

**Microbiological and Geochemical
Dynamics of the Subsurface:
chemical oxidation and bioremediation
of organic contaminants**

Nora Barbour Sutton

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Thesis

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To my grandmothers, the toughest and most loving women I know

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Summary

Summary

The subsurface is a system comprising organic, inorganic, and microbiological components, which function together to support processes including nutrient cycling, carbon sequestration, and water filtration. Past research focuses on understanding the interplay of these dynamic microbiological, geochemical, and physical processes. Contamination of the subsurface due to urban and industrial activities represents a serious environmental risk, requiring the development of efficient remediation technologies that can restore these sites into functional areas. However, the presence and treatment of these contaminants also offer a scientifically advantageous system for investigation of more fundamental topics of microbial ecology and soil geochemistry. This dissertation investigates *in situ* chemical oxidation (ISCO) and *in situ* bioremediation (ISB) of organic contaminants, with a primary focus on illuminating the dynamic microbiological and geochemical processes that occur during remediation ([Chapter 1](#)).

Strong oxidants applied during ISCO significantly change contaminant and soil characteristics, and influence microbial community size, structure, and function. When properly applied, ISCO can be a beneficial pretreatment prior to ISB. A review of previous research indicates that investigations focused on (1) optimizing the chemical oxidant treatment to aid a subsequent biodegradation step, and (2) determining amendments required to support bioremediation following ISCO ([Chapter 2](#)). In some cases, full bioremediation is inhibited either by bioavailability of the contaminant or oxidation-reduction potential of the subsurface, both of which can be ameliorated with ISCO treatments. A survey of bioremediation efficiency is performed using multiple diesel-contaminated soils comprising four soil matrix types ([Chapter 3](#)). By incubating soils under optimized biodegradation conditions, performing molecular analysis of microbial biodegradation capacity based on an alkane monooxygenase gene (*alkB*), and monitoring diesel bioavailability, it is demonstrated that full biodegradation is most likely inhibited by bioavailability rather than nutrient abundance, electron acceptor availability, or microbial capacity. Additionally, analysis of microbial community composition based on pyrosequencing of the 16S rRNA gene fragment is performed to better understand the interplay among soil geochemical parameters, diesel characteristics, and microbial diversity at a contaminated location ([Chapter 4](#)). Results reveal a significant reduction in diversity in contaminated samples, and a strong association between the presence of diesel and the abundance of strict anaerobes, indicating that oxidation-reduction

potential forms a notable hurdle for natural attenuation. Together, these sections provide convincing support for the importance of applying ISCO prior to ISB.

The coupling of chemical oxidation with aerobic bioremediation of diesel-contaminated soil is investigated in both laboratory and field experiments. Treatment with Fenton's reagent and modified Fenton's reagent focuses on understanding the geochemical changes occurring due to chemical oxidation that impact a subsequent bioremediation step ([Chapter 5](#)). Chemical oxidation degrades soil organic matter, thereby releasing dissolved nutrients and organic carbon, the latter of which competes with diesel as a substrate during biodegradation. Changes in microbial community structure and diesel degradation capacity are investigated during bioremediation following treatment with Fenton's reagent, modified Fenton's reagent, permanganate, and persulfate in two different diesel-contaminated soils ([Chapter 6](#)). Overall remediation efficiencies are dependent on the soil type; mobilization of diesel due to chemical oxidation causing reduced remediation efficiencies in peat, as compared to fill, where limited contaminant mobilization is observed. DGGE fingerprinting indicates the development of dissimilar microbial communities between treatments, with quantification of 16S rRNA and *alkB* gene abundance showing enrichment in biodegradation capacity in chemically treated soils, as compared to the biotic control. Finally, microbiological and geochemical dynamics of ISCO with persulfate followed by a nutrient-stimulated ISB are investigated in a diesel contaminated field location ([Chapter 7](#)). Groundwater parameters such as pH and oxidation-reduction potential indicate inhospitable conditions for microbial growth following ISCO, which is reflected in a 1-2 orders of magnitude decrease in both microbial community size (16S rRNA gene) and biodegradation capacity (*alkB*). While there is attenuation of acidic groundwater and regeneration of the microbial community, alterations to soil organic matter structure and oxidation of metal sulfides are irreversible changes to soil components.

The coupling of a chemical oxidation step with anaerobic bioremediation for the treatment of chlorinated solvents is investigated in laboratory and field experiments. Organohalide respiration (OHR) rates are researched in liquid microcosm experiments with permanganate treatment of tetrachloroethene (PCE; [Chapter 8](#)). Results indicate that mild permanganate treatments appear to slightly stimulate biodegradation, as determined by OHR rates, while strong permanganate doses disrupt PCE degradation to vinyl chloride and cause a 2-4 orders of magnitude decrease in the abundance of organohalide respiring bacteria (OHRB) and reductive dehalogenase genes (*rdh*). Microbial community composition based on pyrosequencing of 16S rRNA gene

fragments indicates that chemical oxidation appears to select for *Deltaproteobacteria*, especially *Geobacter*, and *Epsilonproteobacteria*, especially *Sulfurospirillum*. The abundance of OHRB and *rdh* gene abundance is also followed during ISCO treatment with persulfate at a PCE and trichloroethene (TCE) contaminated location ([Chapter 9](#)). While natural attenuation capacity is measured prior to treatment, ISCO caused a significant drop in the abundance of OHRB and *rdh* genes, most likely due to groundwater acidification ($\text{pH} < 3$) and high oxidation-reduction potential (> 500 mV). Though regeneration of the OHRB community is observed six months after ISCO, *Dehalococcoides mccartyi* and *rdh* genes are absent, indicating a long-term disruption of full microbial natural attenuation capacity.

The outcomes of this dissertation provide insight into the dynamics of subsurface microbiological and geochemical processes ([Chapter 10](#)). The value of coupling ISCO with ISB is supported by the improved remediation efficiencies achieved when chemical oxidation is applied rather than bioremediation alone. Studies with chemical treatment illuminate microbial resilience, as microbes are not only able to regenerate following harsh oxidation, but in many cases a slight enrichment in biodegradation capacity is also observed. Chemical oxidation causes changes to subsurface geochemistry, including organic matter structure, mineralogy, and groundwater composition, which can have irreversible implications on microbial community structure and function. Therefore, molecular tools are required in order to properly illuminate the impact of contamination and remediation on microbial communities. Such knowledge on the resilience and sensitivity of soil and groundwater ecosystems to stress conditions thus furthers the field of microbial ecology. Properly executed research on these subsurface systems will go beyond contributing to the field of soil remediation to provide a meaningful exploration of essential microbiological and geochemical processes in the environment. The results of this dissertation are an important new step towards examining contaminated locations and remediation from a fundamental scientific perspective.

Chapter 1

General Introduction:

**Researching *in situ* remediation technologies for
organic contaminants in subsurface systems**

Preface

The importance of the subsurface for aboveground activities is often underappreciated. Soil is commonly acknowledged as a physical platform for building infrastructure, and as a resource essential to food and biomass production. However, the subsurface is also a habitat for a diverse ecosystem of both simple and complex organisms. Some portions of the subsurface ecosystem play a role in nutrient fixation and cycling. Soil is an essential compartment for carbon dioxide sequestration, thereby mitigating climate change. Water infiltration during heavy rainfall events reduces flooding and groundwater extraction provides clean drinking water resources. The capacity for the subsurface to transform or sequester contaminants and pathogens is an important process in the water cycle.

The complexity of the subsurface supports the aforementioned functions while spurring scientific interest into the processes that occur belowground. The organic and inorganic components of soil are either found below the groundwater level or in the vadose zone, with physical-chemical laws dictating the transport and distribution of compounds between the solid, liquid, and gas phases [74]. The interconnectedness of these portions of the subsurface means that changes in groundwater level or flow, oxidation-reduction potential, erosion or deposition, and groundwater composition affect the entire system. Alterations to any component of the system in turn impact microbial diversity, which has implications for nutrient reservoirs, contaminant degradation, and survival of soil microflora and fauna and above ground plants and animals. Finally, whereas surface systems can be readily accessed, the inaccessibility of the subsurface requires innovative and extensive monitoring to understand these processes [124].

Contamination threatens the capacity of the subsurface to fulfill many of its essential functions. High concentrations of pollutants prevent the use of land for food and building purposes, interrupt habitats, and overwhelm the natural degradation capacity of the subsurface [260, 261]. The environmental and economic losses associated with contamination support research into soil remediation and expansion of the remediation industry. However, from a scientific perspective, contamination is merely an additional component in the subsurface system. In some cases, pollutants may simplify the system, by executing environmental pressure in terms of toxicity or homogeneous electron donor or acceptor availability. On the other hand, the transport, distribution, transformation, and full degradation of contaminants add additional layers of complex interactions to be considered [214]. The practical

problems of contamination may drive the development of soil remediation techniques [239]. However, contaminated locations and remediation sites offer a tremendous opportunity to conduct fundamental research into geochemical and microbial responses to these extreme perturbations. New fundamental knowledge may in turn also offer insight into improving current, or designing new, technological approaches. This dissertation presents investigations into *in situ* remediation of organic contaminants, giving balanced and integrated insights towards improved remediation technologies based on advancing the fundamental understanding of subsurface processes in response to artificially introduced, drastic changes.

