong partitioning into the residual DNAPL. The nBA persisted for up to 80 pore volumes in column effluent at concentrations greater than 1% of those initially injected (Cápiro et al., 2011). A one-year field-scale PED implementation test using the PED nBA was performed in a TCE DNAPL source zone in Cape Canaveral, FL, where high TCE concentrations were detected in a low permeability clay layer approximately 40 feet below land surface (Bartlett, 2016). The primary research objective was to evaluate the feasibility of using a PED for bioremediation of a TCE source zone. Results indicated mass removal of TCE DNAPL and ethene production (indicating microbial reductive dechlorination) as well as an increase in *Dhc* biomass over the course of the test, which were not observed during a baseline flux evaluation phase that was performed prior to the PED injection. A noted shortcoming of the field test was the absence of a parallel field demonstration utilizing a commonly-employed electron donor substrate in order to compare the results obtained using the PED to a standard substrate (Bartlett, 2016). A quantitative comparison of the rates of bioenhanced dissolution and extent of

dechlorination of chlorinated solvent DNAPL achieved using a PED with those obtained using a water-soluble electron donor is needed to determine whether PEDs are as effective as other potential electron donors and is the research focus of Chapter 4.

## **2.2. Engineered Nanoparticles and their transport in the subsurface**

Nanoparticles (NPs) are defined as materials having at least two dimensions between 1 and 100 nm. The nomenclature of NPs often varies depending on the media within which they occur; in air, for example, NPs have traditionally been called ultra-fine particles (UFPs) and extensive research has been conducted on the occurrence, sources, formation, and health effects of UFPs (e.g., Brown et al., 2003; Junkermann and Hacker, 2018; Kulmala et al., 2004; Oberdorster, 2001). In soil and aquatic systems, the term “colloid” is often applied to naturally-occurring nano-sized materials falling in the 1 nm to 1  $\mu$ m size range (Klaine et al., 2008); examples include soil colloids, metal NPs produced by a broad range of bacteria such as IRB (Ebrahiminezhad et al., 2017; Law et al., 2008; Narayanan and Sakthivel, 2010 ), humic and fulvic acids (HAs and FAs, respectively), proteins, peptides, & inorganic macromolecular species (Klaine et al., 2008). Engineered nanomaterials (ENM) including engineered nanoparticles (ENPs) have been produced using a wide array of different materials that can be broadly classed into groups including carbonaceous, metal oxide, semiconductor (e.g., quantum dots), zero-valent metal (e.g., Fe, Ag, Au) and dendrimer ENPs (Klaine et al., 2008).

ENPs have unique properties due to their size, including optical, magnetic, thermal/conductive, mechanical, and catalytic properties (Klaine et al., 2008) and as such are being used in numerous commercial, medical, and environmental industries (Dulińska-Litewka et al., 2019; Khan et al., 2019; Klaine et al., 2008; Wang et al., 2016). Navarro (2008) estimated

that the market value of nanotechnology-related products would approach \$1 trillion annually from 2011 to 2015. The significant production volume and widespread use of ENMs makes their entry into the environment inevitable, whether through manufacturing, transportation or consumer use of ENMs, or certainly by their disposal or application *in situ* for contaminant remediation. The potential for health and/or environmental risks of various classes of ENMs (Auffan et al., 2009; Baun et al., 2008a; Biswas and Wu, 2005; Boxall et al., 2007; Brumfiel 2003; Bystrzejewska-Piotrowska et al., 2009; Colvin, 2003; Klaine et al., 2008; Maynard et al., 2006; Moore, 2006; Nel et al., 2006; Nowack and Bucheli, 2007; Service, 2003) and their increasing use for contaminant remediation makes understanding the environmental behavior and fate of ENMs of paramount importance (Lin et al., 2010). Entry of ENMs such as nanoparticles into the environment may proceed via air, water, or soilborne routes, as well as their exchange between these media (e.g., Lin et al., 2010). While there is limited evidence that manufactured ENMs may become airborne (e.g., Schneider and Jensen, 2009; Stahlmecke et al., (2009), this is likely not a dominant pathway of environmental entry by ENMs.

### **2.2.1 Fate and transport of ENPs in the environment**

The stability of ENPs that enter the aqueous environment is a critical determinant of their fate: significant particle aggregation will lead to sedimentation while particles that stabilize in solution will subsequently be transported in the direction of water flow. Important determinants of ENPs in suspension include the pH, ionic strength, and electrolyte composition of the solution and the surface charge of the ENPs. Solution pH impacts the surface charge distribution and zeta potential of ENMs and is therefore a critical determinant of ENM transport through porous media (Wang et al., 2016). When pH of a given solution is below the point of zero charge (PZC)-

equivalent pH ( $\text{pH}_{\text{PZC}}$ ) of a given set of suspended particles, the particle surfaces will be positively charged and, conversely, if solution pH is above the PZC-equivalent pH of the particles, their surfaces will be negatively charged (Wang et al., 2016). It has been demonstrated that, irrespective of whether the pH is above or below the PZC for a given set of particles, as the value of the absolute difference between solution pH and PZC-equivalent pH increases, so too does the stability of the particles (Wang et al., 2016). When the solution pH is equal to the PZC of the particles, their surfaces become zwitterionic, facilitating aggregation and reducing transport through porous media (Godinez and Darnault, 2011). PZC values are specific to the type of ENM being considered (e.g., Guzman et al., 2006; Lanphere et al., 2015). The presence of surfactants and natural organic matter (NOM) can reduce the effects of pH on ENP transport through porous media (Godinez and Darnault, 2011).

The behavior of suspended colloids in the presence of electrolytes can largely be predicted by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Derjaguin and Landau 1941; Verwey and Overbeek, 1948), which describes the effects of the opposing, but independent, van der Waals and electrostatic forces acting on the particles. The DLVO theory posits that particles with charged surfaces will be surrounded by a sphere of ions from the surrounding fluid, creating a repulsive electrostatic double layer which acts to stabilize the particles, while the attractive van der Waals force can destabilize the particles. The van der Waals and electrostatic forces can be summed for a given distance between two particles (Fig 2.4.) and can approximate inter-surface forces to a distance of ca. 5 nm (Adair et al., 2001). The range of the double layer interaction is determined by the ionic strength of the suspension, while the magnitude of the van der Waals attraction is an inverse power law function of the separation distance.

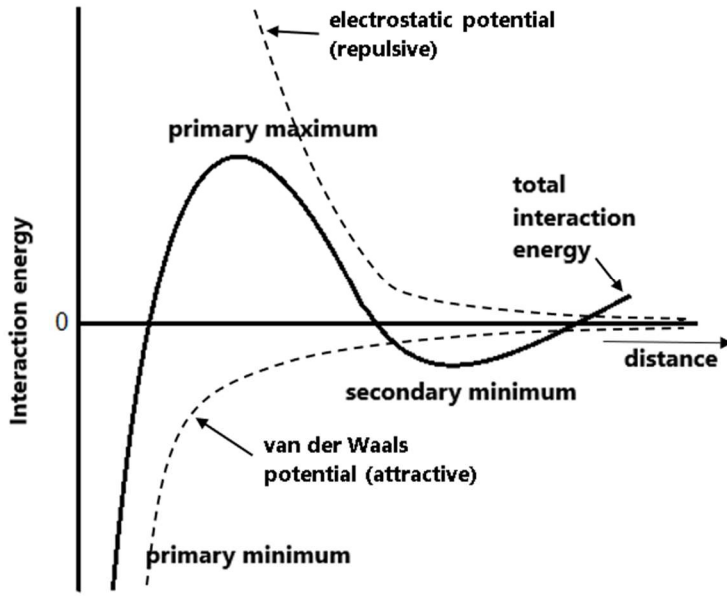


**Fig 2.4. Contributions from electrostatic and van der Waals forces to total interaction energy as described by DLVO theory.** adapted from Ietskleiner, distributed under a CC-BY-SA-4.0 license (<https://creativecommons.org/licenses/by-sa/4.0/>)

The strength of the van der Waals attraction between two particles with identical radii can be calculated according to (Adair et al., 2001):

$$W(D) = \frac{-A_{pmp}R}{12D} \quad (\text{Eq. 2.3})$$

Where  $W$  is the interaction energy,  $D$  is the particle separation distance,  $R$  is the particle radius, and  $A_{pmp}$  is the Hamaker constant, which is specific to the materials comprising the particles ( $p$ ) and suspension medium ( $m$ ). The magnitude of the electrostatic force between two particles decays as a function of particle distance and can be approximated assuming flat surfaces with evenly distributed charges and relatively small (less than 25 mV) surface potentials according to (Adair et al., 2001):

$$\Psi(x) = \Psi_s e^{-\kappa x} \quad (\text{Eq. 2.4})$$

where  $\Psi$  is the electrical potential (as a function of separation distance  $x$  between the particles),  $\Psi_s$  is potential distribution from the outer edge of the adsorbed species layer (“Stern plane”), and  $\kappa^{-1}$  is the double layer thickness (see Horn, 1990) which is dependent on the solution ionic strength. Under DLVO theory, that two particles would have moderate separation distances and experience a net repulsion under low ionic strength (unless the thermal energy of the system was large enough for the particles’ kinetic energy to exceed the energy barrier imposed by the electrostatic repulsive force). In higher ionic strength solutions, two particles would be expected to have a net attractive force under larger separation distances, and aggregation would be more likely in this scenario due to the existence of a secondary minimum of the energy barrier (Fig 2.4.). DLVO theory is also relevant to interactions between ENP and porous media (e.g., sand grains).

Solution pH can modify the surface charges of porous media through which ENMs may be transported, which is another way that pH may affect ENM transport through porous media. Electrostatic double-layer repulsive forces will be an important determinant of ENP transport in cases where the pH of a transport solution exceeds the PZC values of both ENMs and the potential collector surfaces (porous media) through which the ENMs are passing, thereby enhancing particle transport (Wang et al., 2016). For example, anionic ENMs with a  $\text{pH}_{\text{PZC}} \approx 8$  in a circum-neutral aqueous solution would be expected to exhibit low retention in clean quartz sand, which generally has a negative surface charge distribution in circum-neutral conditions primarily due to the dissociation of terminal silanol groups (Behrens and Grier, 2001). Many natural soils, on the other hand, exhibit a heterogeneous charge distribution, therefore the effects of solution pH will depend on the proportion of minerals such as metal oxyhydroxides that contribute to surface charge heterogeneity in soils. The heterogeneous charge distribution

associated with natural soils is mainly associated with minerals such as metal oxyhydroxides occurring in the soils (Tian et al., 2012). Retention of ENPs in natural soils is almost always greater compared to homogenous media, in part to the heterogeneity of surface charge distribution across soil particles (Cornelis et al., 2014). Other factors that can determine particle behavior and fate in soil/groundwater environments include particle size and surface chemistry, characteristics of the solid matrix in which they are in contact with (pore throat size, organic matter content, soil aggregate size; sand and clay content, etc.), and other physiochemical properties of the transport fluid (dissolved organic matter [DOM], pH, etc.) (Braun et al., 2015; Klaine et al., 2008; Lin et al., 2010; Sagee et al., 2012; Wang et al., 2016).

ENPs may be modified or degraded by a variety of environmental processes and/or materials, e.g., photodegradation, dissolution, oxidation, surface coating modification and subsequent passivation, etc. (Lin et al., 2010; Klaine et al., 2008; Wang et al., 2016); these transformations will also determine the fate of ENPs in environmental media. Upon entry into ground- or surface waters, ENMs are likely to encounter numerous aqueous-phase materials including different types of natural colloids as well as bacteria. Buffle et al. (1998) suggested that colloidal aggregation behavior could be described and understood by grouping natural colloids into several classes including (i) compact inorganic colloids, (ii) pedogenic fulvic compounds (FC) and aquagenic refractory organic matter (AROC), and (iii) large, rigid biopolymers. The analysis and classification of naturally-occurring colloids presented by Buffle et al. (1998) is useful not only in understanding potential interactions between ENMs and naturally-occurring colloids in aquatic systems, but also in predicting aggregation of ENMs that possess similar physiochemical properties to colloids (Lin et al., 2010).

Surface adsorption of FCs and other types of DOM can increase stability of some types of ENMs: Aqueous suspensions of carbon nanotubes (CNTs) are stabilized by Suwannee River natural organic matter (NOM) (Hyung et al., 2007; Hyung and Kim, 2008) and tannins (Lin and Xing, 2008). Tannic acid has been shown to stabilize gold nanoparticles (Tsai et al., 2004) and Suwannee River HA increased transport of fullerene NPs through soil (Wang et al., 2010), and both HA and, to a lesser degree, FA increased the stability of fullerene in water (Chen and Elimelech, 2007; Terashima and Nagao, 2007). Tipping and Ohnstad (1984) observed that naturally-occurring NOM-coated colloidal iron oxide present in a lake was stabilized by the NOM, except at high concentrations of  $\text{Ca}^{2+}$ , in which case flocculation bridging and destabilization were observed.

Conversely, heteroaggregation of ENMs has been observed in the presence of inorganic colloids: the stability of surfactant/CNT solutions was affected in variable ways by clay minerals, depending on both surfactant and clay type (Han et al., 2008). Rigid biopolymers such as structural, fibrillar polysaccharides or peptidoglycans have been shown to destabilize inorganic colloids (Buffle et al., 1998) and sulfate polystyrene latex particles (Nguyen et al., 2007) and may therefore also destabilize ENMs possessing similar surface chemistries. Polysaccharides promote aggregation and subsequent deposition via bridging (Singh et al., 2000; Nguyen et al., 2007). ENPs in natural aquatic systems that also contain NOM may therefore be stabilized and subject to transport.

### 2.2.2 Impact of microbial communities on ENP fate and transport

In contrast to the well-characterized effects of solution chemistry, porous media physiochemical properties and macromolecular interactions on ENP fate and transport, there are relatively few reports on the impacts of microbial communities on the transformations and transport and of ENPs in the subsurface.

#### *Microbial biodegradation of ENPs*

Some data suggests that aerobic organisms can modify or degrade ENPs. Surface-oxidized single-walled carbon nanotubes (SWNTs) can be degraded by plant peroxidases (Allen et al., 2008), while a fungal ligninolytic enzyme (manganese peroxidase) was observed to degrade SWNTs, except when the SWNTs contained surface oxygen, in which case the particles became recalcitrant to enzymatic degradation (Zhang et al., 2014). Ligninolytic enzymes are produced by numerous organisms including bacteria such as *Pseudomonas* sp. (Bansal and Kanwar, 2013). ENP surface coatings may also be subjected to biotic (and abiotic) transformations, including degradation of electrostatically-adsorbed ionic species (Cornelis et al., 2014) and covalently-bound materials (Kirschling et al., 2011). In general, the most easily-disrupted interactions in NP systems will dictate their degree of vulnerability to microbial or other environmentally-mediated degradation mechanisms, for instance the coordinate covalent bonds between quantum dot capping ligands and core surface atoms (Aldana et al., 2001; Kirchner et al., 2005; Mahendra et al., 2008; Navarro et al., 2011). Kirschling et al., (2011) investigated the bioavailability of covalently-bound polymer coatings by a polyethylene oxide (PEO)-degrading enrichment culture using hydrophilic PEO brush-coated nanoparticles with

divinyl benzene cross-linked cores. The PEO-coated NPs were exposed to a PEO-degrading culture in experiments in which the NPs were the only carbon source provided, and the investigators determined that the bacteria did indeed degrade the coatings, which led to particle aggregation. The PEO-degrading enrichment culture was isolated from urban river water and included *Pseudomonas* species. Several *Pseudomonas* species have been observed to degrade PEO (Kawai, 2010). Oxidation by biogenic reactive oxygen species (Navarro et al., 2011) or by abiotically-formed oxidants with subsequent dissolution (Auffan et al., 2010; Labille et al., 2010) may reduce NP mobility or possibly enhance it, depending on whether the surface coatings are replaced by other materials such as NOM (Labille et al., 2010).

### **2.2.3 Potential for microbially-mediated reduction of iron oxide nanoparticles IONPs**

Iron oxide nanoparticles (IONPs) are an important class of ENPs both because of their applications in medicine (Acharya et al., 2017; Menon et al., 2017; Mihai et al., 2017; Shams et al., 2019; Zhao and He, 2010), but also because of their use in consumer products (Ali et al., 2016; Campos et al., 2015; Moahapatra and Anand, 2010; Zhang et al., 2015), wastewater treatment and environmental remediation (Fan et al., 2011; Yavuz et al., 2006; Yean et al., 2005), and their potential use as subsurface contrast agents (Bagaria et al., 2013; Li et al., 2014; Park et al., 2012; Ryoo et al., 2012). Despite the many potential applications of IONPs that could lead to either intentional or unintentional release into the subsurface, information on the potential for microbially-mediated reduction of IONPs is scarce. Bose et al. (2009) monitored Fe-reduction of hematite NPs of varying morphologies and sizes (with diameters ranging from 11 to 93 nm) by the DIRB *Shewanella oneidensis* MR-1. The highest surface-area-normalized rates of reduction for the largest particles. Subsequent images of bacteria-NP associations captured using TEM revealed that NP-bacteria attachment modes varied according to NP morphology. The

authors theorized that both the bioreduction mechanisms and electron transfer modes used by IRB species impacted the observed reduction rates and these, in turn depended on the NP's aggregation, size, shape and exposed crystal faces. Yan et al., (2008) investigated Fe-reduction rates of different-sized hematite NPs by *Geobacter sulfurreducens* and found that smaller particles (10-30 nm) had higher Fe(III) reduction rates than larger (50 nm) particles, but the surface-area normalized rate was highest for the 30 nm particle, this observation was in agreement with a previous model indicating that bacteria-particle contact area was an important determinant of reduction rate. Yan et al., (2008) also noted that observed initial reduction rates were similar across the pH range (7-8) and at both H<sub>2</sub> partial pressures (0.01 & 1 atm) investigated.

The potential for microbially-mediation iron reduction of surface-coated IONPs has not previously been reported, despite the fact that surface coatings are commonly applied to IONPs to stabilize particles and prevent surface passivation and loss of superparamagnetism (Li et al., 2014; Zhu et al., 2018). In Chapter 5, the susceptibility of oleic acid coated IONPs to iron reduction by a dissimilatory IRB is investigated. The IONPs used in Chapters 5 (and 6) are surface-coated with an oleic acid bilayer, an anionic, electrostatically-stabilized surface coating, prepared as described by Li et al. (2014)

#### **2.2.4. Biofilm effects on transport of ENPs**

IONPs released unintentionally or injected deliberately into the subsurface are likely to encounter bacteria and other microorganisms, many of which form biofilms (Cai et al., 2019). For example, IONPs in municipal sludge applied to agricultural lands could encounter saprophytic, biofilm- and biosurfactant-producing bacteria, many of which play important

phytoprotective roles for plants including agricultural species (Couillerot et al., 2009; Ganeshan and Arthikala, 2005).

Biofilms can impact both physical (roughness, pore size) and chemical (charge, hydrophobicity) properties of porous media (Mitzel et al., 2016; Xiao and Wiesner, 2013; He et al., 2017; Crampon et al., 2018; Peszynska, et al., 2016), thereby altering flow paths, increasing straining potential and available attachment sites, and increasing ENP retention. Although there have not been any studies of the impacts of biofilms on the transport of IONPs through porous media, there have been a number of investigations on the impact of biofilms on the transport of other ENPs, with differing results. Several investigators have reported an increase in ENP retention in the presence of biofilm-coated media relative to clean (uncoated) media (He et al., 2015; 2017; Mitzel et al., 2016, Tong et al., 2010; Tripathi et al., 2012; Xiao and Wiesner (2013), while others have reported little to no influence of biofilm on ENP transport (Xiao and Wiesner, 2013; Kurlanda-Witek et al., 2015; Lerner et al., 2012), or even decreased retention (Mitzel et al., 2014) of ENPs in the presence of biofilms.

The largest factor controlling ENP transport through biofilms appears to be ENP stabilization type (i.e. no surface stabilization, electrostatic, steric, or electrosteric stabilization) (Liu et al., 2019; Mitzel et al., 2016; Xiao and Wiesner, 2013). In the presence of biofilms, uncoated and/or electrostatically-stabilized ENPs generally exhibit increased retention in porous media compared to transport through clean (uncoated) media (Jiang et al., 2013; Mitzel et al., 2016, Tong et al., 2010; Tripathi et al., 2012; Xiao and Wiesner, 2013).

### ***Biofilm impacts on transport of electrostatically- stabilized and uncoated ENPs***

Electrostatic and uncoated ENPs used in biofilm transport investigations include sulfate-modified polystyrene latex ENPs (Mitzel et al., 2016; Tripathi et al., 2012), carboxyl-modified polystyrene latex ENPs (Mitzel et al., 2016; Tripathi et al., 2012), and carboxyl-modified CdSe/ZnS quantum dots (Tripathi et al., 2012), fullerene (nC60) (Tong et al., 2010; Xiao and Wiesner, 2013), fullerol (Xiao and Wiesner, 2013), graphene-oxide nanoparticles (GONPs) (He et al., 2015; 2017), zinc oxide (ZnO) (Jiang et al., 2013), and citrate-coated nano-silver (nAg-CIT) (Xiao and Wiesner, 2013).

Several mechanisms have been suggested to explain the increased retention of electrostatically-stabilized and uncoated ENPs observed in the presence of biofilms, including hydrophobicity of nanoparticles and/or biofilms. Xiao and Wiesner (2013) observed positive correlations between ENP retention and the degree of ENP hydrophobicity relative to biofilm hydrophobicity. In contrast, Mitzel et al. (2016) concluded that particle surface hydrophobicity was not a major determinant of overall ENP transport potential but did suggest that ENP deposition dynamics may vary between hydrophilic and hydrophobic biofilms. Increased retention of uncoated and/or electrostatically-stabilized ENPs in the presence of biofilms has also been associated with surface hydroxylation of ENPs (in the case of fullerol ENPs; Xiao and Wiesner, 2013), hydrogen bonding between charged ENP surface sites and biofilm or other macromolecular biofilm associations such as the ability of large polysaccharides to “collect” small nanoparticles (Espinasse et al., 2007; Xiao and Wiesner, 2013). Tripathi et al. (2012) attributed physical straining to increased retention of 1  $\mu$ M sulfonated polystyrene latex beads in the presence of biofilm-coated sand when compared to clean quart sand experiments.

### ***Biofilm impacts on transport of sterically- or electrosterically-stabilized ENPs***

Steric and electrosterically-stabilized ENPs used to characterize the effect of biofilm on transport through porous media include polyvinylpyrrolidone (PVP)-coated nAg (Xiao and Wiesner, 2013; Mitzel and Tufenkji, 2014), and polyacrylic acid (PAA)-coated nano-scale zero valent iron (nZVI) (Lerner et al., 2012). Crampon et al., (2018) investigated biofilm impacts on the transport of stabilized nZVI in a commercially-prepared suspension using either uncoated or coated ENPs containing an oxidic surface layer. The details of the exact type of surface stabilization used were not provided although PAA was present either in the nZVI coating or in the commercially-prepared suspension.

The transport of sterically- or electrosterically-stabilized ENPs is typically unaffected (Lerner et al., 2012; Xiao and Wiesner, 2013) or even enhanced (Mitzel and Tufenkji, 2014) in the presence of biofilms. Steric repulsion between polymer-coated ENPs and biopolymers present in biofilms is a potential stabilizing mechanism explaining the low retention of sterically-stabilized ENPs in biofilms (Xiao and Wiesner, 2013). In electrosterically-stabilized ENPs, transport has been observed to either not be impacted by biofilms (Lerner et al., 2012; Xiao and Wiesner, 2013) or to be enhanced (Mitzel and Tufenkji (2014) relative to uncoated porous media at low ionic strengths. However, transport behavior of eletrosterically-stabilized ENPs is altered as solution ionic strength increases, as electrostatic repulsive forces associated with both charged surface moieties anchored to steric coating materials and biofilm biopolymers are impacted by the ion content of the transport solution (Lerner et al, 2012; Mitzel and Tufenkji, 2014).

## Biofilm-forming species used in ENP-biofilm transport investigations

Biofilm type (e.g., bacterial species or strain, gram-positive vs gram-negative, etc.) seems to be less important than particle surface chemistry in predicting ENP transport. He et al. (2015) found that transport of GONPs was significantly retarded through sand coated with either gram-positive (*Bacillus subtilis*) or Gram-negative (*Pseudomonas putida*) biofilm compared to clean sand when 1 to 50 mM NaCl was used as the transport fluid and that BTCs were remarkably similar for both biofilm types. In a related study, He et al. (2017) again found that *B. subtilis* or *P. putida* biofilm-coated sand inhibited GONP transport compared to clean sand when circum-neutral 0.1 to 1 mM  $\text{CaCl}_2$  was used as the transport solution. Compared to transport observed using NaCl as the transport fluid, BTCs were less similar between the two biofilm types when  $\text{CaCl}_2$  was used as the transport solution. Almost no transport was observed at 0.5 and 1 mM  $\text{CaCl}_2$  solutions when either type of biofilm was present.

By far, *Pseudomonas aeruginosa* has been the most commonly-employed biofilm-forming bacterial species used to study ENP transport through biofilm-coated porous media (Lerner et al., 2012; Mitzel and Tufenkji, 2014; Mitzel et al., 2016; Tripathi et al., 2012; Xiao and Wiesner, 2013). Other biofilm-forming bacteria which have been used to examine ENP transport in the presence of biofilms (and/or biofilm EPS) include *P. putida* (He et al., 2015; 2017), *Escherichia coli* (Tong et al., 2010; Jiang et al., 2013), *Bacillus cereus* (Xiao and Wiesner, 2013), *B. subtilis* (He et al., 2015; 2017), and a naturally-occurring anaerobic biofilm dominated by *Pseudomonas stutzeri* (83%) and *Exiguobacterium* sp. (6%) (Crampon et al., 2018).

Previously-published studies of biofilm impacts on ENP transport have been conducted at relatively high pore water velocities (4 to 33 m d<sup>-1</sup>), and none have examined the impacts of

biofilm on the transport of ENPs in the presence of the widely-distributed environmentally-relevant biofilm-forming bacteria *P. fluorescens*.. In Chapter 6, transport of surface-stabilized IONPs through *P. fluorescens* biofilm-coated porous media was characterized under realistic groundwater flow velocities (ca. 0.4 and 4 m d<sup>-1</sup>) and using two different biofilm-formation protocols. While there have also not been prior studies of IONP transport through biofilm-coated porous media, the oleic acid bilayer used to stabilize the IONPs used in Chapters 5 and 6 results in a surface carboxylic acid functionality (Li et al., 2014), which makes the transport experiments comparable to other biofilm-ENP studies that have been performed using carboxyl-modified ENPs (Mitzel et al., 2016; Tripathi et al., 2012).

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### **3. Microbial reductive dechlorination of tetrachloroethene under iron- and sulfate-reducing conditions**

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This work has been prepared for submission to Environmental Engineering Science. Co-author Samuel Gaeth designed and initiated the column experiments presented as Experiment No. 1. The Supplementary Material to be submitted for publication with this work is provided as Appendix A.

#### **3.1 Abstract**

Biologically-mediated abiotic degradation of chlorinated solvents by reduced iron minerals and sulfur species has been documented; however, the ability of organohalide respiring bacteria to perform reductive dechlorination under these conditions is poorly understood. The effects of reduced iron and sulfate products on microbial reductive dechlorination (MRD) of tetrachloroethene (PCE) were studied in two experiments consisting of two flow-through columns packed with iron oxide-coated sand and flushed with reduced medium containing 300  $\mu\text{M}$  PCE, 5 or 7 mM sulfate, 5mM lactate, and 5mM acetate. In the first experiment, one column was inoculated with a PCE-to-ethene dechlorinating consortium (BDI); the other column received both BDI and an iron-reducing bacterium (IRB). Throughout the experiment, 14.8% more dechlorination (as chlorine number averaged over 85 pore volumes) was measured in the IRB-containing column, which was 21% more reduced (measured as oxidation/reduction potential) and produced 6.5% more ethene compared to the BDI-only column over the duration of the experiment. In the second experiment, both columns were inoculated with BDI, IRB, and a sulfate-reducing bacterium; sulfide and additional sulfate were supplied to the columns to assess the sulfide inhibition threshold on MRD in the system. Reduced production of vinyl chloride and ethene (and a 21% increase in chlorine number) were measured when total calculated sulfide (sum of sulfide added to column influent and sulfate reduced as measured in column effluent)

exceeded 10.8 mM, more than twice the concentration previously reported to inhibit MRD. The results indicate that iron reduction benefits dechlorinating bacteria by creating more reduced conditions and by offsetting sulfide inhibition of MRD.

### 3.2 Introduction

Bioremediation is commonly employed to address the widespread problem of subsurface contamination by chlorinated ethenes such as trichloroethene (TCE) and tetrachloroethene (PCE). To promote bioremediation, aquifers are often biostimulated through the addition of a fermentable electron donor substrate such as lactate, injected into the subsurface to encourage microbial reductive dechlorination (MRD) of chlorinated contaminants. Numerous microorganisms can reductively dechlorinate PCE and/or TCE to the dechlorination daughter product *cis*-1,2-dichloroethene (*cis*-DCE) (Löffler et al., 2013a; Lohner and Spormann, 2013; Stroo et al., 2014; Krzmarzick et al., 2018); however, only a few species can reduce PCE and TCE beyond *cis*-DCE to vinyl chloride (VC) and ethene, the most-studied of which are *Dehalococcoides mccartyi* (*Dhc*) strains (Schipp et al., 2013; Parthasarathy et al., 2015; Löffler et al., 2013b; Maymó-Gatell et al., 2001). The consumption of electron donor by non-target bacteria such as iron- and sulfate-reducing bacteria is a potential obstacle to the MRD of chlorinated ethenes in the subsurface not only because it reduces the amount of electron donor available for MRD, but also because the unintentional stimulation of sulfate-reducing bacteria (SRB) results in the production of sulfide, which has been shown to inhibit the activity of *Dhc* at a concentration of approximately 5 mM in batch reactor (Mao et al., 2017), microcosm (Hoelen and Reinhard, 2004) and chemostat (Berggren et al., 2013) studies. Sulfate (SO<sub>4</sub>) is one of the most common anions found in groundwater and sulfide production is commonly measured during *in situ* bioremediation; however, inhibition of MRD is not always detected at sites where substantial sulfate reduction is measured (Aziz et al., 2013; Lee et al., 1998; Aulenta et al., 2006;

Mao et al., 2017). Some investigators (Hoelen and Reinhard, 2004; Hyun and Hayes, 2009; Mao et al., 2017) have speculated that ferrous iron may serve to offset sulfide inhibition of MRD by removing sulfide from solution. Under both iron- and sulfate-reducing conditions, ferrous iron will immediately react with sulfide, forming iron(II) sulfide (FeS) minerals. Owing to the ubiquity of both SRB and iron-reducing bacteria (IRB) in the subsurface, the formation of reduced FeS minerals is common (Schieber, 2011). Biologically mediated abiotic degradation (BMAD) of chlorinated ethenes, including *cis*-DCE, has been demonstrated for numerous iron-bearing minerals including magnetite, mackinawite, pyrite, green rusts, and others (Fan et al., 2017; Lee and Batchelor, 2002a; 2002b; Tobiszewski and Namieśnik, 2012) as well as ferrous mineral-bearing soils under both anoxic (Schaefer et al., 2017; 2018a) and oxic (Schaefer et al., 2018a) conditions.

Some studies have observed or speculated that bioavailable Fe(III) in sediments within contaminated aquifers can hinder organohalide respiration of chlorinated ethenes due to competition for electron donor between iron-reducing bacteria and dechlorinators (Lu et al., 2001; McLean et al., 2006; Shani et al., 2013; Zaa et al., 2010). However, Fe(III) reduction has also been observed to have no impact (Wei and Finneran, 2011; 2013) or even to enhance MRD (Wei and Finneran, 2011). To our knowledge, there have been no reports on the effects of sulfide inhibition on dechlorinating microbes in the presence of active iron reduction in a flow-through system. Since *in situ* MRD in contaminated aquifers realistically occurs under conditions of dynamic groundwater flow and in the presence of concurrent microbial processes including iron- and sulfate-reduction, it is desirable to understand the performance of MRD under these conditions.

We hypothesized that under both Fe- and SO<sub>4</sub>-reducing conditions, sulfide inhibition of MRD would be reduced or eliminated. Therefore, in this work, the performance of MRD of tetrachloroethene (PCE) was evaluated in the presence of iron reduction alone and accompanied by sulfate-reduction. Biologically-active columns packed with iron-coated sand were flushed continuously with a reduced medium containing 300 µM PCE, 5 mM sulfate, and 5mM lactate and acetate (electron donor and carbon sources). Two experiment sets were performed, each consisting of two columns operated in parallel. In the first experiment, the effects of biological iron reduction by *Geobacter sulfurreducens* on dechlorination were measured by inoculating one column with a PCE-to-ethene dechlorinating consortium, Bio-Dechlor Inoculum-SZ (BDI), while the second column received both BDI and an iron-reducing bacterium (IRB). Concentrations of chlorinated ethenes, ferrous and total iron, and sulfate were measured in column influent and effluent samples. In the second experiment, MRD was monitored in the presence of both iron and sulfate reduction by inoculating both columns with BDI, IRB, and the sulfate-reducing bacterium (SRB) *Desulfovibrio vulgaris* Hildenborough, and monitoring concentrations of chlorinated solvents, ferrous and total iron, sulfate and sulfide in column influent and effluent samples. The second experiment consisted of two phases: (1) assessing the effects on PCE dechlorination of both iron reduction and sulfate reduction at different (5 mM and 7mM) sulfate concentrations, and (2) identifying the sulfide threshold at which PCE MRD was inhibited in the experimental system. The sulfate concentrations of 5 and 7 mM supplied to the columns were selected to be equal to and above the 5 mM sulfide concentration previously reported to be inhibitory of MRD. During phase two of the second experiment, sulfide was also added to the column influent solutions because observed microbial sulfate reduction was insufficient to achieve the target sulfide levels ( $\geq 5$  mM).

### **3.3 Materials and Methods**

#### **3.3.1 Chemicals**

Sodium hydroxide and ferric chloride (Sigma-Aldrich, St. Louis, MO, USA), ferric nitrate (Thermo Fisher; Waltham, MA), and nitric acid (Fisher Scientific, Pittsburgh, PA) were used for the iron-coated and/or acid-washed sand procedures, acid digestion of solid phase iron samples, and sample analysis via inductively coupled plasma optical emission spectrometry. Ammonium acetate, ammonium hydroxide, hydroxylamine hydrochloride (Sigma-Aldrich), and FerroZine™ iron reagent (Aldrich Chemical, Milwaukee WI) were used during UV-spectrophotometric analysis of iron. Sulfuric acid and ferric chloride (Fisher Scientific) and N-N-dimethyl-p-phenylenediamine (Tokyo Chemical Industry Co.; Tokyo, Japan) were used for the measurement of sulfide concentrations.

Sodium sulfate, sodium lactate (60% w/w), sodium acetate, and high-performance liquid chromatography (HPLC)-grade tetrachloroethene (PCE), and resazurin were purchased from Sigma-Aldrich and added to the reduced mineral salts medium as described later. Sodium sulfide nonahydrate (Sigma-Aldrich) was used to make sulfide standards and was added to the reduced medium as described later. Ferric citrate (Sigma-Aldrich) was used as an electron acceptor to maintain microcosms containing iron-reducing bacteria inocula. Sodium bromide (Fisher Scientific) was used as a conservative tracer. All chemicals used in sample preparation and calibration were of reagent grade or higher purity, and all solutions were prepared using 18.2 MΩ/cm deionized water (EMD Millipore; Billerica, MA). As needed, some equipment was sterilized using >70% isopropyl alcohol (Sigma-Aldrich).

### 3.3.2 Medium preparation and inocula

The column background solution consisted of a reduced mineral salts medium prepared as described previously (Löffler et al., 2005). The reduced medium was amended with 5mM sodium lactate and 5mM sodium acetate as the electron donor and carbon sources, and either 5 mM or 7 mM sodium sulfate as an electron acceptor for the sulfate-reducing bacterium. A redox indicator (resazurin, 1  $\mu$ M) was added to the medium to confirm that the column influent was anoxic ( $\text{ORP} \leq -100$  mV).

The PCE-to-ethene dechlorinating consortium BDI is a dechlorinating consortium that contains fermenters, homoacetogens, and multiple dechlorinators, including three *Dehalococcoides mccartyi* strains and *Geobacter lovleyi* strain SZ (Amos et al., 2009; Cápiro et al., 2014). BDI was grown in the reduced medium described above amended periodically with 5 mM sodium lactate (fermentable electron donor substrate and carbon source),  $\sim 300 \mu\text{M}$  PCE (electron acceptor), and 20-50 mL  $\text{H}_2$  (direct electron donor). The IRB used in the experiments was *Geobacter sulfurreducens*, an anaerobic, dissimilatory sulfur- and metal-reducing  $\delta$ -*Proteobacterium* capable of using acetate as an electron donor (Caccavo et al., 1994). The *G. sulfurreducens* growth medium was amended periodically with 5 mM ferric citrate (electron acceptor) and 5 mM sodium acetate (electron donor and carbon source), and 20-50 mL  $\text{H}_2$  (alternative electron donor). *Desulfovibrio vulgaris* Hildenborough is a well-characterized anaerobic dissimilatory sulfur- reducing  $\delta$ -*Proteobacterium* (Heidelberg et al., 2004; Santana et al., 2012) that has previously been used as a model SRB for studies on bioremediation (Lovley et al., 1993; Michel et al., 2001; Payne et al., 2002). *D. vulgaris* Hildenborough was used as the SRB in this investigation and was grown and maintained in the reduced medium described above

amended periodically with 5mM sodium sulfate (electron acceptor), 5mM sodium lactate (electron donor), and 5 mM sodium acetate (carbon source), and 20-50 mL H<sub>2</sub> (alternative electron donor).

### **3.3.3 Porous media, column preparation and inoculation procedure**

Federal Fine Ottawa (FFO) quartz sand (US Silica Company; Frederick, MD, USA) was used to pack the columns. FFO has a mean diameter of 0.32 mm, intrinsic permeability of  $4.2 \times 10^{-11} \text{ m}^2$ , and an organic carbon content of 0.001 (Suchomel et al. 2007). Prior to being used in the columns, the Federal Fine Ottawa (FFO) quartz sand was acid washed and coated with iron-oxide coating according to the procedure of Benjamin et al. (1996), with the following modifications: Bulk FFO sand was acid washed using 1 M nitric acid to remove any background iron present on the surface of the sand grains. The sand was soaked for 12 h in the acid solution and then rinsed with 18 M $\Omega$ /cm deionized (MilliQ) water until the rinse water pH was equivalent to the measured pH of the MilliQ (approximately 5.7), and then oven dried at 100°C. Following an initial ferric chloride coating and oven drying (at 100°C) procedure, the Benjamin et al. (1996) method called for the sand to be heated to 550°C for 3 h before a final ferric nitrate coating; however, this step was omitted as it resulted in excessive smoking/burning and created a solid that could not be removed from the heating container. The final ferric nitrate coating was carried out as described in Benjamin et al. (1996).

The total Fe content of acid-washed FFO sand and iron-oxide-coated sand (IOCS) were determined using acid digestion and inductively coupled plasma optical emission spectrometry, described under Analytical Methods (Appendix A). After determining the Fe content of both types of sand, they were combined in proportions such that the total Fe content of the resultant mixture was 1.5% by weight. Column packing and water saturation protocols, and the pore

volume (PV) determination method (which included a non-reactive tracer test) are described in Appendix A.

Following complete water saturation, 5 mM sulfate reduced mineral salts medium containing a target concentration of 300  $\mu$ M PCE was flushed through the columns at 0.5 mL/min using a Harvard Apparatus syringe pump (PHD 2000, Holliston, MA) for 10 PV to establish reduced conditions suitable for anaerobic microbial growth. After the 10 PV flush, the columns were inoculated with the experiment-specific microbes (detailed below). For all columns, the inoculation was accomplished by injecting 8mL of inoculum into both the influent, effluent, and each of the three sampling ports using a Fusion 200 Two-Channel Syringe Pump (Chemyx Inc; Stafford, TX) for a total inoculum volume of 40 mL.

For the first experiment, one column was inoculated with the PCE-to-ethene dechlorinating BDI consortium (BDI-only column), while the other was inoculated with both BDI and IRB (BDI + IRB column). The BDI-only column was inoculated with a mixture of 20 mL BDI inoculum and 20 mL of sterile 5 mM sulfate reduced medium, while the BDI+IRB column was inoculated with a mixture of 20 mL BDI inoculum, 10 mL of *Geobacter sulfurreducens* culture, and 10 mL of sterile 5 mM sulfate reduced medium. For the second experiment, both columns were inoculated mixture containing 10 mL each of BDI inoculum, *G. sulfurreducens*, and *Desulfovibrio vulgaris* Hildenborough cultures plus 10 mL of sterile 5mM sulfate reduced medium. Following inoculation, the columns were sealed and maintained without flow for 24 hours to facilitate attachment of cells to the porous media.

### **3.3.4 Column operation, sampling, and evaluation**

At the end of the acclimation and cell attachment period, reduced medium was supplied to each column at flowrate of 0.015 mL min<sup>-1</sup> (0.22 m  $\pm$  0.03 m day<sup>-1</sup>) using a 50 mL glass

gastight syringes (Hamilton Company; Reno, NV) aligned in parallel. Each syringe was filled manually with a mixture of the sulfate medium without PCE and PCE-supersaturated medium to obtain a target concentration of 300  $\mu\text{M}$  PCE. To maintain effluent redox conditions and to prevent volatilization of the chlorinated solvents, each column effluent was connected to a 25 mL glass bulb (G. Finkenbeiner, Inc., Waltham, MA). Both influent and effluent samples were collected every 1.5 PVs and analyzed for concentrations of PCE and dechlorination transformation products, volatile fatty acids (VFAs), and sulfate concentrations, while pH and ORP measurements were taken every 3-4 PVs. Iron and sulfide analyses were measured periodically (generally every 10-15 PVs) during the first experiment, and every 3-4 PVs during the second experiment. In both experiments, periodic flow interruptions ranging in length from 26-72 h were performed to evaluate the effect of residence time on PCE dechlorination. Analytical methods describing the measurement of concentrations of chlorinated ethenes and ethene, sulfate, VFAs, iron and sulfide, and measurements of pH and ORP can be found in Appendix A.

The extent of PCE degradation that occurred during the experiments was determined by measuring the amount of PCE that was converted into dechlorination daughter products (e.g., TCE, cis-DCE, etc.) over time, expressed as chlorine number (Borden, 2017), which reflects the degree to which PCE has been reduced to benign daughter products (ethene) relative to an initial PCE loading. Chlorine number is calculated as:

$$\text{Chlorine \#} = \frac{4[PCE] + 3[TCE] + 2[DCE] + [VC]}{[PCE] + [TCE] + [DCE] + [VC] + [eth]}$$

Where concentrations of each chlorinated ethene and ethene are expressed in molar units (moles/L). The chlorine number ranges from 0 to 4 representing complete and no dechlorination measured, respectively.

Two-sided t-tests were used to verify whether observed differences in chlorine number were significant between columns within a given experiment. Prior to running the t-tests, F-tests were performed on each variable tested. When the results of the F-tests indicated that variance was not significantly different (for  $\alpha = 0.01$ ), variances were pooled for the t-test calculations. Statistical analyses were performed using R statistical software version 3.2.2 (R Core Team, 2017).

### **3.3.5 Column operation: Experiment 1, Effects of iron reduction on MRD**

During the course of the first experiment, the BDI-only and BDI+IRB columns were operated identically and sampled as described above. The columns were operated until the soil permeability was reduced to the point that the injection syringes could no longer pump influent through the columns due to plugging, which could have been caused by biomass formation and/or FeS formation (as the reduced medium supplied to the columns contained 0.2 mM sulfide and active iron reduction occurred during the experiment). The flow rate remained constant until the final 2-3 samples collected, at which point the flow rate dropped rapidly until the pumps could no longer deliver flow through the columns. The maximum operating pressure listed for the syringe pump equipped with 100 mL gas-tight syringes was ca. 10-15 PSI (Harvard Apparatus), however the flow delivery failure manifested as an influent leak from the gas-tight influent syringes, therefore the actual column permeability-related pressure drop that occurred during the end of the experiment was undetermined.

### **3.3.6 Column operation: Experiment 2, Effects of iron and sulfate reduction on MRD**

The second experiment was performed in four phases. During phase one of the second experiment, both columns received 5 mM  $\text{SO}_4$  until measurable iron- and sulfate- reduction had been achieved, and vinyl chloride (VC) and ethene were the only dechlorination products measured in the column effluents. From this point forward (Phase *II*), one column ( $A_{f5}$ ; “f” represents final influent sulfate concentration) continued to receive the 5 mM sulfate reduced mineral salts medium, while the other column ( $B_{f7}$ ) was supplied with 7 mM sulfate reduced mineral salts medium (both media still contained  $\sim 300 \mu\text{M}$  PCE). In Phase *III* of this experiment, sulfide (starting at 2.3 mM; in addition to the sulfate reduction occurring in the column) was incrementally added to the influents of each column to evaluate the sulfide threshold above which PCE dechlorination, iron, and/or sulfate reduction were inhibited. The columns were operated until the soil permeability was reduced to the point that the injection syringes could no longer pump influent through the columns due to plugging, which was attributable to visible  $\text{FeS}$  formation (as iron and sulfate reduction occurred during most of the experiments and influent sulfide was also provided to the columns), however biomass clogging may also have contributed to the permeability reduction. Column flow rates were unaffected until the last 2-3 samples collected during this stage of the experiment, at which point the flow rate dropped rapidly until the pumps could no longer deliver flow through the columns. As described in Section 3.3.5 (above), the change in permeability arising from clogging in the column could not be determined.

## **3.4 Results**

### **3.4.1 Experiment 1, Effects of iron reduction on MRD of PCE**

In experiment 1, MRD of PCE was compared in the presence and absence of iron reduction, achieved by inoculating one column with only the BDI dechlorinating consortium and

the second with both BDI and an IRB. The chlorine number (Figure 3.1) of the column inoculated with BDI + IRB declined from 4 to 2 (indicating transformation of PCE to *cis*-DCE) 35% earlier (PV compared to the BDI-only column. Similarly, the transition from *cis*-DCE being the most abundant effluent VOC detected (relative to total effluent VOCs) to VC being the main dechlorination product occurred earlier in the IRB-containing column (PV 14 vs PV 25; Figure A.1); between PVs 14 and 25, VC was produced at average rates of  $0.84 \pm 0.44$  and  $3.68 \pm 1.37$   $\mu\text{mol PV}^{-1}$  in the BDI-only and BDI + IRB columns, respectively. Averaged over the duration of the experiment, both the chlorine number (Figure 3.1) and ORP value (Figure 3.2) in the BDI + IRB column (1.28 and -138, respectively) were significantly lower ( $P < 0.001$  for both tests) compared to the BDI-only column, 1.49 and -114, respectively); The BDI + IRB column also produced 6.5% more ethene compared to the BDI- only column over the duration of the experiment. The BDI + IRB column had a significantly ( $P < 0.001$ ) higher average pH (Figure 3.2) (7.53) and compared to the BDI-only column (7.39). Reduction of iron(III) oxides has been demonstrated to increase both pH and alkalinity in anaerobic systems using dissimilatory (Dong et al., 2017) and fermentative (Howell et al., 1998) IRB, and could explain why the average pH of the BDI + IRB column effluent was higher than that observed in the BDI-only column. Iron reduction was measured in effluent samples from both columns (data not shown), and there was an average of 3.5 times more ferrous iron detected in the effluent of BDI-IRB column  $22.6 \pm 16.9$   $\text{mg L}^{-1}$ ) compared to the BDI-only column ( $6.4 \pm 6.5 \text{mg L}^{-1}$ ). From PV 1.5-25.6, the average chlorine number in the BDI + IRB column (1.45) was 23.0% lower relative to the average chlorine number in the BDI only column (1.89), while during the remainder of the experiment, the average chlorine number in the BDI + IRB column (0.75) was 13.5% lower relative to the average chlorine number in the BDI only column (0.87). Therefore, the period over which the

BDI+IRB column saw the largest gains in dechlorination occurred relatively early in the experiment. However, the results of the statistical tests showed that the differences between dechlorination performance were significant over the entire course of the experiment, therefore the increased dechlorination performance achieved in the presence of the IRB are not solely attributable to start-up effects between the columns. Additionally, paired t-test results performed using only data collected after PV 26 in the columns revealed that the observed differences in chlorine number were still significantly different between the columns ( $P < 0.001$ ), strengthening the conclusion that benefits conferred to dechlorinating microbes in the presence of iron reduction were not limited to start up effects.

Following a series of six flow interruptions which occurred between PVs 22 and 67 (Fig. A1, Appendix A), chlorine numbers fell by an average of  $114.3 \pm 8.2\%$  and  $13.1 \pm 9.9\%$  in the BDI-only and BDI+IRB columns, respectively. The increased dechlorination observed between samples collected immediately before and after the flow interruptions was attributable to the longer PCE:microbe contact time in the column, consistent with findings from previous studies (Amos et al., 2009; Cápiro et al., 2015; Haest et al., 2011). A linear least-squares regression performed using the fractional change in chlorine number (relative to pre-flow interruption chlorine number) versus the duration of the flow interruption for the BDI-only and BDI + IRB columns suggested that the magnitude of change in the chlorine numbers following the flow interruptions did not correlate with the duration of the flow interruptions ( $R^2$  0.06). Experiment 1 was terminated at ~PV 85, when the permeability of both columns was reduced, possibly due to a combination of biomass growth and FeS formation (since 0.2 mM sulfide was added to the anaerobic transport fluid as a reducing agent) and the influent syringe pumps could no longer maintain the target flow rate of  $0.015 \text{ mL min}^{-1}$ .

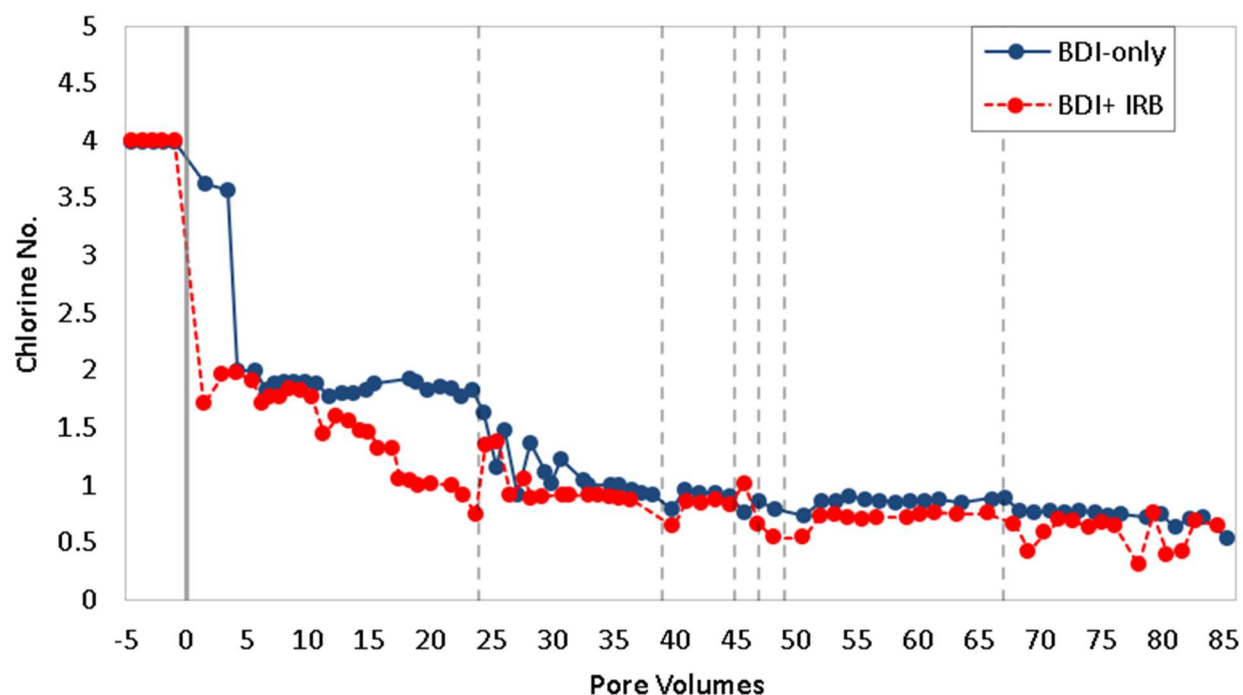


**Figure 3.1** Chlorine number as a function of pore volume in the BDI-only and BDI + IRB column experiment. Dashed lines indicate flow interruptions. PV 0 corresponds to column inoculation (vertical line).

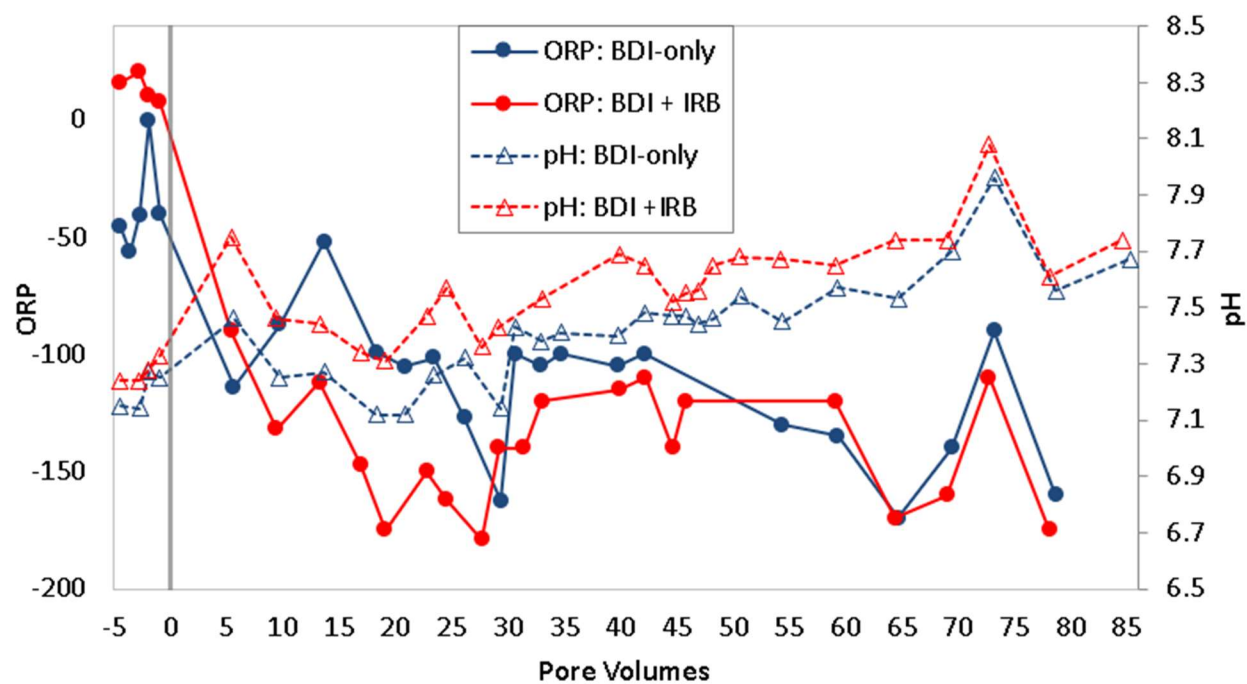


**Figure 3.2** Oxidation/Reduction Potential (ORP; circles) and pH (triangles) as a function of pore volume in the BDI-only and BDI + IRB column experiment. Dashed lines indicate flow interruptions. PV 0 corresponds to column inoculation (vertical line).

### 3.4.2 Experiment 2: Effects of iron and sulfate reduction on MRD

*Phase I: 5 mM sulfate supplied to both columns*

The experiment #2 columns, each containing all three inocula (BDI, IRB, and SRB), were both initially provided with reduced medium containing 5 mM sulfate in phase *I* of the experiment. Dechlorination of PCE to mainly *cis*-DCE (as indicated by chlorine numbers of 2.29 and 1.93 in columns A<sub>f5</sub> and B<sub>f7</sub>, respectively) occurred simultaneously in both columns, immediately after inoculation (PV 0, Fig. 3.3). While *Geobacter lovleyi* strain SZ, which was present in the column inocula, is capable of PCE reduction to *cis*-DCE (Sung et al., 2006), the transition from *cis*-DCE to VC production is only attributable to *Dhc* activity. VC and ethene were both first detected immediately following the first flow interruption (27 hrs at PV 11, Figure A.2); average rates of VC and ethene production between the first and second flow interruptions were 0.09±0.03 and 0.03±0.02 µmole PV<sup>-1</sup> in column A<sub>f5</sub>, respectively, and 0.08±0.02 and 0.04±0.02 µmole PV<sup>-1</sup> in column B<sub>f7</sub>, respectively. Between the second (36 hrs at PV 17.5) and third (41 hrs at PV 35) flow interruptions, average rates of VC and ethene production were 1.32±1.03 and 0.04±0.01 µmole PV<sup>-1</sup> in column A<sub>f5</sub>, respectively, and 0.58±0.30 and 0.04±0.02 µmole PV<sup>-1</sup> in column B<sub>f7</sub>, respectively. Ethene levels remained below 1% of total effluent VOC molar concentrations in both columns until the third flow interruption, after which VC became the predominant (i.e., > 50% of total VOCs) VOC measured in both columns (at PV 35 and 37 in the A<sub>f5</sub> and B<sub>f7</sub> columns, respectively; Fig. A.2), and ethene concentrations steadily increased from 14.8 to 44.1 and from 11.6 to 48.4 µM.

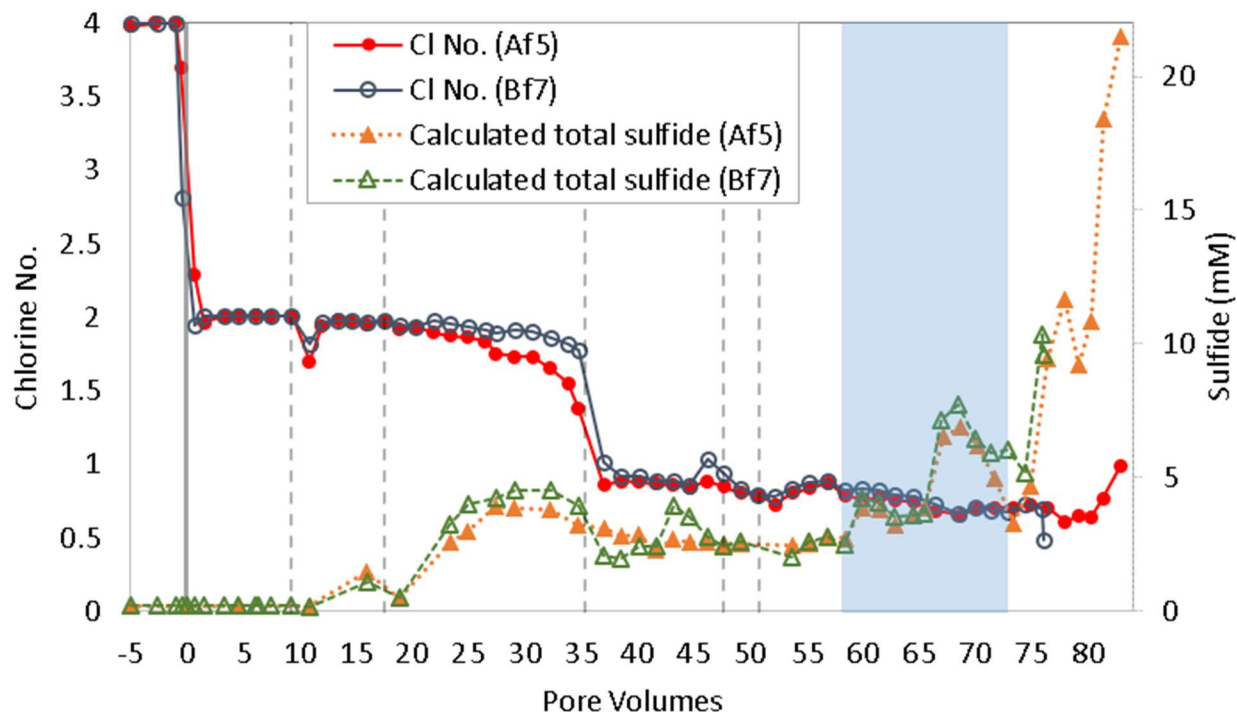


**Figure 3.3** Experiment 2 Chlorine number and calculated total sulfide input (sum of reduced sulfate measured in column effluent and sulfide added to column influent) versus pore volume in the 5 mM (Af5) and 7 mM (Bf7) sulfate experimental columns. Dashed lines indicate flow interruptions. PV 0 corresponds to column inoculation (vertical line). 7mM lactate treatment is denoted by blue shading.

Ferrous iron (Fig. 3.4) was first detected above the method detection limit ( $0.5 \text{ mg L}^{-1}$ ) in effluent from both columns at PV 5, and increased to  $11 \pm 0.45$  and  $13 \pm 0.53 \text{ mg L}^{-1}$  by PV 18.9 and 20.4 in columns Af5 and Bf7, respectively. After this first peak in effluent iron concentrations, sulfate reduction (Figure 3.4) increased, with sulfide peaking and plateauing at  $3.9 \text{ mM}$  (PV 27.5) and  $4.5 \text{ mM}$  (PV 32.3) in columns Af5 and Bf7, respectively, during which time iron concentrations declined to  $1.8 \pm 0.07$  and  $2.3 \pm 0.09 \text{ mg L}^{-1}$ , respectively. The transition to predominantly VC production occurred just after the period of peak sulfide production in both columns. This transition could be related to the decline in sulfide production or because sufficient *Dhc* colonization had occurred in the columns at this point. After the peak and plateau in sulfate reduction, there was a subsequent decrease to 2-3 mM of sulfate reduced

(sulfide production) for much of the rest of the experiment. Aulenta et al. (2007) observed coexistence of MRD and sulfate reduction, and noted that MRD rates did not increase after depletion of  $\text{SO}_4$ , suggesting that dechlorinators can effectively compete with SRB for electron donors, which may explain the lack of sulfate reduction observed following this initial temporary increase in  $\text{SO}_4$  reduction.

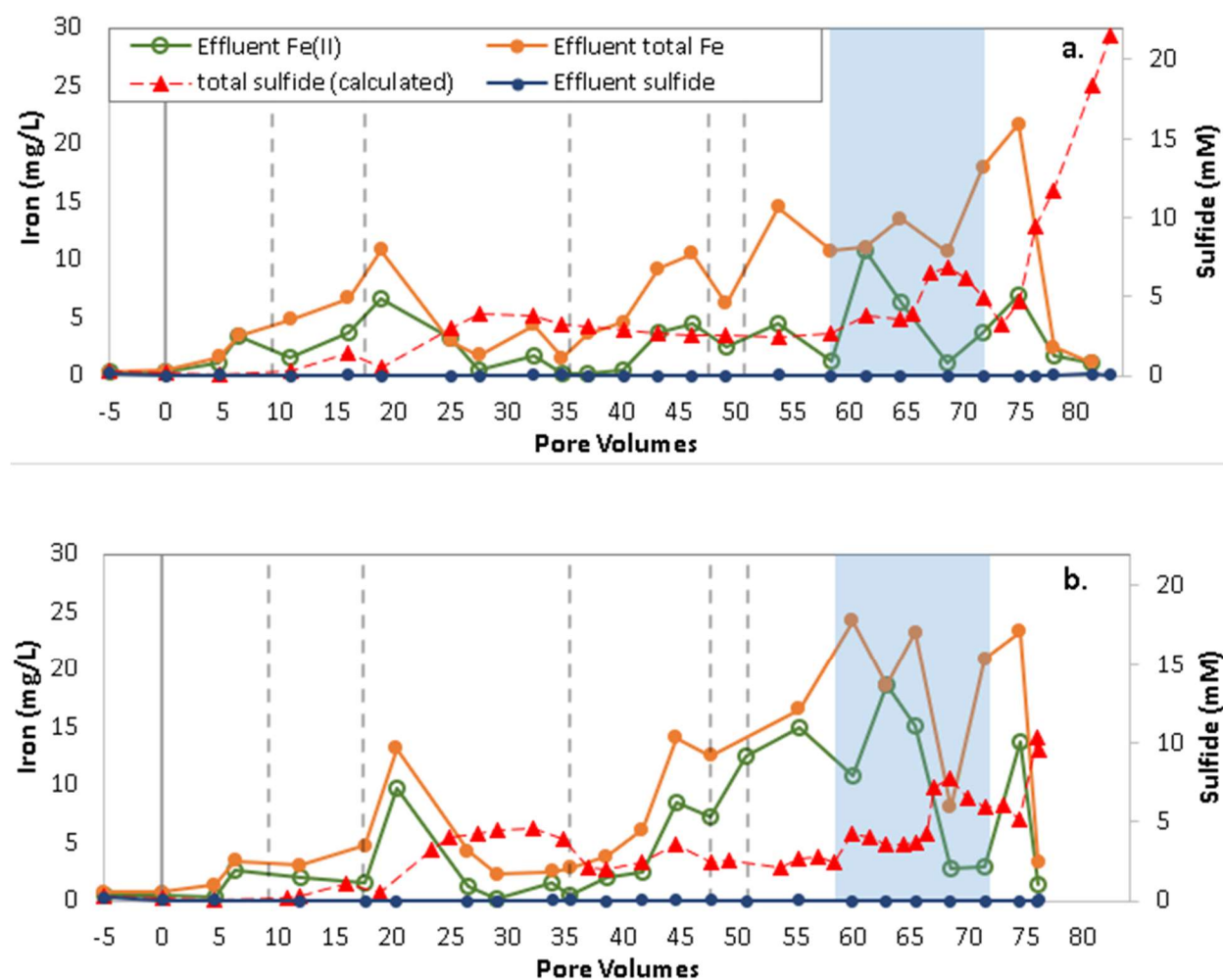


**Figure 3.4.** Experiment 2 ferrous and total iron concentrations in effluent samples and total calculated sulfide (i.e., sum of influent sulfate reduced and sulfide added to column influent) versus pore volume in the 5 (Af5) and 7 (Bf7) mM sulfate experimental columns (panels a and b, respectively). Column inoculation occurred at PV 0 (solid line). 7 mM lactate treatment is shaded in blue; dashed gray lines denote flow interruptions.

## *Phase II: Elevated sulfate treatment*

The elevated sulfate treatment (i.e., 7 mM influent sulfate provided to the B<sub>f7</sub> column) was initiated at PV 42. Following the sulfate treatment, there was a temporary increase in sulfide formation (from 2.4 to 3.9 mM) in the B<sub>f7</sub> column effluent; however, within 6 PVs, sulfide production had returned to 2.4 mM, and thereafter, was nearly identical to the level of sulfate reduction measured in the A<sub>f5</sub> sulfate column effluent. After 3 PVs of the elevated sulfate treatment, the chlorine number in column B<sub>f7</sub> increased from 0.85 to 1.03 for 3 PVs before dropping to 0.83, while during this same 3 PV period, column A<sub>f5</sub> (5 mM SO<sub>4</sub> treatment) had an average chlorine number of  $0.84 \pm 0.03$ . While the total amount of sulfate reduced in the two columns was similar during this part of the experiment ( $2.56 \pm 0.11$  and  $2.54 \pm 0.27$  mM in columns A<sub>f5</sub> and B<sub>f7</sub>, respectively), the percentage of influent sulfate reduced in the column B<sub>f7</sub> ( $36 \pm 4\%$ ) was lower than that reduced in column A<sub>f5</sub> ( $51 \pm 2\%$ ), since more total sulfate was being supplied to column B<sub>f7</sub>.

From the beginning of the elevated sulfate treatment (PV 42) to the end of Phase II of this experiment (PV 66), effluent ferrous and total iron concentrations (Fig. 3.4) generally increased in both columns, with ferrous and total iron increasing from  $3.8 \pm 0.16$ - $10.8 \pm 0.44$  mg L<sup>-1</sup> and  $9.3 \pm 0.38$ - $13.5 \pm 0.55$  mg L<sup>-1</sup>, respectively, in column A<sub>f5</sub>, and from  $8.5 \pm 0.35$ - $18.6 \pm 0.76$  mg L<sup>-1</sup> and  $14.0 \pm 0.57$ - $24.3 \pm 1.0$  mg L<sup>-1</sup>, respectively in column B<sub>f7</sub>. Following two 24 hour flow interruptions (PVs 47.6 and 50.8), the chlorine numbers of columns A<sub>f5</sub> and B<sub>f7</sub> decreased by 14 and 17%, respectively, then temporarily increased by 16 and 11%, respectively, over the next 4.6 PVs presumably due to rebounding to steady-state following the flow interruption (Amos et al., 2009). Because sulfide production in these systems stalled below the 5 mM threshold previously shown to inhibit dechlorination (Hoelen and Reinhard, 2004; Mao et al., 2017; Berggren et al.,

2013), a pulse of 7 mM of lactate was added to the influent of both columns beginning at PV 57 in an attempt to stimulate sulfate reduction to the 5 mM level, as lactate was never detected in column effluent samples and *D. vulgaris* is known to use lactate directly as an electron donor (Santana et al., 2012). After initiation of the 7 mM lactate pulse, sulfide production increased from 2.7 to 3.8 mM and from 2.5 to 4.2 mM in the A<sub>f5</sub> and B<sub>f7</sub> columns, respectively, but never reached the 5 mM target. During the elevated lactate pulse, the chlorine numbers in the A<sub>f5</sub> and B<sub>f7</sub> columns decreased, by 20 and 23%, respectively. The elevated lactate treatment was terminated after 11 PVs because sulfate reduction was still below the 5 mM target, despite the presence of excess electron donor in column effluent samples (Fig. A.3). At this point, the chlorine numbers of the two columns leveled out at  $0.69 \pm 0.04$ .

#### *Phase III: sulfide addition to target 5 and 7 mM levels and beyond*

Starting at PV 66, sulfide was added to the influent of both columns for 10 PVs to supplement the amount of sulfide being produced via sulfate reduction and attain target sulfide levels of 5 and 7 mM in the A<sub>f5</sub> and B<sub>f7</sub> columns, respectively. The amount of sulfide provided to each column was calculated based on the difference between the sulfate measured in influent and effluent samples; however, actual sulfide concentrations in the columns were difficult to control due to the volatility of sulfide and the dynamic nature of the sulfate reduction in the columns. The total calculated amount of sulfide in the columns (i.e., sum of reduced sulfate and supplied sulfide) was 5.2 ( $\pm 1.4$ ) and 6.0 ( $\pm 1.3$ ) mM in the A<sub>f5</sub> and B<sub>f7</sub> columns, respectively (Fig. 3.4). The chlorine number during this time (PV 65-75) remained close to 0.69; thus, there was no apparent effect on dechlorination activity following the supplemental addition of sulfide (Figure 3.3).

Ethene production reached a maximum of 33 and 32% of total effluent VOC molar concentrations at PV 69 and 70 in the A<sub>f5</sub> and B<sub>f7</sub> columns, respectively. At PV 76, the B<sub>f7</sub> column began to clog, due to a combination of biomass growth and FeS production. At this point, the influent syringe could no longer maintain the target flow rate (0.015 mL min<sup>-1</sup>); therefore, this column was shut down and the remainder of the experiment was performed using only the A<sub>f5</sub> column. Iron oxides are good adsorbents for a number of metals (Benjamin et al., 1996) and can be reduced by sulfide to produce FeS (Niyuhire et al., 2019; Hansel et al., 2015). Therefore, FeS formation on the surface of the iron oxide-coated sand used in this study could have contributed to the observed column plugging: visual observations indicated FeS formation in both columns (A<sub>f5</sub> and B<sub>f7</sub>).

Since sulfide levels of 5-7 mM had no observable effects on dechlorination of PCE after 10 PVs, the concentration of sulfide delivered to column A<sub>f5</sub> was increased to ca. 10.8 mM starting at PV 76 to determine the threshold level of sulfide required to impact dechlorination in this system. Exposure to an average calculated sulfide concentration of 10.8 mM for 5 PVs (with a maximum of 11.7 mM, including up to 3 mM contributed from sulfate reduction), over twice the threshold shown to inhibit dechlorination in previous studies, had no apparent effect on dechlorination (Figure 3.3) as the average chlorine number ( $0.67 \pm 0.06$ ) in the column during the ca. 10.8 mM sulfide treatment was slightly lower than the average chlorine number during the 5 mM sulfide treatment ( $0.69 \pm 0.02$ ). Sulfate reduction was affected by the 10.8 mM sulfide treatment and declined from an average of  $2.9 \pm 1.2$  to  $0.9 \pm 1.3$  mM after total sulfide exceeded 10mM, which has also been reported in microcosms (Mao et al., 2017). Effluent iron concentrations dropped to between  $1 \pm 0.04$  and  $4 \pm 0.16$  mg L<sup>-1</sup> when total sulfide exceeded 5 mM. However, ferrous iron produced during this time would be expected to rapidly react with

sulfide and be removed from solution, therefore effects on iron reduction cannot be evaluated. As sulfide influent concentrations were increased above 11 mM, there was a concomitant increase in effluent pH from ca. 7.6 to ca. 8.7. VC production did not decrease until influent sulfide exceeded 25 mM and effluent pH exceeded 8.5 (PV 85; not shown). Ethene production, on the other hand, began to decline when influent sulfide was between 11 and 18 mM (pH 8.3).

### **3.5 Discussion**

#### **3.5.1 Experiment 1, Effects of iron reduction on MRD**

In the presence of IRB, MRD of PCE by dechlorinating bacteria was more extensive and produced more ethene compared to the column experiment conducted in the absence of IRB. This could be due to more reduced conditions in the IRB-containing column, as reducing conditions become increasingly important for MRD of the less-chlorinated PCE daughter products, DCE and VC (Chapelle, 1996; Bradley, 2000; Badin et al., 2016). Although iron-reduction was measured in both columns, reduced iron was measured earlier in the effluent of the BDI+IRB column, which also yielded twice as much iron as the BDI-only column over the course of the experiment. Paul et al. (2013) observed competitive inhibition of TCE dechlorination from IRB in the presence of poorly-crystalline Fe(III) minerals, but no inhibition of TCE MRD in the presence of crystalline Fe(III) minerals including goethite and hematite. The iron-oxides present on the coated sand used in this study were found to have a poorly crystalline structure (Benjamin et al., 1996); however, the results of this study indicate that dechlorination was increased in the presence of IRB.

### 3.5.2 Experiment 2, Effects of iron and sulfate reduction on MRD

Although *Dhc* inhibition has been observed at 5 mM sulfide in microcosm studies (Hoelen and Reinhard, 2004; Mao et al., 2017), active MRD of PCE and TCE has been measured at lower sulfide exposures (2-4 mM; Aulenta et al., 2007), and sulfate reduction never reached the 5 mM level during the experiment. In the A<sub>f5</sub> and B<sub>f7</sub> columns, complete reduction of the influent sulfate supplied to the columns was never achieved; maximum sulfate conversions were 81.4 % (PV 27) and 91.2% (PV 32), respectively, and these maxima occurred during Phase I of the experiment when both columns received 5 mM of sulfate. Electron donor limitation may not have been a factor in the amount of sulfate reduced in either column, as excess VFAs (mainly in the form of acetate) were always present in column effluent samples (Fig. A.3) with the exception of a single sample collected from the columns at PV 32 when effluent VFAs were below the instrument detection limit in both columns. However, following the elevated lactate pulse, sulfate reduction did increase by 41 and 68% in the 5 and 7 mM SO<sub>4</sub> columns, respectively. In a microcosm study evaluating the use of various electron donors for MRD and the occurrence of relevant side reactions (i.e., VFA formation, consumption of electron donor in competing terminal electron-accepting processes), Aulenta et al., (2007) observed that the fermentation pathway of lactate was dependent on the presence of SO<sub>4</sub>. Under sulfidogenic conditions, only acetate was formed, however when available SO<sub>4</sub> was consumed, both acetate and propionate were formed. The authors used thermodynamic calculations to show that the production of both acetate and propionate would be the energetically-favorable pathway of lactate fermentation unless H<sub>2</sub> partial pressures were very low (on the order of 1 nM). At such low H<sub>2</sub> concentrations, the acetate-only production pathway becomes more electronegative. In this experiment, both propionate and acetate were only measured in column effluent samples during the first 25 PVs, just prior to the onset of maximum sulfate reduction. Thereafter, acetate

was the sole VFA detected in column effluents (except for small amounts of propionate measured in the S7 column between PVs 37-40). Some studies have suggested that in the presence of both sulfate and reducible iron, IRB can competitively exclude SRB by maintaining electron donors including acetate and hydrogen at sufficiently low thresholds (Anderson et al., 2003; Chapelle and Lovley, 1992). *Desulfovibrio* strains produce acetate during the incomplete oxidization of lactate, complicating the interpretation of the VFA data (Santana et al., 2012). Although H<sub>2</sub> concentrations were not measured in the present study, acetate was always present in column effluent samples, except in a single sample collected from each column (mentioned above), which occurred at the end of the period of peak sulfate reduction in both columns. These observations suggest the possibility that as iron reduction progressed, H<sub>2</sub> concentrations may have been low enough to partially inhibit sulfate reduction. Given that *Dhc* is known to have a low H<sub>2</sub> utilization threshold (Löffler et al., 1999), and the fact that chlorine numbers declined throughout the experiments (until the elevated sulfide treatment), electron donor limitation is unlikely to have impacted MRD. MRD also was not apparently impacted by sulfide levels up to 10.8 mM (Phase *III* of the experiment, 5 & 7 mM sulfide treatments); reduced transformation to VC and ethene only began during Phase *IV* (elevated sulfide treatment) of the experiment.