1.1 Subsurface Contamination

While natural deposition of contaminants such as arsenic [63] and methane [38] does occur, most environmental risks are related to human release of pollutants. In industrial, urban, and agricultural areas, soil and groundwater contamination often occurs due to anthropogenic activity. In industrial areas, accidental spillage, leakage of surface or underground storage tanks and pipes, and intentional discarding of contaminants through effluents and chemical waste into the environment have polluted the subsurface. A broad spectrum of industries and activities contribute to the problem, with the distribution and prevalence of these contamination sources varying regionally (Figure 1.1). In The Netherlands, more than 600000 potentially polluted sites were identified in 2004 and, as of 2009, between 4800 and 7000 urgent locations required further remediation [211].

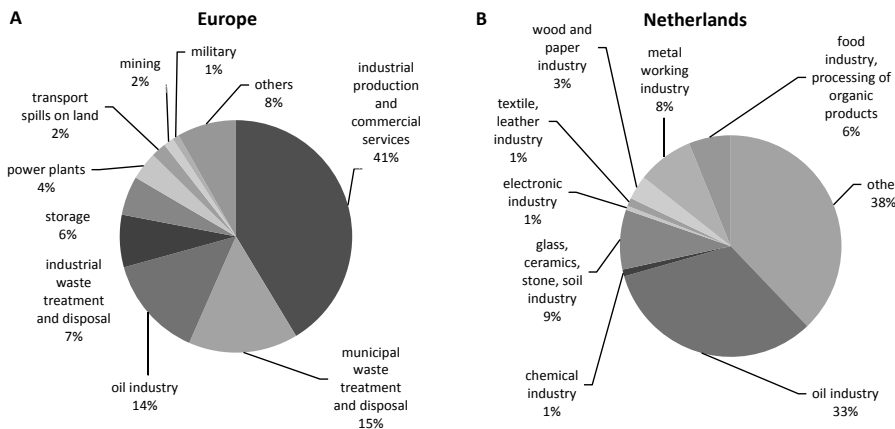


Figure 1.1 Activities causing contamination of the subsurface in (A) Europe and (B) The Netherlands. The main sources of contamination are related to industrial activities, the energy industry, and waste treatment and disposal [58].

The types of chemicals encountered in the subsurface are as diverse as the sources for their origin. While the prevalence of inorganic contaminants such as heavy metals poses a significant environmental risk (Table 1.1), the emphasis in this dissertation will be placed on organic contaminants. These include petroleum derived hydrocarbons (so-called total petroleum hydrocarbons; TPH); mono-aromatic compounds such as benzene, toluene, ethylbenzene, and xylenes (BTEX compounds); polycyclic

aromatic hydrocarbons (PAHs); and chlorinated hydrocarbons. The behavior and environmental fate of each compound depends upon the physical properties of both the contaminant and the subsurface. Contaminants are predominantly encountered dissolved in groundwater, as pure product, or sorbed to soil. Each compound's properties, such as solubility and hydrophobicity, control the migration and fate of these pollutants in the subsurface. At high concentrations, organic contaminants form pure product pools known as non-aqueous phase liquids (NAPLs; [279]). Depending on the relative density of the chemical compared to water, contaminants may either form a pool floating on top of the groundwater table (light non-aqueous phase liquids; LNAPLs) or sink through the aquifer to accumulate on impermeable layers (dense non-aqueous phase liquids; DNAPLs; [111]). With groundwater flow, NAPLs slowly dissolve and are transported, thus creating a source which can continuously feed a plume of contaminated groundwater [111]. Additionally, soil characteristics, such as soil organic matter, clay content, and hydraulic conductivity, control the partitioning between the solid phase and aqueous and NAPL phases. In organic-rich soil contaminated with hydrophobic contaminants, sorption onto soil organic matter is an important sequestration process.

Table 1.1 Relative prevalence of different contaminant types throughout Europe, in The Netherlands, and at US Superfund sites [57, 233].

Contaminant	Prevalence (%)		Contaminant	United States Percentage of total NPL sites ^g
	Europe	The Netherlands		
Heavy Metals	37	10	Arsenic	68
Mineral Oil	34	8	PAHs ^a	42
PAHs ^a	13	13	Benzene	59
BTEX ^b	6	6	Ethylbenzene	49
Phenols	4	15	Xylenes	51
Chlorinated Hydrocarbons	2	3	PCB ^c	31
Cyanides	0	14	PCE ^d	54
Others	4	31	TCE ^e	60
			VC ^f	37

^a PAH: polycyclic aromatic hydrocarbon

^b BTEX: benzene, toluene, ethylbenzene, xylenes

^c PCB: polychlorinated biphenyl

^d PCE: tetrachloroethene

^e TCE: trichloroethene

^f VC: vinyl chloride

^g National Priorities List ("Superfund sites")

1.2 Remediation Technologies

1.2.1 General Introduction

Owing to the prevalence, environmental and health risks, and economic consequences of subsurface contamination, a large market exists for the remediation of these locations [239]. In some areas, remediation is mandated to mediate toxicological risks or to prevent contamination of important receptors such as drinking water supplies and surface waters. Additionally, contamination often impedes further redevelopment of brownfields; that is, neglected sites previously used for industrial or commercial purposes. The higher costs associated with the remediation activities required at a brownfield, compared to developing agricultural or natural greenfields into commercial or industrial areas can prohibit redevelopment of these sites. To stimulate redevelopment, low-cost, robust technologies are required that can rapidly and predictably remediate a location.

Soil and groundwater remediation techniques are based on physical, chemical or biological processes which can be performed *in situ* or *ex situ*. The advantages and disadvantages of each technology depend upon the site, contaminant characteristics, and remediation goals. Traditional *ex situ* techniques such as soil washing, biopiles, landfarming, and incineration, often include excavation and disposal of at least a portion of the contaminated soil. *Ex situ* technologies are applied under controlled conditions, allowing good process monitoring and estimation of remediation efficiencies [106].

In the last 15-20 years, the application of *in situ* technologies has increased, accounting for approximately 43% of remediation projects in Europe (Figure 1.2; [59, 111]). *In situ* remediation techniques can be more cost-effective and sustainable than conventional technologies. Additionally, there is more flexibility in the design and application of *in situ* treatments, thereby allowing tailoring of the treatment to reduce disruption of infrastructure. This final point is especially important when dealing with urban brownfields, which often contain significant aboveground structures and belowground infrastructure (such as pipes and conduits).

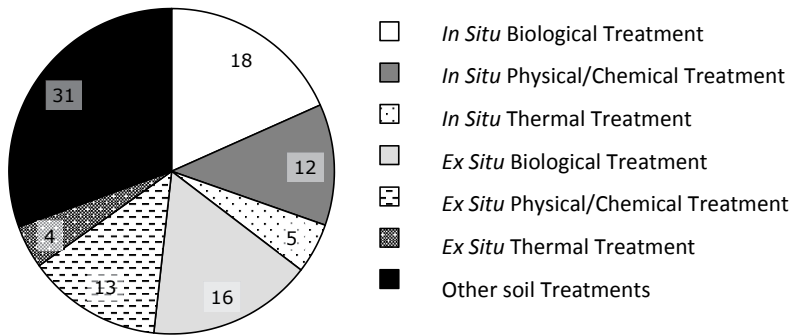


Figure 1.2 Relative application of different remediation technologies across Europe given as percentage. All *ex situ* treatments assume excavation prior to physical, chemical, biological, or thermal treatment [59].

In situ technologies can also be better integrated into projects focusing on risk-based remediation. Whereas previously remediation projects often aimed at complete removal of all contaminants with excavation, goals have been refocused with the realization of the economic and environmental costs associated with such extensive clean-up [281]. Risk-based remediation focuses on mediating acute exposure and toxicity hazards while attempting to determine and reduce long-term impacts [216]. In source areas, this can mean reducing the mass of pure product present in order to interrupt dissolution and expansion into the plume [281]. Similarly, receptors can be protected from contamination with technologies which remove or degrade pollutants immediately upstream from receptors, such as reactive barriers [110]. *In situ* remediation technologies are especially fit for these applications due to the breadth of techniques and design options available.

1.2.2 *In situ* chemical oxidation (ISCO)

Technologies using strong chemical oxidants for treatment of organic residuals in water were initially developed for above-ground technologies such as *ex situ* pump-and-treat of contaminated groundwater [233]. Commercial application of *in situ* chemical oxidation (ISCO) started in 1984 with the treatment of formaldehyde with hydrogen peroxide [28]. Since then, ISCO has developed into a full-fledged commercially viable remediation technology for the rapid degradation of organic contaminants (Figure 1.3; [233]). A wide variety of chemical oxidants is available with differing oxidation potentials, application requirements, and modes of oxidation (Table 1.2). Oxidants such as permanganate directly gain electrons in the oxidation of

organic contaminants [96]. On the other hand, some ISCO treatments employ radicals produced by activation of the oxidant. Examples include activation of persulfate to produce the sulfate radical ($\text{SO}_4^{\cdot-}$), and Fenton's reagent, in which hydroxide radicals OH^{\cdot} are produced by activating hydrogen peroxide with ferrous iron [12, 96]. The reactivity of each ISCO treatment towards specific contaminants differs, with certain oxidants more applicable to a wider range of contaminant types (Table 1.2; [112]). Differences in reactivity are to some extent related to the mode of oxidation and the stability of the oxidant in the field. However, contaminant characteristics such as solubility, soil-water partitioning, and extent of weathering should be considered when choosing a chemical oxidant [233]. Finally, soil properties such as organic matter content, soil oxidant demand, soil buffering capacity, permeability, and mineralogy also determine the efficiency of an ISCO treatment [111, 233].