#### *Elevated sulfide treatment*

As noted previously, the elevated sulfide (> 10.8 mM) added to the column influent solution caused the effluent pH to increase above 8. Although VC production was not impacted until sulfide exceeded 25 mM and pH exceeded 8.5, ethene levels dropped as pH reached 8.3 (at sulfide concentrations between 11-18 mM). Optimal *Dhc* growth conditions are within the pH range of 6-8 (Stroo et al., 2014; Löffler et al., 2013a); however, some evidence (e.g., Parsons, 2004; Delgado et al., 2012) suggests that biotransformation to *cis*-DCE using dechlorinating

microbes other than *Dhc* (e.g., *Sulfurospirillum* multivorans, Yang et al., 2017a) can be implemented at sites with pH values as low as 5 or as high as 9. Sulfate-reducing bacteria have a wider pH range within which growth is possible, with an upper-limit of 9.5 (Huang and Kao, 2016). Nonetheless, the elevated pH caused by the addition of high levels of sulfide to the column influent prevented identification of the threshold at which MRD of PCE was inhibited by sulfide in this system, as the observed impacts on dechlorination when sulfide concentrations exceeded 10 mM could also be attributable to the elevated pH.

### **3.5.3 Comparison of dechlorination performance between column experiments 1 and 2**

The columns from experiments 1 and 2 were inoculated and operated at different times, however, a qualitative comparison between the extent of dechlorination across all four experimental columns used in this study reveals the following: Vinyl chloride and ethene were detected earliest in the columns inoculated with BDI dechlorinating consortium, IRB, and SRB (experiment 2) and these compounds also appeared at the same time in both columns. In the BDI-only and BDI-IRB columns (experiment 1), VC was first detected at PV 7 and 3, respectively, while ethene was first detected at PV 27 and 23, respectively. The BDI-IRB column from experiment 1 exhibited the earliest drop in chlorine number, followed by the BDI-only column, however, VC and ethene appeared first in the BDI-IRB-SRB columns. The columns in experiment 2 (A<sub>f5</sub> and B<sub>f7</sub>) exhibited remarkably similar trends in dechlorination performance as expressed by chlorine number as well as in sulfate reduction, while the differences between the BDI-only and BDI-IRB columns from experiment 1 were statistically significant. In contrast, paired t-tests showed that the chlorine numbers of the A<sub>f5</sub> and B<sub>f7</sub> columns were not statistically different over the course of experiment 2 ( $P = 0.99$ ) except for between PVs 29-37, when the average chlorine No. in column B<sub>f7</sub> ( $1.71 \pm 0.35$ ) was significantly ( $P = 0.0016$ ) higher than the

average chlorine No. in column A<sub>f5</sub> ( $1.48 \pm 0.33$ ). These observations support the hypothesis that the addition of the IRB was responsible for the enhanced dechlorination performance observed in the BDI-IRB column, as opposed to random variability between columns. During the last 10 PVs of each experiment, ethene comprised 27.6 and 42.6% of total effluent VOCs in the BDI-only and BDI+IRB columns, respectively, while ethene comprised 30.0 and 29.4% of total effluent VOCs in the A<sub>f5</sub> and B<sub>f7</sub> columns, respectively, during the last 10 PVs in which both columns were operated. In a chemostat study involving a PCE-dechlorinating enrichment culture, Berggren et al. (2013) observed that the addition of 1 mM sulfide reduced the rate of VC dechlorination relative to a sulfide-free chemostat. Since sulfide was never detected in column effluent, sulfide was likely removed from solution, either through reaction with ferrous iron generated by IRB or via surface reaction/adsorption to the IOCS, since sulfide can reduce iron oxides to produce FeS (Niyuhire et al., 2019; Hansel et al., 2015). In either case, these results confirm microcosm-level observations (Hoelen and Reinhard, 2004) that the presence of reactive iron may remove sulfide from solution and thus negate its inhibitory effect on MRD, but also show benefit of active iron reduction to dechlorinating bacteria even in the absence of sulfate reduction. Therefore, encouraging iron reduction during biostimulation of MRD at chlorinated solvent-contaminant sites may be advisable, particularly when elevated sulfate is observed as a co-contaminant. The presence of reducible iron in aquifer materials should be assessed prior to initiation of bioremediation; reducible (ferric) iron could be added as an amendment during biostimulation in the absence sufficient geogenic iron-bearing minerals.

### **3.5.4 Implications of column plugging for field biostimulation**

The field implications of the clogging and reduced permeability observed at the end of both column experiments would depend, in part, on aquifer type. In unconfined aquifers, reduced permeability following biostimulation would likely result in flow bypass, which could translate

to decreased treatment and/or contaminant displacement. In confined aquifers, clogging could impede delivery of amendments and necessitate high-pressure injections which could increase remediation cost. Additionally, the nature of the clogging (i.e., biomass-related or arising from FeS-formation) would also determine implications for *in situ* biostimulation. Pulsed injections of electron donors could be used if biomass clogging was expected to be a large contributor to reduced permeability. If formation of FeS was expected to be the dominant process resulting in reduced permeability (such as observed most prominently during experiment 2, when both columns visibly turned black as relatively large amounts of sulfide were being supplied to the columns), subsurface heterogeneity (e.g., with regard to distribution of iron-bearing minerals), which is not represented by the column conditions used in this study, could make clogging less of a concern. Additionally, the formation of FeS in the treatment zone could increase the potential for abiotic degradation of the contaminants, as discussed below.

### **3.5.5 Potential for abiotic and biotic remediation of chlorinated ethenes**

Some researchers have either suggested or observed (e.g., Lampron et al., 2001; Darlington et al., 2008; Badin et al., 2016; Schaefer et al., 2018b) that naturally-occurring biotic (bioremediation via microbial reductive dechlorination i.e., MRD) and biologically-mediated abiotic degradation (BMAD) processes can be used in concert to reach remediation goals. FeS-bearing minerals can degrade a range of groundwater contaminants, including PCE and TCE (Butler and Hayes, 1999; Evans et al., 2014). In environments typical of many aquifers, Fe-S phases are formed only in solution (Schieber, 2011), and the specific phases formed depend on pH, redox state, and other factors such as sulfide concentration, presence of buffers, etc. (He et al., 2010; Harmandas and Koutsoukos, 1996; Lemos et al., 2007; Wan et al., 2017). BMAD occurs on mineral surfaces and is enhanced by sorbed Fe(II), (Lee and Batchelor, 2002a; Culpepper et al., 2018); therefore, precipitation of FeS in systems in which ferrous iron is

stoichiometrically in excess of sulfide could result in Fe(II)-surface complexes and thus, a favorable environment for BMAD contaminant degradation. Hyun and Hayes, (2009) noted that TCE reactivity of FeS precipitates was substantially impacted by solution pH (and Fe:S ratio). Fe-S precipitates formed at pH ~ 9 were more reactive with respect to TCE than precipitates formed at pH ~8, while those formed under circumneutral conditions were nearly non-reactive. He et al. (2010) observed an increase in the reactivity of mackinawite as pH increased from 5.4 to 8.3, and Butler and Hayes (1998) observed an exponential increase in the first-order reaction rate constant for the FeS-mediated reduction of hexachloroethane as pH increased from ~7-9.5, while Lee and Batchelor (2002b) found a linear increase in the pseudo-first order initial rate constant describing abiotic TCE degradation by green rust sulfate as pH increased from ~7-10.

In practice, the conditions that support efficient MRD may not necessarily overlap with the conditions under which BMAD is an efficient contaminant degradation process; growth of *Dhc* and MRD have been observed at pH values ranging from ~6-8 (Delgado et al., 2012; Löffler et al., 2013a), with reduced dechlorination observed at higher or lower pH levels (Holliger et al., 1993; Yang et al., 2017b; Zhuang and Pavlostathis, 1995), whereas BMAD may be most efficient at pH values  $\geq 8$ . While MRD of chlorinated subsurface contaminants may be the dominant degradation pathway at circum-neutral pH and at low sulfide concentrations, increases in sulfide and/or pH accompanied by active iron reduction in Fe-bearing aquifers creates the potential for contaminant degradation via BMAD, and/or a reduction in sulfide toxicity to dechlorinating organisms through FeS formation. These results demonstrate that iron reduction can benefit MRD and be protective of dechlorinators when sulfide is present in a flow-through system under circum-neutral pH conditions. However, if the pH of a system increases beyond the threshold above which MRD can occur, BMAD as an alternate contamination biodegradation

pathway may be favorable. These findings can inform remediation strategies employed at sites co-contaminated with sulfate and chlorinated ethenes in which iron-bearing minerals are present.

### **3.6 Acknowledgements**

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## 4. Use of the Partitioning Electron Donor n-Butyl-Acetate to Promote Organohalide Respiration and Bioenhanced Dissolution of PCE-DNAPL

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This work has been prepared for submission to Biodegradation. Co-author Danielle Sylvia set up and operated the abiotic column experiments and initiated two of the biotic column experiments presented in this chapter. The Supplementary Material to be submitted for publication with this work is provided as Appendix B.

### 4.1 Abstract

Biostimulation with a partially-soluble partitioning electron donor (PED) such as n-butyl acetate (nBA) in chlorinated solvent nonaqueous phase liquid (NAPL) source zones is a potential approach to promote more efficient microbial reductive dechlorination (MRD). PEDs favorably partition into the NAPL phase and can be released back into the aqueous phase at aqueous:NAPL interfaces, where they could conceivably support dechlorinating bacterial biomass. The objective of this research was to evaluate the performance of PEDs relative to conventional electron donor substrates (i.e., lactate) under biologically-active and dynamic (flow-through) conditions. A series of one-dimensional columns packed with Federal Fine Ottawa sand containing a uniform distribution of a mixed (tetrachloroethene) PCE (0.25 mol/mol)-hexadecane (0.75 mol/mol) NAPL source zone were used to quantify NAPL-water partitioning coefficients ( $K_{NW}$ ) and suitability to support biologically-enhanced NAPL dissolution as a function of electron donor substrate type within abiotic and biotic systems, respectively. In the abiotic column experiments, effluent breakthrough of the electron donor substrates revealed that a two-pore volume (PV) pulse of nBA persisted approximately 9 times longer than the same volume of a soluble electron donor (lactate) and had a  $K_{NW}$  value 63 times greater than lactate (48.5 vs. 0.77). In biotic

columns inoculated with a PCE-to-ethene dechlorinating microbial consortium, three electron donor delivery approaches were compared: (1) continuous injection of lactate (5 mM), (2) pulsed injections of the food-grade PED nBA (40 mM), and (3) pulsed injections of lactate (5 mM). After approximately 90 PVs, the maximum dissolution enhancement factors (relative to values expected from abiotic dissolution) of the continuous injection lactate, pulsed-injection nBA and lactate columns were 8.3, 11.5, and 1.8, respectively, and the cumulative dissolution enhancement factors were 3.6, 3.0, and 1.8, respectively. These findings demonstrate the utility of nBA as a promising PED that persisted in a NAPL source zone with intermittent pulsed additions and supported MRD, leading to biologically enhanced NAPL dissolution.

## 4.2 Introduction

Due in part to the low cost of biological processes relative to physical-chemical treatment approaches, bioremediation, including monitored natural attenuation (MNA, which often relies on natural biological transformations), biostimulation and bioaugmentation, is the most common technology used to remediate polluted soils and groundwater (Pandey et al., 2009). Over the last decade, bioaugmentation and biostimulation have been particularly successful in treating chlorinated solvents (Lyon and Vogel, 2013), in part due to an improved understanding of *Dehalococcoides mccartyi* strains that can detoxify chlorinated ethenes to benign ethene (Löffler et al. 2013a; b). Numerous studies have shown that dechlorinating microorganisms are capable of reductive dechlorination in close proximity to dense nonaqueous phase liquid (DNAPL) source zones (Adamson et al., 2003; Cápiro et al. 2015; Isalou et al., 1998; Nielsen and Keasling, 1999; Yang and McCarty, 2000), and may enhance tetrachloroethene (PCE) or trichloroethene (TCE) dissolution (Amos et al., 2008; 2009; Cápiro et al., 2015; Carr et al., 2000; Cope and

Hughes, 2001; Sleep et al., 2006). Bioenhanced dissolution has the potential to accelerate depletion of source zone contaminant mass, thereby reducing source zone longevity and remediation cost. The successful application of *in situ* microbial reductive dechlorination often requires the delivery of fluids, including electron donor, to the contaminated zone, followed by contact and/or reactivity with the target contaminant(s).

The current practice for electron donor delivery to enhance DNAPL bioremediation typically involves the injection of either miscible electron donor(s) (e.g., lactate, methanol, ethanol) (Carr and Hughes, 1998) or immiscible viscous or emulsified fluids, from which electron donor dissolves slowly into groundwater such as the polylactate ester HRC® (Adamson et al., 2003) and emulsified vegetable oil (EVO) (Harkness and Fisher, 2013; Hiortdahl and Borden, 2013; Newman and Pelle, 2006). Miscible electron donors (e.g., methanol and ethanol) have been reported to increase PCE-DNAPL dissolution rates by 2- to 3-fold (Sleep et al., 2006); however, the supplied electron donors were readily consumed in competing microbial processes, thus limiting efficacy and requiring repeated electron donor additions. Commercially available immiscible separate phase (e.g., EVO, molasses) electron donors have been widely used to stimulate bioremediation in the field (Adamson et al., 2003; Da Silva et al., 2006; Newman and Pelle, 2006), but can reduce hydraulic conductivity and alter groundwater flow paths (Coulibaly and Borden, 2004; He et al., 2018). Additionally, it is difficult to deliver amendments to adjust the biogeochemical conditions (e.g., pH) in the reaction zone after the initial injection of these insoluble substrates (Henry, 2010) due to the reduced permeability resulting from the separate phase liquid.

More recently, partitioning electron donors (PEDs) have been investigated as an alternative means of electron donor delivery to the DNAPL:water interface over traditional

soluble and separate phase electron donors. PEDs are relatively water-soluble organic compounds (e.g., n-butyl acetate; nBA) that also partition into organic liquid phases (DNAPL), thus incorporating the desirable attributes of both conventional electron donor delivery methods. Abiotic studies have shown that PEDs and contaminant are simultaneously released into the aqueous phase, resulting in sustained concentrations of dissolved phase PED and DNAPL contaminants in close proximity (Cápiro et al., 2011). The dissolved PED can then be hydrolyzed or fermented to yield fatty acid substrates that either directly serve as an electron donor (e.g., acetate) to support PCE-to-cis-1,2-dichloroethene dechlorinating bacteria (e.g., *Desulfuromonas michiganensis*, *Geobacter lovleyi* strain SZ) or undergo further fermentation to generate additional electron donors, including hydrogen, that support dechlorination of cis-1,2-dichloroethene to ethene by organohalide-respiring bacteria (e.g., *Dehalococcoides mccartyi* strains) (Fennell et al., 1997; He et al., 2002). This strategy is promising because in contrast to the rapid fermentation of lactate that yields abundant hydrogen, which is often consumed in alternative terminal electron-accepting processes, fatty acid fermentation of compounds such as propionate or butyrate is thermodynamically constrained. The fermentation of fatty acids such as propionate or butyrate proceeds slowly, because under standard conditions, these reactions are endergonic. However, cooperation between syntrophic bacteria create reactions with net energy generation which remain exergonic for the first partner only through maintenance of a low hydrogen partial pressure by the second partner (ca 1-25 nM; Aulenta et al., 2007; Fennell et al., 1997; Schink et al., 1997; 2002). Competition for hydrogen with other microbial groups is reduced because methanogens are sensitive to the elevated solvent concentrations expected near DNAPL source zones and organohalide-respiring microbes outcompete methanogens at low

hydrogen partial pressures (DiStefano et al., 1992; Fennell et al., 1997; Loffler et al., 2000; Smatlak et al., 1996; Yang and McCarty, 1998).

Consequently, PEDs provide several advantages over conventional viscous or emulsified fluids, including a lower viscosity-aqueous phase that enables rapid transport in unconfined aquifer formations without reducing groundwater flow or the need for high-pressure delivery. Despite the potential utility of PEDs to support sustained enhanced bioremediation in DNAPL source zones, few laboratory (Harkness et al., 2012) and field evaluations (Bartlett, 2016) of PED dechlorination performance have been completed. Enhanced mass removal of TCE DNAPL and ethene production were confirmed using nBA during a one-year field-scale PED implementation test; an increase in Dhc biomass was also measured during the test, which were not observed during a baseline flux evaluation phase that was performed prior to the PED injection (Bartlett, 2016). However, a noted shortcoming of the demonstration was the need for a quantitative comparison between the amount of DNAPL bioenhanced dissolution and extent of dechlorination achievable using a PED versus using a conventional electron donor (Bartlett, 2016). A side-by-side comparison between PEDs and conventional electron donors under conditions that incorporate groundwater flow has not been completed.

To examine the potential utilization of a PED compared to lactate to support MRD and bioenhance NAPL dissolution, three biotic columns containing residual PCE-DNAPL were operated in which each column was supplied with a different electron donor or a different electron donor delivery scheme. In one column, lactate was continuously supplied in a reduced medium, in a second column nBA was supplied in pulses, and in the third column lactate was provided in pulses. Two abiotic columns were also operated to determine the aqueous phase:NAPL partitioning coefficient ( $K_{NW}$ ) of the electron donors in the mixed PCE: hexadecane

NAPL, and to quantify the residence time of the nBA and lactate electron donors in the columns following pulsed injection without microbial transformation. Data obtained from these experiments were used to quantitatively compare dechlorination performance obtained using a PED versus a water-soluble electron donor, in terms of both rate and extent of PCE dechlorination and in terms of bioenhanced dissolution (relative to abiotic dissolution) of PCE from a mixed DNAPL. The mixed-NAPL was used because the expected aqueous phase concentration of PCE in this mixture (50 mg/L) is nontoxic to the dechlorinating microbial population (Amos et al. 2007, Amos et al. 2008).

## **4.3 Experimental Protocols**

### **4.3.1 Chemicals and Porous Media**

High-performance liquid chromatography (HPLC)-grade tetrachloroethene (PCE) was purchased from Acros Organics (Fisher Scientific, Waltham, MA), hexadecane (HD: 99% purity), sodium lactate (60% w/w), and resazurin (a redox indicator) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PCE and HD (with densities of 1.63 g/cm<sup>3</sup> and 0.77 g/cm<sup>3</sup>, respectively) were used to prepare a mixed PCE-HD NAPL at a molar ratio of approximately 0.25:0.75 with a calculated mixture density of 0.86 g/mL that was used for both the abiotic and biotic columns. The aqueous solubilities of PCE and HD are 200 mg/L and 0.0036 mg/L, respectively, and the aqueous phase solubility of the PCE in the PCE:HD mixture was approximately 50 mg/L based on Raoult's Law. HPLC grade n-butyl acetate (nBA) was also purchased from Sigma-Aldrich and has a density of 0.88 g/cm<sup>3</sup> and an aqueous solubility of 6,800 mg/L (at 20°C; Lide and Haynes, 2009). Sodium bromide (NaBr), HPLC-grade methanol, and Oil-Red-O (a hydrophobic dye) were purchased from Fisher Scientific. All chemicals used in sample preparation and calibration solutions were of reagent grade or higher purity, and all

solutions were prepared using 18.2 M $\Omega$ /cm deionized water (EMD Millipore; Billerica, MA) and/or HPLC grade methanol.

Federal Fine Ottawa (FFO) quartz sand (30-140 mesh) obtained from US Silica Company; Frederick, MD, USA was used as the porous media in the columns. FFO has a mean grain size of 0.32 mm, intrinsic permeability of  $4.2 \times 10^{-11} \text{ m}^2$ , and an organic carbon content of 0.001% by weight (Suchomel et al., 2007).

#### **4.3.2 Medium Preparation and Microbial Inoculum**

The column background solution was a reduced mineral salts medium prepared as described previously (Cápiro et al., 2015) with the addition of NaHCO<sub>3</sub> (5 mM, Fisher Scientific) to increase the buffering capacity of the system and provide an additional electron acceptor to support homoacetogenesis (Delgado et al., 2012). Where indicated, the medium was amended with 5 mM (445 mg/L) lactate or 40 mM (4500 mg/L) nBA as the electron donor source. The half reactions for complete oxidation from lactic acid (Ni et al., 2014) and nBA to CO<sub>2</sub> result in the production of 6 and 16 moles of hydrogen, respectively, and since 4 moles of hydrogen are required to reduce 1 mole of PCE, the theoretical molar PCE reducing equivalents of lactate and nBA are 1.5 and 4, respectively. The concentration of nBA used in the column experiments (40 mM, 4,500 mg/L) was selected to maximize the amount of PED delivered to the NAPL while being sufficiently below the aqueous solubility of nBA (6800 mg/L); the lactate concentration was selected to be in the range of dissolved organic carbon recommended for field applications (1.3-8 mM; Henry, 2010). Biotic columns were inoculated by wet-packing (as described in the supplementary materials) with the BDI-SZ inoculum, a well-characterized PCE-to-ethene dechlorinating consortium that contains multiple dechlorinators, including three

*Dehalococcoides mccartyi* (*Dhc*) strains and *Geobacter lovleyi* strain SZ (Amos et al., 2009; Cápiro et al., 2014). The inoculum was maintained in reduced medium amended with 0.33mM PCE and 10 mM sodium lactate as described previously (Cápiro et al., 2014).

#### **4.3.3 Column Preparation and Setup**

A total of five column experiments, two abiotic and three biotic, were completed to assess the partitioning behavior of lactate or nBA into the mixed NAPL and the utilization of these electron donors to support organohalide respiration for bioenhanced dissolution and transformation of a mixed PCE-NAPL, respectively. All experiments were conducted in identical borosilicate glass columns (15 cm length x 4.8 cm dia., Kontes Glass Company; Vineland, New Jersey) equipped with Teflon end plates, packed with FFO sand as described in the supplementary materials, which also contains additional descriptions of the column preparations (e.g. determination of pore volume-PV). After packing, porosity in the columns ranged from 0.37-0.39. An anoxic delivery system was employed as described in Appendix B to ensure that both column influent and effluent solutions were anoxic and to prevent volatilization of chlorinated solvents and nBA prior to sampling.

Following packing, 3-4 PVs of reduced medium was continuously flushed through the columns at 0.15 mL/min or a seepage velocity of 0.3 m/day in all columns. For the biotic columns, the elution of *Dhc* cells was monitored during this flushing period by collecting biomass from 5-15 mL column effluent samples. Biomass samples were pelleted by centrifugation and frozen at -20°C until quantitative real-time polymerase chain reaction (qPCR) analysis, as described briefly in Appendix B and in greater detail in Cápiro et al. (2014). These samples, along with column inocula samples for each column (details in Appendix B) were used to quantify the approximate number of *Dhc* cells remaining in the columns at the end of the

microbial elution phase of the biotic column experiments. At the conclusion of the microbial elution period, 45%, 36%, and 25% of the *Dhc* cells initially added to the continuous lactate, nBA, and pulsed lactate nBA columns, respectively, had eluted from the columns prior to the introduction of NAPL into the columns. When adjusted for the dry mass of sand in each column, it was calculated that there were  $5.7 \times 10^6$ ,  $3.3 \times 10^7$ , and  $1.5 \times 10^7$  cells  $\text{g}^{-1}$  dry sand remaining in the continuous lactate, nBA, and pulsed lactate columns, respectively.

After the flushing phase, pre-NAPL saturation bromide tracer tests were performed in each column by flushing approximately 2 PVs of reduced medium containing 10 mM sodium bromide and either lactate or nBA (supplied as electron donor) through the columns at 0.15 mL/min, or a seepage velocity of  $0.3 \text{ m d}^{-1}$ , using a peristaltic pump (Minipuls 3, Gilson Inc., Middleton, WI). Following the initial 2 PV pulse of bromide tracer, a bromide-free reduced medium was continuously supplied to the columns. The bromide tracer samples were collected using a fraction collector (Spectra/Chrom CF-2, Spectrum Labs, Rancho Dominguez, CA, USA) and analyzed to confirm the pre-NAPL saturation PV and column Peclet number as described in Section 4.3.6 below and in the supplementary materials and to confirm the absence of immobile water and stagnant (i.e., low or no-flow) zones in the column as completed in prior studies (Cápiro et al., 2011).

Next, an autoclaved mixed PCE-HD NAPL was added to each column using a Harvard Apparatus Syringe Infusion Pump 2.2 (Holliston, MA) at 2.5 mL/min. The PCE-HD mixture was dyed with 0.4 mM Oil-Red-O to visually confirm that NAPL was evenly distributed in the columns following saturation. Between 80 and 100 mL of NAPL were introduced from top to bottom in each column followed by flushing with the reduced medium background solution (at a flow rate of 0.15 mL/min) until no NAPL was visible exiting in the column effluent. Column

NAPL saturation was determined by using the volume of NAPL added to the column less the amount recovered during the NAPL elution period and dividing this value by the pre-NAPL-saturation PV (Wilson et al. 1990). Average NAPL saturation of the columns was  $13.1 \pm 2.1$ . The column was flushed until 4-5 PVs free of visible NAPL had eluted; a post-NAPL saturation bromide tracer test was performed as described above to determine post-saturation PV and to confirm the absence of immobile water and stagnant zones in the column according to previously established methods (Cápiro et al., 2011).

#### **4.3.4 Abiotic Column Operation**

Two abiotic columns were operated to determine the mass transfer of electron donor in the mixed NAPL under flow-through conditions and to quantify how long the electron donors persisted in column effluent and at what concentrations following pulsed injections. A previous study of PED partitioning into a pure TCE DNAPL measured equilibrium aqueous-phase:NAPL partitioning coefficients ( $K_{NW}$ ) values that were ca. 6.6-12.2 times higher using batch reactors as compared to values modeled under dynamic conditions in column experiments (Cápiro et al., 2011), suggesting rate-limited partitioning of the PED into the DANPL in the columns. Equilibrium  $K_{NW}$  values of 187 and 16 were calculated for nBA and lactate, respectively, in batch reactor experiments using the same mixed NAPL and reduced medium as used in this study (Sylvia, 2016). Lactate (5 mM) or nBA (40 mM) was introduced as a 2 PV pulse, followed by flushing with unamended medium. Flushing and monitoring of the effluent for PCE, lactate or nBA (plus byproducts of nBA hydrolysis) continued until the compounds were no longer detected in the column effluent, at which point these experiments were terminated. The effluent lactate and nBA breakthrough curves (BTCs) were plotted versus the number of dimensionless PVs of aqueous column eluent and analyzed using equilibrium and non-

equilibrium transport models as described in Section 4.3.6. As determined in prior work by Cápiro et al. (2011), BTCs for nBA through similarly packed columns with FFO sand coincided with non-reactive tracer curves in the absence of NAPL; therefore, partitioning of nBA into the immobile mixed PCE-HD NAPL was considered to be the dominant process influencing the transport and release of nBA (Cápiro et al., 2011).

#### **4.3.5 Biotic Column Operation and Sample Collection**

Three biotic columns were used to compare rates of bioenhanced NAPL dissolution and extent of PCE dechlorination by the dechlorinating consortium under different electron donors and delivery methods: (1) continuous addition of lactate (5mM) in the reduced medium, (2) pulsed addition of 40 mM nBA, and (3) 5 mM pulsed lactate addition. The pulsed additions were introduced in 2PV increments. The timing of subsequent 2 PV pulses of nBA-amended medium in the nBA biotic column was determined based on monitoring the column effluent for nBA and transformation products. When concentrations of the parent nBA and hydrolysis products, butanol and acetate, were not detected for 1-2 PVs, an additional 2PV pulse of nBA amended medium was introduced. Pulses of lactate-amended medium were timed to occur at the same PV intervals as those supplied to the nBA column to facilitate a comparison of dechlorination/bioenhanced-dissolution performance between nBA and lactate when both electron donors were supplied as pulsed amendments.

During each biotic column experiment, two periods of flow interruption, ranging from 12-72 hours, were employed to assess mass transfer limitations under no-flow conditions and to evaluate the effect of residence time on PCE microbial reductive dechlorination. The first flow interruption lasted for 12 hours and occurred after 11 PVs. Following the first interruption, the column flow rate was reduced from 0.15 mL/min (0.3 m/day) to 0.10 mL/min (ca. 0.2 m/d). The

second interruption lasted for 72 hours and occurred after 30 PVs; following this pause in flow, the column flow rate was reduced again to 0.07 mL/min (ca. 0.15 m/d) and maintained at this rate for the remainder of the experiment. Samples were collected within 1 PV after each flow interruption. Identical operational procedures were used to run all columns to allow for direct comparisons between data sets.

Concentrations of PCE and its daughter products (TCE, cis-dichloroethene [cis-DCE], vinyl chloride [VC], and ethene) in column effluent samples were monitored every 1-2 PVs throughout the course of the experiments, as were concentrations of nBA or lactate. In the biotic columns, pH and oxidation-reduction potential (ORP), concentrations of electron donors and volatile fatty acids (VFAs) were also monitored every 1-2 PVs, and biomass samples were collected from the column effluent according to analytical procedures described in Appendix B. All effluent samples were collected using a syringe connected to a Hamilton valve that pulled from a 25 mL glass sampling bulb that maintained sample redox conditions and minimized disruptions in column flow.

#### **4.3.6 Mathematical simulations**

For each column experiment, relative concentrations ( $C/C_0$ ) of effluent bromide tracer and electron donor (for the abiotic columns) were plotted versus the number of pore volumes of solution eluted from the column. The resulting tracer BTCs were simulated using the mathematical model Code for Estimating Equilibrium Transport Parameters from Miscible Displacement Experiments (CFITM, van Genuchten, 1980) as a part of Studio of Analytical Models (STANDMOD) Version 2.2 (available through USDA-ARS U.S. Salinity Laboratory; <http://www.ars.usda.gov>) to confirm pre- and post-NAPL saturation aqueous pore volumes and Peclet numbers. Lactate and nBA BTCs were also analyzed using CFITM along with another

STANMOD model, Code for Estimating Non-Equilibrium Transport Parameters from Miscible Displacement Experiments (CFITIM, van Genuchten, 1981) as described in Appendix B and in more detail in Cápiro et al. (2011) to determine the retardation factors ( $R_f$ ) and the linear NAPL-water partition coefficients ( $K_{NW}$ ) in the nBA and lactate abiotic columns. Within CFITIM, the electron donor BTC data were modeled by considering for equilibrium and one-site and two-site rate-limited chemical non-equilibrium mass transfer processes to determine the most appropriate fit.

#### 4.3.7 Assessment of Dechlorination Performance and Biological Enhanced Dissolution

The biotic columns were operated for approximately 90 PVs, during which time the extent of PCE degradation was calculated and compared for each of the different electron donor treatments. The extent of PCE degradation was expressed as chlorine number (Parsons, 2004), which reflects the degree to which PCE has been reduced to daughter products (trichloroethene, TCE; cis-dichloroethene, cis-DCE; vinyl chloride, VC; ethene) relative to an initial PCE loading. Chlorine number is calculated as:

$$\text{Chlorine \#} = \frac{4[PCE] + 3[TCE] + 2[DCE] + [VC]}{[PCE] + [TCE] + [DCE] + [VC] + [ethe]}$$

The extent of bioenhanced dissolution of PCE from the mixed NAPL were computed by comparing the expected recovery of PCE (i.e., via expected abiotic dissolution) in the aqueous phase with measured concentrations of PCE and its daughter products for each column. The result is expressed as a cumulative and maximum dissolution enhancement factor, calculated as described briefly in the supplementary materials and in greater detail in Amos et al. (2008).

## 4.4 Results

### 4.4.1 Abiotic Column Results

The abiotic columns were completed to quantify the longevity and breakthrough of the electron donors in the absence of a microbial community in FFO sand containing a uniform mixed PCE:hexadecane NAPL source zone, and to subsequently use this data to determine the  $R_f$  and  $K_{NW}$  of the electron donors with mathematical models. Effluent samples were analyzed for PCE and lactate or nBA, and butanol; measured PCE concentrations in the abiotic columns were an average of  $47.1 \pm 7.5 \text{ mg L}^{-1}$ . Lactate was detected in the column effluent at PV 0.99 in the abiotic lactate column (Figure 4.1) and quickly approached a relative concentration of 1.0, exhibiting similar breakthrough behavior to the nonreactive bromide tracer. However, relative to the non-reactive tracer, there was some lag in the descending limb of the lactate BTC and lactate was detected until PV 3.2, 0.45 PV longer than the bromide tracer. Relative to the mass of lactate supplied to the column, 95.7% was recovered in the column effluent. The one-site non-equilibrium model better-fit the observed tailing of the lactate BTC compared to the equilibrium model indicating non-instantaneous adsorption to attachment sites (Figure 4.1). The Sum of Squared Residuals (SSQ) of the non-equilibrium model (0.277) was also lower than the SSQ for the equilibrium model (1.13). The non-equilibrium model was used to determine  $R_F$ , and  $K_{NW}$  which were calculated to be 1.16 and 0.77, respectively.

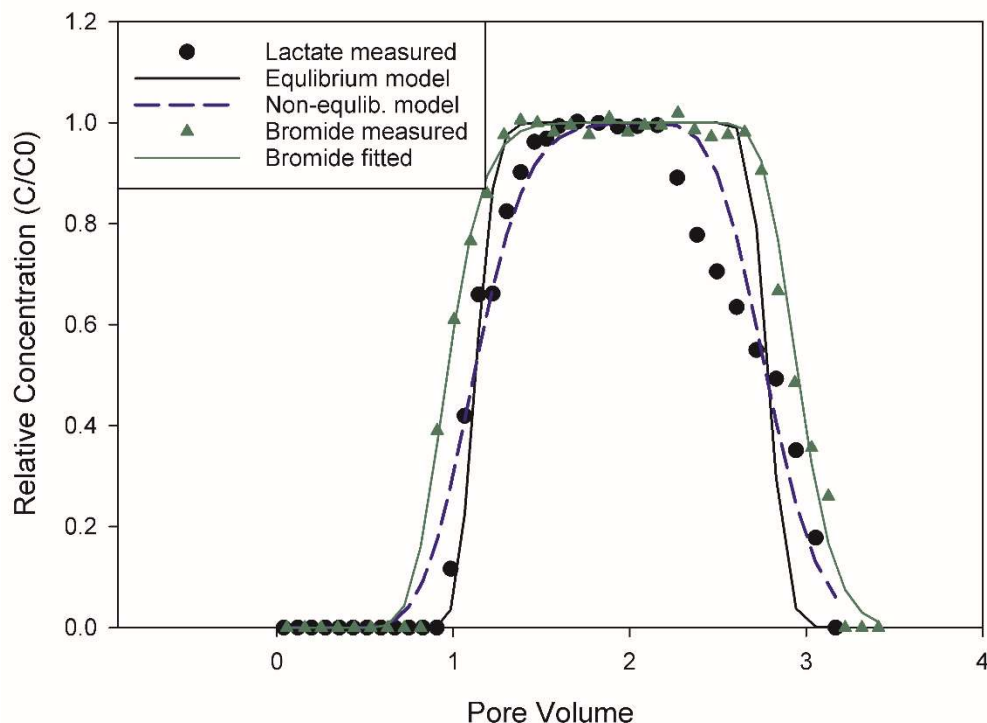


**Figure 4.1.** Measured effluent concentrations of lactate and bromide, simulated equilibrium and non-equilibrium lactate concentrations, and fitted bromide BTC in the abiotic lactate column.

In contrast, breakthrough of nBA was detected at PV 5.2 with measurable concentrations until PV 24.9 (Figure 4.2); therefore, the nBA persisted approximately 9 times longer than lactate in the column. The highest concentration of nBA measured (8.2 mM) was 23.4% of  $C_0$  (35 mM), which occurred at 12.9 PVs. The rising and falling limbs of the nBA BTC were relatively symmetric. Butanol was also measured in the effluent samples indicating that nBA was breaking down to form butanol through hydrolysis. In total, 82.9% of the nBA was recovered; 70.4% was recovered as nBA and the remaining 12.5% was recovered as butanol. Since nBA hydrolyzes to form both butanol and acetate in equal molar proportions (Johannes et al., 1997), it is assumed that acetate also represented 12.5% of the nBA recovered as butanol. The best-fit model for the observed nBA effluent concentrations was the two-site non-

equilibrium model, with an SSQ of 0.008 (compared to an SSQ of 0.041 for the equilibrium model), suggesting that partitioning was instantaneous on some reaction sites and rate-limited on others. Model results indicated that the percentage of potential reaction “sites” at equilibrium was 58%; Cápiro et al. (2011) found that up to 40% of potential reaction sites were at equilibrium in an abiotic mass-transfer column experiment in which the partitioning of nBA into pure TCE-DNAPL was studied. The nBA-TCE  $K_{NW}$  (290.8) was ca. 6 times higher than the  $K_{NW}$  calculated for the partitioning of nBA into the mixed PCE-HD NAPL in this study. Since the partitioning examined in Cápiro et al. (2011) was for a pure-phase TCE DNAPL, a direct comparison is not justified, however the fact that the percentage of equilibrium reaction sites was of the same magnitude as reported by Cápiro et al. (2011) suggests that PED-NAPL partitioning dynamics were similar in nBA-TCE DNAPL and nBA-mixed-phase HD-PCE NAPL systems. The  $R_f$  value for nBA was calculated to be 10.5 and the  $K_{NW}$  value was 48.5; these values are 9 and 63 times larger than the  $R_f$  and  $K_{NW}$  values calculated for lactate, respectively. These findings illustrate the degree of affinity that the PED has for the mixed NAPL and explain why nBA persisted substantially longer in effluent samples compared to lactate following the pulsed injections of the two electron donors.

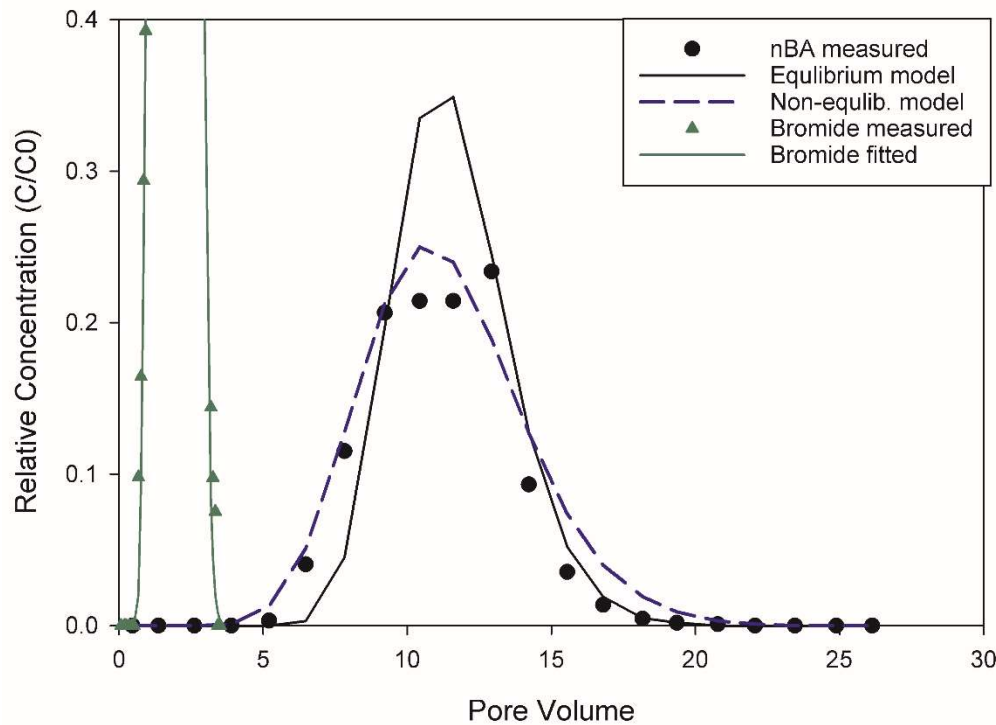


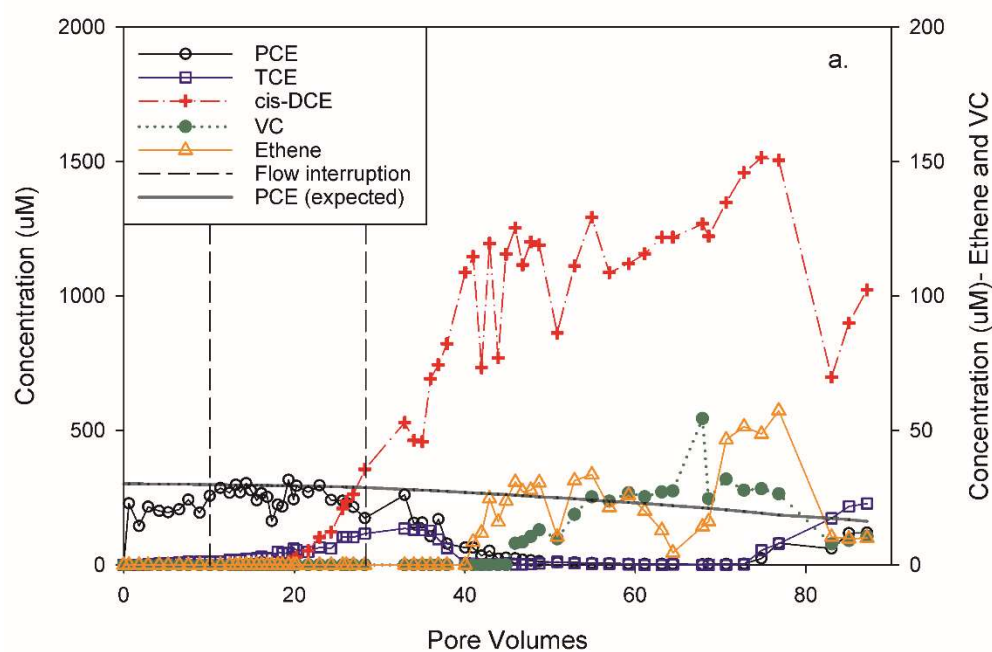
**Figure 4.2.** Measured effluent concentrations of n-butyl acetate (nBA) and bromide, simulated equilibrium and non-equilibrium nBA concentrations, and fitted bromide BTC in the abiotic nBA column.

#### 4.4.2 Biologically-Active Columns

*Phase 1: 0.3 m<sup>-d</sup> pore water velocity*

Continuous-injection lactate, pulsed-injection nBA and pulsed-injection lactate biotic columns inoculated with the PCE-to-ethene dechlorinating BDI-SZ culture were completed using the same initial flow rates as the abiotic columns (0.15 mL/min), and were monitored over time to assess dechlorination (Figures 4.3 and 4.4) and microbial enhancement of PCE dissolution (Figure 5). Prior to the first flow interruption (PV 11; 12 h) and subsequent flow reduction (from 0.3 to 0.2 m d<sup>-1</sup>) chlorine numbers (Figure 4) in the continuous lactate and nBA columns decreased by average values of 0.004 and 0.016 PV<sup>-1</sup>, respectively. Dechlorination was absent in the pulsed lactate column prior to the first flow interruption. In the continuous-injection lactate

and nBA columns, TCE was first measured at PV 2.8 and 1.3, respectively; cis-DCE was first measured in the nBA column at PV4.3. The chlorine number (Figure 4.4) declined earliest in the nBA column (beginning at PV 1.3, compared to PV 2.8 in the continuous lactate column) and had a lower average (Table A1) value (3.90) compared to the continuous lactate column (3.98) during this portion of the experiment. Prior to and just after the first pulse of nBA provided to the nBA column (PV 3.6), the chlorine number decreased by 0.57% (from 3.99 to 3.97).



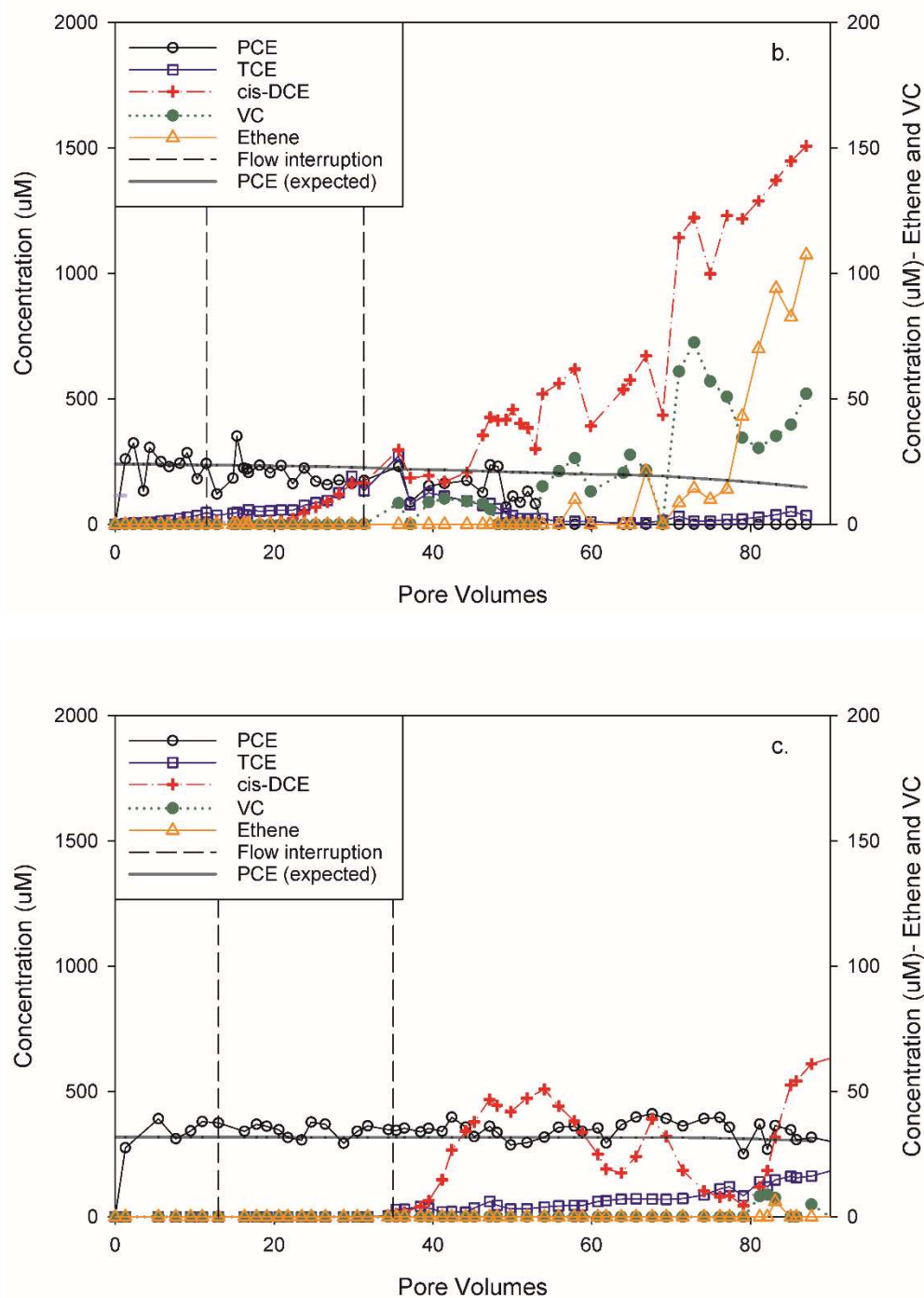


**Figure 4.3.** Concentrations of PCE and its daughter products versus pore volume in (a.) the continuous lactate column, (b.) the nBA column, and (c.) the pulsed lactate column. Duration of flow interruptions (dashed lines) was 12 hrs (1<sup>st</sup> flow interruption, ca. 10 PVs) and 72 hrs (2<sup>nd</sup> flow interruption, ca. 30 PVs). Expected PCE concentrations (solid grey lines) due to abiotic dissolution are shown for comparison.

## Chlorine number vs. Pore volume

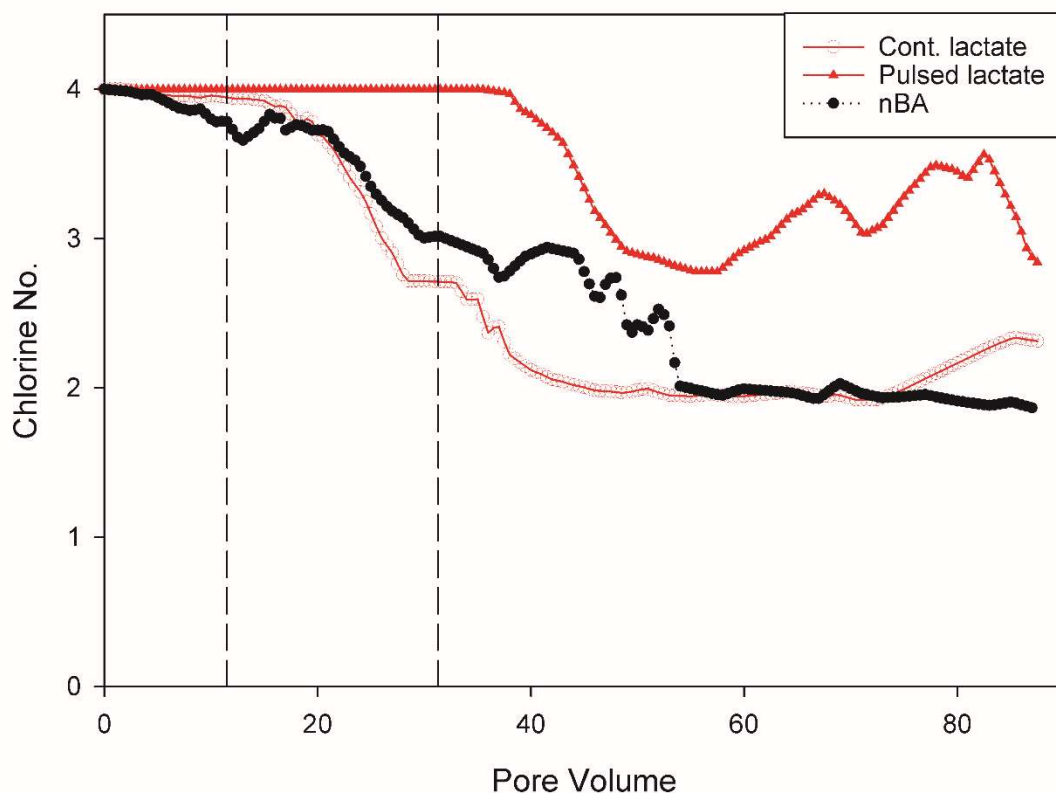


**Figure 4.4.** Chlorine number versus pore volume for the different electron donor treatments. Flow interruptions (each followed by a subsequent reduction in flow rate) are indicated by dashed vertical lines.

### *Phase 2: 0.2 m<sup>-d</sup> pore water velocity*

Between the first (PV 11, 12 h) and second (PV 30, 72 h) flow interruptions (seepage velocity 0.2 m d<sup>-1</sup>), chlorine numbers in the continuous lactate and nBA columns decreased by 31.3% and 20.8%, respectively. In the continuous-injection lactate column, cis-DCE was first measured just after (PV 11.3) the first flow interruption and became the most prevalent byproduct recovered from this column at PV26, comprising 41.5% of total volatile organic compounds (VOCs) recovered at this time. The average chlorine number in the continuous lactate column (3.87) was higher than that in the nBA column (3.76) from PVs 11-20; thereafter

the average chlorine number in the continuous lactate column dropped below the nBA column for the remainder of this phase of the experiment (Table A1). Prior to and just after the second pulse of nBA provided to the nBA column (PV 30), which was completed just before the second flow interruption (PV 30) the chlorine number decreased by 7.48% (from 3.13 to 2.90). Dechlorination was not measured in the pulsed lactate column during this phase of the experiment.

*Phase 3: 0.15 m<sup>d</sup> pore water velocity*

Following the second flow interruption (PV 30; 72 h) and up to the end of the experiment (PV 87), chlorine numbers in the continuous lactate and nBA columns decreased by 16.1% and 34.1%, respectively. In the nBA column, cis-DCE became the dominant VOC (36.7% of total VOCs) measured starting at PV 36, 27.8 % later than the continuous lactate column. The increased dechlorination measured in the nBA column at this time occurred just after the second flow interruption, at which point the flow to the columns was decreased to 0.15 m day<sup>-1</sup>; therefore, the increased dechlorination observed in the nBA column may have been a result of a longer column residence time, a result that has been documented previously (Amos et al., 2009; Cápiro et al., 2015; Haest et al., 2011). The chlorine number continued to drop in the continuous after the second flow interruption (from 2.72 at PV 28 to 2.4 at PV 37). Both cis-DCE and TCE were first measured in the pulsed lactate column at PV 34.6, after the second flow interruption and flow reduction. TCE comprised 10.7 % of the total VOCs recovered in the pulsed lactate column between PVs 34-87, compared to 4.5% and 6.9 % in the continuous lactate and nBA columns, respectively over the same interval. cis-DCE was produced in substantially lower quantities in the pulsed lactate column (comprising 42.1 % of the total VOCs recovered between

PVs 34-87) compared to the other columns (accounting for 88.9 % and 80.1 % of total VOCs recovered in the continuous lactate in the nBA columns, respectively, over this interval).

The chlorine numbers of the continuous lactate and nBA columns exhibited similar temporal behavior until PVs 37-52, when the chlorine number in the nBA column increased briefly three times by an average value of  $6.2 \pm 1.0$  %. During this 15 PV interval, the average molar mass ( $72.4 \pm 29.4$   $\mu\text{mol}$ ) of total VOCs recovered from each sample in the nBA was still higher than the average molar mass ( $53.2 \pm 14.7$   $\mu\text{mol}$ ) recovered from samples in the preceding 15 PV intervals (i.e., PV 22-37), therefore total dechlorination was still increasing, and thus the chlorine number does not reflect the overall increase in the production of dechlorination daughter products in the nBA column during this period. Fluctuations in PCE dissolution from the mixed PCE:HD NAPL also contributed to this observed variability, as increased PCE concentrations were also observed during this time (Figure 4.3). After the temporary increases, the chlorine number in the nBA column declined starting at PV 52, from 2.52 to within 0.4% of the value observed in the continuous lactate column (1.96) by PV 57 (indicating predominantly cis-DCE production), before dropping below the continuous lactate column from PV 75 until the end of the experiment. After the third pulse of nBA provided to the nBA column (PV 53.8) the chlorine number fell by 17% (from 2.41 to 2.01) while the final nBA pulse (PV 71.0) did not have an obvious impact on chlorine number.

In the pulsed-lactate column, PCE was the most dominant VOC recovered over the course of the experiment, although DCE was dominant (i.e. > 50% of measured VOCs) from PVs 44-59 and again from 84-90, when it comprised 60% and 57% of total recovered VOCs, respectively. The increases ( $98.2 \pm 73.5$  %) in cis-DCE occurred during or within 3-5 PVs of lactate pulses. In general, flow interruptions increased the concentration of dechlorination

daughter products (Figure 4.3), attributable to an increase in contact time between the PED electron donor, NAPL and microbes and an associated increase in microbial activity (e.g., Cápiro et al., 2015; Chen et al., 2013; Haest et al., 2011). At the end of the experiment, PCE accounted for 61% of the total chlorinated ethenes and ethene recovered from the pulsed lactate column, while PCE represented 13% and 18% of total chlorinated ethenes and ethene recovered in the continuous lactate and nBA columns, respectively.

By the end of the experiment, *cis*-DCE accounted for 78.5, 68.8, and 28% of all chlorinated VOCs recovered from the continuous-injection lactate, nBA, and pulsed-injection lactate columns, respectively, while TCE comprised 5.8, 9.0 and 7.7% of the total VOCs recovered from the three columns, respectively. Vinyl chloride and ethene were more abundant in the nBA column compared to the continuous-injection and pulsed lactate columns. Over the last 10 PVs of the experiment, VC and ethene accounted for 2.7 and 4.6% of total VOCs recovered from the nBA column compared to 0.8 and 0.9% of total VOCs recovered from the continuous lactate column, and 0.5 and <0.1% of total VOCs recovered from the pulsed lactate column respectively. To determine whether vinyl chloride or ethene would eventually be produced under a pulsed lactate delivery scheme, this column was operated for an additional 50 PVs after the other experiments were stopped (not shown); by considering these additional 50 PVs, PCE, TCE, and DCE each accounted for ca.1/3 of the VOCs recovered from the column, while vinyl chloride and ethene represented just 0.2 and 0.1 %, respectively.

The rates of production of vinyl chloride and ethene ( $\text{day}^{-1}$ ) relative to the initial mass of PCE present in each column were calculated to facilitate comparisons across columns. The resulting rates were averaged across ca. 10 PV intervals (Table 4.1). Rates were zero or one to two orders of magnitude lower in the pulsed lactate column than in the other two columns.

Compared to the continuous-injection lactate column, VC appeared 29% earlier in the nBA column and was produced at higher rates from PVs 30-47 (likely due to temporary increases after the flow interruption and nBA pulse) and again from PVs 70-90 (a combination of increased residence time and stimulation following an nBA pulse), while ethene appeared 41% later in the nBA column and production was slower than the continuous lactate column until the last 10 PVs of the experiment when ethene production was 10 times higher in the nBA column. At the end of the experiment (PV 90), the continuous-supply lactate column had a chlorine number of 2.3 (8.6% PCE, 16.4% TCE, 73.6% cis-DCE, 0.8% VC and 0.7% ethene [mole/mole]), while the nBA and pulsed lactate columns had chlorine numbers of 1.9 (0% PCE, 2.1% TCE, 88.5% cis-DCE, 3.1% VC and 6.3% ethene) and 2.9 (28.9% PCE, 14.9% TCE, 55.7% cis-DCE, 0.4% VC and 0% ethene), respectively.

**Table 4.1.** Chlorine number, dissolution enhancement factors, and production rate of vinyl chloride and ethene<sup>†</sup>.

| PV range           | Chlorine number |      |      | Enhanced dissolution factor |      |      | Vinyl chloride production rate <sup>†</sup> |         |        | Ethene production rate <sup>†</sup> |         |        |
|--------------------|-----------------|------|------|-----------------------------|------|------|---------------------------------------------|---------|--------|-------------------------------------|---------|--------|
|                    | cLAC            | pLAC | nBA  | cLAC                        | pLAC | nBA  | cLAC                                        | pLAC    | nBA    | cLAC                                | pLAC    | nBA    |
| 0-11 <sup>a</sup>  | 3.98            | 4.00 | 3.90 | 0.98                        | 1.00 | 1.18 | 0                                           | 0       | 0      | 0                                   | 0       | 0      |
| 11-20 <sup>b</sup> | 3.87            | 4.00 | 3.76 | 1.06                        | 1.21 | 1.17 | 0                                           | 0       | 0      | 0                                   | 0       | 0      |
| 20-30 <sup>b</sup> | 3.14            | 4.00 | 3.35 | 1.71                        | 1.24 | 1.49 | 0                                           | 0       | 4.6E-5 | 0                                   | 0       | 0      |
| 30-40 <sup>c</sup> | 2.47            | 3.97 | 2.90 | 3.35                        | 1.22 | 2.49 | 0                                           | 0       | 4.1E-5 | 0                                   | 0       | 0      |
| 40-50 <sup>c</sup> | 2.01            | 3.32 | 2.75 | 4.39                        | 2.04 | 2.54 | 3.3E-5                                      | 0       | 9.3E-5 | 1.7E-4                              | 0       | 0      |
| 50-60 <sup>c</sup> | 1.96            | 2.83 | 2.13 | 4.72                        | 2.90 | 2.73 | 1.5E-4                                      | 0       | 1.3E-4 | 1.8E-4                              | 0       | 1.2E-5 |
| 60-70 <sup>c</sup> | 1.96            | 3.14 | 1.97 | 5.92                        | 2.69 | 2.85 | 2.3E-4                                      | 0       | 4.7E-4 | 1.0E-4                              | 0       | 4.5E-5 |
| 70-80 <sup>c</sup> | 2.01            | 3.27 | 1.94 | 8.21                        | 2.66 | 6.81 | 2.1E-4                                      | 0       | 3.4E-4 | 3.8E-4                              | 0       | 1.5E-4 |
| 80-90 <sup>c</sup> | 2.27            | 3.28 | 1.89 | 7.17                        | 2.98 | 9.77 | 6.7E-5                                      | 2.8 E-5 | 4.6E-5 | 7.4E-5                              | 5.9 E-6 | 7.6E-4 |

Values represent averages across 10 PV intervals in the continuous lactate (cLAC), pulsed lactate (pLAC), and nBA columns.

<sup>a</sup>Phase I of experiment (pore water velocity 0.3 md<sup>-1</sup>). <sup>b</sup>Phase II of experiment (pore water velocity 0.2 md<sup>-1</sup>). <sup>c</sup>Phase III of experiment (pore water velocity 0.15 md<sup>-1</sup>), PV 30-90.

<sup>†</sup> Production rates for vinyl chloride (VC) and ethene are in units of fraction of initial column PCE mass transformed to VC or ethene day<sup>-1</sup>.

#### 4.4.3 Effect of flow interruptions on electron donor concentrations

After the first flow interruption (PV 11) and subsequent flow reduction, nBA and butanol concentrations dropped by 12% and 29%, respectively, in the nBA column, while acetate increased by 265%. The second flow interruption occurred just prior to a pulse of nBA, so effects on nBA could not be determined; however, acetate decreased from 0.8 mM to non-detectable levels after this flow interruption. Lactate was never measured in the effluent of the continuous lactate column, but acetate concentrations decreased by 3% after the first flow interruption, and by 54% after the second flow interruption. In the pulsed lactate column, lactate fell from 0.3 mM to non-detectable levels after the first flow interruption, and there was no lactate or acetate detected in this column just before or after the second flow interruption. Cápiro et al. (2011) investigated the partitioning behavior of two PEDs including nBA into TCE-DNAPL in abiotic columns at pore water velocities of 1.2 to 6 m d<sup>-1</sup> and observed that, depending on when the flow interruption occurred (i.e. during rising vs. falling limb of the PED BTC), flow interruptions increased PED partitioning into the DNAPL phase, suggesting that mass transfer of PED into DNAPL was rate-limited at the flow rates investigated. While the water velocities used in the present study (0.15-0.3 m d<sup>-1</sup>) were lower than those used in the Cápiro et al. (2011) study, the observed decline in effluent nBA concentrations following the first flow interruption (which occurred near the peak of the rising limb of the nBA BTC) could also have been caused by increased partitioning of the nBA into the mixed NAPL during the no-flow period. A mass transfer rate of nBA into pure PCE DNAPL of 0.126 min<sup>-1</sup> was measured in a series of batch reactor experiments (Sylvia, 2016). While the mass transfer rate of nBA into a mixed HD-PCE NAPL was not determined during that study, by applying the mass transfer rate measured for nBA into pure PCE DNAPL measured by Sylvia (2016) to the present study, > 99.9% of the nBA would have partitioned into the NAPL phase within 54 minutes, therefore at the end of the

12 h flow interruption, virtually all of the nBA would be expected to have partitioned into the mixed NAPL.

#### **4.4.4 Biological Enhanced Dissolution**

The maximum dissolution enhancement factor (Figure 4.5) represents the ratio ( $\mu\text{M}/\mu\text{M}$ ) between the total molar concentration of VOCs measured for each sample and the aqueous PCE molar concentration expected under solely abiotic dissolution (shown on Figure 4.3), while the cumulative dissolution enhancement factor (Fig A.1) represents the mass ratio ( $\mu\text{mol}/\mu\text{mol}$ ) between the total recovered VOC mass versus expected PCE mass recovery calculated cumulatively over time (described in the Supplementary Materials and in more detail in Amos et al., 2008). These enhancement factors were impacted by flow interruptions and subsequent changes in seepage velocities. Prior to the first flow interruption and flow reduction (seepage velocity =  $0.3 \text{ m d}^{-1}$ ), the average dissolution enhancement factor ( $\mu\text{M}/\mu\text{M}$ ; Figure 4.5) and cumulative dissolution enhancement factor ( $\mu\text{mol}/\mu\text{mol}$ ; Fig A.1) in the continuous lactate column was approximately 1 (i.e. no enhancement) for the first 11 PVs; during the same interval, these values were  $1.20 \mu\text{M}/\mu\text{M}$  and  $1.10 \mu\text{mol}/\mu\text{mol}$  in the nBA column, respectively. Between the first and second flow interruptions and flow reductions (seepage velocity  $0.2 \text{ m d}^{-1}$ ), the dissolution enhancement and cumulative dissolution enhancement factors increased to  $2.26 \mu\text{M}/\mu\text{M}$  and  $1.22 \mu\text{mol}/\mu\text{mol}$ , respectively, in the continuous lactate column and to  $2.27 \mu\text{M}/\mu\text{M}$  and  $1.26 \mu\text{mol}/\mu\text{mol}$  in the nBA column, respectively. There was no dechlorination (and thus no bioenhanced dissolution) observed in the pulsed lactate column prior to the two flow interruptions/flow reductions. After the second flow interruption (PV 30) and flow reduction (seepage velocity  $0.15 \text{ m d}^{-1}$ ), the enhancement factor increased in all three columns. By the end of the biotic column experiment, the maximum and cumulative enhancement factors reached final values of  $8.68 \mu\text{M}/\mu\text{M}$  and  $3.69 \mu\text{mol}/\mu\text{mol}$ , respectively, in the continuous lactate column;

11.50  $\mu\text{M}/\mu\text{M}$  and 3.02  $\mu\text{mol}/\mu\text{mol}$  respectively, in the nBA column; and 4.69  $\mu\text{M}/\mu\text{M}$  and 1.77  $\mu\text{mol}/\mu\text{mol}$ , respectively, in the continuous lactate column.

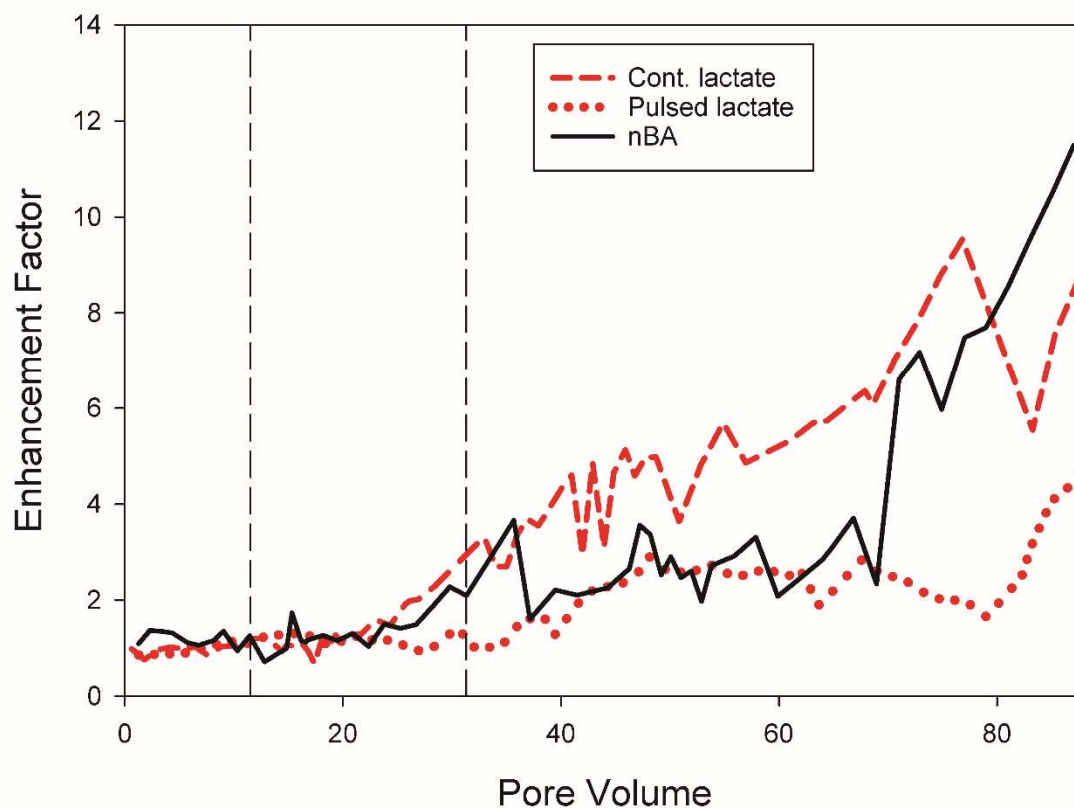


**Figure 4.5.** Maximum enhanced dissolution factors versus pore volume for the different electron donor treatments. Flow interruptions (each followed by a subsequent reduction in flow rate) are indicated by dashed vertical lines.

In the nBA column, enhancement factors were also impacted by the nBA pulses. A small impact on dissolution enhancement was observed in the nBA column after the 1st pulse of nBA provided to that column (PV 3.6); the dissolution enhancement factor increased from 1.09 to 1.34 in the samples collected prior to and just after the pulse. The second pulse of nBA coincided with the second flow interruption/flow reduction; the samples collected just prior to and after the nBA pulse/flow interruption revealed that the dissolution enhancement factor increased by 98%

(from 1.85 to 3.66), possibly due to both the flow interruption and the nBA pulse. The impacts from nBA pulses persisted beyond just the initial samples collected following the pulse: Maximum dissolution enhancement increased by 61% (from 2.27 to 3.66) and 68% (from 1.97 to 3.31) relative to pre-interruption values in the 5 PVs following the second and third nBA pulses, respectively (PVs 29.8 and 53.8). After the last nBA pulse (PV 71), dissolution enhancement increased rapidly from 4.5 to 7, fell by 15% for 2 PVs, and then continued to increase sharply for the remainder of the experiment. From PV 36-71, the dissolution enhancement factor in the nBA column ( $2.76 \pm 1.15$ ) was 43% lower than the continuous-supply lactate column value measured during this interval ( $4.86 \pm 1.76$ ), before recovering and then outpacing the continuous lactate column from PV 80 onward. The pulsed lactate column exhibited some enhanced PCE dissolution, with an average maximum enhancement factor of 2, but dissolution was generally lower than in the other columns, except between PVs 45-64 when the average enhanced dissolution factor ( $2.53 \pm 0.26$ ) was similar to what was observed in the nBA column ( $2.57 \pm 0.86$ ) during this time. The dissolution enhancement factors calculated for each column were averaged across ca. 10 PV intervals and are presented in Table A1.

#### **4.5 Implications for Bioremediation**

The results presented here demonstrate that use of nBA as a PED when supplied in pulsed inputs can result in similar or superior dechlorination and bioenhanced dissolution compared with continuously-supplied or pulsed additions of lactate for treatment of a PCE-NAPL source zone. The utility of nBA partitioning into the NAPL phase was illustrated by the superior dissolution observed under pulsed injections of nBA compared to pulsed injections of lactate: 71% more dissolution was observed in the pulsed nBA column relative to the pulsed lactate column at the end of the experiment. Additionally, at the end of the experiments, the maximum dissolution enhancement factor was 3.2 times higher in the nBA column and 44 times

more VC + ethene were produced (as a fraction of total VOCs detected) in the nBA column compared to the pulsed lactate column. The nBA column also produced 10% more VC and ethene over the course of the experiment and 275% more ethene relative to the continuous lactate column during the last 10 PVs of the experiment, suggesting that nBA is a fortuitous electron donor to support *Dhc* and therefore, resulted in a superior product distribution relative to lactate. Based solely on bioenhanced dissolution or chlorine number, dechlorination performance was comparable between the pulsed nBA and continuously-supplied lactate columns; additionally, the total molar amounts of nBA (36 mmol) and lactate (40 mmol) used over the course of these experiments were similar. These chemicals are also comparably priced (nBA: \$0.84-\$0.89/lb (ICIS, 2020); sodium lactate: \$1.10/lb (Terra Systems, 2020)). Therefore, there is approximate price parity between nBA and continuous sodium lactate as amendments to support biostimulation of NAPL-phase PCE. Although continuous injection of lactate is an option for source zone bioremediation (Stroo et al., 2014) the possibility of biofouling is increased when amendments are continuously supplied; biofouling is a common problem associated with biostimulation via well injection (Chopra et al., 2005; Simpkin and Norris, 2010). Pulsed additions of amendments such as electron donors has been recommended as an engineering solution to curtail injection well fouling (Faris and Vlassopoulos, 2003; Simpkin and Norris, 2010). Additionally, pulsed amendments of nBA offer a promising treatment alternative at NAPL-containing sites where other more costly (e.g., emulsified zero valent iron [ZVI]) or hazardous (chemical oxidants) amendments might otherwise be used, or where high-pressure injection of slurries (ZVI with carbon substrate) is not desirable. The use of nBA to stimulate MRD would be ideal for source zone remediation due to PED partitioning into chlorinated solvent DNAPL and at sites that are situated near sensitive receptors such as surface waters or

subsurface utilities (e.g., storm sewers) since nBA is a food-grade additive and fully water-soluble, in contrast to emulsified oils, which are persistent and can lead to adverse impacts from hydrophobic properties of the oil (Stroo et al., 2014). Additionally, the use of nBA to remediate dissolved-phase chlorinated solvent plumes is another potential application in place of a pulsed lactate addition due to the higher number of reducing equivalents per mole of nBA compared to lactate.

nBA exhibited low, but non-negligible partitioning (3.1-5.5% of added mass) into soils with organic matter (OM) contents ranging from 0.7-2% to in a batch study (unpublished data). Since dissolved phase chlorinated solvents emanating from residual DNAPL would also be expected to be sorbed to soils in areas containing residual DNAPL, it is possible that the use of a PED such as nBA could promote enhanced mass removal in locations containing organic carbon. Further characterization of microbial reductive dechlorination using a PED such as nBA in high-organic carbon, low permeability media with sorbed contaminant mass is warranted. While lactate soil partitioning was not investigated in the aforementioned batch study, the octanol-water partitioning coefficient ( $\log K_{ow}$ ) for lactate (-0.72; Hansch et al., 1995) is substantially lower than that of nBA (1.78, Hansch et al., 1995), therefore soil OM partitioning would be expected to be negligible for lactate.

*In situ* bioremediation occurs in the groundwater matrix where competition for electron donor by non-dechlorinating microbes such as methanogens would be expected to occur; however, it is known that dechlorinating microbes such as *Dhc* have a competitive advantage over methanogens at low hydrogen partial pressures (Fennell et al., 1997; Smatlak et al., 1996). In solution, nBA hydrolyzes to form acetic acid and butanol; the fermentation of these products expected to proceed more slowly than lactate fermentation and result in lower hydrogen partial

pressures (Aulenta et al., 2007; Fennell et al., 1997; Schink, 2002). Future work should focus on dechlorination performance observed between PEDs and soluble donors in a more realistic field setting and in the presence of competitor bacteria. Despite limited field investigations, field-scale bioremediation of a source zone contaminated with TCE DNAPL and biostimulated with nBA has been documented (Bartlett, 2016). The results of the present study illustrate that the PED nBA performs comparably or even favorably in terms of dechlorination performance, bioenhanced dissolution, and source zone longevity compared to a conventional soluble electron donor.

The PED approach offers a cost-effective alternative to currently employed biostimulation techniques, and may be particularly fortuitous to support *Dhc* activity based on the increased production of VC and ethene production measured in the nBA column relative to the lactate columns towards the end of the biotic column experiment. Through this targeted delivery approach, the potential for bioenhanced dissolution is increased due to the simultaneous availability of sustained electron donor (i.e., PED and associated byproducts) and electron acceptor (i.e., chlorinated solvent) at the DNAPL:water interface, which is likely to promote the growth of dechlorinating biomass in close proximity to the DNAPL source zone.

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## 5. Impacts of anaerobic soil microbiological processes on nanoparticle fate and transport: Potential for microbially-mediated transformations of oleic acid-coated IONPs

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### 5.1 Abstract

Iron oxide nanoparticles (IONPs) are an important class of engineered nanoparticles (ENP) with numerous applications entailing the potential for both unintentional and intentional release into subsurface environments. While there is limited evidence that IONPs may be degraded by iron-reducing bacteria (IRB), the susceptibility to iron reduction of surface-stabilized IONPs by IRB- or by exposure to sulfide is unknown. Additionally, the potential for biodegradation of ENP surface coatings is largely unknown. In this study, the susceptibility of oleic acid-coated IONPs to iron reduction by the IRB *Geobacter sulfurreducens* was quantified, and the potential for sulfide-mediated reduction and biodegradation of OA-coated IONPs was qualitatively examined. The presence of the OA coating reduced the susceptibility of the IONPs to IRB-mediated iron reduction by up to 70%. OA-coated IONPs also appeared to be impacted by exposure to sulfide and subject to biodegradation by aerobic bacteria, however more rigorous investigations are required to quantify and further characterize these impacts.

### 5.2 Introduction

Iron oxide nanoparticles (IONPs) are an important class of engineered nanomaterials (ENMs) with numerous applications ranging from consumer products (e.g., coatings/paints for automotive, construction, and industrial coatings; in plastics and rubber materials and as print inks, in electronics, batteries, sporting goods and cosmetics, etc.; Ali et al., 2016; Campos et al., 2015; Moahapatra and Anand, 2010; Zhang et al., 2015), biomedicine (Arias et al., 2018; Boyer et al., 2010; Ling et al., 2015), contaminant remediation and wastewater treatment (Fan et al.,

2011; Yavuz et al., 2006; Yean et al., 2005), chemical/biological sensing (Jiang et al., 2018), and as contrast agents for subsurface remote sensing (Bagaria et al., 2013; Li et al., 2014; Park et al., 2012; Ryoo et al., 2012), among others. Environmental release of ENMs in general can theoretically occur during production, manufacturing, use and disposal, although sludge application, wastewater, and waste incineration are likely to be the dominant pathways for unintentional release of ENMs (Gottschalk et al., 2011).