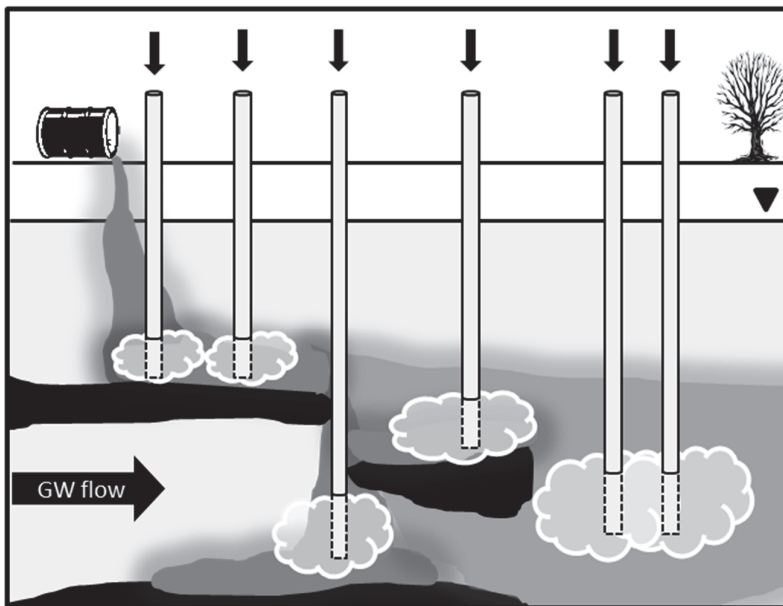


Figure 1.3 Schematic of contaminated location treated with ISCO. The contaminant sinks through the subsurface and pools as DNAPL (dark gray) on impermeable layers (black), feeding a dissolved plume (light gray). Chemical oxidants (white) are applied via wells in source and plume areas.

When properly designed, ISCO treatments have a number of advantages over other *ex situ* and *in situ* technologies. ISCO treatments are:

- (1) rapid, with treatment times ranging from days to months (Table 1.2; [112]),
- (2) versatile, allowing simultaneous remediation of a number of pollutants at a multi-contaminant site [112],
- (3) robust, meaning that, with proper site characterization and treatment design, good remediation efficiencies can be reproducibly achieved [233],
- (4) complete, allowing full conversion of organic contaminants to innocuous compounds such as CO₂ and Cl⁻ [96].

That said, chemical oxidants continue to be associated with high economic and environmental costs. While ISCO treatments are designed to optimize contaminant degradation, chemical oxidants also react with soil organic matter, resulting in a higher price for additional remediation product, degradation of the soil matrix, and a decrease in the sustainability of the overall treatment [238]. Additionally, certain chemical oxidants can require non-neutral pH (for example, Fenton's reagent and alkaline activated persulfate), cause groundwater acidification (persulfate), or result in reduced soil permeability (permanganate, due to precipitation of MnO₂), all of which have implications for long-term soil function [96, 235, 272]. Finally, proper monitoring of ISCO treatments is challenging, as both displacement of contaminated groundwater with oxidants and rebound due to dissolution of residual contaminants can preclude proper estimates of treatment efficiency [173, 193]. The increasing frequency with which ISCO is applied in the field, and the rising number of scientific publications contribute to improvements in site characterization, application techniques, and performance monitoring, thereby furthering the technical and practical knowledge of ISCO [233].

Table 1.2 Overview of chemical oxidant characteristics and contaminant reactivity [12, 96, 112].

Oxidant	Chemical Formula	Oxidation Potential (V)	Applicable Contaminants	Not reactive	Chemical activity
Fenton's Reagent	H ₂ O ₂ activated with Fe ²⁺	OH [•] : 2.8	(chloro)ethenes, (chloro)ethanes, BTEX ^a , light fraction TPH ^b , PAH ^c ; free cyanides, phenols, phthalates, MTBE ^d	heavy fraction TPH ^b , higher alkanes, heavy fraction PAH ^c , PCB ^e , complex cyanides	less than 1 day
Ozone	O ₃	Peroxide activated (OH [•]): 2.8 Inactivated (O ₃): 2.6	(chloro)ethenes, BTEX ^a , TPH ^b , PAH ^c , free cyanides, phenols, phthalates, MTBE ^d	heavy fraction, PAH ^c , PCB ^e , complex cyanides	1-2 days
Persulfate	S ₂ O ₈ ²⁻	Activated (SO ₄ ^{•-}): 2.6 Inactivated (S ₂ O ₈ ²⁻): 2.0	(chloro)ethenes, (chloro)alkanes, BTEX ^a , light fraction PAH ^c ; free cyanides, phenols, phthalates, MTBE ^d	heavy fraction PAH ^c , PCB ^e	weeks to months
Permanganate	MnO ₄ ⁻	MnO ₄ ⁻ : 1.7	Chloroethenes, TEX ^a , phenols	Benzene, (chloro)alkanes, TPH ^b , PAH ^c , PCB ^e , cyanides	weeks

^a BTEX (or TEX): benzene, toluene, ethylbenzene, xylenes

^b TPH: total petroleum hydrocarbon

^c PAH: polycyclic aromatic hydrocarbon

^d MTBE: methyl tert-butyl ether

^e PCB: polychlorinated biphenyl

1.2.3 *In situ* bioremediation (ISB)

The innate microbial capacity to biodegrade a variety of organic contaminants is widespread and increasingly being harnessed for the remediation of contaminated soil and groundwater. Given the proper subsurface conditions, many contaminants are degraded without intervention through natural attenuation (NA; [111]). However, amendments such as electron donors, electron acceptors, nutrients, and bio-augmentation are often added to enhance degradation conditions and ensure biodegradation capacity. The choice of amendments depends on contaminant and site characteristics. As microbial metabolic capacity is often determined by oxidation-reduction potential, treatments most often focus on the addition of electron acceptors or electron donors [75].

Bioremediation of aliphatic and aromatic hydrocarbon is most effective under oxic conditions via aerobic pathways (Table 1.3; [111, 167]). The first step in degradation of aliphatic hydrocarbons is hydroxylation catalysed by hydroxylases or oxygenase enzymes (Figure 1.4; [276]). A complete analysis of TPH degradation pathways, transition products, and enzymes is challenging, as diesel, oil, and fuel consist of many different chemical compounds. Thus, biomolecular assays have been designed to focus on determining microbial capacity for the initial hydroxylation step, which is essential to further biodegradation of aliphatic hydrocarbons. Investigations often aim at quantifying the capacity for biodegradation based on the abundance of specific hydroxylases or oxygenase enzymes, rather than determining microbial diversity or identifying specific microorganisms involved in biodegradation [199].

Biodegradation of chlorinated hydrocarbons takes place under strictly anoxic conditions [167]. In contrast to TPH, which is an electron donor, chlorinated solvents act as electron acceptors during biodegradation in a process known as organohalide respiration (OHR). Degradation of tetrachloroethene (PCE) occurs via sequential substitution of chlorine atoms for hydrogen atoms to produce daughter products trichloroethene (TCE), dichloroethene (DCE), vinyl chloride (VC) and finally ethene (Figure 1.5; [163]). Degradation steps are either metabolic or co-metabolic, whereby transformation transpires without producing energy. A limited number of microorganisms are able to perform OHR, with *Dehalococcoides mccartyi* the only organism able to completely transform PCE to ethane [143]. Significant research focuses on identifying degraders, known as organohalide respiring bacteria (OHRB), and the genes encoding for reductive dehalogenase enzymes (*rdh*). As a result, a number of biomolecular assays are available to monitor OHRB and *rdh* in the environment to determine the capacity for degradation (using DNA-template) and degradation activity (by measuring mRNA; [153]).

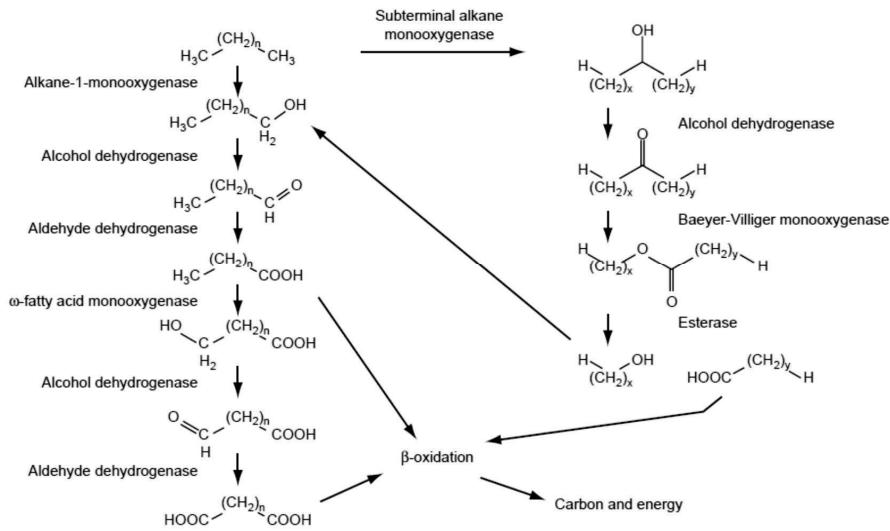


Figure 1.4 Aerobic degradation pathways for alkanes. The initial step involves either terminal or subterminal oxidation via an alkane-monoxygenase. (Figure from [277]).