IONPs may be released specifically into the subsurface through either unintentional or intentional pathways, depending on their particular application. Possible unintentional releases of IONPs into the subsurface could occur via the disposal of IONP-containing consumer products that would subsequently enter solid and/or liquid waste streams, point releases from exterior IONP-containing paints/coatings, application of municipal sludge to agricultural land, landfill leachate and the use of IONPs in wastewater treatment. Applications entailing intentional subsurface releases of IONPs include environmental remediation, the application of IONPs as chemical and biological sensors and as subsurface contrast agents. The intended application of a particular class of IONP should determine the degree to which transport and/or degradation of the IONPs is desirable upon entry into the subsurface environment. For example, transport with little to no degradation or surface passivation would be ideal for IONPs used as subsurface contrast agents, while transport into contaminant zones with subsequent reactivity would be required for IONP remediation applications. On the other hand, little to no transport and high degradability would be desirable for IONPs released into the subsurface unintentionally, such as through landfill leachate or via municipal sludge application.

IONPs released or injected into the subsurface are likely to encounter a variety of subsurface microorganisms including metal-reducing or sulfate-reducing bacteria, as well as

generalist bacteria that could potentially degrade certain coating materials added to IONP surfaces as stabilizing agents. Therefore, it is important to characterize the degree to which IONPs are susceptible to microbially-mediated metal reduction and/or degradation of surface coating materials. Information on the potential for transformations of IONPs by dissimilatory iron-reducing bacteria (DIRB) is scant; however, limited studies have observed iron reduction of hematite nanoparticles (Bose et al., 2009; Yan et al., 2008). The size and morphology of IONPs, along with bioreduction mechanisms (such as electron transfer mechanisms) used by a given bacterial species are likely to influence the observed reduction rate. Yan et al., (2008) investigated Fe-reduction rates of different-sized hematite NPs by the DIRB *Geobacter sulfurreducens* and found that smaller particles (10-30 nm) had higher Fe(III) reduction rates than larger (50 nm) particles, but that bacteria-particle contact area was the most important determinant of reduction rate than particle surface area.

Previous studies of reduction of IONPs by iron-reducing bacteria have considered only uncoated IONPs, which makes the prospect for iron-reduction of surface-coated IONPs purely speculative. Similarly, while the chemical reduction of magnetite by dissolved sulfide is well known (e.g., Kostka and Nealson, 1995; Shah et al., 2014), the potential for sulfide to reduce coated magnetite particles has not been reported. Sulfidation of iron oxides can also have benefits depending on the intended use of the resultant products, for instance in contaminant degradation (Du et al., 2016a; b; Guo et al., 2010). Kostka and Nealson (1995) observed bacterial reduction rates of magnetite by *Shewanella putrefaciens* to be of the same order-of-magnitude as those estimated for reduction by sulfide.

Surface coatings are commonly applied to ENPs to increase stability and enhance transport; particle surface characteristics are likely to control the susceptibility of ENPs to

microbial transformations. Production of oil-soluble IONPs is an effective way to obtain electrostatically-stabilized monodisperse IONPs, and oleic acid (OA) is one of the most common organic compounds used to produce oil-soluble IONPs due to its ability to produce highly uniform IONPs with high saturation magnetization values (Wu et al., 2015). Little is known about whether OA-coated IONPs are recalcitrant to microbial iron or sulfide reduction, or about the susceptibility of different ENP surface coating materials to biodegradation (Cornelis et al., 2014).

To address these knowledge gaps, a series of batch reactor experiments were performed to (1) quantify the susceptibility of OA-coated IONPs to iron reduction by a model DIRB, *Geobacter sulfurreducens*, (2) determine the potential for reduction of OA-coated IONPs by sulfide and (3) evaluate whether exposure to ambient, airborne bacteria can lead to degradation of OA-coating materials. To achieve research objective No. 3, a concentrated stock solution of OA-IONPs was exposed to ambient aerobic environmental conditions and evaluated for susceptibility to biodegradation. The research presented in this study will increase the understanding of the environmental fate of IONPs, an important but under-studied class of IONPs, and help to inform IONP particle design for IONP applications in which *in situ* subsurface transport is desirable.

## **5.3. Materials and Methods**

### **5.3.1 Chemicals**

Sodium sulfate, sodium lactate (60% w/w), sodium acetate, sodium fumarate dibasic, and resazurin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and added to the reduced mineral salts medium as described later. Sodium sulfide nonahydrate (Sigma-Aldrich) was added

to some reactors as described later. Ferric citrate (Sigma-Aldrich) was used as an electron acceptor to maintain iron-reducing bacteria inocula. All chemicals used in sample preparation and calibration were of reagent grade or higher purity, and all solutions were prepared using 18.2 MΩ/cm deionized water (EMD Millipore; Billerica, MA). As needed, experimental equipment was sterilized either via autoclaving or using >70% isopropyl alcohol (Sigma-Aldrich). Hydrochloric acid (HCl; Certified ACS Plus, Fisher Chemical) was used for iron digestion. Ammonium acetate, ammonium hydroxide, hydroxylamine hydrochloride (Sigma-Aldrich), and FerroZine™ iron reagent (Aldrich Chemical, Milwaukee WI) were used during UV-spectrophotometric analysis of ferrous and total dissolved iron. Iron(II) chloride tetrahydrate (Strem Chemicals, Inc., Newburyport, MA) was used to prepare ferrous and total iron standards.

### 5.3.2 Engineered Nanoparticles

IONPs utilized in the experiments consist of a magnetite (Fe<sub>3</sub>O<sub>4</sub>) core coated with an oleic acid bilayer (Li et al., 2014), which is an anionic surface coating under circum-neutral pH conditions. The oleic acid bilayer is homogenous, consisting of a hydrophilic headgroup (carboxylic acid functionality) which renders the nanoparticles water-stable (Li et al., 2014). Magnetite NPs have been suggested for use in a range of applications and are thus environmentally relevant (Laurent et al., 2008; Li et al., 2014; Roca et al., 2006; Sun and Zeng, 2002; Yavuz et al., 2006; Zhou et al., 2015). The IONPs have a nominal diameter of 11 nm in neutral MilliQ water.

### 5.3.3 Inocula

Studies on the interactions between metal-reducing bacteria and ENPs were performed using *G. sulfurreducens*, a dissimilatory sulfur- and metal-reducing  $\delta$ -*Proteobacterium* that is

generally considered a strict anaerobe (e.g., Caccavo et al., 1994), although it has been observed to use O<sub>2</sub> as an electron acceptor at low O<sub>2</sub> concentrations (Lin et al., 2004). The *G. sulfurreducens* growth medium described in Sections 3.3.2 and 4.3.2 and in Löffler et al. (2005) was used in these experiments and in reactors used to maintain the inoculum, in which case it was amended with 5 mM ferric citrate and 5 mM sodium acetate, and periodically amended with 20-50 mL H<sub>2</sub>.

### 5.3.4 Experimental design

#### *Metal reduction of OA-coated IONPs by a DIRB*

In this experiment, the potential for metal reduction of OA-coated IONPs was examined using the DIRB *G. sulfurreducens* in a series of biotic batch reactors. Batch reactors consisted of 160 mL serum bottles (Wheaton; Millville, NJ) containing 50 mL of reduced mineral salts medium (Löffler et al., 2005) amended with sodium acetate (5 mM) as the electron donor and carbon source and (for some reactors) sodium fumarate (5 mM) as the electron acceptor, respectively. Reactors were sealed with butyl rubber stoppers (Chemglass Life Sciences, Vineland, NJ) and crimped with an aluminum crimp cap (Chemglass Life Sciences) and then autoclaved prior to addition of DIRB, IONPs, or both according to the experimental matrices presented in Tables 5.1 and 5.2. The batches were maintained in the dark at ambient temperature (22-25°C). Each experimental treatment and abiotic control was repeated in triplicate to assess reproducibility of results, while biotic positive controls containing only medium and bacteria were prepared in duplicate. Experimental reactors were inoculated with 0.5 mL of *G. sulfurreducens* inoculum and provided with IONPs at a concentration of 5 mg L<sup>-1</sup> as Fe, with additional reactors containing either only *G. sulfurreducens* or only IONPs completed as biotic

and abiotic controls, respectively. Biotic positive controls were amended with ferric citrate as a reducible form of iron in order to verify that iron reduction was achievable and could be measured using the experimental set-up. Additionally, biotic positive control reactors containing uncoated magnetite particles were constructed to determine the potential for iron reduction of the magnetite used as the core material for the OA-coated IONPs.

During the course of the experiments, concentrations of ferrous iron and volatile fatty acids were measured and analyzed as described below to monitor iron reduction and microbial growth/activity. In some experiments, average particle size and zeta potential of the IONPs and bacteria were also measured using a Dynamic Light Scattering (DLS) particle analyzer (Zetasizer® Nano, Malvern Panalytical, Westborough, MA) as described later. All reactors were sampled at the same time. Two-sample t-tests were used to verify whether measured differences in DLS-derived IONP size and zeta potential were statistically significant between batch reactor treatments. Prior to running the t-tests, F-tests were performed on each variable tested. When the results of the F-tests indicated that variance was not significantly different (for  $\alpha = 0.01$ ), variances were pooled for the t-test calculations. Statistical analyses were performed using R statistical software version 3.2.2 (R Core Team, 2017).

**Table 5.1.** Experimental matrix design for anaerobic batch reactor experiment 1

| ID     | Objective                                                                                                                                                                                                      | IONP    | DIRB    | e-acceptor (fumarate) | n |
|--------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|---------|-----------------------|---|
| EXP-F  | Determine whether <i>Geobacter</i> can reduce OA-coated IONPs in the presence of an external electron (e-) acceptor (fumarate); determine effects of <i>Geobacter</i> on size and zeta potential (ZP) of IONPs | present | present | present               | 3 |
| EXP-NF | Determine whether <i>Geobacter</i> can reduce OA-coated IONP in the absence of external e-acceptor; determine effects of <i>Geobacter</i> on size and ZP of IONPs                                              | present | present | absent                | 3 |
| BC-F   | Biotic control: confirm Fe reduction of supplied ferric iron can be measured in system (e-acceptor present)                                                                                                    | absent  | present | present               | 3 |
| BC-NF  | Biotic control: confirm no Fe reduction by <i>Geobacter</i> in absence of iron in system (no e- acceptor)                                                                                                      | absent  | present | absent                | 3 |
| AC-F   | Abiotic control: Monitor effects of aging of IONPs in anaerobic medium (with e- acceptor) in absence of bacteria using size and ZP measurements                                                                | present | absent  | present               | 2 |
| AC-NF  | Abiotic control: Monitor effects of aging of IONPs in anaerobic medium (no e- acceptor) in absence of bacteria using size and ZP measurements                                                                  | present | absent  | absent                | 2 |

For reasons described in Section 5.4.1, following the completion of the first *Geobacter*-IONP anaerobic batch reactor experiment, a second anaerobic batch reactor experiment was designed using a reductant-free medium and a modified experimental matrix. The modified anaerobic batch reactor experimental design is shown in Table 5.2.

**Table 5.2.** Revised experimental matrix design for anaerobic batch reactor experiment 2

| ID     | Objective                                                                                         | IONP    | DIRB    | e- acceptor (fumarate) | n |
|--------|---------------------------------------------------------------------------------------------------|---------|---------|------------------------|---|
| EXP    | Determine whether DIRB can reduce Fe in freshly-prepared OA-coated IONPs                          | present | present | present initially      | 3 |
| BC-FC  | Biotic control: Confirm Fe reduction of ferric citrate added to reactors                          | absent  | present | present initially      | 3 |
| BC-MAG | Biotic control: Confirm DIRB can reduce the (uncoated) magnetite core material used to make IONPs | MAG**   | present | present initially      | 3 |
| AC     | Abiotic control: Confirm no reduced Fe detected in absence of bacteria                            | present | none    | present                | 2 |

\*IONPs used in this experimental treatment were fortuitously contaminated by an unknown aerobic bacteria

\*\*In these reactors, sterile, uncoated suspensions of the same magnetite powder used to produce the OA-coated IONPs were added to the reactors instead of the OA-coated IONPs

### *Metal reduction of OA-coated IONPs by exposure to sulfide*

In this preliminary experiment, a set of six sterile, abiotic, reductant-free batch reactors were used to determine the potential for iron reduction of the OA-coated IONPs by exposure to environmentally-relevant sulfide concentrations (Aulenta et al., 2007; Mao et al., 2017). Reactors were constructed as described above for the abiotic reductant-free reactors. Sterile sodium sulfide nonahydrate was added to reactors to achieve sulfide concentrations of 0.4 and 2 mM; a set of sulfide-free control reactors was also set up as a control. Sterile IONPs were added to reactors to achieve IONP concentrations of ca. 5 mg L<sup>-1</sup> as Fe and reactors were operated in duplicate. Since any iron reduced in the presence of sulfide would have instantaneously formed FeS in the presence of sulfide, measurements of dissolved ferrous iron were not attempted and instead, impacts of sulfide exposure on OA-coated IONP stability were assessed over the course of 10 days using mean particle diameter and zeta potential measurements performed using a DLS particle analyzer operated as described below.

### *Susceptibility of OA-coated IONPs to aerobic biodegradation*

A portion of the concentrated OA-IONP stock used in the batch reactor experiments was exposed to ambient conditions (and potential airborne bacteria) and subsequent microbial growth within the stock was confirmed through culturing (not shown).. The contaminated IONP stock suspension contained only OA-coated IONPs, neutral MilliQ water, and trace amounts of residual oleic acid (i.e., no other carbon sources were present in the suspension). Initial IONP concentrations were measured in the stock liquid (via acid digestion and UV-Vis Fe analysis, as described below) upon receipt and after aging for 10 weeks.

### **5.3.5 Sampling and analytical techniques**

Samples were collected from anaerobic batch reactors using sterile plastic syringes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Syringes were pre-filled with sterile nitrogen, which was used to displace the liquid volume removed during sampling and keep the reactors under positive pressure, thus preventing oxygen intrusion. Stoppers were flame-sterilized prior to sampling and reactors were sampled inside a bio-safety hood sanitized with  $\geq 70\%$  isopropanol prior to use. Particle size and zeta potential of batch reactor samples were measured using 1 mL samples placed into 1 cm quartz cuvettes and analyzed using a DLS particle analyzer. DLS operating parameters were as follows: refractive index (RI): 2.420; absorption: 0.001; temperature: 22 °C; dispersant: water; dispersant viscosity was assumed to equal the sample viscosity. For zeta potential measurements, the Smolouchowski model was used with an F value of 1.50. DLS measurements were performed to determine whether stability

and zeta potential of stabilized IONPs were affected by the DIRB or by exposure to environmentally-relevant sulfide levels.

Concentrations of dissolved ferrous iron were determined via ultraviolet–visible (UV-Vis) spectrophotometric analysis using a UV-Vis spectrophotometer (Shimadzu Corp., model UV-1800, Kyoto, Japan) using a modified version of the ferrozine method (Viollier et al., 2000) as follows: 300  $\mu$ L of reagent A (identical to the Ferrozine reagent used in Viollier et al., 2000) was added to a cuvette, followed by 25  $\mu$ L of sample. After swirling the cuvettes and allowing color to develop for 2 minutes, 2.5 mL of MilliQ water was added to the cuvette and the solution was rested for 10 minutes, at which point absorbance at 562 nm was measured to determine ferrous iron concentrations. Next, 200  $\mu$ L of reagent B (similar to the reducing agent used by Viollier et al., 2000 except with a concentration of 1.24 M hydroxylamine hydrochloride in a 1.73 M hydrochloric acid solution) was added to the cuvette and allowed to mix for 10 minutes. Finally, 50  $\mu$ L of reagent C (identical to the buffer solution used in Viollier et al., 2000) was added to the cuvette before re-measuring absorbance at 562 nm to quantify total dissolved iron. Linear calibration curves were generated using a stock solution of iron (II) chloride tetrahydrate at concentrations ranging from 1 to 30 mg/L iron.

Concentrations of volatile fatty acids (VFAs) including acetate, propionate, and fumarate were determined using a Dionex ICS-2000 (Thermo Fisher) ICS-2100 ion chromatography system (IC). The IC was equipped with a Dionex IonPac AS11-HC analytical column (Thermo Fisher) maintained at 35 °C with an anion self-regenerating suppressor operated at 149 mA. The IC was operated with a constant flow rate of 1.0 mL/min and a variable potassium hydroxide eluent concentration that was held at an initial concentration of 1 mM for 25 min, ramped up to 30 mM from 25 to 54 min, then ramped down to 1 mM until the run ended at 56 min. Calibration

standards were prepared by diluting a stock solution containing 50 mM of each VFA in MilliQ water. The method detection limit for VFAs listed above was about 1  $\mu$ M (Marcet et al., 2018).

## 5.4 Results

### 5.4.1 IRB-IONP batch reactors

DLS-derived zeta potentials and dominant (“peak 1”) particle sizes for the reactors recorded over the course of the first anaerobic batch reactor experiment are shown in Figure 5.1. The DLS-derived zeta potentials and dominant (“peak1”) particle sizes of the various experimental treatments were initially similar; however, over the course of the experiment the zeta potentials measured in the batch reactors containing IRB and fumarate increased by  $58 \pm 1\%$ , while the zeta potentials of all other experimental treatments remained lower than that of the anaerobic medium alone (background conditions, shown as a red point in Figure 5.1). The zeta potential of the anaerobic medium was  $27 \pm 2\%$  lower than the fumarate-containing biotic batches (regardless of the presence of IONPs) and was 19-49% higher than all other treatments. Zeta potential values were least negative in fumarate-containing biotic control and experimental reactors, which were significantly ( $\alpha = 0.01$ ) less-negative than all other experimental treatments, except fumarate-containing abiotic controls, which had a temporary increase in zeta potential values in samples collected on day 5. Differences between zeta potentials in biotic control reactors and experimental control reactors without fumarate were not significantly different from each other but were significantly ( $\alpha = 0.01$ ) lower than fumarate-containing biotic reactors and abiotic reactors. Zeta potentials in fumarate-containing reactors were therefore probably representative of cells and not IONPs. Abiotic control reactors had significantly smaller dominant (peak 1) particle sizes ( $499 \pm 91$  nm) compared to all other reactors, which likely

reflected the size of IONP aggregates in the anaerobic medium, while biotic controls (with or without fumarate) and fumarate-containing abiotic controls all had similar average particles sizes ( $698 \pm 92$  nm) that were not significantly different from each other, and likely represented cells in these reactors.

The dominant (“peak 1”) particles in the experimental treatments consistently made up 83-100% of recorded particles in the samples, with the smallest peak 1 diameters observed in the abiotic batches, which contained only IONPs (Figure 5.2). The largest peak 1 diameters were observed in the biotic batches and were generally largest when both IONPs and fumarate were present. These results suggest that the IRB grew when electron acceptor was present and/or that bacteria-NP aggregates may have formed in the reactors. Zeta potential measurements from biotic reactors (regardless of the presence of IONPs) likely reflected zeta potentials of the IRB cells, while measurements from abiotic reactors most likely represented IONP aggregates. Similarly, DLS-derived size measurements in biotic controls, experimental reactors, and abiotic controls likely reflected of the average sizes of cells, cell-IONP aggregates, and IONP aggregates, respectively. While the DLS measurements performed during the first batch reactor experiment gave a picture of how zeta potential and size parameters varied over time, it was determined that these data were not necessary for subsequent IRB-IONP batch reactor experiments and were therefore only collected for the first set of reactors completed.

Over the course of the first anaerobic batch reactor experiment, one of the first observations made was the near-instantaneous formation of a visible black precipitate upon addition of the IONPs to the batch reactors (Fig C1). It was speculated that the sulfide (added to the anaerobic growth medium as a reductant) in the reactors was forming iron sulfide (FeS) with the iron from the IONPs despite the presence of the OA coating around the IONPs. It should be

noted that iron (0.42 mg/L) in the form of iron(II) dichloride tetrahydrate is a minor component of the anaerobic medium and therefore the formation of a small amount of FeS is often observed at the bottom of reactors as the sulfide and iron react, however the FeS present in the IONP reactors was visibly more extensive than what is typically observed in the absence of IONPs. Ferrous iron was never observed in any of the experimental treatments over the course of the first two experiments (not shown); however, FeS formation became more pronounced as the experiments progressed, and it was determined that if iron reduction was occurring in the reactors, it could not have been measured using the dissolved ferrous iron method because the reduced medium used in the reactors contained sulfide in stoichiometric excess of the iron added to the reactors in the form of IONPs. These initial batch reactors contained ~0.2 mM sulfide, but only ~0.09 mM Fe (from IONPs), and when both sulfide and reduced iron are present, FeS forms near-instantaneously, removing ferrous iron from solution and preclude its measurement using a dissolved ferrous iron technique. Additionally, residual Fe from the inoculum (which contained ferric citrate) was added to the reactors during inoculation, further complicating the task of attributing iron measured during the experiment to reduction of the IONPs.

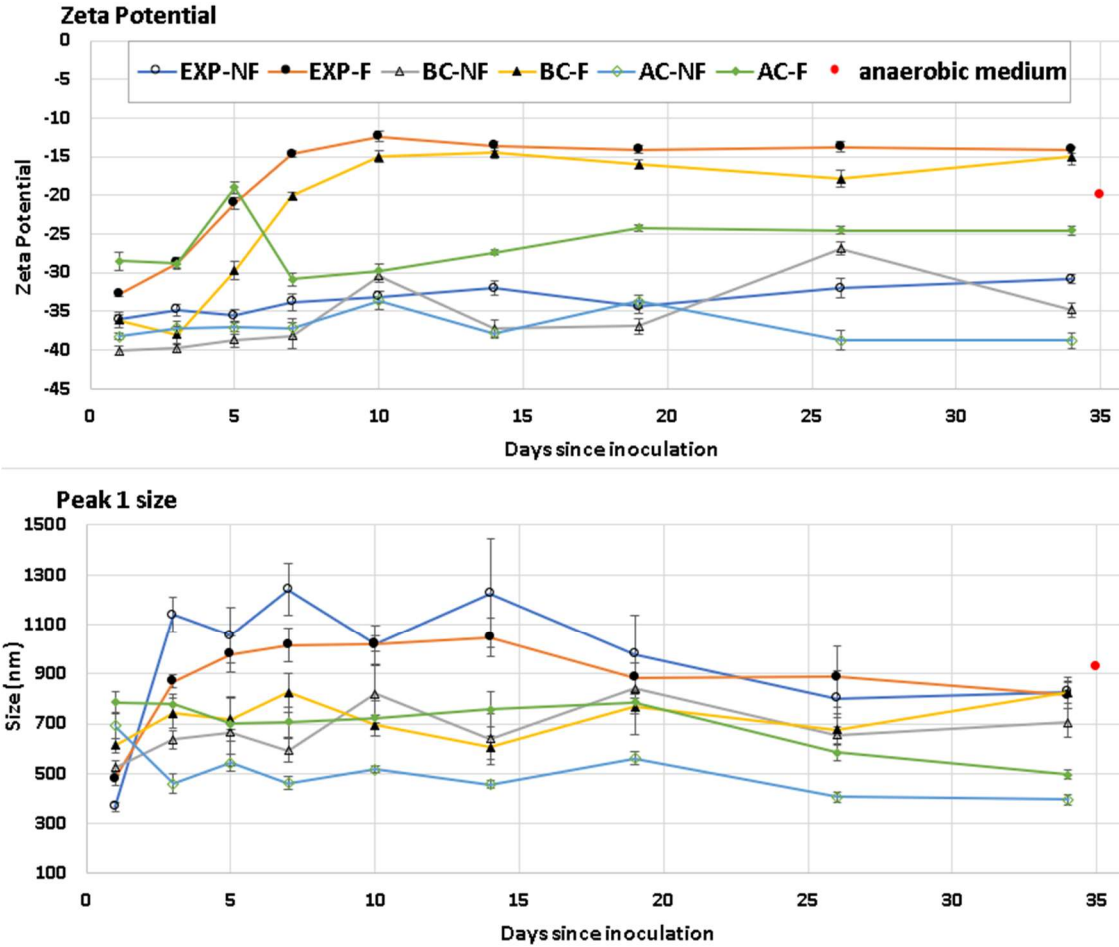


**Figure 5.1.** Zeta potential (top) and dominant peak 1 particle size (bottom) results over the first anaerobic batch reactor experiment. Experimental reactors containing both IONPs and DIRB are denoted “EXP”; biotic and abiotic control reactors are denoted “BC” AND “AC”, respectively; reactors with and without fumarate are denoted by “-F” and “-NF”, respectively. The mean size and zeta potential measured using aerobic medium is indicated as a red dot. Error bars indicate standard deviations across triplicates, or ranges across duplicates (for biotic control reactors)

**Table 5.3** Results\* of paired t-tests for DLS-derived peak 1 zeta potential (shaded cells) and size (unshaded cells) between batch reactor treatments.

|       | EXPNF        | EXPF             | BCNF             | BCF           | ACNF             | ACF              |
|-------|--------------|------------------|------------------|---------------|------------------|------------------|
| EXPNF |              | <b>&lt;0.001</b> | 0.1007           | <b>0.0036</b> | <b>0.0099</b>    | <b>0.0008</b>    |
| EXPF  | 0.1395       |                  | <b>&lt;0.001</b> | 0.004         | <b>&lt;0.001</b> | 0.0173*          |
| BCNF  | 0.0113*      | <b>0.0037</b>    |                  | <b>0.0008</b> | 0.5358           | <b>&lt;0.001</b> |
| BCF   | 0.0216*      | 0.0182*          | 0.2701           |               | <b>0.0012</b>    | 0.2818           |
| ACNF  | <b>0.003</b> | <b>0.0012</b>    | <b>0.0069</b>    | <b>0.0019</b> |                  | <b>&lt;0.001</b> |
| ACF   | 0.0227*      | 0.0259*          | 0.6038           | 0.7475        | <b>&lt;0.001</b> |                  |

\*Values represent *P* values. Bold values and asterisks represent significant differences for at  $\alpha = 0.01$  and  $0.05$ , respectively. Experimental batches containing both bacteria and IONPs are indicated by “EXP”; biotic and abiotic control reactors (containing only bacteria or only IONPs, respectively) are denoted as “BC” and “AC”, respectively; batches containing fumarate as an electron acceptor are indicated as “-F”, while batches without fumarate are denoted as “-NF”.

To solve this problem, another set of anaerobic batch reactors was constructed using a modified low-sulfide medium containing L-cysteine as an alternate reductant, with the rationale that if iron reduction occurred in the reactors, ferrous iron would not be expected to be lost to FeS formation. The amount of IONPs were also doubled in this experiment (~0.18 mM as Fe, equivalent to 10 mg/L) relative to the earlier experiment to ensure that if iron reduction occurred, it would be detectable. The second set of anaerobic reactors all contained fumarate as an initial electron acceptor in order to encourage growth of the IRB, since VFA analyses performed during the first anaerobic experiment (not shown) indicated that all fumarate was removed during the first 10 days of the experiment, but only 25-30% of the electron donor (acetate) was consumed during this time. Esteve-Núñez et al. (2004) showed that *G. sulfurreducens* growth yield is three times higher when fumarate is provided as an electron acceptor compared with ferric iron. This ensured that there would be sufficient biomass to observe iron reduction as well as ample electron donor present after the initial electron acceptor (fumarate) had been depleted. Finally, the *G. sulfurreducens* inoculum was transferred into a ferric citrate-free medium prior to

inoculation, thereby reducing the possibility that ferrous iron detected during the experiment originated from ferric citrate.

Results of reduced (ferrous) iron analyses performed during the second anaerobic batch experiment (not shown) indicated little to no ferrous iron production, although up to ~1.2 mg/L was observed in the biotic IONP-containing batches (day 25) and up to 1 mg/L was present in one of the abiotic control batches (day 43). The method detection limit for Fe is ~0.5 mg/L. Even with the use of L-cysteine as an alternate reductant, evidence of FeS formation was still observed in the low-sulfide, elevated L-cysteine reactors, leading to a final iteration of anaerobic reactors which were made without the addition of any reductant.

#### *Reductant-free medium reactors*

A third set of anaerobic batch reactors were constructed without reductant, using freshly-synthesized, sterile IONPs. This set of batch reactors included three reactors containing DIRB and magnetite powder ( $5 \text{ mg L}^{-1}$  as Fe) of the same type used to produce the coated IONPs, to confirm that *G. sulfurreducens* are capable of iron reduction of the uncoated core material used to make the particles. Results indicated that the DIRB are viable in the reductant-free anaerobic medium, as evidenced by fumarate and acetate consumption in all biotic reactors (not shown) and by reduction of ferric citrate that was added to a set of biotic control reactors (not shown). The DIRB were also capable of completely reducing the uncoated magnetite used in the production of the OA-coated IONPs within 11 days (Figure 5.2). Iron reduction of the OA-IONPs was also observed over the course of this experiment, although only  $29.4 \pm 9.4\%$  of the iron from the IONPs was reduced over the course of 60 days. VFA analyses (not shown) confirmed that electron donor and carbon source (acetate) was still present in all of the reactors

at the end of the experiment, therefore the incomplete Fe-reduction observed in the IONP experimental reactors was not caused by a limitation in available electron donor or carbon source. Concentrations of ferrous iron in abiotic reactors were within the limit of uncertainty associated with the ferrous iron method ( $0.5 \text{ mg L}^{-1}$ ).

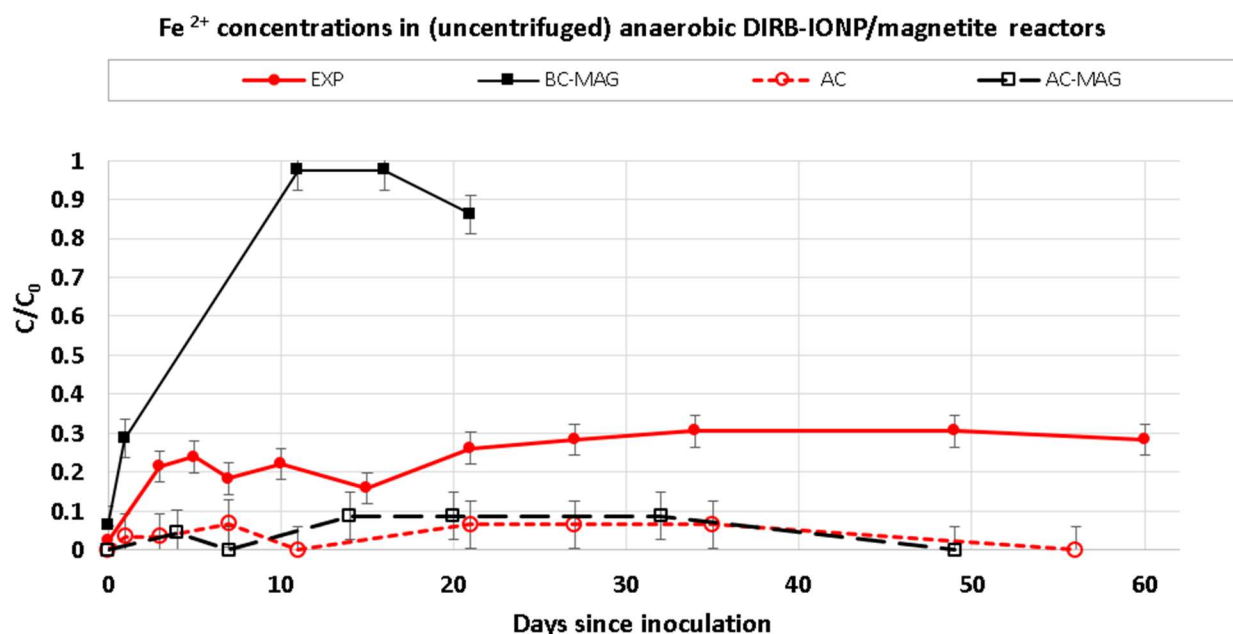


**Figure 5.2.** Ferrous iron observed during the third anaerobic batch reactor experiment. The red line denoted as “Fe addition” shows the day on which IONPs were added to the experimental reactors and ferric citrate was added to the biotic control reactors.

#### 5.4.2 Microbial colonization of exposed nanoparticle stock

The presence of microbial growth in the IONP stock that had been exposed to ambient air demonstrated the susceptibility of the OA-coated IONPs to microbial degradation and allowed for a qualitative evaluation of the effects of bacterial growth on the IONP suspension. The microbially-colonized IONP stock had an initial iron concentration of ca  $130 \text{ mg L}^{-1}$ . Growth of microbial colonies were clearly visible on the surface of the reservoir containing the IONP

suspension after 10 days (Fig C2). After 10 weeks, most of the IONPs had visibly sedimented out of suspension (Fig C2) and the iron concentration of the remaining suspension had decreased by 97%. Concentrations of iron in sterile IONP suspensions measured over the same time period did not change. Since there was no other carbon source present in the IONP stock suspensions, it is likely that the unknown bacteria were using the oleic acid coating material as a carbon source, and that the biodegradation of the OA coating de-stabilized the IONPs, leading to aggregation and sedimentation. These are preliminary observations and a more rigorous, structured approach is required to quantify the potential for biodegradation of OA-coating materials under ambient environmental conditions, however these observations do indicate that this coating material is susceptible to biodegradation, which is unsurprising considering that oleic acid is a commonly-occurring fatty acid.

#### **5.4.3 Exposure of OA-IONPs to sulfide**

DLS-derived measurements of particle size and zeta potential in abiotic reactors exposed to 0, 0.4, or 2 mM sulfide (Figure 5.3) indicate that sulfide exposures up to 2 mM did not substantially impact the zeta potential of the IONPs; although average zeta potentials recorded in the 2 mM reactors ( $-23.8 \pm 6.1$  mV) were somewhat less-negative than in the sulfide-free control reactors ( $-29.8 \pm 1.2$  mV), these were approximately equal to average zeta potentials recorded for 0.4 mM sulfide reactors ( $-29.9 \pm 3.4$  mV). Average particle size increased in both the 2 mM sulfide (from  $1254.3 \pm 94.7$  to  $2935 \pm 96.4$ ) and no-sulfide reactors (from  $131.8 \pm 4.6$  to  $1212 \pm 361$ ) over the course of one week but decreased in the 0.4 mM reactors (from  $881.7 \pm 98.4$  to  $510.8 \pm 15.5$ ). Based on a visual assessment of the reactors (Fig C1) it appears that exposure to sulfide resulted in FeS formation in the reactors, which would likely impact the behavior of IONPs in the subsurface, but the use of DLS-derived measurements did not offer substantial insight into the

nature of these potential impacts, therefore conclusions cannot be made regarding the impact of sulfide exposure on OA-coated IONPs based on these data.

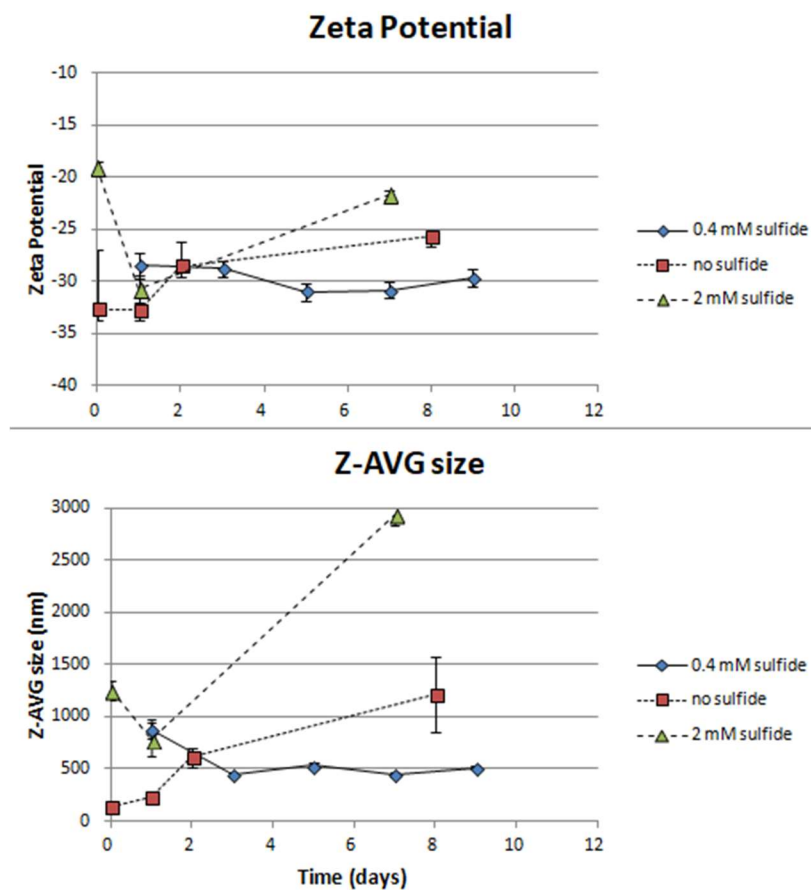


**Figure 5.3.** DLS-derived zeta potential (top) and mean particle size (bottom) results for abiotic sulfide batch reactors

## 5.5 Discussion and Engineering recommendations

In this work, we have demonstrated that oleic acid-coated IONPs are susceptible to microbially-mediated iron reduction, although it appears that the presence of the OA bilayer coating decreases the degree to which the IONPs can be reduced by the DIRB *G. sulfurreducens*. Biodegradation of OA surface coatings has also been qualitatively demonstrated here, but a more

systematic characterization is warranted. Although exposure to sulfide visibly altered OA-coated IONPs even at modest (0.2 mM) concentrations, DLS-derived measurements of particle size and zeta were of limited use in characterizing the potential impacts of sulfide exposure on IONP fate and transport. Sulfide is commonly observed in subsurface environments under reducing conditions and therefore when IONPs are being considered for applications in which subsurface transport is desirable (e.g. as subsurface contrast agents for aquifer characterization), the potential for sulfide reduction and/or surface passivation of IONPs should be explored using appropriate analytical approaches (i.e., X-Ray diffraction, scanning and/or transmission electron microscopy, transport columns, etc.) to confirm that desirable properties of IONPs (e.g. superparamagnetism) are conserved following exposure to sulfide.

Many of the properties of IONPs (saturation magnetization, anisotropy, surface activity, etc.) depend on their size and shape; parameters which are controlled by the mode of IONP synthesis and starting materials used. There are numerous available techniques for the synthesis of IONPs, ranging from relatively simple, common, and cost-effective methods such as co-precipitation to more expensive and/or involved techniques (e.g., sol-gel synthesis, laser pyrolysis). Surface coatings are commonly applied to IONPs to prevent homoaggregation and preserve surface activity and superparamagnetic properties, as well as to functionalize the particles for biomedical use and ensure biocompatibility (Ali et al., 2016; Wu et al., 2015).