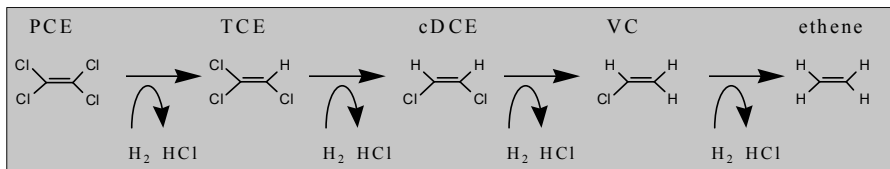


Figure 1.5 OHR of tetrachloroethene (PCE) to harmless ethene [241].

ISB has gained widespread acceptance as a low-cost, sustainable technology that can provide full remediation of a contaminated location. With the advent of molecular techniques to monitor microbial diversity, the abundance of degraders, and degradation capacity based on quantification of genes specific for contaminant conversion, prediction of bioremediation potential and field monitoring have been improved [153]. The main limitation of ISB is the moderate rate of microbial biodegradation, with full remediation often necessitating years even under optimal conditions [233]. Additionally, ISB is only applicable to the bioavailable portion of the contaminant. Compounds strongly sorbed to the soil matrix or present as NAPL must first become bioavailable through dissolution into the water phase before biodegradation can occur [229, 230]. Finally, in the case of chlorinated ethenes, toxic

intermediates can accumulate [233]. Nonetheless, ISB remains popular due to the flexibility of design, versatility of application, and low remediation costs [233].

Table 1.3 Biodegradation of organic contaminants (adapted from [111] with input from [275]).

Contaminant	Electron acceptor					OHR	co-metabolic reductive dechlorination
	oxygen	nitrate	iron(III)	sulfate	carbon dioxide		
Benzene	++	+	+	+	+		
Toluene	++	+	+	+	+		
Ethylbenzene	++	+	+	+	+		
Xylenes	++	+	+	+	+		
naphthalene	++	+	~	+	~		
higher PAH	+	-	-	-	-		
Alkanes	++	+	+	+	+		
PCE ^b	-	-	-	-	-	++	+
TCE ^c	(+) ^a	-	-	-	-	++	+
DCE ^d	(+) ^a	?	?	-	-	+	+
VC ^e	++	+	+	?	?	+	+

++ easily degradable, + degradable, ~ possibly degradable, - not degradable, ? unknown or inconclusive

^a co-metabolic degradation

^b PCE: tetrachloroethene

^c TCE: trichloroethene

^d DCE: dichloroethene

^e VC: vinyl chloride

1.3 Coupling ISCO and ISB

In addition to improvements in application of single technologies, increasing focus is being placed on treatment trains whereby multiple remediation technologies are applied at one site. Owing to the flexibility in design, treatment trains can, when properly implemented, be cost-saving and highly efficient. The application of each individual technology can be tailored to optimize treatment of a certain pollutant or contaminated area, thereby improving the overall remediation efficiency. Additionally, such biphasic treatment is a unique system in which to investigate the impacts of remediation on microbial ecology and subsurface processes. By approaching research from a more fundamental perspective studying geo- and biochemical perturbations and responses, knowledge is gained that can be translated beyond the field of soil

remediation into interactions between geochemical changes and microbiological population dynamics.

1.3.1 Advantages of coupling ISCO and ISB for remediation of organic contaminants

Treatment trains coupling ISCO and ISB have gained attention in the past decade. Initial interest was sparked by the realization that microbial communities were able to regenerate in field systems without amendment. While it was assumed that the harsh conditions during chemical oxidation were inhospitable to degraders, it has been observed that microorganisms were resilient towards ISCO treatments [172, 217]. With this came the recognition that a combination of chemical oxidation with bioremediation has a number of advantages.

Coupling ISCO with ISB has the potential to provide significant cost-savings. Chemical oxidation is relatively expensive, both in terms of consumables such as chemical oxidants and activators, and personnel costs for skilled workers required to perform and monitor injection [238]. In contrast, ISB projects are generally less costly [233]. Thus, if a portion of the contamination can be handled with ISB technology, the ISCO treatment can be minimized, thereby reducing the overall cost of remediation.

Improved remediation efficiencies can also be achieved when a bio-polishing step is performed following ISCO treatment to biodegrade residual contaminants. Research has shown that chemical oxidation can actually stimulate bioremediation by improving the bioavailability and biodegradability of the contaminant [121, 176]. Partial oxidation of especially non-polar contaminants (such as oil and diesel) increases the hydrophilic content of the compounds to be treated, thereby improving the overall remediation through increased dissolution and improved microbial degradation. Additionally, bio-polishing may be used to deal with rebound of contaminants in groundwater or soil vapor once all chemical oxidants have been consumed. Following ISCO, rebound of groundwater concentrations can occur due to either desorption of sorbed contaminants or dissolution of residual NAPL pools [172, 173]. For both processes, bioremediation of this residual contamination would mitigate risk and ensure continued degradation of rebound contaminants [172, 217]. Overall, a bio-polishing step following ISCO has the potential to yield higher remediation efficiencies than either chemical or biological treatment alone.

Finally, proper design and implementation of a coupled ISCO with ISB has the potential to be a more sustainable treatment, a topic of increasing importance [66, 82, 237]. The advantages of reducing the quantity of chemical oxidants used and the number of injection events are clear for reduction of greenhouse gas emissions

and preservation of the soil matrix [238]. Also, initiating an ISB step through the use of amendments following ISCO can be beneficial for improving soil quality. The negative effects of ISCO, such as changes in subsurface geochemistry and disruption of normal soil function, can to some extent be reversed through microbial activity. Thus, coupling ISCO with ISB has important implications for improved sustainability of the treatment as such and for maintaining subsurface function.

1.3.2 Research opportunities related to coupling ISCO and ISB

With the proven feasibility of consecutive chemical oxidation and bioremediation, a number of research opportunities are available in this field. Investigations into further optimizing biphasic treatment are necessary to improve application and thus remediation efficiencies. Such work is, by nature, applied science; however, a more fundamental research approach can be implemented. The impact of ISCO on subsurface geochemistry and microbiology is significant. Harsh oxidizing conditions, groundwater acidification or alkalization, and changes to the oxidation-reduction potential can have implications for microbial community size and biodegradation potential [51, 172, 217, 272]. Additionally, irreversible degradation of the soil matrix through oxidation of soil organic matter, and reduced inorganic constituents after chemical oxidation, can have long-term implications for the availability of substrates and nutrients for microorganisms [104, 185, 240, 289]. These changes naturally have consequences for remediation efficiency and cost-effectiveness. However, in-depth research into the perturbations to the subsurface from chemical oxidation can go beyond refining soil remediation practices towards an improved understanding of subsurface processes. As described in the preface, the subsurface is a complex, dynamic system. Contamination, and *in situ* treatment thereof, add an additional dimension to the system. Investigations can provide insight on soil mineralogy and structure, microbial ecology, and the dynamics of geochemical interactions between soil and groundwater. Thus, when properly approached, studies on contamination and remediation offer a variety of research opportunities in fundamental scientific fields.

To this end, research should consider the subsurface as an integrated system where soil, groundwater, microorganisms, and contaminants are interacting compartments (Figure 1.6). In contrast, previous studies investigating contaminated soil systems or *in situ* remediation techniques often focus on only certain aspects of the system. In general, laboratory-based studies often aim only at improving remediation efficiencies [72, 121, 271]. Conclusions on more fundamental knowledge gaps, such

as changes in subsurface geochemical parameters and shifts in microbial community structure, are often neglected or even avoided in remediation-optimization oriented studies, even though these may also influence remediation efficiencies. For example, in some studies, the impact of oxidation on soil, groundwater, and contaminant parameters is considered, however, these results are not extrapolated to consider the impact of such changes on the microbial community [72, 176, 183, 274]. Similarly, investigations often focus either on the detrimental effect of ISCO on microbial communities or on improvements in bioavailability and biodegradability after oxidation, without integrating the two subjects [103, 128, 176, 188, 189, 299]. These studies do not consider the subsurface as an integrated system and thus cannot be used to further our understanding of subsurface processes. The results from such compartmentalized investigations give information on certain parameters within the described experimental conditions. However, as the system is not investigated in an integrated manner, it is difficult to extrapolate results to other environmental conditions or translate conclusions into general knowledge on subsurface processes. These limitations in experimental design and research focus preclude further advancing our fundamental understanding of biphasic soil treatment.

Soil contamination and remediation thereof provide unique opportunities to investigate the impact of alterations in subsurface parameters on the microbial community. Changes in substrate availability, pH, oxidation-reduction potential, and nutrient concentrations, in addition to oxidative stress from chemical oxidation, have implications for community structure and function. Properly-designed investigations into the impact of contamination and chemical oxidation on microbial communities can contribute to the field of microbial ecology. While microbiological analyses have been included in chemical oxidation studies, previous experimental setups focused on culture-dependent techniques such as determining the number of heterotrophic bacteria (colony forming units; CFUs; [103, 105, 177]. This line of research provides little information on the entire system and does not contribute to our fundamental understanding of soil microbial community structure and function. The application of culture-independent molecular tools for the illumination of full microbial diversity and degradation capacity contributes to our fundamental understanding of microbial response to changes in environmental conditions [2, 55, 155]. Diversity analyses, based on the 16S rRNA gene, such as denaturing gradient gel electrophoresis (DGGE) and next generation sequencing (NGS), and capacity assays, such as quantitative PCR to assess the abundance of specific degraders or metabolic genes, are becoming increasingly routine and cost-effective. Such tools are essential to fully appreciate

the impact of chemical oxidation on the microbial community, and for designing treatments that support regeneration. Moreover, contaminated locations are often well characterized, with both extensive monitoring wells in place and long-term monitoring results available. By integrating molecular results with more traditional measurements of contaminant concentrations and geochemical constituents, general knowledge on microbial resilience to chemical oxidation and regeneration thereafter can be attained. Such knowledge will prove integral in further optimization and monitoring of coupled treatment, while simultaneously giving new insight into microbial community structure and function.

Locations contaminated with chlorinated solvents present an especially interesting system for investigation. OHR, an extensively studied biodegradation pathway, is performed by a specific set of well-characterized microorganisms under strictly anaerobic conditions. ISCO treatments can stimulate bioremediation of alkanes and aromatic hydrocarbons by the addition of electron acceptors such as sulfate (from persulfate treatment), manganese oxides (from permanganate), iron (used as a catalyst), or oxygen (from hydrogen peroxide or ozone treatment; [172]). In contrast, an increase in the oxidation-reduction potential interrupts OHR and can actually be fatal for OHRB. Considering the prevalence of locations contaminated with chlorinated solvents and the frequency with which ISCO is used to remediate these locations [57, 233], implementation of a bio-polishing step following chemical oxidation should be investigated to improve state-of-the-art soil remediation practices. Preliminary work with column studies indicates that regeneration of degradation capacity is possible, supporting development of biphasic treatment for chlorinated solvents [87, 218]. However, investigations on the impact of chemical oxidation on OHRB and OHR activity are also attractive from a pure scientific perspective. OHRB, an anomaly in microbial metabolic capacity, has been investigated extensively, yielding a good understanding of the genes and enzymatic systems involved in OHR and biomolecular assays available for monitoring. System requirements such as electron donor and vitamin concentrations as well as degradation rates and products are well established. Impacts of chemical oxidation such as oxidative stress, decrease in substrate availability, and increase in the oxidation-reduction potential are environmental stressors that give important information on OHRB resilience. In systems where regeneration of OHR activity occurs following chemical oxidation, molecular tools for illuminating community structure and for quantifying OHRB and *rdh* genes should be applied. By thoroughly characterizing shifts in the microbial community following chemical oxidation and during subsequent bioremediation,

insights are gained into OHRB resilience and the symbiotic processes required for regeneration. In addition to improving biphasic soil remediation, such investigations would contribute significantly to the field of microbial ecology.

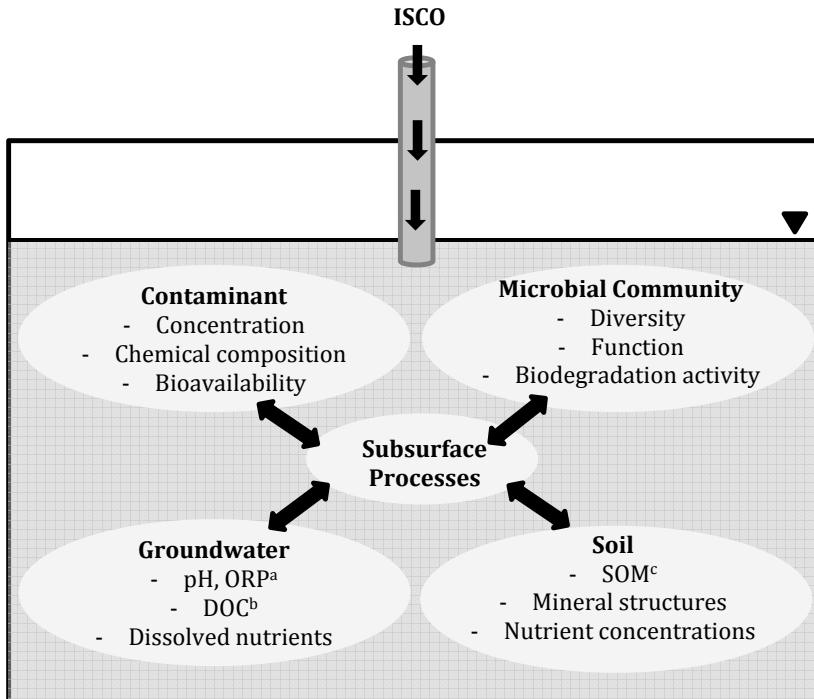


Figure 1.6 Schematic of the interconnectedness of different subsurface components. An integrated approach to research on the impact of ISCO treatments must consider the contaminant, microbial community, groundwater, and soil characteristics as portions of one system, all of which play a role in subsurface processes.

^a oxidation-reduction potential

^b dissolved organic carbon

^c soil organic matter

Finally, in addition to work with microcosms and column experiments under controlled lab conditions, field investigations are required. Often, observation of regeneration of biodegradation capacity following chemical oxidation is a chance circumstance; full field investigations into ISCO and ISB are limited [232]. Bioremediation is rarely an integrated component of either the treatment design or monitoring plan in soil management practice and is not reported in peer-reviewed scientific literature [148, 149]. Finally, the use of molecular tools to monitor microbial communities before, during, and after field ISCO treatment is sparse. Field investigation into the post-oxidation impacts of chemical oxidation on subsurface processes and microbial community give insights into the dynamics of an open system, where groundwater flow can neutralize pH changes, transport substrates and nutrients, and reintroduce endogenous bacteria affected by ISCO treatment. Such knowledge from field demonstrations is required to better understand subsurface processes and can, in turn, improve the design of combined chemical and biological soil treatment.

1.4 Structure of this research

As described above, coupling of ISCO with ISB into a treatment train is a potentially cost-effective and sustainable technology offering higher remediation efficiencies than either treatment alone. Moreover, such biphasic treatments offer a unique and interesting system to research subsurface processes. Chemical oxidation changes groundwater, soil, contaminant, and microbial community characteristics, which can be investigated to improve knowledge on subsurface processes (Figure 1.6). Improved versatility, predictability, and acceptability of coupling ISCO with ISB is required to fully exploit the advantages of biphasic treatment. This fundamental understanding of the system is required to further develop such applications into a robust cost-effective technology. When properly executed, the results of such investigations will go beyond applications in soil remediation to contribute to such fields as soil science, geochemistry, and microbial ecology. This work must approach the system in a multi-disciplinary manner. The experimental methodology should be designed from the outset to integrate results of microbiological analyses using molecular tools with the characterization of contaminant, soil, and groundwater properties before and after remediation. Additionally, emphasis should also be placed on more challenging research areas such as coupling chemical oxidation and bioremediation for the treatment of chlorinated solvents and full-fledged field experiments.

This dissertation focuses on the aforementioned research opportunities, approaching the work from a more fundamental integrated investigation of the system (Figure 1.7). The experimental designs aim to improve coupling of chemical oxidation with bioremediation while providing insight into more fundamental questions on subsurface processes, soil and groundwater interactions, and subsurface biology. Investigations include two major classes of contaminants, namely TPH and chlorinated solvents, in order to determine the impact of chemical oxidation on either an aerobic or anaerobic biodegradation step.

While traditional degradation experiments coupling chemical oxidation with bioremediation are performed, factors such as contaminant bioavailability, microbial diversity and degradation capacity, and changes in soil and groundwater geochemical parameters are considered individually, and then integrated to understand the entire system. To this end, a wide array of analytical methods from a variety of disciplines are included in laboratory experiments under controlled conditions, and in field investigations at actual contaminated locations. Through utilizing an assorted toolbox of techniques in diverse experimental setups, this research aims to come to an integrated understanding of the processes occurring during and following chemical oxidation. The results do give multi-disciplinary recommendations on improved application of biphasic treatment. However, this outcome is a secondary coincidental factor to primary research questions on the subsurface system and its dynamic processes.

Part One of this dissertation aims at further examining the motivation for performing ISCO followed by bioremediation. First of all, [Chapter 2](#) provides a thorough review of the current literature in order to both ascertain efforts to optimize treatment and identify knowledge gaps in our understanding of the system. Thereafter, limitations to bioremediation of TPH (diesel) contaminated soil are investigated in the lab in [Chapter 3](#) and in the field in [Chapter 4](#). Through integrating biodegradation experiments, bioavailability measurements, and advanced molecular tools, it was determined that in certain soil types TPH bioavailability ([Chapter 3](#)) and subsurface oxidation-reduction potential, as illuminated by microbial community analysis ([Chapter 4](#)), restrict bioremediation. Additionally, the integration of soil and contaminant characteristics with NGS results gave insight into the relationships between contamination presence and microbial community structure *in situ*, thereby illuminating the selective pressure of contamination ([Chapter 4](#)). These findings provide overall support for the application of chemical oxidation, as it can improve bioavailability and provide electron acceptors for bioremediation of TPH.