Future investigations should evaluate other potential IONP surface-stabilizing agents for their degree of susceptibility to biodegradation and iron reduction of IONPs. IONPs designed for nonreactive *in situ* environmental applications requiring transport through the subsurface (e.g., as subsurface contrast agents) should be designed with a core-shell structure selected to protect core materials from microbially-mediated reduction, both directly by metal-reducing bacteria and

indirectly by reactive byproducts of microbial redox reactions (e.g., sulfide). Core materials should therefore be impervious to potential reductants and should not be composed of materials that could serve as carbon sources for bacteria. If OA-coated IONPs were used in consumer products and subsequently released into the environment through waste-water discharge, landfill leachate, or sludge application, our results suggest that they would readily degrade in the subsurface. Additionally, magnetite is abundant in the subsurface and oleic acid is a non-toxic food-grade substance, therefore ecotoxicity is not likely to be a concern in the case that OA-coated IONPs are released into the subsurface.

ENP surface coatings may be subjected to both biotic and abiotic transformations, including degradation of electrostatically-absorbed ionic species (Cornelis et al., 2014) and covalently-bound materials (Kirschling et al., 2011). Oxidation by biogenic reactive oxygen species (Navarro et al., 2011) or by abiotically-formed oxidants with subsequent dissolution (Auffan et al., 2010; Labille et al., 2010) may reduce particle mobility or possibly enhance it, depending on whether the surface coatings are replaced by other materials such as natural organic matter (Labille et al., 2010). Microbial transformations including metal reduction or biodegradation of ENP coating materials can also occur. These observations underscore the importance of better understanding IONP interactions with biological communities and other ecosystem components that IONPs may encounter in the subsurface environment.

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## 6. Impacts of aerobic soil microbiological processes on nanoparticle fate and transport: transport of IONPs through biofilm-coated sand

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### 6.1 Abstract

Iron oxide nanoparticles (IONPs) are an important class of engineered nanoparticles (ENP) with numerous applications with the potential for both unintentional and intentional release into subsurface environments, where biofilm forming bacteria are commonly found. While the impacts of biofilm on the transport of a variety of ENPs from several biofilm-forming bacterial species have been previously investigated, there have been no studies using IONPs and also no studies of *Pseudomonas fluorescens* biofilm, despite the fact that *P. fluorescens* is a common and environmentally relevant species. Additionally, previous biofilm-ENP transport studies have been conducted at high seepage velocities ( $> 4 \text{ m d}^{-1}$ ) whereas groundwater flow rates are typically well below  $1 \text{ m d}^{-1}$ . In the present study, the transport of oleic acid (OA)-coated IONPs through saturated porous media in the presence of *P. fluorescens* biofilm was investigated using 10 mM NaCl and an aerobic minimal medium (with ionic strengths of either 35 mM or 50 mM) as transport fluids. Column experiments were performed at both ca. 0.4 and  $4 \text{ m d}^{-1}$  seepage velocities, with biofilms formed using either non-recirculating or recirculating growth medium methods. Transport of OA-coated IONPs was inhibited in the presence of *P. fluorescens* biofilm grown at a high seepage velocity ( $5.6 \text{ m d}^{-1}$ ) using non-recirculated growth medium, even at a low ionic strength (10 mM NaCl), and at a seepage velocity of  $4.2 \text{ m d}^{-1}$ . However, some IONP transport was observed when biofilm was grown under a lower seepage velocity ( $0.42 \text{ m d}^{-1}$ ) using recirculated growth medium. The results indicated that *P. fluorescens* biofilm retained greater than 99% of IONP under all conditions tested, demonstrating the potential for biofilms to mitigate the transport of ENP in porous media. In contrast, IONP retention under abiotic

conditions varied between  $21.8 \pm 0.8\%$  (10 mM NaCl) and  $92.2 \pm 1.8\%$  (50 mM aerobic minimal medium) at seepage velocities of ca.  $0.42 \text{ m d}^{-1}$ .

## 6.2 Introduction

Iron oxide nanoparticles (IONPs) are an important class of ENPs because of their numerous applications including potential uses in medicine as both therapeutic and diagnostic agents (Acharya et al., 2017; Menon et al., 2017; Mihai et al., 2017; Shams et al., 2019; Zhao and He, 2010; ) and as subsurface contrast agents (Bagaria et al., 2013; Li et al., 2014; Park et al., 2012). Since IONPs are also used in consumer products (Ali et al., 2016; Campos et al., 2015; Moahapatra and Anand, 2010; Zhang et al., 2015) and show promise in removal of various metals from wastewaters (Lei et al., 2014; Li and Zhang, 2006; Liang et al., 2013a; Lu et al., 2016; Ngomsik et al., 2012; Shipley et al., 2013), a potentially significant route of unintentional entry of IONPs into the terrestrial/subsurface environment is via waste water discharge or application of waste water sludges to agricultural lands (Cornelis et al., 2014; Gottschalk et al., 2009; Johnson et al., 2011; Tong et al., 2007). IONPs may also be intentionally released into the subsurface as contrast agents for subsurface remote sensing because of their superparamagnetic properties (Bagaria et al., 2013; Li et al., 2014; Park et al., 2012; Ryoo et al., 2012) or for contaminant remediation, due to their ability to sorb and/or catalytically degrade a variety of potential subsurface contaminants such as oils, divalent and heavy metals, pesticides, and pharmaceuticals (Araújo et al., 2015; Gutierrez et al., 2017; Jiang et al., 2018; Mohammed et al., 2017).

IONPs released or injected into the subsurface are likely to encounter subsurface bacteria, many of which form biofilms (Cai et al., 2019). For example, IONPs in municipal sludge applied to agricultural lands could encounter saprophytic, biofilm- and biosurfactant-producing bacteria,

many of which play important phytoprotective roles for plants including agricultural species (Couillerot et al., 2009; Ganeshan and Kumar, 2005). Biofilms can impact both physical (roughness, pore size) and chemical (charge, hydrophobicity) properties of porous media (Mitzel et al., 2016; Xiao and Wiesner, 2013; He et al., 2017; Crampon et al., 2018; Peszynska, et al., 2016), thereby altering flow paths, increasing straining potential and available attachment sites, and increasing ENP retention. Although there are no published studies of biofilm impacts on IONP transport through porous media, there have been a number of investigations of biofilm impacts on the transport of other ENPs. Several investigators have reported an increase in ENP retention in the presence of biofilm-coated media relative to clean (uncoated) media (He et al., 2015; 2017; Mitzel et al., 2016, Tong et al., 2010; Tripathi et al., 2012; Xiao and Wiesner, 2013), while others have reported little to no influence of biofilm on ENP transport (Xiao and Wiesner, 2013; Kurlanda-Witek et al., 2015; Lerner et al., 2012), or even decreased retention (Mitzel et al., 2014) of ENPs in the presence of biofilms.

A key controlling ENP transport through biofilm-coated porous media appears to be the approach used to stabilize ENP in solution (i.e., no surface stabilization, electrostatic, steric, or electrosteric stabilization) (Liu et al., 2019; Mitzel et al., 2016; Xiao and Wiesner, 2013). In the presence of biofilms, uncoated and/or electrostatically-stabilized ENPs generally exhibit increased retention in porous media compared to transport through clean (no biofilm) media (Jiang et al., 2013; Mitzel et al., 2016, Tong et al., 2010; Tripathi et al., 2012; Xiao and Wiesner, 2013), while the transport of sterically- or electrosterically-stabilized ENPs is typically unaffected (Lerner et al., 2012; Xiao and Wiesner, 2013, ) or even enhanced (Mitzel and Tufenkji, 2014) in the presence of biofilms. Steric repulsion between polymer-coated ENPs and biopolymers present in biofilms is a stabilizing mechanism that could explain the low retention

of sterically-stabilized ENPs in biofilms (Xiao and Wiesner, 2013). For electrosterically-stabilized ENPs, transport has been observed to either be unimpacted by biofilms (Lerner et al., 2012; Xiao and Wiesner, 2013) or to be enhanced (Mitzel and Tufenkji (2014) relative to transport in uncoated porous media at low ionic strengths. However, transport eletrosterically-stabilized ENPs is reduced as solution ionic strength increases because electrostatic repulsive forces associated with both charged surface moieties anchored to ENP coating materials and biofilm biopolymers are impacted by the ion content of the transport solution (Lerner et al, 2012; Mitzel and Tufenkji, 2014). Several mechanisms have been suggested to explain the increased retention of electrostatically-stabilized and uncoated ENPs observed in the presence of biofilms, including hydrophobic interactions between nanoparticles and biofilm. Xiao and Wiesner (2013) observed positive correlations between ENP retention and the degree of ENP hydrophobicity relative to biofilm hydrophobicity. In contrast, Mitzel et al. (2016) concluded that particle surface hydrophobicity was not a major determinant of overall ENP transport potential but did observe that NP deposition dynamics vary between hydrophilic and hydrophobic biofilms. Specifically, ENP retention was increased to a larger extent as ionic strength increased in the presence of a hydrophilic biofilm compared to under hydrophobic biofilm conditions, suggesting a greater for ionic destabilization of hydrophilic biofilms (Mitzel et al., 2016). Increased retention of uncoated and/or electrostatically-stabilized ENPs in the presence of biofilms has also been associated with surface hydroxylation of fullerol ENPs (Xiao and Wiesner, 2013), hydrogen bonding between charged ENP surface sites and biofilm or other macromolecular biofilm associations such as the ability of large polysaccharides to “collect” small nanoparticles (Espinasse et al., 2007; Xiao and Wiesner, 2013). Tripathi et al. (2012) attributed physical straining to increased retention of 1  $\mu$ M sulfonated polystyrene latex beads in the presence of

biofilm-coated sand when compared to clean quartz sand experiments, and Jiang et al. (2013) suggested pore size constriction contributed to increased retention of zinc oxide (ZnO) in *Escherichia coli*-coated quartz sand-packed columns.

Biofilm type (e.g., bacterial species or strain, gram positive vs gram-negative, etc.) appears to be less important than particle surface chemistry in predicting ENP transport. He et al. (2015) found that transport of graphene-oxide nanoparticles (GONPs) was significantly retarded through sand coated with either gram-positive (*Bacillus subtilis*) or gram-negative (*Pseudomonas putida*) biofilm compared to clean sand when 1-50 mM NaCl was used as the transport fluid. In a related study, He et al. (2017) reported that *B. subtilis* or *P. putida* biofilm-coated sand inhibited GONP transport compared to clean sand when circumneutral 0.1 to 1 mM CaCl<sub>2</sub> was used as the transport solution. Compared to transport observed using NaCl as the electrolyte, BTCs were less similar between the two biofilm types when CaCl<sub>2</sub> was used as the transport solution. Nearly complete retention of GONP was observed at 0.5 and 1 mM CaCl<sub>2</sub> solutions when either type of biofilm was present.

*Pseudomonas aeruginosa* has been the most commonly employed bacterial species in the study of ENP transport through biofilm-coated porous media (Lerner et al., 2012; Mitzel and Tufenkji, 2014; Mitzel et al., 2016; Tripathi et al., 2012; Xiao and Wiesner, 2013). Other biofilm-forming bacteria which have been used to examine ENP transport in the presence of biofilms (and/or biofilm EPS) include *P. putida* (He et al., 2015; 2017), *E. coli* (Tong et al., 2010; Jiang et al., 2013), *B. cereus* (Xiao and Wiesner, 2013), *B. subtilis* (He et al., 2015; 2017), and a naturally-occurring anaerobic biofilm dominated by *P. stutzeri* (83%) and *Exiguobacterium* sp. (6%) (Crampon et al., 2018). *P. fluorescens* is a commonly-occurring and widely-distributed aerobic biofilm- and biosurfactant-forming gram-negative

Gammaproteobacteria that primarily produces the lipopeptide biosurfactant viscosin (Bonnichsen et al., 2015; Neu et al., 1990; Laycock et al., 1991; Raaijmakers et al., 2006), while the better-studied biofilm-forming pseudomonad *P. aeruginosa* produces a class of glycolipid biosurfactants known as rhamnolipids (Déziel et al., 2003; Soberón-Chávez et al., 2005; Varjani and Upasani, 2017). *P. fluorescens* can use a wide variety of carbon sources including glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils and are (Abouseoud et al., 2007; 2008; Desai and Banat, 1997; Laycock et al., 1991) and has a range of known habitats, including soil/rhizosphere, plant tissue and various aquatic environments including wastewaters (Moore et al., 2006; Vasileva-Tonkova et al., 2006). *P. fluorescens* is a commonly known phytoprotectant saprophyte (Sakthivel and Gnanamanickam, 1987; Stutz et al., 1986) and is adapted to survive in low-oxygen environments including the rhizosphere and plant nodules (Pitcher and Watmough, 2004; Moore et al., 2006). Because of the diverse habitats of *P. fluorescens* and its ability to produce extracellular biofilms, *P. fluorescens* represents an environmentally-relevant microbe; however, there have not been previously-published studies of ENPs transport through *P. fluorescens* biofilm-coated porous media.

Electrostatic and uncoated ENPs used in previous biofilm transport investigations include sulfate-modified polystyrene latex NPs (Mitzel et al., 2016; Tripathi et al., 2012), carboxyl-modified polystyrene latex NPs (Mitzel et al., 2016; Tripathi et al., 2012), and carboxyl-modified CdSe/ZnS quantum dots (Tripathi et al., 2012), fullerene (nC60) (Tong et al., 2010; Xiao and Wiesner, 2013), fullerol (Xiao and Wiesner, 2013), graphene-oxide nanoparticles (GONPs) (He et al., 2015; 2017), ZnO (Jiang et al., 2013), and citrate-coated nano-silver (*n*Ag-CIT) (Xiao and Wiesner, 2013). Production of oil-soluble IONPs is an effective way to obtain electrostatically-stabilized monodisperse IONPs, and oleic acid (OA) is one of the most common organic

compounds used to produce oil-soluble IONPs due to both its ability to produce highly uniform IONPs with high saturation magnetization values (Wu et al., 2015), however biofilm impacts on the transport of OA-coated IONPs have not yet been investigated. Two previous investigations of biofilm impacts on ENP transport using particles with similar surface chemistries as OA-coated ENPs (Mitzel et al., 2016; Tripathi et al., 2012) were performed at seepage velocities: ca. 5 to 6 m d<sup>-1</sup> and found that *P. aeruginosa*-derived biofilm increased retention of ENPs in the presence of biofilm. However other factors (particle size; Tripathi et al., 2012) and solution chemistry (Mitzel et al., 2016), exerted larger influences over particle transport than the biofilm itself.

All of the previously-published studies ENP-biofilm transport used relatively high (ca. 4 to >20 m d<sup>-1</sup>) seepage velocities; however, groundwater velocities are commonly under 1 m d<sup>-1</sup> (Kunkel and Wendland, 1997). Braun et al. (2015) found that the retention of surfactant-stabilized *n*Ag through loamy sand increased from 9% to 97% when velocity was decreased from 2.9 to 0.05, m day<sup>-1</sup>, and no transport was achieved through silty loam at a flowrate of ~0.02 m day<sup>-1</sup>. Taghavi et al., (2013) noted that at low transport velocities, single collector contact efficiency can increase, leading to enhanced retention. There have been no studies of the potential impacts of biofilm on the transport of IONPs, nor on any class of ENPs under condition representative of realistic groundwater flow rates (< 1 m d<sup>-1</sup>). Additionally, no prior studies have investigated ENP transport through *P. fluorescens*-derived biofilms despite the fact that this species is commonly detected in numerous subsurface environments.

In this study, column transport experiments were performed to quantify impacts of the common biofilm- and biosurfactant-producing bacteria *P. fluorescens* on OA-coated IONPs transport through saturated porous media under groundwater-relevant seepage velocities (ca. 0.4 m d<sup>-1</sup>). Two sets of transport experiments were performed using clean (acid-washed) and

biofilm-coated acid-washed sand: In experiment 1, a total of seven column experiments were performed using two solutions at circumneutral pH ( $7.1 \pm 0.03$ ): 10 mM NaCl ( $n=4$ ) and an aerobic minimal medium with an ionic strength of 50 mM ( $n=3$ ). One of the biofilm column experiments using 10 mM NaCl as a transport fluid was performed at a higher flow rate ( $4.2 \text{ m d}^{-1}$ ) to facilitate a comparison of our results with previous studies of ENP transport through biofilm-coated porous media and to characterize the effect of flow rate on IONP transport through biofilm-coated porous media. The biofilms used in experiment 1 were grown using non-recirculated aerobic minimal medium amended with  $5 \text{ g L}^{-1}$  as a carbon source supplied to inoculated columns at a seepage velocity of  $5.6 \text{ m d}^{-1}$ . In experiment 2, three column transport experiments were performed using aerobic minimal medium with an ionic strength of 35 mM and a pH of 6.5 as the transport fluid and biofilm growth solution. In this experiment, biofilm was grown under a realistic seepage velocity ( $0.42 \text{ m d}^{-1}$ ) using recirculated medium amended with  $5 \text{ g L}^{-1}$  as a carbon source. All three transport experiments were performed at ca.  $0.42 \text{ m d}^{-1}$  in experiment 2. Previously published studies of ENP transport through biofilm-coated porous media have used relatively high flow rates ( $\geq 4 \text{ m d}^{-1}$ ) during biofilm establishment and have generally not used recirculated medium during biofilm establishment. However, Tong et al., (2010) used a recirculating, carbon-free synthetic mineral salt solution to form *E. coli* biofilm for use in fullerene ( $n\text{C}_{60}$ ) transport experiments. By evaluating the differences in ENP transport through biofilms grown under varying conditions, the biofilm-related endpoints between which subsurface ENP transport may be expected to be impacted can be defined.

## 6.3 Materials

### 6.3.1 Chemicals

For the aerobic medium, ammonium sulfate (VWR, West Chester, PA), potassium phosphate monobasic (Sigma Life Science, St. Louis, MO), magnesium sulfate heptahydrate (Fisher Chemical, Fairlawn, NJ), calcium chloride dihydrate (Fisher Chemical) and glycerol (Fisher Chemical) were used. Sodium chloride (NaCl; Sigma Life Science) was used to prepare 10 mM NaCl solution used in transport experiments. Sodium hydroxide (Sigma Life Science) was used to adjust the pH of the aerobic medium and NaCl to  $7.1 \pm 0.05$ . Sodium bromide (NaBr; Fisher Chemical) was used for non-reactive tracer tests. Hydrochloric acid (HCl; Certified ACS Plus, Fisher Chemical) was used for iron digestion. For biosurfactant analyses, chloroform and methylene blue were purchased from Acros Organics (Fisher Scientific, Waltham, MA), borate 10-hydrate was obtained from J.T. Baker (Phillipsburg, N. J.), and rhamnolipid standards were purchased from AGAE Technologies, LLC (Corvallis, OR).

### 6.3.2 Engineered Nanoparticles

IONPs consisting of a magnetite ( $\text{Fe}_3\text{O}_4$ ) core were coated with an oleic acid bilayer as described in Li et al. (2014), which is an anionic surface coating under circum-neutral pH conditions. Magnetite NPs have been suggested for use in a range of applications and are thus environmentally relevant (Laurent et al., 2008; Li et al., 2014; Roca et al., 2006; Sun and Zeng, 2002; Yavuz et al., 2006; Zhou et al., 2015). The oleic acid bilayer is homogenous, consisting of a hydrophilic headgroup (carboxylic acid functionality) which renders the nanoparticles water-stable (Li et al., 2014). The IONPs have a nominal diameter of 11 nm in neutral MilliQ water.

### 6.3.3 Porous Media and Aerobic mineral salts medium

The porous media used in column transport experiments was 80-100 mesh size fraction obtained by sieving F70 Ottawa sand (OS; US Silica Company; Frederick, MD, USA). The sand was acid-washed using the nitric acid washing procedure described in Wang et al. (2008) prior to use. The aerobic medium used in the experiments is a mineral salts medium based on Spizizen, (1958) containing ammonium sulfate ( $1 \text{ g L}^{-1}$ ), potassium phosphate monobasic ( $1.5 \text{ g L}^{-1}$ ), magnesium sulfate heptahydrate ( $0.1 \text{ g L}^{-1}$ ) and calcium chloride dihydrate ( $0.05 \text{ g L}^{-1}$ ). Glycerol ( $5 \text{ g L}^{-1}$ ) was added as a carbon source because it has been shown to support biosurfactant production in *Pseudomonas fluorescens* (Stoimenova et al., 2009).

### 6.3.4 Inocula and culture method

*P. fluorescens* Migula 1895 (ATCC 13525) were kept frozen in glycerol stock solution at  $-80^{\circ}\text{C}$ . Approximately 3 days prior to initiating experiments, 1 mL aliquots of the frozen stock were defrosted and used to streak agar plates containing beef extract, (3g/L), peptone (5g/L), and agar (15 g/L). After 24-36 hrs of incubation, a single CFU from the agar plates was transferred into a nutrient broth containing beef extract (3g/L) and peptone (5 g/L). After 24 hours of incubation, the inoculated broth was ready for use in batch reactors and aerobic columns. For biotic column experiments, 0.5 mL of inoculated nutrient broth was added to 0.5 L of aerobic medium and maintained at 26 deg. C for 12 hrs on a shaker table operated at 200 RPM. The inoculated aerobic medium was then used to directly inoculate biofilm columns as described later. For batch reactor experiments, 0.5 mL of inoculated nutrient broth was added to each batch reactor as described below.

### 6.3.5 Column preparation

Transport experiments were performed in borosilicate glass columns (15 cm long, 2.5 cm diameter; Kimble-Chase; Vineland, NJ) with end caps fitted with 40-mesh nylon screens and a 40  $\mu$ m nylon filter to support the solid phase and promote uniform flow. Columns, end-caps, valves, tubing, sand, transport fluids, and packing tools were all sterilized via autoclaving or with 70% isopropanol prior to packing and saturation. Columns were dry-packed in 1.5 cm lifts under gentle vibration. After packing, columns were purged with CO<sub>2</sub> for 60 mins to facilitate dissolution of entrapped gas and ensure complete wetting of the sand during the subsequent saturation phases. Columns were saturated for ~10 pore volumes (PVs) with either aerobic medium or 10 mM NaCl, depending on the transport experiment. Conservative tracer experiments using 10 mM sodium bromide were performed to confirm the aqueous PV, porosity, and dispersivity of the columns and to confirm the absence of regions of immobile water within the columns. Tracer experiments consisted of a 3 PV pulse of bromide tracer followed by 3 PVs of flushing with NaBr-free transport fluid. For 50 mM aerobic medium transport columns, the bromide tracer consisted of aerobic medium containing 10 mM NaBr with concentrations of other salts reduced such that the overall ionic strength of the bromide -containing medium remained at 50 mM. For 10 mM NaCl transport experiments, the bromide tracer consisted of 10 mM NaBr in neutralized milliQ water. During the tracer experiment, effluent samples were collected continuously using a CF2 fraction collector (Spectrum Chromatography Inc., Houston TX) and monitored for bromide concentrations using an ion chromatograph (IC), operated as described in Section 6.3.8.

### 6.3.6 Biofilm columns

For biofilm columns, inoculation and biofilm formation procedures were performed after the bromide tracer test and prior to the NP transport experiments. Two different biofilm establishment protocols were employed: establishment of biofilm under low-flow, recirculating medium conditions and biofilm formation under high-flow conditions with freshly-supplied growth medium. Biofilm columns were inoculated with *P. fluorescens* inocula using 0.5 L of inoculated aerobic medium supplied to the column at a flow rate of  $0.8 \text{ mL min}^{-1}$  (ca.  $5.6 \text{ m d}^{-1}$ ) for 8 h (ca. 13 PVs), with the orientation of the column reversed after 4 h. Following inoculation, the flow to the column was suspended for 3 h to facilitate microbial attachment to the porous media.

#### *Experiment 1 biofilm*

After the microbial attachment period, fresh 50 mM aerobic medium (pH 7.1) was supplied to the column at a flow rate of  $0.8 \text{ mL min}^{-1}$  (ca.  $5.6 \text{ m d}^{-1}$ ) for 60 h (~100 PVs) to promote biofilm formation. The orientation of the column was reversed every 12 h to facilitate even biofilm growth within the column. At the end of the biofilm formation period, columns were equilibrated for 10 PVs by flushing with selected transport fluid (i.e., glycerol-free aerobic medium or 10 mM NaCl), at a flow rate of  $0.8 \text{ mL min}^{-1}$  for 4 PVs, followed by  $0.25 \text{ mL min}^{-1}$  for 6 PVs, during which time an additional bromide tracer test was performed to determine post-biofilm PV and dispersivity. OA-IONP transport experiments were then performed as described in Section 6.2.7.

#### *Experiment 2 biofilm*

After the microbial attachment period, recirculated 35 mM aerobic medium (pH 6.5) from a 1.6 L reservoir was supplied to the column at a flow rate of  $0.06 \text{ mL min}^{-1}$  (ca.  $0.42 \text{ m d}^{-1}$ ) for 60 h (ca. 8 PVs). The orientation of the column was reversed every 12 h to facilitate even biofilm growth within the column. At the end of the biofilm formation period, columns were equilibrated for 10 PVs by flushing with glycerol-free 35 mM aerobic medium at a FR of  $0.8 \text{ mL min}^{-1}$  for 4 PV, followed by  $0.25 \text{ mL min}^{-1}$  for 6 PVs, during which time an additional bromide tracer test was performed to determine post-biofilm PV and dispersivity. OA-IONP transport experiments were then performed as described below.

### **6.3.7 Transport experiments**

Prior to performing each OA-IONP transport experiment, Dynamic Light Scattering (DLS) measurements were performed to determine particle size and zeta potential of the IONP influent solution as described in Section 6.3.8. A 3 PV pulse of OA-IONPs ( $\sim 5 \text{ mg L}^{-1}$  as Fe) was supplied to the columns followed by 3 PVs of particle-free transport fluid. During the NP pulse, a concentrated OA-IONP stock solution ( $110 \text{ mg L}^{-1}$  as Fe) was supplied to a three-way valve at a flow rate of  $0.003 \text{ mL min}^{-1}$  using a Chemyx syringe pump (Fusion 200, Chemyx, Inc., Stafford, TX) and was mixed with particle-free transport fluid (also plumbed into the three-way valve) supplied using a Harvard Apparatus syringe pump (PHD 2000, Holliston, MA) at a flow rate of  $0.057 \text{ mL min}^{-1}$ , for a total influent flow rate of  $0.06 \text{ mL min}^{-1}$  (ca.  $0.42 \text{ m d}^{-1}$ ). The OA-IONP influent solution was passed through several cm of tubing to facilitate mixing before entering the column.

After the NP pulse, the flow rate of the particle-free transport fluid was increased to 0.06 mL min<sup>-1</sup>. Effluent samples were continuously collected using a fraction collector and were monitored for particle breakthrough as described in Section 6.3.8. An additional biofilm transport experiment was performed at a seepage velocity of ca. 4.2 md<sup>-1</sup>, using the 5.6 m d<sup>-1</sup> non-recirculated medium biofilm formation procedure and 10 mM NaCl (pH 7.12) as the transport fluid. Other than transport fluid velocity, all other parameters used in the high flow rate experiment were identical to those used in the Experiment 1 transport columns.

Abiotic control experiments were performed using the same column materials, NPs, porous media, background solutions, and flow rates (ca. 0.42 md<sup>-1</sup>), and the results from the biotic and abiotic column experiments were compared to assess the effects of biofilm on transport of OA-IONPs. For abiotic columns, the OA-IONP transport experiment was performed following the initial bromide tracer test. After the completion of each transport experiment, columns were dissected into 10 1.5 cm segments and dried at 55 °C for 72 h. Retained IONPs within each column segment were quantified by acid digestion of a known portion of each column segment in a 33% HCl solution followed by UV-Vis analysis. Background iron present in the sand was also measured using clean acid-washed sand. Iron retention profiles were adjusted for background iron present in the porous media. Prior to analysis, samples were sonicated for 2 hrs using a bath sonicator (FS 20, Fisher Scientific). For biofilm columns, the remaining portions of each dried column segment were used to determine biomass distribution in the column. The segments were combined into 5 sections, each representing 3cm column increments, pre-weighed, and then baked at 800 °C for 24 h in a muffle furnace (Isotemp, Fisher Scientific), and post-weighed for biomass determination. Biomass (the difference between pre- and post-furnace exposure) was adjusted for the contribution from clean (i.e., biofilm-free) sand

samples that were subjected to the same bake-out regime, and results were expressed as mg biofilm g<sub>DM</sub><sup>-1</sup> sand, where the subscript DM denotes dry weight.

### 6.3.8 Analytical methods

For the bromide tracer tests, a Dionex IC (ICS-2100, Thermo Fisher) equipped with a Dionex IonPac AS11-HC analytical column (Thermo Fisher) maintained at 35 °C with an anion self-regenerating suppressor operated at 149 mA was used. The IC was operated with a constant flow rate of 1.0 mL/min and a variable potassium hydroxide eluent concentration held at an initial concentration of 1 mM for 25 min, ramped up to 30 mM from 25 to 54 min, then ramped down to 1 mM until the run end at 56 min. The IC was calibrated with the same bromide transport fluid used for the tracer experiment, with bromide standards ranging from 0.2 to 10 mM. Bromide concentrations detected in the column effluent samples were expressed as  $C/C_0$  and used to characterize aqueous pore volume and dispersivity of the columns and to confirm the absence of regions of immobile water in the columns using the Code for Estimating Equilibrium Transport Parameters from Miscible Displacement Experiments (CFITM) (van Genuchten, 1980), a module of the Studio of Analytical Models (STANMOD) program version 2.08.1130 (Šimůnek et al., 1999).

Particle size and zeta potential of IONP-influent solutions were measured using 1 mL samples placed into 1 cm quartz cuvettes and analyzed using a Dynamic Light Scattering (DLS) particle analyzer (Zetasizer® Nano, Malvern Panalytical, Westborough, MA). DLS operating parameters used to analyze OA-IONP stability were as follows: refractive index (RI): 2.420; absorption: 0.001; temperature: 22 °C; dispersant: water; dispersant viscosity was assumed to

equal the sample viscosity. For zeta potential measurements, the Smoluchowski model was used with an F value of 1.50 (Smoluchowski, 1921).

For determination of total dissolved iron, UV-Vis absorbance at 562 nm was recorded using 1 cm polystyrene cuvettes and concentrations were determined using a variation of the spectrophotometric ferrozine method (Stookey 1970; Viollier et al. 2000) as described in Chapter 5. Linear calibration curves for iron were obtained using iron (II) chloride tetrahydrate stock solutions at concentrations ranging from 1-10 mg L<sup>-1</sup>.

## **6.4 Results**

A total of 10 IONP transport columns were completed, including 6 abiotic control columns and 4 *P. fluorescens* biofilm columns. Transport fluid and column parameters (fluid type, seepage velocity, pH, ionic strength, Peclet No., porosity), DLS-derived IONP influent size and zeta potential values, and transport and retention results for Experiments 1 and 2 are summarized in Tables 6.1 and 6.2, respectively.

**Table 6.1** Experiment 1 column operating parameters, DLS results, and IONP transport and retention results for column transport experiments. Column names starting with “B” denote biofilm transport experiments

| name | T. fluid <sup>1</sup> | I <sup>2</sup> (mM) | pH   | FR <sup>3</sup> (m d <sup>-1</sup> ) | Diam. <sup>4</sup> (σ) | Z.P. <sup>5</sup> (σ) | Peclet No. | φ <sup>6</sup> | % BT <sup>7</sup> | % Ret. <sup>8</sup> | Max C/Co <sup>9</sup> | IONP conc. as Fe <sup>10</sup> |
|------|-----------------------|---------------------|------|--------------------------------------|------------------------|-----------------------|------------|----------------|-------------------|---------------------|-----------------------|--------------------------------|
| A50a | AE                    | 50                  | 7.1  | 0.42                                 | 85.3 (2.2)             | -24.6 (0.4)           | 140        | 0.39           | 7.3               | 90.4                | 0.12                  | 4.4                            |
| A50b | AE                    | 50                  | 7.08 | 0.43                                 | 72.2 (3.0)             | -31.6 (1.0)           | 241        | 0.38           | 12.8              | 94                  | 0.22                  | 4.7                            |
| B50  | AE                    | 50                  | 7.12 | 0.42                                 | 70.8 (3.6)             | -35.8 (0.3)           | 186        | 0.37           | 0.2               | 105                 | 0.01                  | 5.7                            |
| N10a | NaCl                  | 10                  | 7.1  | 0.44                                 | 63.8 (1.1)             | -44.3 (3.4)           | 163        | 0.37           | 78                | 22.6                | 0.81                  | 4.5                            |
| N10b | NaCl                  | 10                  | 7.08 | 0.44                                 | 61.3 (0.4)             | -57.1 (2.0)           | 234        | 0.37           | 81.2              | 21                  | 0.94                  | 5.3                            |
| B10a | NaCl                  | 10                  | 7.14 | 0.42                                 | 63.3 (4.9)             | -45.9 (1.9)           | 185        | 0.38           | 0                 | 99.9                | 0                     | 5.1                            |
| B10b | NaCl                  | 10                  | 7.14 | 4.2                                  | 63.7 (0.6)             | -65.6 (3.4)           | 199        | 0.38           | 0                 | 97.7                | 0                     | 4.8                            |

<sup>1</sup>Transport fluid (\*AE denotes aerobic medium). <sup>2</sup>Ionic strength. <sup>3</sup>Seepage velocity. <sup>4</sup>Mean particle diameter. <sup>5</sup> Zeta Potential. <sup>6</sup>Porosity. <sup>7</sup>Percent IONP breakthrough. <sup>8</sup>Percent IONP retained in column. <sup>9</sup>Maximum influent IONP concentration relative to influent concentrations. <sup>10</sup>Influent IONP concentrations (as Fe, mg L<sup>-1</sup>)

**Table 6.2** Experiment 2 column operating parameters, DLS results, and IONP transport and retention results for column transport experiments. Column names starting with “B” denote biofilm transport experiments

| name | T. fluid <sup>1</sup> | I <sup>2</sup> (mM) | pH  | FR <sup>3</sup> (m d <sup>-1</sup> ) | Diam. <sup>4</sup> (σ) | Z.P. <sup>5</sup> (σ) | Peclet No. | φ <sup>6</sup> | % BT <sup>7</sup> | % Ret. <sup>8</sup> | Max C/Co <sup>9</sup> | IONP conc. as Fe <sup>10</sup> |
|------|-----------------------|---------------------|-----|--------------------------------------|------------------------|-----------------------|------------|----------------|-------------------|---------------------|-----------------------|--------------------------------|
| A35a | AE*                   | 35                  | 6.5 | 0.425                                | 69.1 (1.0)             | -44.5 (0.9)           | 386        | 0.37           | 54.7              | 44.9                | 0.73                  | 5.2                            |
| A35b | AE                    | 35                  | 6.5 | 0.42                                 | 66.9 (0.4)             | -36.1 (6.0)           | 507        | 0.38           | 39.3              | 54.8                | 0.63                  | 4.6                            |
| B35  | AE                    | 35                  | 6.5 | 0.42                                 | 68.1 (2.2)             | -39.8 (1.5)           | 263        | 0.39           | 24.4              | 79.8                | 0.46                  | 6.3                            |

<sup>1</sup>Transport fluid (\*AE denotes aerobic medium). <sup>2</sup>Ionic strength. <sup>3</sup>Seepage velocity. <sup>4</sup>Mean particle diameter. <sup>5</sup> Zeta Potential. <sup>6</sup>Porosity. <sup>7</sup>Percent IONP breakthrough. <sup>8</sup>Percent IONP retained in column. <sup>9</sup>Maximum influent IONP concentration relative to influent concentrations. <sup>10</sup>Influent IONP concentrations (as Fe, mg L<sup>-1</sup>)

#### 6.4.1 Experiment 1 results

A total of three IONP-biofilm transport experiments were performed in *P. fluorescens* biofilms grown under seepage velocities ca.  $5.6 \text{ m d}^{-1}$  using non-recirculated aerobic growth medium, with four accompanying abiotic IONP transport experiments performed for comparison. Two abiotic transport column experiments were performed in circum-neutral pH 10 mM NaCl, and another two were run using circum-neutral pH 50 mM aerobic medium as a transport fluid. All abiotic transport experiments were run at seepage velocities ca.  $0.42 \text{ m d}^{-1}$ . One biofilm transport experiment was performed in each of the two transport fluids at seepage velocities  $0.42 \text{ m d}^{-1}$ , while a third biofilm transport experiment was run at a seepage velocity of  $4.2 \text{ m d}^{-1}$  in 10 mM NaCl to allow for comparison to previous studies operated under similar flow and ionic strength conditions.

IONP transport measured in the abiotic columns (Figure 6.1) indicates a strong effect of ionic strength and electrolyte composition on IONP transport, with 6.9 times more transport ( $79.6 \pm 1.6\%$ ) and 76% less retention ( $21.8 \pm 0.8\%$ ) observed in the 10 mM NaCl columns compared to the 50 mM aerobic medium columns ( $10.1 \pm 2.8\%$  and  $92.2 \pm 1.8\%$ , respectively). Zeta potentials and mean particle diameters of IONPs in the abiotic 10 mM NaCl influent ( $-50.7 \pm 6.4 \text{ mV}$  and  $62.6 \pm 1.3 \text{ mV}$ , respectively) indicated that particles were ca. 21% smaller and had ca. 45% lower zeta potentials compared to IONPs in the abiotic 50 mM aerobic medium influent ( $-28.1 \pm 3.5 \text{ mV}$  and  $78.8 \pm 6.6$ , respectively), therefore the increased transport observed in the 10 mM NaCl abiotic columns was not surprising. The rising limbs of the abiotic NaCl BTCs had initially steep slopes followed by a gradual increases to maximum values, and the falling limbs of the NaCl BTCs dropped off steeply following termination of the IONP pulses, suggesting irreversible attachment to the porous media. The BTCs in the abiotic 50 mM aerobic medium columns, on the other hand, exhibited gentler rising limb slopes, indicative of higher

retention capacities. The shape of the abiotic 50 mM aerobic medium BTC was similar in both shape and magnitude to BTCs measured using carboxylate-modified latex ENPs (which have a similar surface chemistry to the OA-IONPs) in a 10 mM  $\text{CaCl}_2$  transport solution (Mitzel et al., 2016) suggesting that the presence of  $\text{Ca}^{2+}$  ions in the transport fluid may have caused Ca-bridging and subsequent retention of the IONPs.