In **Part Two**, the coupling of chemical oxidation with aerobic bioremediation for the treatment of TPH contaminated soils is investigated. Laboratory and field studies examine the influence of chemical oxidation on soil and groundwater geochemical parameters as well as the microbial community in order to understand the short- and long-term implications of each for the whole system. In [Chapter 5](#), the impact of soil organic matter degradation due to chemical oxidation with either Fenton's reagent or Modified Fenton's reagent on dissolved organic carbon and aqueous nutrient concentrations is examined. Through monitoring dissolved carbon and nitrogen species following chemical oxidation and during extended bioremediation, the influence of soil degradation on a subsequent bio-polishing step is investigated. In order to understand the effect of different chemical oxidants on microbial community dynamics, in [Chapter 6](#), a survey of four chemical oxidation treatments coupled with bioremediation is performed. Here, molecular tools are utilized to determine the impact of each ISCO treatment on the microbial community and monitor regeneration of diversity and degradation capacity. In [Chapter 7](#), the approaches from [Chapters 5 and 6](#) are integrated into a field experiment in which a persulfate ISCO treatment is followed by enhanced ISB with nutrient amendment. Extensive monitoring of groundwater parameters, changes in soil organic matter and mineralogy, and quantification of microbial community size and degradation capacity give insight into subsurface processes.

In **Part Three**, research is performed on the coupling of chemical oxidation with anaerobic bioremediation for the treatment of chlorinated hydrocarbons. Here, molecular tools for the quantification of OHRB and *rdh* genes involved in the degradation of chlorinated ethenes and NGS analysis of microbial community diversity are used to ascertain the impact of chemical oxidation on biodegradation capacity. In [Chapter 8](#), liquid microcosm experiments are used to determine changes in the microbial community upon oxidation with permanganate. By combining diversity analyses of the entire community with quantification of specific degraders and degradation genes, this work gives insight into microbial resilience under oxidative stress, and identifies portions of the microbial consortium essential for regeneration of OHR activity following chemical oxidation. In [Chapter 9](#), field monitoring of OHRB and *rdh* genes is performed at a site contaminated with PCE and TCE that is treated with persulfate. By determining NA potential before treatment and measuring the immediate and long-term impact of ISCO, this research gives insight into microbial regeneration of OHR capacity in an open field system.

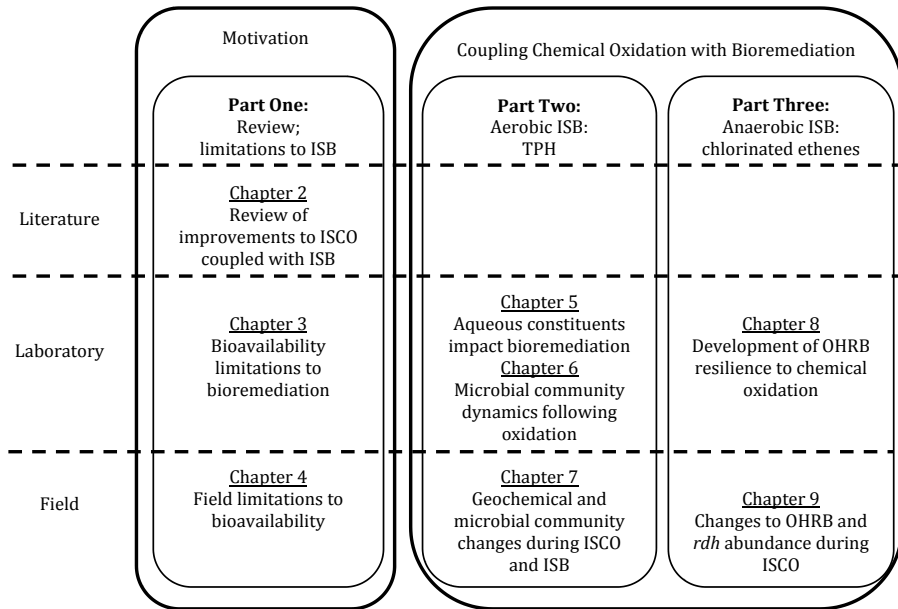


Figure 1.7 Schematic representation of research areas covered in this dissertation

Finally, in Chapter 10, a synthesis of the results of this work is presented. This dissertation presents a novel and unique approach to soil remediation research. As a whole, the work includes scientifically-driven experimental design and integration of lab and field investigations aimed at a fundamental understanding of subsurface processes during *in situ* soil remediation. In Chapter 10, the results of each chapter are unified and interpreted, giving knowledge on subsurface processes, microbial resilience and regeneration, and the application of molecular tools to guide biphasic treatment. The major outcomes of this dissertation provide specific recommendations for continued research on the microbial and geochemical dynamics in biphasic subsurface treatment systems as well as general guidelines for the further optimization of commercial application of ISCO followed by ISB.

Part One

Chapter 2

Efforts to improve coupled *in situ* chemical oxidation with bioremediation: a review of optimization strategies

Chapter 3

Biodegradation of aged diesel in diverse soil matrixes: impact of environmental conditions and bioavailability on microbial remediation capacity

Chapter 4

Impact of long-term diesel contamination on soil microbial community structure

Chapter 2

Efforts to improve coupled *in situ* chemical oxidation with bioremediation: a review of optimization strategies

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Abstract

In order to provide highly effective yet relatively inexpensive strategies for the remediation of recalcitrant organic contaminants, research has focused on *in situ* treatment technologies. Recent investigation has shown that coupling two common treatments – *in situ* chemical oxidation (ISCO) and *in situ* bioremediation – is not only feasible, but in many cases provides more efficient and extensive cleanup of contaminated subsurfaces. However, the combination of aggressive chemical oxidants with delicate microbial activity requires a thorough understanding of the impact of each step on soil geochemistry, biota, and contaminant dynamics. In an attempt to optimize coupled chemical and biological remediation, investigations have focused on elucidating parameters that are necessary to successful treatment. In the case of ISCO, the impacts of chemical oxidant type and quantity on bacterial populations and contaminant biodegradability have been considered. Similarly, biostimulation, that is, the adjustment of redox conditions and amendment with electron donors, acceptors, and nutrients, and bioaugmentation have been used to expedite the regeneration of biodegradation following oxidation. The purpose of this review is to integrate recent results on coupled ISCO and bioremediation with the goal of identifying parameters necessary to an optimized biphasic treatment and areas that require additional focus. Although a biphasic treatment consisting of ISCO and bioremediation is a feasible *in situ* remediation technology, a thorough understanding of the impact of chemical oxidation on subsequent microbial activity is required. Such an understanding is essential as coupled chemical and biological remediation technologies are further optimized.

2.1 Introduction

Subsurface and groundwater contamination of recalcitrant organic compounds has created a large industry for technologies able to clean up polluted sites. The international market for the remediation sector is valued at US\$ 50–60 billion [239]. Conventional technologies focus on *ex situ* or on-site removal of contaminants through excavation or by so called pump-and-treat remediation of groundwater. However, the high costs and health risks associated with removal of contaminated material have spurred a shift towards *in situ* technologies, where decomposition of xenobiotic compounds via chemical or biological pathways is contained within the subsurface environment [62]. Of the pool of remediation strategies, *in situ* bioremediation and, more recently, *in situ* chemical oxidation (ISCO) are arguably the most commonly used *in situ* treatments, adaptable to a variety of subsurface conditions and contaminant types.

During an ISCO treatment, chemical oxidants are pumped into the subsurface to oxidize organic pollutants. This aggressive treatment removes source zone contaminants either sorbed to soil organic matter or pure product present as nonaqueous phase liquids [231, 292]. Typical treatments include Fenton's Reagent with hydrogen peroxide and ferrous iron, the less acidic Modified Fenton's Reagent, permanganate, persulfate, and ozone injection (Table 2.1). A number of review articles provide insight into the chemistry and proper application of oxidation regimens [68, 119, 210, 231, 268, 292]. ISCO has been successfully used to oxidize and thus lower the concentration of petroleum derived hydrocarbons (total petroleum hydrocarbons: TPH; [41, 68, 89, 103, 129]), polyaromatic hydrocarbons (PAH) [128, 162, 176, 291], and chlorinated hydrocarbons [88, 89, 92, 181, 187, 227, 234, 282, 293]. ISCO is a versatile remediation strategy, providing rapid and extensive removal of both light and dense recalcitrant compounds in pure phase.

Table 2.1 Conditions and reactions associated with common ISCO treatments [96, 265, 272].

ISCO Treatment	Important Reactions	Optimal pH	Oxidation Potential (V)	Type of Injection
Fenton's Reagent	$H_2O_2 + Fe^{2+} \rightarrow OH \cdot + OH^- + Fe^{3+}$	3-4	2.8	Liquid
Modified Fenton's Reagent	$H_2O_2 + OH^- \rightarrow HO_2 \cdot + H_2O$ $HO_2 \cdot \rightarrow O_2^{\cdot -} + H^+$ $2H_2O_2 \rightarrow 2H_2O + O_2$	neutral	Hydroxyl radical (OH \cdot)	Liquid
Activated Persulfate:				
Metal Activated	$M^{n+} + S_2O_8^{2-} \rightarrow SO_4^{\cdot -} + SO_4^{2-} + M^{n+1}$	3-4	2.6	
Heat Activated	$S_2O_8^{2-} + \text{heat} \rightarrow 2SO_4^{\cdot -}$	neutral	Sulfate radical (SO $_4^{\cdot -}$)	Solution
Alkaline Activated	Reaction unknown	>10.5		
Ozone	$O_3 + OH^- \rightarrow O_2 + OH \cdot$	neutral	2.1	Gas
Permanganate	$MnO_4^- + 2H_2O + 3e^- \rightarrow MnO_2(s) + 4OH^-$ $2KMnO_4 + C_2HCl_3 \rightarrow 2CO_2 + 2MnO_2(s) + 3Cl^- + H^+ + 2K^+$	neutral	1.7	Solution

Biological based remediation technologies require longer time spans than chemical oxidation techniques and are more appropriate for the bioavailable plume section of a contaminated site [239]. Whereas fungi and plants alike can mediate removal of recalcitrant compounds, here we focus on biodegradation by bacterial populations. The ubiquity of hydrocarbon-utilizing bacteria indicates that Natural Attenuation, that is, the inherent biodegrading ability of contaminated soils, will, in most cases, occur without intervention [253]. That said, a number of technologies aim to increase the rate of bioremediation, either by increasing the bioavailability of contaminants through the application of surfactants [166, 270, 286] or by creating conditions ideal for general microbial growth and for specific reactivity to a compound. In the case of petroleum based hydrocarbons, these include biostimulation with nutrients [67, 166] and electron acceptor addition, for example, by bioventing to provide oxygen to accelerate aerobic degradation pathways [81, 152]. Microbial dechlorination of highly chlorinated compounds occurs under anaerobic conditions. Thus, stimulation includes the addition of an appropriate electron donor and nutrients to create ideal (redox) conditions [9, 80, 245]. Bioaugmentation with a bacterial enrichment or culture able to break down the contaminant of interest has been used to improve bioremediation of (chlorinated) hydrocarbon contaminants [47, 203, 219, 242, 247]. This strategy is most commonly used to accelerate conversion of tetrachloroethene to harmless ethene through the addition of cultures enriched with *Dehalococcoides spp.*, the only bacterial group known to perform the complete chain of metabolic halorespiration reactions to produce ethene [3, 85, 118, 151, 224, 263].

Conventional wisdom dictates that chemical oxidation is not compatible with biological-based remediation techniques. The oxidative stress, increase or decrease in pH (persulfate and Fenton's Reagent, respectively) and change in redox conditions caused by ISCO treatments significantly alter subsurface conditions and are toxic to microbial populations [31, 149, 169]. However, recent work indicates that, although chemical oxidation can temporarily reduce microbial activity, bacterial populations do regenerate contaminant degradation ability both in the field [100, 117, 123, 146, 148, 249] and in laboratory experiments [10, 84, 107, 121, 177]. In many cases, it has been concluded that ISCO pretreatment appears to improve overall remediation by: (1) decreasing the concentration of pollutants to levels less toxic for the soil biota [37]; (2) improving bioavailability of the parent compound, [121, 169]; (3) producing bioavailable and biodegradable oxidized daughter compounds [128, 159, 169, 176]; or (4) providing oxygen for aerobic biological transformation of contaminants [121]. It has recently been suggested that, as ISCO treatment is not able to access and

oxidize all residual contaminants, biological polishing is required to fully remediate a site [35].

This statement reflects the new direction of coupled chemical and biological treatment. Previous reviews echoed the “wait and see” mentality common to earlier experiments, where, following chemical oxidation, the regeneration of biomass was merely monitored without stimulation [217, 288]. However, in the open system encountered *in situ*, even local sterilization with chemical oxidants will eventually be reversed as groundwater upstream will always transport indigenous microorganisms [27]. This acceptance of microbial resilience is reflected in a recent shift in the literature towards more studies investigating which parameters are essential to a biphasic remediation strategy and how these parameters can be manipulated. Here, we first consider the ISCO step, summarizing work in which aspects of chemical oxidation are tested for their influence on biodegradation and thus the overall remediation process. Secondly, we will review literature in which parameters essential to the rebound of an actively biodegrading microbial population are examined.

2.2 Improving ISCO treatment to enhance bioremediation

Conditions created during ISCO can significantly influence the effectiveness and efficiency of subsequent biodegradation of residual contaminants. As summarized in Table 2.1, chemical oxidation can create adverse environments in terms of pH and oxidation potential that inhibit general microbial growth and function. Recent work has indicated that the type [35, 121, 299] and quantity [30, 103, 188, 218, 274] of chemical treatment have repercussions on soil geochemistry and bacterial populations, and thus on the success of continued bioremediation. Additionally, the ISCO step oxidizes a specific fraction of the contamination, producing a range of oxidized substrates. The variety of compounds remaining after chemical oxidation must be bioavailable and biodegradable in order to ensure an effective bioremediation step.

2.2.1 Impact of chemical oxidant type on subsequent bioremediation

It has been shown that known ISCO treatments are versatile, able to oxidize a wide variety of substrates. Thus, rather than choosing chemical oxidation regimens based primarily on the contaminant type, treatments can be designed that cater to the requirements of the microbial population. This strategy generally improves the overall removal efficiency.

For example, the high sulfate concentrations that remain following persulfate oxidation can stimulate sulfate-reducing bacteria. To avoid the evolution of the toxic H_2S , Cassidy and Hampton investigated the potential of activating persulfate with calcium peroxide at alkaline pHs, which favor production of the dissolved hydrosulfide ion (HS^-) [34]. This strategy not only reduced H_2S production, but also minimized the presence of toxic mercury ions through precipitation of stable mercury sulfide (HgS).

Studies have been performed to screen various oxidants to determine their impact on biological activity. Treatments of permanganate, Modified Fenton's Reagent, a calcium peroxide-based product, and ferric iron activated persulfate were used to oxidize tetrachloroethylene- and ethylbenzene-spiked samples [26]. From overall contaminant removal and cell number, it was concluded that calcium peroxide resulted in the largest mass removal of the two contaminants, 96% and 95%, respectively, and an order of magnitude increase in the number of culturable heterotrophic microbes.

However, in most cases, the ISCO treatment providing the highest mass removal shows the largest negative impact on microbial activity, and thus on the overall remediation efficiency (Figure 2.1). Cassidy et al. tested the feasibility of coupling preoxidation by ozone, Modified Fenton's Reagent, or sodium persulfate with bioremediation of soil contaminated with 2,4-dinitrotoluene (2,4-DNT) [35]. It was determined that, although sodium persulfate less effectively oxidized 2,4-DNT, overall remediation occurred within 14 days, as compared to 30–90 days for Modified Fenton's Reagent and ozone, respectively. This result was explained by the fact that preoxidation with persulfate showed a minimal impact on the 2,4-DNT-degrading portion of the microbial population, both in terms of number of degraders and the time required for bacterial degradation to rebound. In experiments where permanganate, Fenton's Reagent, or an oxygen-release compound containing MgO_2 were paired with bioremediation on jet fuel contaminated soil, similar results were found [299]. Permanganate was the oxidant that showed the highest mass reduction, but did not provide the greatest overall remediation efficiency. Rather, by using the less aggressive oxygen-release compound, minimal disruption of microbial activity was observed, allowing for overall removal of up to 80% of the oil-derived hydrocarbons.

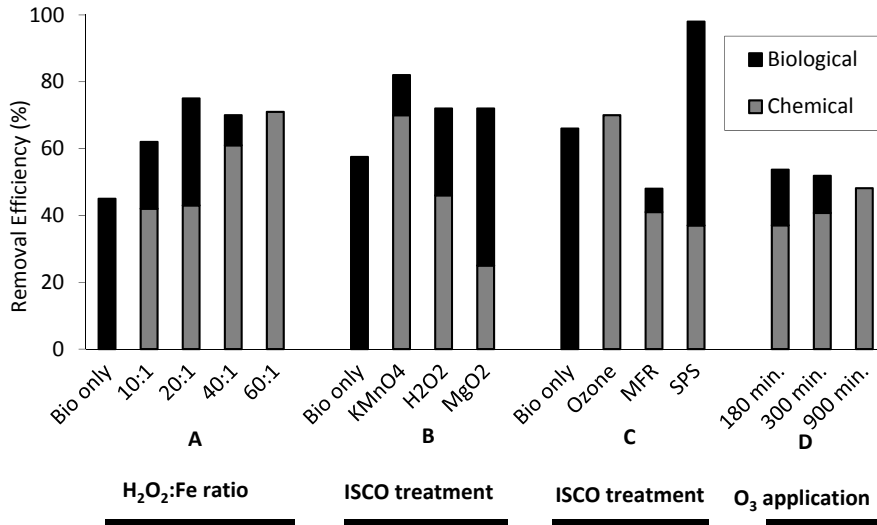


Figure 2.1 Summary of removal efficiency for combined chemical and biological remediation in instances where a variety of ISCO treatments were tested. Data gleaned from (A) (Valderrama et al. 2009), where labels indicate the H₂O₂:Fe ratio used for treatment [274]; (B) (Xie and Barcelona 2003), where labels indicate the type of chemical oxidant used prior to incubation for 134 days [299]; (C) (Cassidy et al. 2009), where labels indicate the type of chemical oxidant, including Modified Fenton's Reagent (MFR) and iron-activated sodium persulfate (SPS), and data are for the first 20 days of experimentation [35]; and (D) (Jung et al. 2005), where labels indicate the duration of ozone treatment [103].