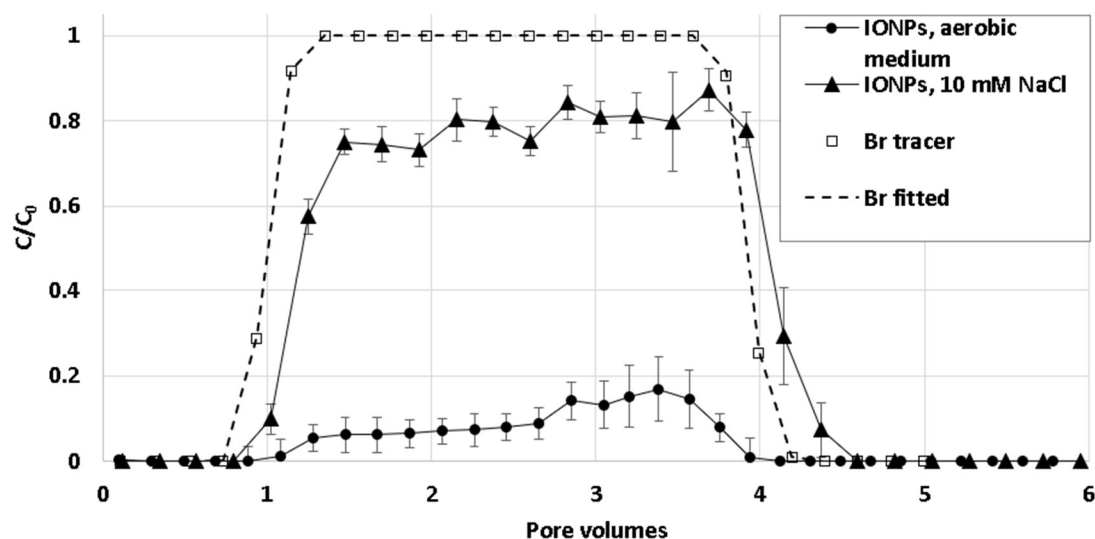


**Figure 6.1.** IONP breakthrough and measured and fitted bromide (Br) tracer results for the abiotic transport experiments run in circumneutral pH 50 mM ionic strength aerobic medium and 10 mM NaCl. Experiments were run at a seepage velocity of  $0.42 \pm 0.01 \text{ m d}^{-1}$ . Error bars represent range of observed values across duplicate columns.

Transport of IONPs was not observed in “high flow” biofilm experiments run at  $0.42 \text{ m d}^{-1}$  (Figure 6.2) in either transport fluid, nor was transport observed for the “high flow” biofilm transport experiment performed at a seepage velocity of  $4.2 \text{ m d}^{-1}$  (Figure 6.3), despite the fact that mean particle diameters and zeta potential values for the IONP-influent suspensions used in the biofilm experiments were very similar to values recorded for the abiotic experiments (Table 6.2). These results are in contrast with previous studies that have observed transport of electrostatically-stabilized ENPs at seepage velocities  $\geq 4 \text{ m d}^{-1}$  using a variety of different

biofilm-forming bacteria (e.g., Mitzel et al., 2016, Tripathi et al., 2012; Xiao and Wiesner, 2013). It has been shown that at low transport velocities, single collector contact efficiency can increase, leading to enhanced retention (Braun et al., 2015; Taghavy et al., 2013). Yet even at a relatively high seepage velocity ( $4 \text{ m d}^{-1}$ ), OA-coated IONPs were completely retained in the presence of *P. fluorescens* biofilm.

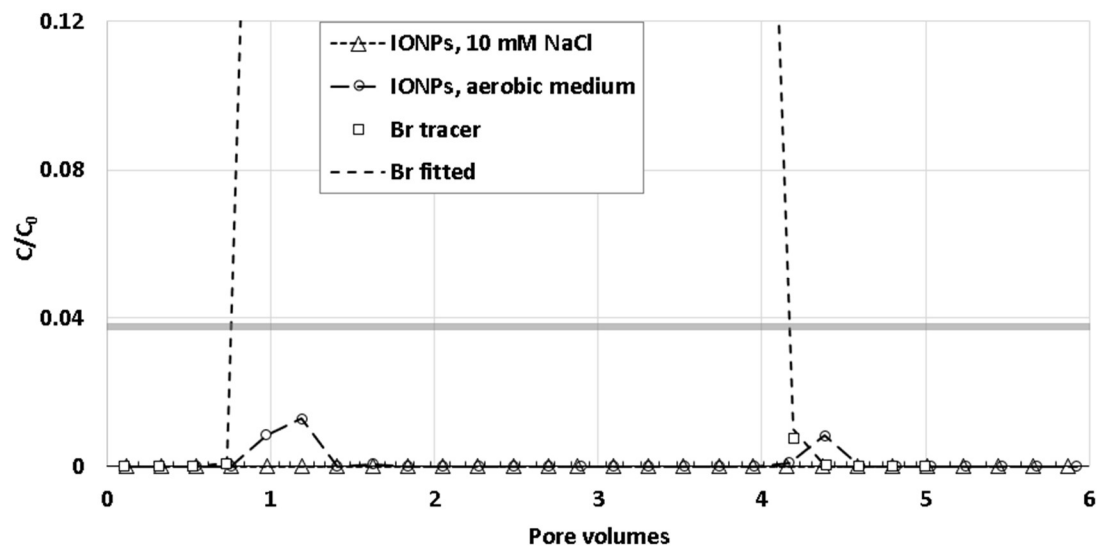


**Figure 6.2.** IONP breakthrough and non-reactive measured and fitted bromide (Br) tracer results for Experiment 1 biofilm transport columns run in circumneutral pH 50 mM ionic strength aerobic medium and 10 mM NaCl. Experiments were run at a seepage velocity of  $0.42 \pm 0.01 \text{ m d}^{-1}$ . Method detection limit is shown as horizontal grey line.

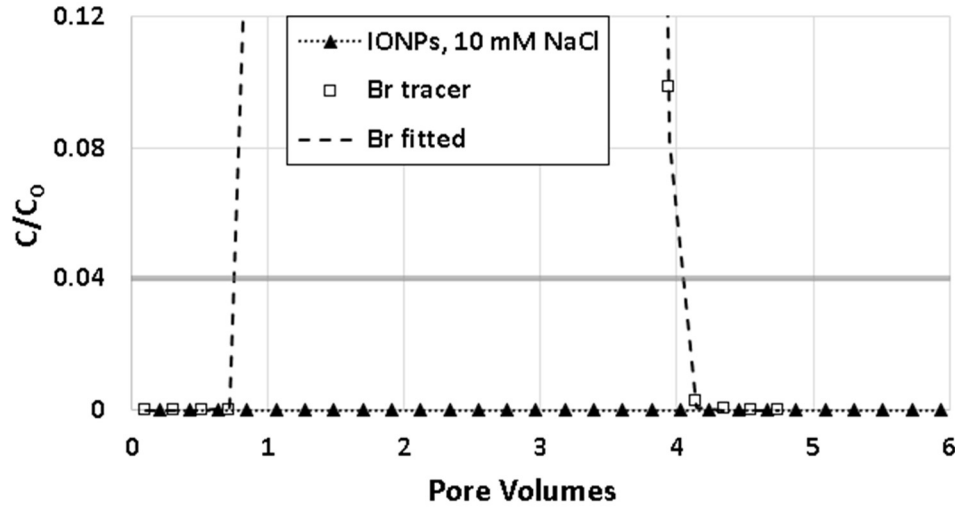


**Figure 6.3.** IONP breakthrough and non-reactive tracer results for the “high flow” biofilm transport experiment run in circumneutral pH 10 mM NaCl at a seepage velocity of 4.2 m d<sup>-1</sup>. Method detection limit is shown as horizontal grey line.

IONP retention data for abiotic columns run using 10 mM NaCl and 50 mM aerobic medium are shown in Figure 6.4. Retention was highest in the inlet-adjacent segments of the aerobic medium columns, while there were no obvious patterns in retention between duplicate abiotic 10 mM NaCl columns, indicating that ionic strength and electrolyte composition impacted the retention of the IONPs. IONP retention data for biofilm columns run using 10 mM NaCl and 50 mM aerobic medium are shown in Figure 6.5. Retention was highest near column inlets for all biofilm columns, with the highest inlet Fe concentrations observed in the biofilm column run in 50 mM aerobic medium, in which all of the measured Fe was detected in the column segment nearest to the inlet. Interestingly, retention profiles for the 10 mM NaCl biofilm column operated at a seepage velocity of 0.42 m d<sup>-1</sup> (“N10a”) indicated that more of the IONPs were transported beyond the inlet-adjacent column segment relative to the 10 mM NaCl biofilm column operated at a seepage velocity of 4.2 m d<sup>-1</sup> (“N10b”). This implies that transport velocity was not a critical determinant of observed transport and retention behavior in the presence of the biofilm. It should be noted that the interpretation and modeling of ENP-biofilm transport can be complicated by the

fact that biofilm coatings on porous media grains may not be uniform, leading to increased heterogeneity of potential collector surfaces (Lerner et al., 2012; Mitzel and Tufenkji, 2014; Xiao and Wiesner, 2013).

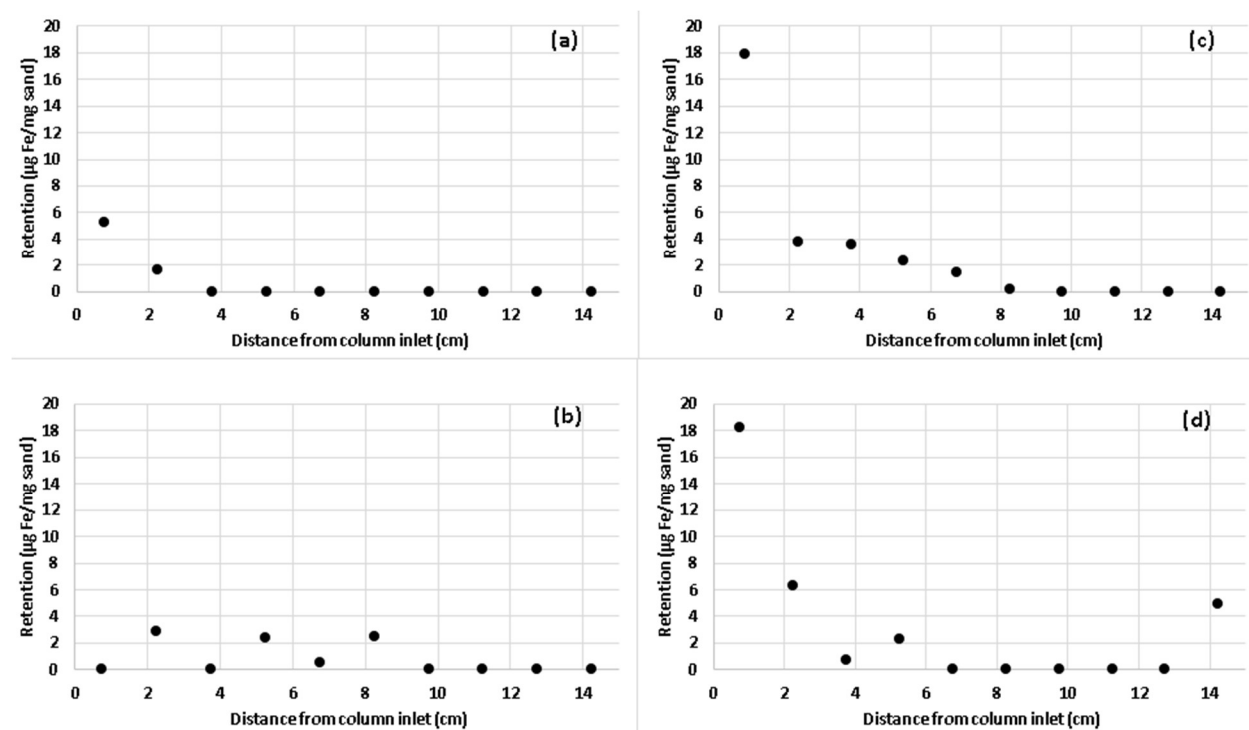


**Figure 6.4.** IONP retention in abiotic 10 mM NaCl (left) columns N10a (a) and N10b (b) and 50 mM aerobic medium (right) columns A50a (c), and A50b (d).

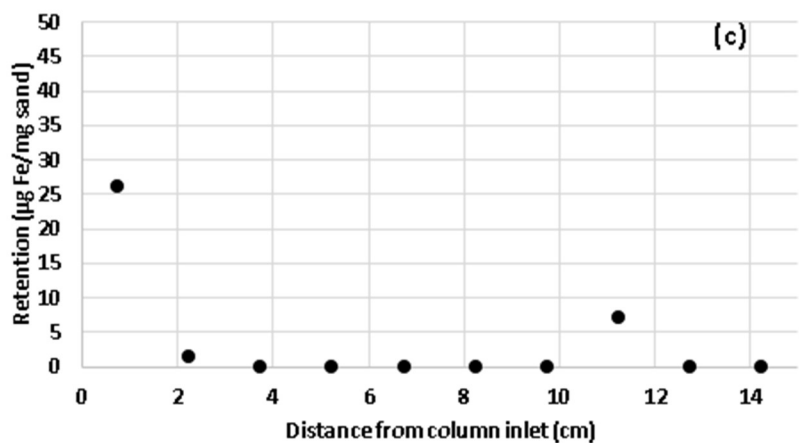
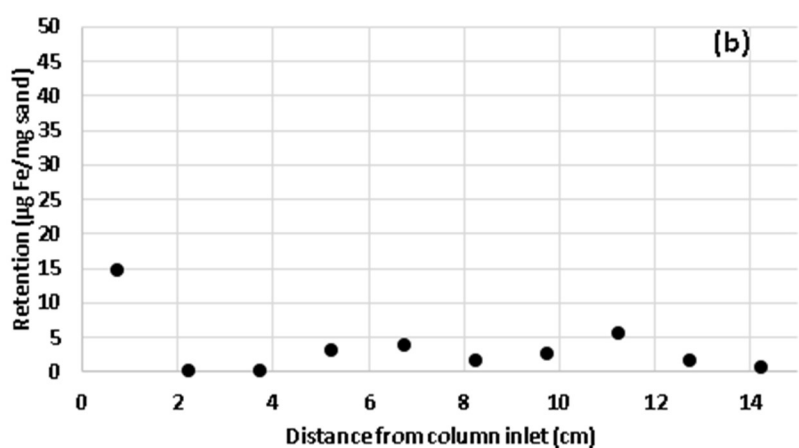
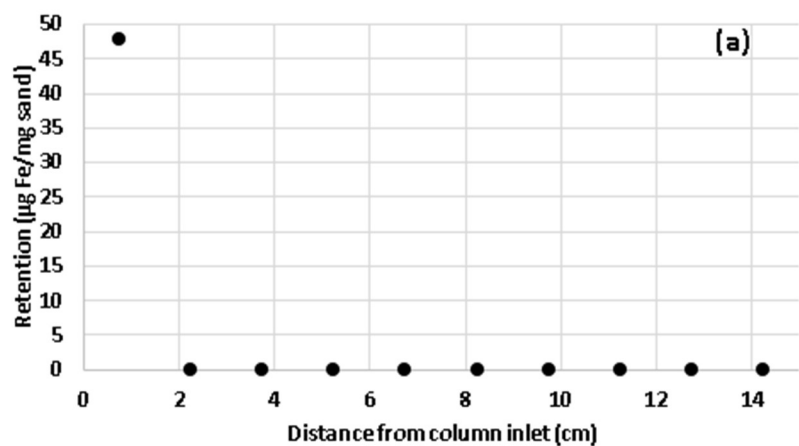


**Figure 6.5.** IONP retention in Experiment 1 biofilm columns performed in 50 mM aerobic medium “B50” (a) 10 mM NaCl at 0.42 m d<sup>-1</sup> “B10a” (b) and 10 mM NaCl run at 4.2 m d<sup>-1</sup> “B10b” (c).

### *Biomass distribution*

Average biomass distribution for the Experiment 1 biofilm columns is shown in Figure 6.6.

Average biomass density in column B10a ( $0.74 \pm 0.1 \mu\text{g G}_{\text{DW}}$ ) was somewhat higher than the mean biomass density in columns B10b ( $0.59 \pm 0.2 \mu\text{g G}_{\text{DW}}$ ) and B50 ( $0.54 \pm 0.1 \mu\text{g G}_{\text{DW}}$ ).

Therefore, the increased IONP retention measured in the inlet-adjacent segment of the aerobic medium biofilm column (B50) was likely a result of the increased ionic strength and presence of multivalent ions in the transport fluid. Compared to the  $0.42 \text{ m d}^{-1}$  NaCl column (“B10a”), IONP retention was slightly more concentrated near the inlet of the  $4.2 \text{ m d}^{-1}$  NaCl column (“B10b”), and since the  $4.2 \text{ m d}^{-1}$  column also had slightly lower average biomass density, the flowrate was therefore not an important determinant of IONP retention in the presence of *P. fluorescens* biofilm. Compared to other biofilm-ENP transport studies reporting biomass density using the  $800^{\circ}\text{C}$  furnace method, our biomass density values are similar to values reported for *P. aeruginosa* biofilm columns (Tripathi et al, 2012; Mitzel et al., 2016). Biomass was relatively evenly distributed throughout the columns, likely due to the periodic reversal of the influent growth medium flow direction during the biofilm formation period. Tripathi et al. (2012) did not reverse flow direction during the biofilm growth phase of their experiment and resultant biomass densities decreased as a function of distance from the column inlet.

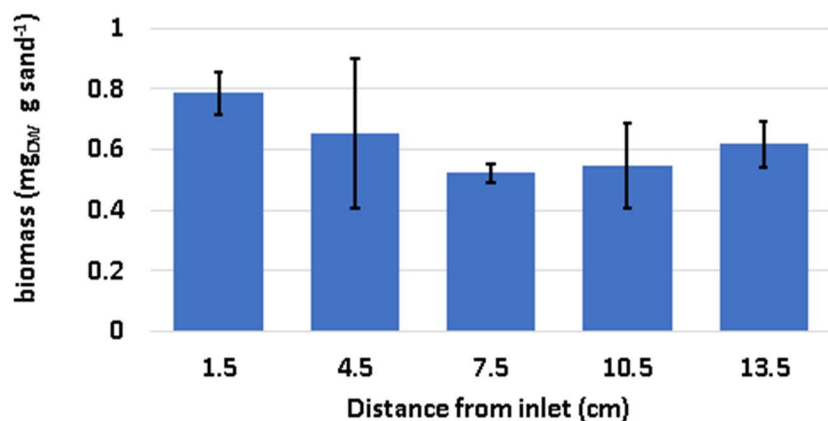


**Figure 6.6.** Average biomass distributions for Experiment 1 biofilm columns. Error bars represent standard deviations across three biofilm columns

#### 6.4.2 Experiment No. 2 transport results

One transport column experiment was performed in the presence of a *P. fluorescens* biofilm grown under realistic seepage velocities ( $0.42 \text{ m d}^{-1}$ ) and using recirculated medium (Figure 6.3). Two abiotic control column transport experiments were performed to elucidate the impacts of the low-flow biofilm on IONP transport. For both abiotic and biofilm transport experiments, the BTCs exhibited concave rising limbs, suggesting a maximum retention capacity of the porous media, and sharply declining falling limbs, indicating irreversible IONP attachment to the sand. Average breakthrough observed in the abiotic columns was  $47\% \pm 15.4\%$ . The presence of biofilm reduced IONP transport by ca. 48%. Average IONP particle diameters and zeta potentials in the 35 mM, pH 6.5 aerobic medium influent solutions used in this set of transport experiments were  $68.0 \pm 1.1 \text{ nm}$  and  $-40.1 \pm 4.2 \text{ mV}$ , indicating that the IONPs were stable and mobile in the transport fluid. Unlike to observed BTC shape in the abiotic 50 mM aerobic medium columns, the BTCs for the 35 mM aerobic medium columns were similar in shape and closer in magnitude to the BTCs observed in the abiotic 10 mM NaCl transport columns. It is possible that calcium was removed from the transport fluid during the de-salting that occurred in the 35 mM aerobic medium columns, eliminating the potential for Ca-bridging

and leading to BTCs with similar shapes (but lower magnitudes) to those observed in the 10 mM NaCl columns. Biomass was not measured in the 35 mM aerobic medium biofilm column, therefore differences in transport and retention observed between Experiment 1 and 2 biofilm columns cannot be attributed definitively to differences in biomass density between the columns.

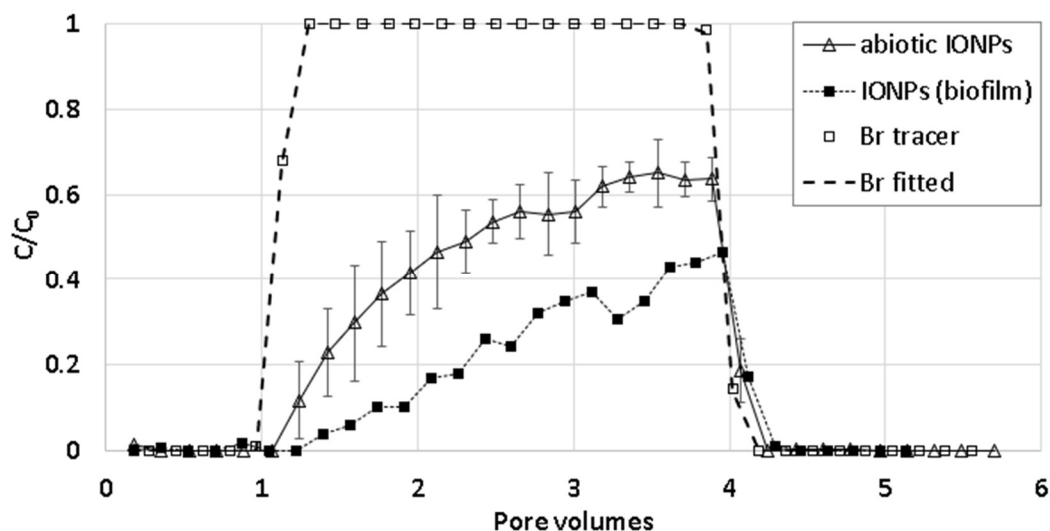


**Figure 6.7.** IONP breakthrough and measured and fitted bromide (Br) tracer results for the transport experiments run in 35 mM ionic strength aerobic medium at pH 6.5 for abiotic and “low-flow,” re-circulated medium biofilm transport columns. Error bars represent range of observed values across duplicate columns (abiotic columns only).

Retention of IONPs (Figure 6.8) was similar in the duplicate abiotic columns ( $49.9 \pm 5.0\%$ ), with ~15% of the IONPs injected retained near the column inlets, and lower but variable IONP concentrations in segments farther from the inlets. Although the presence of the biofilm increased the retention of IONPs in the “low flow” biofilm column by ca. 30% relative to the 35 mM pH 6.5 aerobic medium abiotic control columns, the retention of IONPs in the biofilm column (79.8%) was more even throughout the length of the column compared to the abiotic experiments. Since *P. fluorescens* is a known biosurfactant-producing bacteria, it is possible that

some biosurfactant was produced which increased transport distance in the biofilm column, while the biofilm itself may have led to overall increased retention.

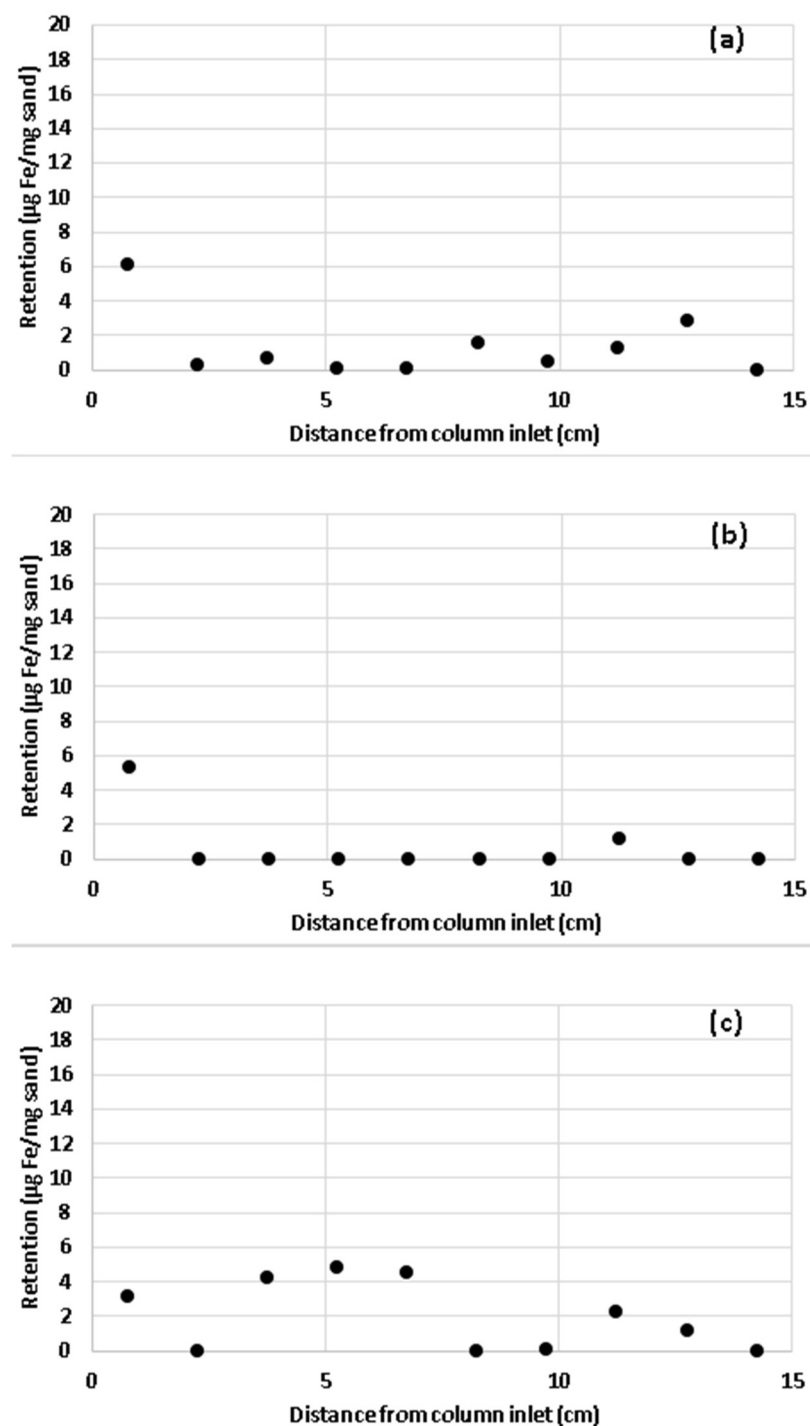


**Figure 6.8.** IONP retention in abiotic 35 mM aerobic medium transport columns A35a (a), A35b (b) and 35 mM aerobic medium biofilm column B35(c).

## 6.5 Discussion and Conclusions

In this study, we demonstrated that biofilms derived from *P. fluorescens*, an environmentally-relevant biofilm- and biosurfactant-producing bacteria, impede transport of oleic acid-coated IONPs, even at relatively high flow rates and in a low ionic strength solution (10 mM NaCl). While there have not been previous column transport studies of ENP transport in *P. fluorescens* biofilm-coated porous media, Peulen and Wilkinson (2011) found that both NP charge and size were important factors in diffusion behavior of various ENPs through *P. fluorescens* biofilm. Negatively-charged ENPs exhibited significant decreases in self-diffusion coefficients in dense *P. fluorescens* biofilms (Peulen and Wilkinson, 2011). Additionally, *P. fluorescens* biofilms grown under Fe-limited (0.01 mM) conditions formed much denser structures compared to biofilms cultivated in high-Fe (0.01 mM) conditions: The effective pore size of *P. fluorescens* biofilm was ca. 50 nm for biofilms grown under high Fe conditions but < 10 nm for denser biofilms grown under Fe-limited conditions. As Fe was not added to the growth medium used to cultivate the biofilms in this study, it is possible that the biofilms in this study, especially those grown under optimal “high flow” conditions were considerably denser than biofilms grown under “low flow” conditions. Additionally, the size of the IONPs used in the present investigation ( $68.4 \pm 6.9$  nm) would exclude them from dense *P. fluorescens* biofilms similar to those investigated by Peulen and Wilkinson (2011).

Hydrophobic interactions between IONPs and biofilm are not suspected to play a role in the observed transport behavior in this study since the surface of the oleic acid bilayer used to stabilize the IONPs in the present study (Li et al., 2014) is homogenous and consists of a hydrophilic headgroup (carboxylic acid functionality) which renders the nanoparticles water-stable. However, increased retention of ENPs with non-neutral surface charges and polar surface

functional groups have been observed in biofilms exhibiting a high degree of hydrophobicity, and a higher degree of biofilm hydrophobicity has also been shown to correlate with a higher biofilm protein:polysaccharide ratio (Xiao and Wiesner, 2013). Although biofilm composition was not determined in this study, most strains of *P. fluorescens* have been shown to produce lipo-protein biosurfactants, while *P. aeruginosa*, the most-studied Pseudomonad in terms of biofilm-NP transport, produces predominantly rhamnolipid, a polysaccharide-based biosurfactant. It is therefore possible that *P. fluorescens* produces a relatively hydrophobic biofilm which led to the high retention of OA-stabilized IONPs observed in this investigation. Attachment efficiencies ( $\alpha$ ) for hydrophobic ENPs have been found to be positively correlated with the degree of biofilm hydrophobicity, while  $\alpha$  for hydrophilic ENPs (Ag-CIT and fullerol) were not well-explained by hydrophobic interactions (Xiao and Wiesner, 2013). It has been suggested that enhanced affinity of charged ENPs to biofilms could be attributable to hydrogen bonding between surface hydroxyl groups (in the case of fullerol) formed with the collector surface or other macromolecular biofilm associations such as the ability of large polysaccharides to “collect” small nanoparticles (Espinasse et al., 2007; Xiao and Wiesner, 2013). Considering that the oleic acid coating is biodegradable (as illustrated in Chapter 5), and that biofilms are likely to contain viable cells, it is also possible that the OA-coated particles were destabilized via transformation or removal of the OA coating by *P. fluorescens*, which have been shown to grow on various hydrocarbons (Abouseoud et al., 2007; 2008; Desai and Banat, 1997).

The results of this study indicate that (1) OA-coated IONPs released into the subsurface are not likely to be transported if dense *P. fluorescens* biofilms are present and (2) biofilm growth regimes can affect transport of ENPs (e.g., retention ranged from ca. 80% under conditions of biofilm grown at a low seepage velocity using recirculated medium to ca. 100%

when biofilms were grown at a high seepage velocity using non-recirculated medium), therefore it is critical to understand and characterize *in situ* biofilm structure and occurrence if laboratory-based investigations are to be used to predict the fate and transport of ENPs in the subsurface. Future work should investigate the transport of IONPs through biofilms representative of *in situ* conditions as well as through biofilm-coated soils. Finally, if subsurface transport of IONPs is desirable (e.g., in applications such as subsurface remote sensing), alternative surface-stabilization chemistries and approaches should be evaluated in order to maximize IONP stability and transport in environments where biofilms may occur.

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## 7. Key Findings, publications, and recommendations for future work

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### 7.1 Key Findings

The goals of the studies reported herein were to improve bioremediation performance in chlorinated solvent source zones, to enhance our understanding of microbial reductive dechlorination (MRD) under iron- and sulfate-reducing conditions, and to improve our understanding of the impacts of iron-reducing and biofilm-forming bacteria on the subsurface fate and transport of oleic acid (OA)-coated iron oxide nanoparticles (IONPs). The work has specifically focused on: (1) examining the effects of iron reduction on PCE MRD under dynamic (i.e., flow-through) conditions in the presence and absence of sulfate-reduction/sulfide production, (2) optimization of fermentable electron donor substrate delivery to support tetrachloroethene (PCE) MRD in PCE source zones, (3) quantifying the potential for microbially-mediated iron reduction of OA-stabilized IONPs, and (4) characterizing the effects of an environmentally-relevant biofilm-forming bacteria (*Pseudomonas fluorescens*) on OA-IONP transport through saturated porous media. The results of this research can assist *in situ* bioremediation (ISB) practitioners determine the likelihood of successful ISB of chlorinated solvent contamination at sites containing high iron(III) and/or sulfate concentrations and make decisions regarding selection of fermentable electron donor substrates when dense non-aqueous phase liquid (DNAPL)-phase chlorinated solvent contamination is present at a site. This research will also be of use to industries and/or decision makers interested in the subsurface fate and/or transport of IONPs, for example manufacturers of consumer goods containing IONPs, regulators considering the likely impacts of IONPs released unintentionally into the subsurface, or

enterprises exploring the viability of utilizing IONPs as subsurface contrast agents in aquifer characterization or for petroleum discovery.

### **7.1.1 Microbial reductive dechlorination of PCE under iron- and sulfate-reducing conditions**

- Iron-reducing bacteria (IRB) benefit dechlorinating bacteria by creating more reduced conditions, leading to increased MRD.
  - In the presence of both a PCE-to-ethene dechlorinating consortium (BDI) and a model IRB, 14.8% more dechlorination (as average chlorine number) was measured compared to BDI-only conditions.
  - Conditions were 21% more reduced (measured as oxidation/reduction potential) when both BDI and IRB were present relative to BDI-only conditions.
- Iron-reducing bacteria (IRB) also benefit dechlorinating bacteria under conditions of sulfate-reduction because of the production of reduced iron(II) products, which react with sulfide to form iron(II) sulfide that protects dechlorinators from the inhibitory effects of dissolved-phase sulfide.
  - When both reduced iron and sulfur products were present, effects on dechlorination (e.g., 21% increase in chlorine number due to reduced production of vinyl chloride and ethene) were not detected until sulfide concentrations exceeded 10.8 mM, more than twice the concentration previously reported to inhibit MRD in the absence of reduced iron products.

### 7.1.2 Use of the Partitioning Electron Donor n-Butyl-Acetate (nBA) to Promote Organohalide Respiration and Bioenhanced Dissolution of PCE-DNAPL

- Under abiotic conditions, in flow-through 1-D columns, the partitioning electron donor (PED) nBA persisted longer than lactate in a mixed NAPL source zone composed of 25:75 (mole/mole) PCE-Hexadecane, respectively.
  - nBA persisted approximately 9 times longer than the same volume of lactate and had a NAPL-water partitioning coefficient value (48.5) 63 times greater than lactate (0.77) in the mixed NAPL.
- The use of nBA resulted in a superior distribution of dechlorination end-products relative to both continuously supplied or pulsed-injection lactate, findings which suggest that nBA particularly benefits *Dehalococcoides* bacteria
  - Over a 1-D column study duration of approximately 90 PVs, the nBA column produced 44 times more VC + ethene (as a fraction of total VOCs detected) compared to the pulsed lactate column
  - The nBA column also produced 10% more VC and ethene over the course of the experiment and 275% more ethene relative to the continuous lactate column during the last 10 PVs of the experiment
  - By the end of the 90 PV study, rates of ethene production ( $d^{-1}$ , expressed as a fraction of initial PCE mass) were ca. 10.3 and 128.8 times higher when nBA was used as a fermentable electron donor substrate compared to rates observed when lactate was supplied continuously or as a pulsed input, respectively.
- Pulsed amendments of nBA increase the potential for biologically-enhanced dissolution of PCE from source zones and result in superior or comparable dissolution enhancement

relative to continuous or pulsed injection of lactate, depending on the dissolution enhancement metric considered.

- After approximately 90 PVs, the maximum bioenhanced dissolution enhancement factor (relative to values expected from abiotic dissolution) under pulsed-injection of nBA was 11.5, compared to maximum enhancement factors of 8.3 and 1.8 under conditions of continuously-supplied and pulsed-addition lactate, respectively.
- After approximately 90 PVs, the cumulative dissolution enhancement factor (relative to expected abiotic dissolution) under pulsed-injection of nBA was 3.0 compared to enhancement factors of 3.7 and 1.8 under conditions of continuously-supplied and pulsed-addition lactate, respectively.

### **7.1.3 Impacts of anaerobic soil microbiological processes on nanoparticle fate: Potential for microbially-mediated transformations of OA-coated IONPs**

- Susceptibility of IONPs to microbially-mediated iron reduction is reduced, but not eliminated by the presence of an OA surface coating.
  - Iron reduction of OA-coated IONPs by the dissimilatory iron-reducing bacteria *Geobacter sulfurreducens* was reduced by up to 70% in the presence of the OA surface coating relative to iron reduction of the uncoated iron oxide material used to produce the coated IONPs.

### **7.1.4 Transport of OA-IONPs through *P. fluorescens* biofilm-coated porous media**

- Transport of OA-coated IONPs through saturated quartz sand was completely inhibited in the presence of *P. fluorescens* biofilm grown at a relatively high pore-water velocity (5.6 m d<sup>-1</sup>) and provided with fresh growth medium.

- No transport of OA-coated IONPs through saturated porous quartz sand in 10 mM NaCl or 50 mM mixed electrolyte (mono- and di-valent) transport fluids at circumneutral pH was measured through *P. fluorescens* biofilm-coated quartz sand at seepage velocities of  $0.41 \pm 0.1 \text{ m d}^{-1}$  or through 10 mM NaCl at seepage velocities of  $4.2 \text{ m d}^{-1}$
- Under biofilm-free conditions,  $10.1 \pm 2.8$  to  $79.6 \pm 1.6\%$  of IONP mass injected was transported through saturated porous quartz sand at seepage velocities of  $0.41 \pm 0.1 \text{ m d}^{-1}$  in 10 mM NaCl and 50 mM mixed mono- and di-valent electrolyte transport fluids at circumneutral pH, respectively.

## **7.2 Implications for bioremediation and IONP subsurface fate and transport**

### **7.2.1 Implications for bioremediation of chlorinated solvents when reducible iron and sulfate are abundant in a contaminated aquifer**

- When ISB is selected as a treatment remedy in chlorinated solvent-contaminated aquifers, MRD may benefit by co-stimulation of iron reduction if reducible iron-bearing minerals are also present due to the potential for more reduced conditions that are caused by the formation of reduced iron products.
- In chlorinated solvent-contaminated aquifers containing high sulfate concentrations, the increase in sulfide production expected when biostimulation of MRD is applied as a treatment remedy may not result in MRD inhibition if reducible iron-bearing minerals (and IRB) are also present.
- If the capacity for indigenous iron-reduction is low at a high-sulfate site, bioaugmentation with IRB and/or ferric iron delivered as co-amendments during bioaugmentation and/or

biostimulation may be protective against sulfide inhibition of organohalide respiring bacteria.

### 7.2.2 Implications for bioremediation of chlorinated solvent source zones

- nBA may be a particularly fortuitous electron donor for supporting cis-DCE dechlorination by *Dhc* based on the increased rates of ethene production measured in this study relative those achieved using a conventional water-soluble electron donor (lactate); greater production of ethene reduces the need to manage hazardous dechlorination daughter products such as cis-DCE and VC, which could translate to reductions in remediation costs.
- Because nBA dissociation products include fatty acids, which ferment less rapidly compared to some conventional fermentable electron donors (e.g. lactate), the use of nBA may result in lower hydrogen partial pressures, benefitting dechlorinating species such as *Dhc* which have lower hydrogen thresholds compared to many competitor bacteria. This behavior, along with the ability to target delivery of nBA DNAPL:aqueous interfaces and maintain electron donor levels to support dechlorinating biomass in these regions, could reduce remediation costs, because lower quantities of electron donor would be required relative to current practices in which excess donor amendments are supplied to compensate for electron donor consumption by non-target microbes.
- Use of a fermentable PED substrate such as nBA can increase the rate of mass removal in chlorinated solvent source zones through bioenhanced NAPL dissolution and is a less-hazardous, cost-effective amendment relative to toxic (e.g., chemical oxidants) or costly (zero valent iron slurry) source zone remediation options.

### 7.2.3 Implications for fate and transport of OA-coated IONPs in the subsurface

- OA-coated IONPs contain naturally-occurring materials (oleic acid, magnetite) that are biodegradable and are not likely to experience significant mobility upon release into the subsurface; for applications entailing unintended subsurface release of OA-IONPs (e.g. in consumer products that may eventually enter waste streams), ecotoxicity or other adverse impacts, such as those associated with other types of engineered nanoparticles that may be released into the subsurface (Farré et al., 2009; Gupta et al., 2017; Pereira et al., 2011) are unlikely.
- For applications in which subsurface transport of IONPs is desirable (e.g. subsurface remote sensing/aquifer mapping and characterization), particle surface stabilization materials should be non-biodegradable and steric stabilization techniques may be optimal.
- *In situ* biofilm characteristics and occurrence should be determined for sites in which IONPs subsurface transport applications are being considered: Conditions under which biofilms are formed can substantially impact transport behavior of IONPs.

## 7.3 Publications and Presentations

The research in this dissertation is the subject of three manuscripts currently in preparation for publication. Research presented in chapter 4 of this dissertation has also been previously presented as a poster presentation.

### 7.3.1 Manuscripts in preparation for publication

1. Duhl, T. R., Sylvia, D. F., Pennell, K. D., Cápiro, N. L., (in preparation). Use of the Partitioning Electron Donor n-Butyl-Acetate to Promote Organohalide Respiration and Bioenhanced Dissolution of PCE-DNAPL, Manuscript in preparation for submission to Biodegradation.

2. Duhl, T. R., Gaeth, S. P., Pennell, K. D., Cápiro, N. L., (in preparation). Microbial reductive dechlorination of tetrachloroethene under iron- and sulfate-reducing conditions, Manuscript in preparation for submission to Environmental Engineering Science.
3. Duhl, T. R., Liao, S., Ghosh, A., Fortner, J. D., Pennell, K. D., Cápiro, N. L., (in preparation). *Pseudomonas fluorescens* biofilm inhibits transport of oleic acid-coated iron oxide nanoparticles, Manuscript in preparation (journal TBD).

### **7.3.2 Other presentations**

1. Duhl, T. R., Gaeth, S. P., and Capiro, N. L. “Microbial reductive dechlorination of tetrachloroethene under iron- and sulfate-reducing conditions”, presented at the ACS National Meeting, Boston, MA, 19-23 August, paper ID 2999073, 2018.

## **7.4 Recommendations for Future Research**

The research presented herein has validated the use of a novel partitioning electron donor substrate to support chlorinated solvent source-zone bioremediation, demonstrated that MRD can benefit from iron-reduction including under conditions of elevated sulfate reduction, and quantified of susceptibility of OA-IONPs to iron-reducing bacteria and the substantial impacts of an environmentally-relevant biofilm-forming bacteria on OA-IONP subsurface fate and transport. However, additional research is warranted to expand upon the findings presented above.

In Chapter 3, the use of the PED nBA to stimulate MRD and bioenhanced PCE NAPL dissolution in a mixed PCE-HD NAPL source zone was evaluated against a water-soluble electron donor, however the performance of a PED relative to an insoluble donor such as emulsified vegetable oil could yield additional insight about PED performance relative to other electron donor substrates commonly-used in chlorinated solvent source zone bioremediation.

Since ISB often entails competition for electron donor by non-dechlorinating microbes such as methanogens, future research could also focus on the performance of nBA relative to conventional electron donor substrates under more realistic field settings and in the presence of competitor bacteria. The benefits of using nBA may be greater than anticipated since the fermentation of nBA hydrolysis products is expected to proceed more slowly than lactate fermentation and because dechlorinating microbes such as Dhc have a competitive advantage over methanogens at low hydrogen partial pressures.

In Chapter 4, MRD performance under conditions of iron- and sulfate-reduction was studied, and reduced iron products were shown to be protective against the inhibitory effects of sulfide on dechlorinating bacteria. The upper-limit of sulfide concentrations identified before adverse impacts on MRD were observed (10.8 mM) may actually be higher than reported in this study because elevated pH effects on MRD from increased sulfide levels could not be disentangled from effects of the increased sulfide concentrations alone. Therefore, future research efforts could focus on sulfide-related MRD inhibition thresholds under conditions of iron reduction in well-buffered systems.

In chapter 5, the susceptibility of OA-IONPs to iron reduction by the dissimilatory IRB *Geobacter sulfurreducens* was investigated and quantified. Additionally, it was demonstrated that OA-IONPs are susceptible to aerobic biodegradation and likely also chemical reduction of the IONPs following exposure to even low (0.2 mM) concentrations of sulfide. Additional studies are needed to quantify the nature and rates of (1) aerobic biodegradation and (2) chemical reduction of OA-IONPs if these particles are to be used for environmental applications. Additionally, potential end-users of IONPs would benefit from investigations of the impacts of sulfide exposure on IONP transport and superparamagnetic properties.

Finally, in Chapter 6, the effects of *Pseudomonas fluorescens* biofilm on the transport of OA-IONPs were studied, and results suggested that the methods used to cultivate the biofilm substantially impacted resultant transport. Additional studies that more systematically explore biomass density and other characteristics resulting from varying biofilm formation techniques are required to elucidate potential impacts of pH, nutrient availability, and flowrate on biofilm formation and subsequent impacts on IONP transport. Additionally, transport and retention behavior of IONPs with alternate surface chemistries and/or in the presence of different biofilm-forming species should be investigated.

## 7.5 References

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# **Appendix A: Supplementary Material for Chapter 3: Microbial reductive dechlorination of tetrachloroethene under iron- and sulfate-reducing conditions**

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## **A.1 Supplementary Chapter 3 Methods**

### **A1.1 Column packing, saturation, and pore volume determination**

Two identical borosilicate glass columns (Kimble-Chase; Vineland, NJ), each 15 cm in length equipped with Teflon end plates were used for the experiments. Each contained 3 side ports located 4, 8, and 12 cm from the column inlet used for microbial inoculation. Columns were dry-packed in 2 cm lifts with the iron-oxide coated sand (IOCS). The inlet two centimeters of each column were packed with acid washed sand to limit the potential for clogging by iron sulfide (FeS) formation at the column inlet. All materials used to pack the columns including the porous media were autoclaved or washed in 70% isopropanol prior to use. Packed columns were flushed with Ultra High Purity CO<sub>2</sub> for at least 60 minutes to facilitate the dissolution of entrapped gas prior to saturation.

The column (PV) was calculated using two techniques. In the first approach, the PV was calculated based on the packed column weight before and after saturation, taking into account the volume of water contained in end cap tubing and valves. In the second approach, a conservative tracer experiment was performed in which a 3 PV pulse of reduced mineral salts medium containing 10 mM sodium bromide was introduced to the columns followed by flushing with bromide-free medium and continuous collection of effluent samples using a CF2 fraction collector (Spectrum Chromatography Inc., Houston TX). Column effluent bromide

concentrations (analyzed as described below) were expressed as  $C/C_0$  and used to characterize aqueous pore volume and dispersivity of the columns using the Code for Estimating Equilibrium Transport Parameters from Miscible Displacement Experiments (CFITM) (van Genuchten, 1980) as a part of Studio of Analytical Models (STANMOD) version 2.08.1130 (Šimůnek et al., 1999).

### **A.1.2 Analytical Methods**

Concentrations of chlorinated ethenes and ethene from 1 mL aqueous samples were measured using an Agilent model 7890 gas chromatograph equipped with Teledyne-Tekmar HT3 headspace autosampler (Thousand Oaks, CA) and an HP-64 column (60 m by 0.32 mm; film thickness, 1.8  $\mu\text{m}$  nominal) connected to an FID as described previously (Amos et al., 2007; Cápiro et al., 2014; Marcet et al., 2018a). Analytical standards were completed as described previously (Gossett, 1987; Amos et al., 2007; Marcet et al., 2018a). Concentrations of bromide, sulfate, and VFAs including lactic, acetic, and propionic acids were determined using a Dionex ICS-2000 (Thermo Fisher) ICS-2100 ion chromatography system (IC). The IC was equipped with a Dionex IonPac AS11-HC analytical column (Thermo Fisher) maintained at 35 °C with an anion self-regenerating suppressor operated at 149 mA. The IC was operated with a constant flow rate of 1.0 mL/min and a variable potassium hydroxide eluent concentration that was held at an initial concentration of 1 mM for 25 min, ramped up to 30 mM from 25 to 54 min, then ramped down to 1 mM until the run ended at 56 min. Calibration standards were prepared by diluting a stock solution containing 100 mM of sulfate and 40 mM of each VFA in MilliQ water. The method detection limit for VFAs listed above was about 1  $\mu\text{M}$  (Marcet et al., 2018b). An Orion Triode Ag/AgCl combination pH/ATC electrode connected to an Orion 3-star pH meter

(Thermo Scientific) was used to measure pH, and ORP was measured using an Accumet platinum pin Ag/AgCl combination electrode (Fisher Scientific).

### ***Solid-phase total iron***

Total solid phase iron content was determined by mixing 5 g of dry material in 10 mL of concentrated nitric acid followed by acid digestion in a Discover SP-D microwave digester (CEM Corp., Matthews, NC). A 3 mL aliquot of the resultant acid solution was then diluted 4:1 in water and analyzed for aqueous phase iron using an inductively coupled plasma optical emission spectrometer (ICP-OES; Optima 7000, Perkin Elmer, Waltham, MA). The plasma intensity was correlated to the concentration of iron in solution, and a linear calibration curve was obtained by preparing standards with iron concentrations of 0.1 to 30 mg iron/L. Argon was the carrier gas, which operated at approximately 18 L/min.

### ***Aqueous-phase iron analysis***

Aqueous-phase ferrous and total iron concentrations were determined using a variation of the spectrophotometric ferrozine method (Stookey 1970; Viollier et al. 2000) and measured with a UV-Vis Shimadzu UV 1800 spectrophotometer (Shimadzu Scientific; Marlborough, MA). Three reagents were prepared to facilitate the colorimetric detection of iron. First, 300  $\mu$ L of a solution of 100 mM ammonium acetate and 10 mM ferrozine iron reagent was added to a 1 cm polystyrene cuvette (Fisher Scientific), followed by 25  $\mu$ L of sample. After allowing the mixture to rest for 2 mins, 2.5 mL of MilliQ water was added to the cuvette and the solution was rested for 10 minutes to allow for color development, after which ferrous iron content was determined by recording absorbance at 562 nm. Next, 200  $\mu$ L of a solution containing 1.2 M hydroxylamine hydrochloride in 1.7 M hydrochloric acid was added to the cuvette, and the solution was rested

for an additional 10 minutes. Finally, 50  $\mu$ L of a buffering solution of 10 M ammonium acetate (adjusted to a pH of 9.55 using a 28% ammonium hydroxide solution) was added to the cuvette, and the total iron concentration was determined by recording absorbance at 562 nm. A linear calibration curve was obtained using a stock solution of iron (II) chloride tetrahydrate at concentrations ranging from 1 to 30 mg/L iron.

### ***Aqueous phase sulfide analysis***

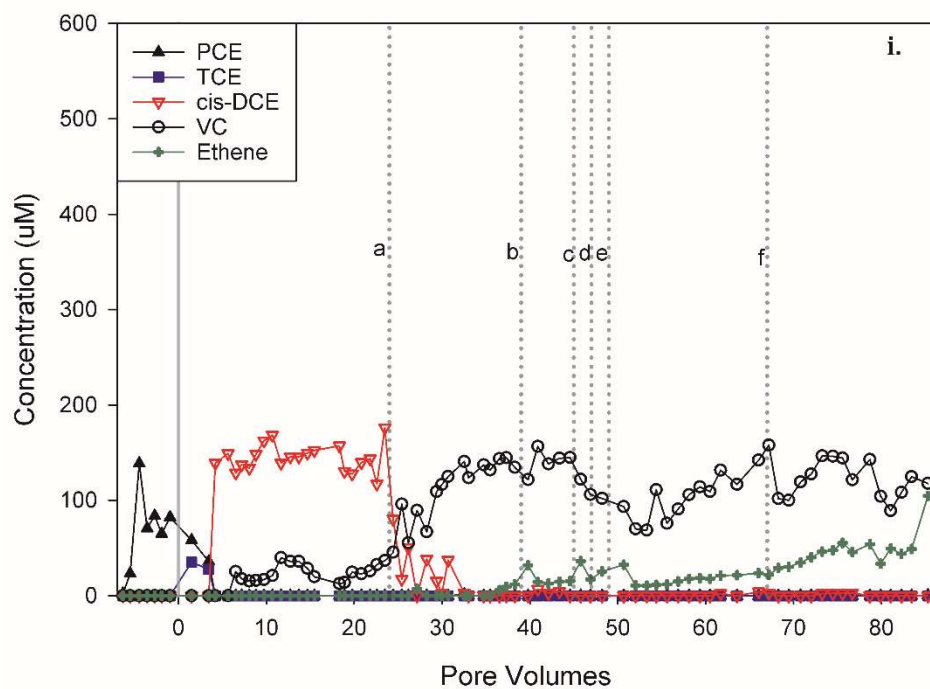
Determination of aqueous sulfide concentration was accomplished using a variant of the method of Haddad and Heckenberg (1988). One mL of sample was added to a 1 cm cuvette, followed by one mL of 22 mM N-N-dimethyl-p-phenylenediamine in 1M sulfuric acid. A single drop of saturated iron (III) chloride was then added and the cuvette was gently inverted and rested for 5 mins to facilitate color development. Even in the presence of low (5 mg/L) sulfide levels, the color was very dark and dilution was warranted. Therefore, both samples, blanks, and standards were diluted by adding 50  $\mu$ L of solution to a new cuvette containing 2mL of nitrogen-sparged MilliQ water. After 5 mins, sulfide concentrations were determined by measuring absorbance at 664 nm using a UV-1800 UV-Vis Spectrophotometer. A calibration curve containing a linear range between 5-100 mg sulfide/L was used to quantify sulfide concentrations.

### ***Statistical analyses***

Two-sample t-tests were used to verify whether observed differences in chlorine number were significant between columns within a given experiment. Prior to running the t-tests, F-tests were performed on each variable tested. When the results of the F-tests indicated that variance was not

significantly different (for  $\alpha = 0.01$ ), variances were pooled for the t-test calculations. Statistical analyses were performed using R statistical software version 3.2.2 (R Core Team, 2017).

## A.2 Supplementary Chapter 3 Figures



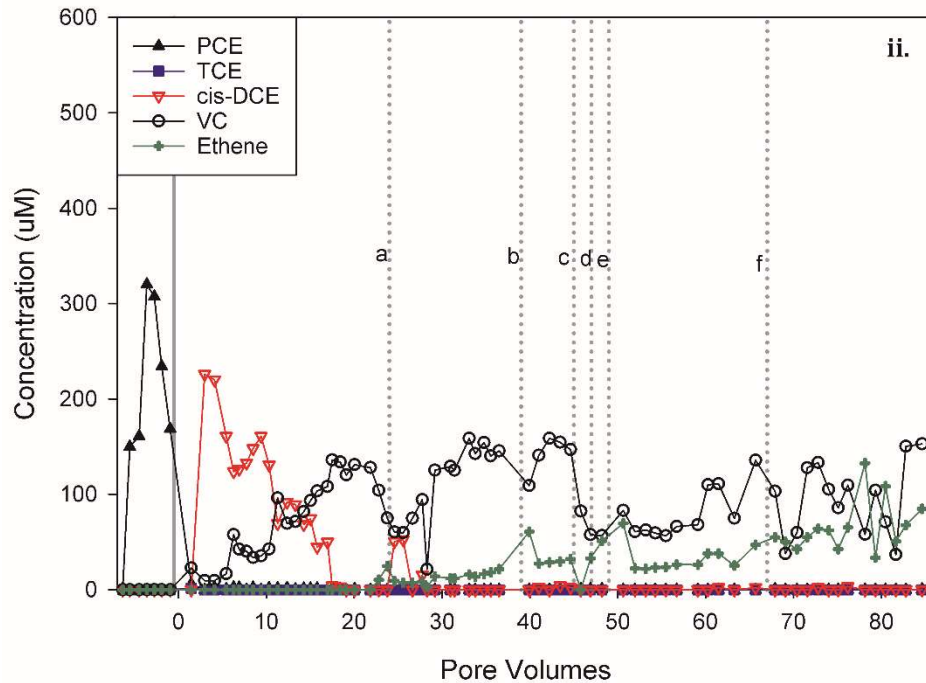


**Figure A.1.** Temporal evolution of PCE its dechlorination daughter products measured in effluent samples from the BDI-SZ-only (i) and BDI + IRB (ii) experimental columns. Letters correspond to flow interruptions (dotted lines) of the following durations (in hours): a, 48; b and c, 72; d, 46; e, 66; f, 26. PV 0 corresponds to column inoculation (solid vertical line).

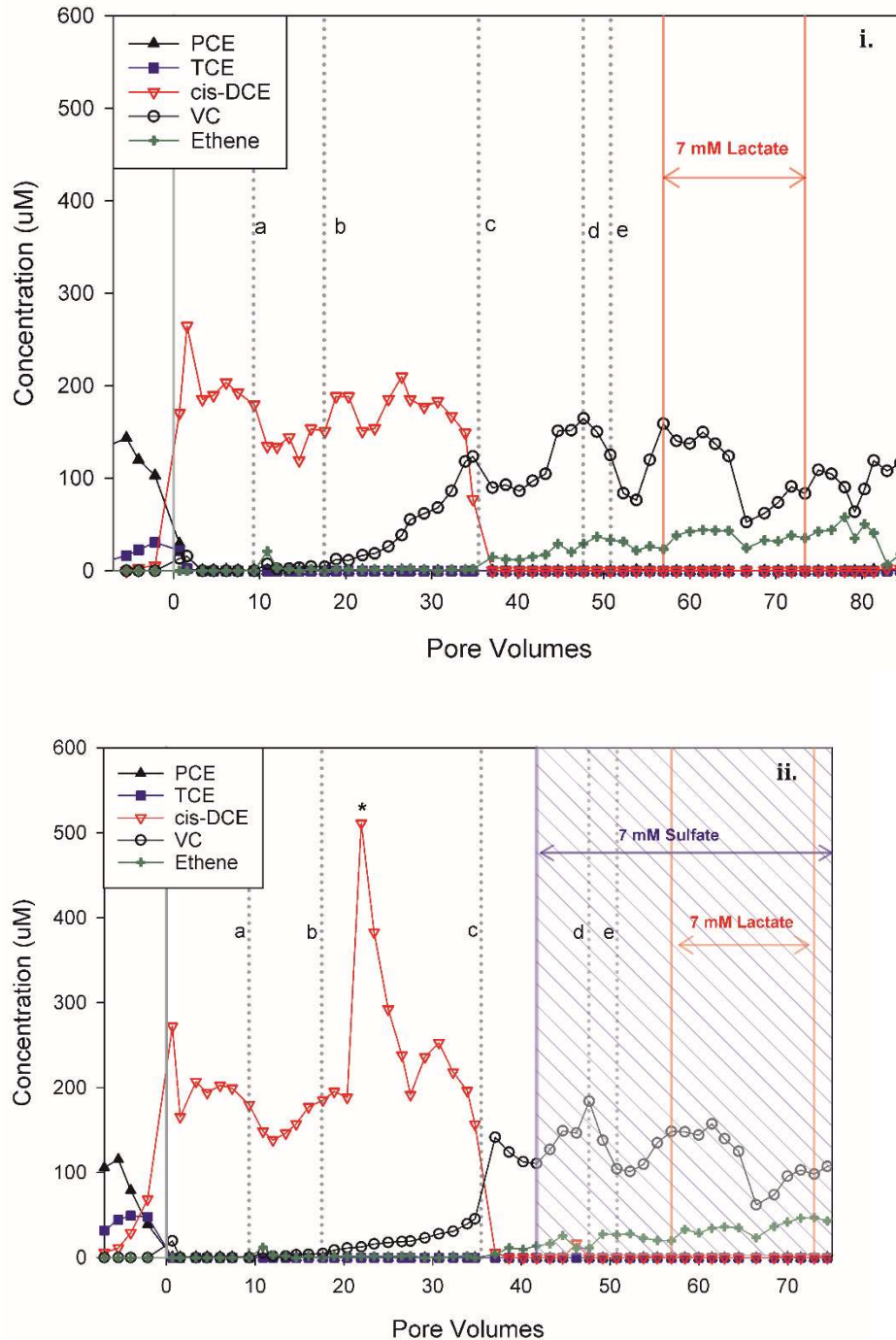


**Figure A.2.** PCE and its dechlorination daughter products measured in effluent samples from the 5 mM (A<sub>5</sub>; i) and 7 mM (B<sub>7</sub>; ii) sulfate experimental columns. The timing and duration of 7 mM sulfate influent treatment (for column B<sub>7</sub>) and 7 mM lactate treatment (both columns) are indicated with text, lines, and arrows. Letters correspond to flow interruptions of the following durations (in hours): a, 27; b, 36; c, 41; d and e, 24. \*: the cis-DCE spike in the B<sub>7</sub> plot was caused by a small amount of PCE non-aqueous-phase liquid in the influent syringe during syringe filling, which led to increased concentrations of cis-DCE in column effluent samples.

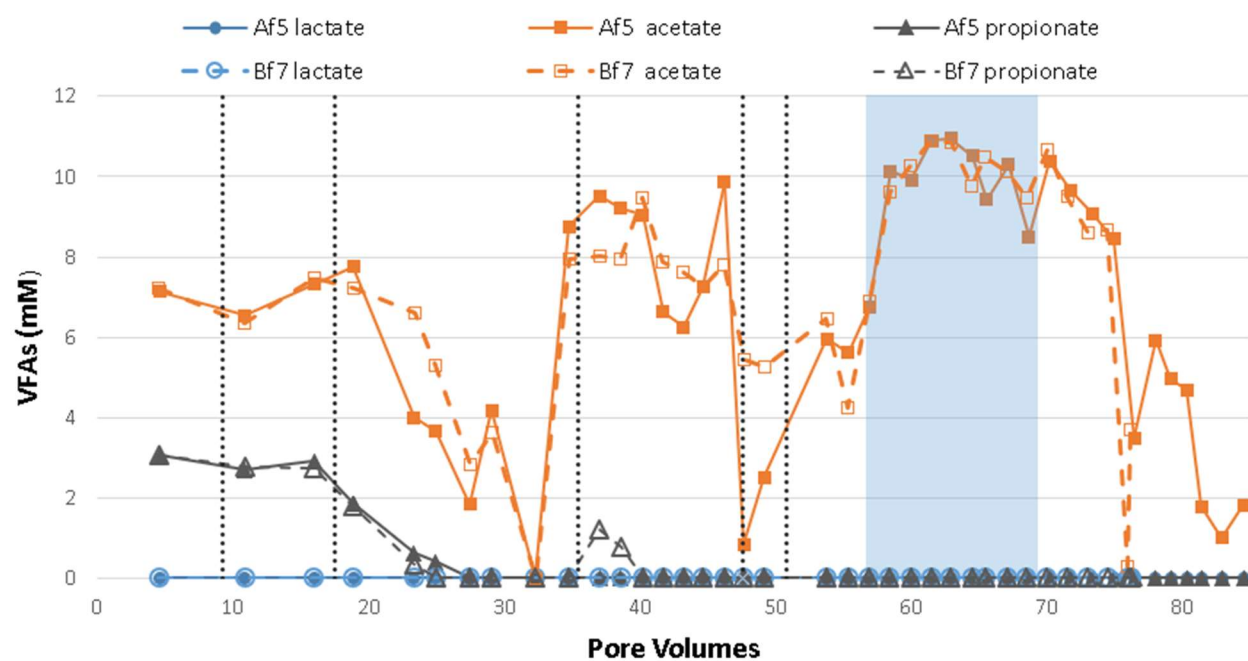


**Figure A.3.** Effluent VFA concentrations of lactate (blue circles), acetate (orange squares) and propionate (grey triangles) detected in 5 mM sulfate (“Af5”, solid symbols and lines) and 7 mM sulfate (“Bf7”, open symbols, dashed lines) columns. Flow interruptions are denoted by vertical dotted lines; the 7 mM lactate pulse represented by the region shaded in gray.

### A.3 Supplementary Chapter 3 References

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# Appendix B: Supplementary Material for Chapter 4: Use of the Partitioning Electron Donor n-Butyl-Acetate to Promote Organohalide Respiration and Bioenhanced Dissolution of PCE-DNAPL

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## B.1 Supplementary Chapter 3 Methods

### B.1.1 Column preparation and packing

All materials used to pack the columns, including the porous media were autoclaved or washed in  $\geq 70\%$  isopropyl alcohol prior to use. Columns were wet-packed in  $\sim 2$  cm lifts with autoclaved FFO sand and 90-110 mL of reduced mineral medium in an anoxic glove box with an atmosphere of nitrogen and  $< 2\%$  hydrogen (Coy Laboratory Products; Grass Lakes, MI). For the packing of the biotic columns, the reduced medium was amended with the BDI-SZ dechlorinating consortium, and upon completion, columns were capped and kept in the glove box 24-48 hrs to promote microbial attachment. Aqueous *Dhc* biomass samples (5 mL) were collected in triplicate from the BDI-SZ inocula and were prepared and analyzed using pPCR as described below, and in more detail in Cápiro et al. (2014). The inocula used in the continuous lactate, nBA, and pulsed lactate columns contained  $4.6 \times 10^7 \pm 0.3$ ,  $1.9 \times 10^8 \pm 0.02$ , and  $2.7 \times 10^8 \pm 0.5$  *Dhc* cells  $\text{mL}^{-1}$ , respectively. The pre-NAPL-saturation pore volume (PV) for all columns was determined as the difference between the saturated weight of the packed columns (including end-caps, tubing and valves), the weight of the sand used to pack the columns, and the empty weight of the columns, end-caps, and valves, taking into consideration the volume of water in the end cap tubing and shut-off valves and was confirmed using a nonreactive tracer experiment described in the main text.

### **B.1.2 Anoxic column delivery system**

An anoxic delivery system was established to supply reduced medium to the columns from 4L Mariotte bottles connected to peristaltic pumps (Dynamax Model RP-1; Rainin Instruments, Oakland, CA and/or Gilson Minipuls 3, Middleton, WI). The Mariotte bottles (Ace Glass, Vineland, NJ) were continuously flushed with argon gas to prevent oxygen intrusion into the influent reservoir. A redox indicator (resazurin) was added to the medium at a concentration of 1  $\mu$ M to confirm that the column influent was anoxic. Column effluents were connected to a 25 mL glass bulb (G. Finkenbeiner, Inc., Waltham, MA), used to keep the effluent anoxic and to prevent volatilization of chlorinated solvents and nBA prior to sampling.

### **B.1.3 Biomass sample preparation and analysis**

DNA from the frozen, pelleted biomass samples was extracted using the QIAmp DNA Mini Kit (Qiagen, Valencia, California) following the manufacturer-recommended protocol. The extracted DNA was stored at -20°C until qPCR analysis. Bacterial *Dhc* abundance from extracted DNA was measured using an Applied Biosystems Step One Plus qPCR system (Foster City, California) under the standard operating mode and using previously-established procedures (Capiro et al., 2014). Established protocols for TaqMan-based quantification of *Dhc* 16S rRNA genes (Ritalahti et al. 2006) were followed as described previously (Amos et al., 2007). Standard curves were generated following the procedure of Ritalahti et al. (2006) and used a 10-fold dilution series of quantified plasmid DNA. Additional details may be found in Capiro et al. (2014).

#### **B.1.4 Analytical methods**

Concentrations of nBA and PCE were measured using a 7890 Gas Chromatograph (GC) equipped with a liquid autosampler (HP 7683, Agilent Technologies, Santa Clara, CA, USA) and an Agilent DB-5 column (30 m by 0.32 mm OD) connected to a flame ionization detector (FID; Agilent). PCE daughter products were measured using an Agilent model 7890 GC equipped with a Teledyne-Tekmar HT3 headspace autosampler (Thousand Oaks, CA) and an HP-64 column (60 m by 0.32 mm; film thickness, 1.8  $\mu\text{m}$  nominal) connected to an FID as described previously (Amos et al., 2007; Cápiro et al., 2014). Analytical standards were completed as described previously (Costanza, 2007; Gossett, 1987; Löffler et al., 1997). VFA concentrations of lactic, acetic, and propionic acids were determined by adding 0.5 mL of effluent filtered through 0.2  $\mu\text{m}$  nylon filters (Microliter, Wheaton Industries Inc., Millville, NJ) to 2 mL glass vials analyzed by an Agilent 1200 High Performance Liquid Chromatograph (HPLC) equipped with a diode array detector (DAD) operated at 210 nm with an Aminex HPX-87H Ion Exclusion Column (300 mm X 7.8 mm) as described previously (He, Sung et al. 2002). Bromide was monitored using an ion-selective probe (Cole-Parmer; Vernon Hills, IL) connected to an Accumet Model 50 pH/ion/conductivity meter (Fisher Scientific) calibrated with a standard consisting of 5 bromide concentrations ranging from 0.1 to 10 mM, prepared using the same bromide-containing medium that was used to flush the columns. An Orion Triode Ag/AgCl combination pH/ATC electrode connected to an Orion 3-star pH meter (Thermo Scientific; Waltham, MA) was used to measure pH, and ORP was measured with an Accumet Platinum/Ag-AgCl electrode (Fisher Scientific) connected to a pH meter (Orion 420Aplus, Thermo Fisher Scientific, Waltham, MA, USA).

### B.1.5 Mathematical simulations

Bromide and electron donor breakthrough curves (BTCs) were simulated using the mathematical model Code for Estimating Equilibrium Transport Parameters from Miscible Displacement Experiments (CFITM) as a part of Studio of Analytical Models (STANDMOD) Version 2.2 (available through USDA-ARS U.S. Salinity Laboratory; <http://www.ars.usda.gov>). PED BTCs were also analyzed using this model along with another STANMOD model, Code for Estimating Non-Equilibrium Transport Parameters from Miscible Displacement Experiments (CFITIM) as described in Cápiro et al. (2011). Both codes incorporate a 1-D form of the advective-dispersive-reactive (ADR) solute transport equation and provide analytical solutions for semi-infinite columns. The nonequilibrium solutions consider the two-region dual-porosity (bicontinuum) flow model for physical nonequilibrium and the one-site or two-site sorption models for chemical nonequilibrium. When immobile NAPL is present, the dimensionless form of the 1-D ADR solute transport equation may be written as (Cápiro et al., 2011):

$$R_f \frac{\partial C^*}{\partial PV} = \frac{1}{P_e} \frac{\partial^2 C^*}{\partial^2 X} - \frac{\partial C^*}{\partial X} \quad (1)$$

$$R_f = 1 + \frac{K_{NW} S_N}{(1 - S_N)}; C^* = \frac{C}{C_0}; PV = \frac{v_p t}{L};$$

$$P_e = \frac{v_p L}{D_H}; D_H = v_p \alpha; X = \frac{x}{L} \quad (2)$$

where  $R_f$  is the solute retardation factor,  $K_{NW}$  is the linear NAPL-water partition coefficient,  $S_N$  is the volumetric NAPL saturation,  $C$  is the aqueous concentration ( $M L^{-3}$ ),  $C_0$  is the influent

aqueous concentration ( $M L^{-3}$ ),  $PV$  is the dimensionless pore volume,  $v_p$  is the pore-water velocity ( $L t^{-1}$ ),  $t$  is time,  $L$  is the column length,  $Pe$  is the Peclet number,  $D_H$  is the hydrodynamic dispersion coefficient ( $L^2 t^{-1}$ ),  $\alpha$  is dispersivity ( $L$ ), and  $x$  is the distance parallel to flow.

### **B.1.6 Cumulative and Maximum Dissolution Enhancement Factors**

The maximum enhancement factor represents the total concentration of VOCs (i.e., chlorinated ethenes + ethene) measured during a given sampling interval divided by the theoretical concentration of PCE expected to be based on the aqueous solubility of PCE and the mole fraction of PCE in the mixed PCE:HD NAPL calculated at the time of the sample (i.e., the mole fraction is adjusted by the mass of PCE recovered in preceding samples), while the cumulative enhancement factor represents the total molar mass of chlorinated ethenes + ethene eluted during a given sampling interval divided by the theoretical mass expected to dissolve into the aqueous transport medium under abiotic conditions over the sampling interval computed cumulatively, starting at the beginning of the experiment up to the collection of each sample (Amos et al., 2008).

## B.2 Supplementary Chapter 3 Figures

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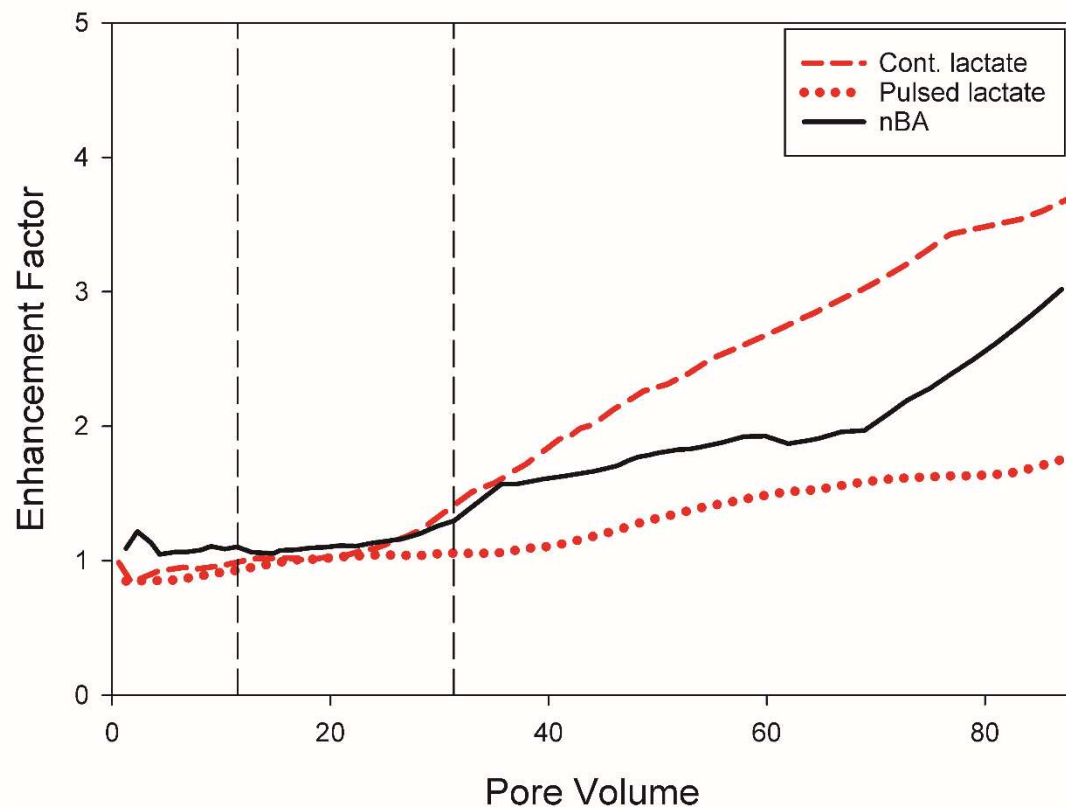


**Figure B.1. Cumulative enhanced dissolution factors versus pore volume for the different electron donor treatments. Flow interruptions (each followed by a subsequent reduction in flow rate) are indicated by dashed vertical lines.**

## Appendix C: Photographic Images of iron sulfide (FeS) in anaerobic IONP batch reactors and microbial colonization of concentrated IONP stock

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### I. FeS formation in batch reactors

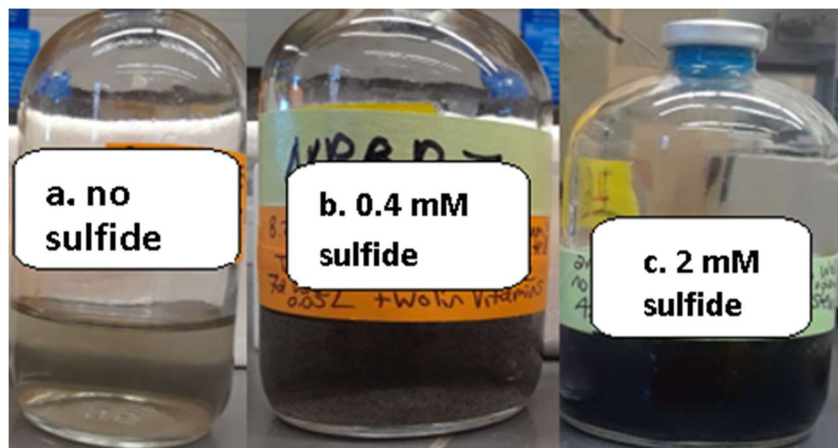


**Figure C.1.** Images taken anaerobic batch reactors containing IONPs and either no reductant (a), 0.4 mM sulfide (b), or 2 mM sulfide (c)

### II. Microbial colonization of IONP stock solution

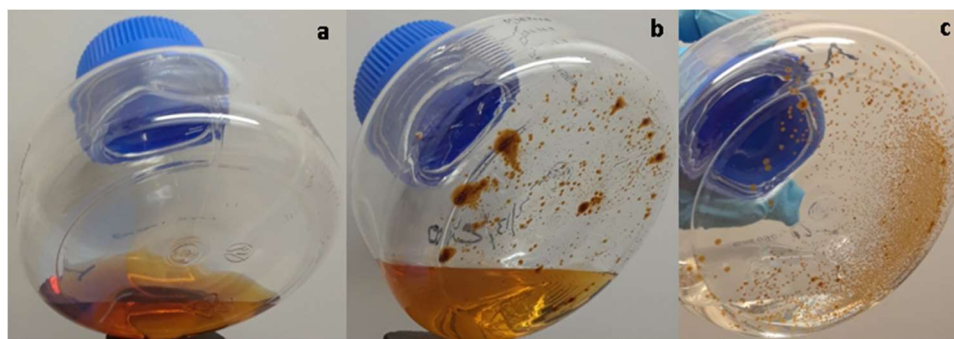


**Figure C.2.** Images taken of (a) sterile IONP stock solution 6 weeks after receipt, (b) IONP stock exposed to ambient aerobic conditions (after 10 d), and (c) exposed IONP solution after 6 weeks