The interaction between various soil matrices, a number of ISCO treatments, and subsequent bioremediation has also been investigated [121]. Sand and peat samples spiked with creosote were treated with ozone and hydrogen peroxide, with and without the addition of ferrous iron. Whereas preoxidation steps showed an overall greater efficiency in organic-poor sand samples, biodegradation of PAH contaminants was greater in peat experiments, especially when ferrous iron was added to the ISCO treatment. It was concluded that traditional Fenton's Reagent was the most effective preoxidation step for PAH-spiked sand, whereas ozone was more effective in the case of peat samples. Although it is known that the efficiency of chemical oxidation is related to the matrix, this work indicates that soil type to some extent dictates the effectiveness of coupled chemical and biological treatment.

2.2.2 Optimizing the quantity of chemical oxidant

Just as the type of chemical oxidant impacts bioremediation to varying degrees, the quantity and concentration of ISCO compounds also influence subsequent biological degradation of recalcitrant contaminants. Similar to the aforementioned section addressing the type of chemical oxidants, investigations into the impact of the amount of oxidants on bioremediation found that less aggressive mass removal in the chemical phase allowed for overall more efficient remediation (see Figure 2.1). In the case of column experiments on diesel-containing soil that was ozonated at 30 mg/L for time intervals between 60 and 900 minutes, the extent of biodegradation was found to be inversely proportional to the ozonation time [103]. Whereas the highest chemical TPH removal was 50% for 900 minutes of ozonation, no further biodegradation occurred upon incubation for 9 weeks. However, the 180 minute treatment oxidized initially only 37% of TPH, but ultimately removed more contamination as a coupled treatment. Similar results were obtained when various ratios of hydrogen peroxide to iron were used to oxidize creosote in aged natural samples [274]. It was determined that a $\text{H}_2\text{O}_2:\text{Fe}$ ratio of 20:1 (mol/mol) was aggressive enough to remove 43% of contaminants without impeding further bioremediation, which led to a total removal of 75% of PAHs.

Column experiments, which may more accurately represent the injection of ISCO chemicals in the field, have highlighted the influence of oxidant concentration and volumetric flux on further bioremediation at a given location in the column. Working with columns containing PAH-contaminated (3,318 mg/kg), iron-rich (16.4 g/kg) soils, Palmroth et al. first applied a total of 0.4 g hydrogen peroxide/g soil over the course of either 4 or 10 days [188]. Subsequently, column sections were separately incubated in batch experiments to determine the potential for bioremediation. Averaging 36% removal of PAH, coupled treatment did not perform significantly better than control setups without chemical oxidation, in which 20–30% of PAHs were biodegraded. However, the largest mass removal (54%) was found in a microcosm-containing soil from the lower part of the column that received the hydrogen peroxide treatment spread over 10 days. A different study with PCE-spiked sand columns, in which the amount of permanganate and velocity of injection were adjusted, investigated the potential for coupled chemical oxidation and reductive microbial dechlorination [218]. The best results were obtained when a large quantity of oxidant with a low concentration was rapidly injected into the column: 31.5 pore volumes of 0.63 mM KMnO_4 at a rate of 120 cm/d. From each of these studies, it can be concluded that through minimizing the amount of direct contact that bacteria has with concentrated

chemical oxidants, the biological impact of oxidation is reduced; in general, less aggressive oxidation improves the overall remediation efficiency.

2.2.3 Bioavailability and biodegradability of contaminants following ISCO

In addition to optimizing the ISCO step to reduce biological impacts, it is essential to understand the influence of chemical oxidation on the biodegradability and bioavailability of the remaining contaminant pool [128, 169, 176]. Although most ISCO treatments increase bioavailability, oxidation with permanganate can reduce bacterial access to contaminants. Upon reaction with organic compounds, precipitation of manganese oxide occurs, which reduces soil permeability [235] and can encapsulate contaminants present as non-aqueous phase liquids [150]. As a result, this portion of the pollutant remains inaccessible to either bacterial degradation or dissolution into the aqueous phase. In previous lab studies, increased bioremediation was associated with setups with a low concentration permanganate solution which was injected at high velocity [218]. Although regeneration of microbial activity could occur more rapidly under less oxidizing conditions, improved overall remediation efficiency may also be attributed to higher bioavailability of the remaining contaminant concentration under lower permanganate concentrations. This work reinforces the importance of considering whether post-ISCO conditions are amenable to bacterial polishing when planning a chemical oxidation strategy.

Whereas chemical oxidation can either positively or negatively impact bioavailability, ISCO generally improves biodegradability of the contaminant pool. A number of studies have investigated which parent and daughter compounds within a mix of substrates are broken down during the chemical or biological remediation steps in an effort to determine how to optimally treat the whole contamination. Some work has suggested that the readily biodegradable fraction of PAHs was chemically removed in the course of Fenton's Reagent oxidation, thus impeding further bioremediation [10]. However, other research has found that pre-ozonation produced short chain daughter compounds that were more easily biodegradable [140]. Similarly, Fenton's Reagent treatment of spiked benz(a)anthracene samples oxidized 43% of the contamination to produce benz(a)anthracene-7,12-dione [128]. During 63 days of incubation, 98% of the daughter compound was removed, as compared to only 12% of the parent compound, for an overall 54% removal of benz(a)anthracene. Manipulation of the ISCO phase to optimize the removal of the less biodegradable fraction of the contaminant was not further discussed.

Work on weathered crude oil samples indicated that pretreatment with permanganate influenced the biodegradation efficiency of different components of the contamination [298]. Biological degradation of the resin and aromatic fractions increased following permanganate treatment, while bioremediation of the aliphatic fraction was reduced by 50-60%. Similarly, when optimizing Fenton's Reagent dosages for subsequent bioremediation of PAH-contaminated soil, it was noted that chemical treatment parameters had an influence on the fraction of ring sizes removed [274]. More of the less biodegradable 5-ring PAHs were chemically oxidized in treatments with higher hydrogen peroxide concentrations. However, optimal oxidant ratios for 5-ring PAH removal (60:1 H₂O₂:Fe) had a negative effect on the regeneration of the bioremediating microbial community. Thus, this study does indicate the potential for designing optimized treatment strategies in which the costs in terms of regeneration of the bacterial population are balanced with the benefits associated with removal of less biodegradable compounds.

2.3 Optimizing microbial regeneration following ISCO

Ideally, the last moment of ISCO coincides with the first moment of bioremediation. In practice, regeneration of microbial activity is a process. Just as the microbiology, geochemistry, and residual contaminant concentrations of soil must be considered during chemical oxidation, a number of parameters can be manipulated at the beginning of bioremediation. After ISCO, the naturally occurring degradation processes inherent to the subsurface, so called Natural Attenuation, have been disturbed. In the process of Enhanced or Stimulated Natural Attenuation, optimal conditions for bioremediation are recreated through the addition of electron acceptors or donors, nutrients, and bacteria (bioaugmentation). Significant work has investigated biostimulation, bioaugmentation, and Natural Attenuation. However, in view of the fact that chemical oxidation can have profound impacts on soil characteristics and biota, we consider here only work in which preoxidation occurs. Biostimulation in terms of the adjustment of redox conditions to promote either aerobic or anaerobic bioremediation (3.1), and amendment with electron donors and nutrients (3.2), are techniques that have been used to ensure the proper activity of biodegrading microbes. Bioaugmentation to re-inoculate soils with depleted microbial populations will also be considered. A summary of conditions set for aerobic and anaerobic bioremediation in chemically pre-oxidized soils is given in Tables 2.2 and 2.3.

Table 2.2 Summary of investigations on the feasibility and optimal conditions for combined chemical oxidation and aerobic bioremediation.

Reference	Contaminant Type	ISCO treatment	Conditions Set For Bioremediation	Removal Efficiency
[35]	2,4-dinitro-toluene	Ozone Modified Fenton's Reagent Iron-activated Sodium Persulfate	Aerobic incubation Amendment with NH ₄ and PO ₄	98%
[189]	PAH	Modified Fenton's Reagent	Aerobic incubation	43-59%
[188]	PAH	Modified Fenton's Reagent	Aerobic incubation	36-54%
[274]	PAH	Fenton's Reagent	Aerobic incubation Amendment with nutrients Bioaugmentation	75%
[103]	Diesel	Ozone	Aerobic incubation	> 50%, exact value not reported
[121]	PAH	Fenton's Reagent Ozone	Aerobic incubation	75-94% (sand samples) 55-75% (peat samples)
[140]	Crude Oil	Ozone	Aerobic incubation Amendment with nutrients Bioaugmentation	35-40% (ozone + bioremediation) 70% (including bioremediation step prior to ozonation)
[299]	Jet Fuel	Permanganate Hydrogen Peroxide Magnesium peroxide (oxygen release compound)	Aerobic incubation Amendment with nutrients, carbon source, and trace elements Bioaugmentation	15-80% dependent upon fraction of oil
[269]	Fuel oil	Fenton's Reagent	Aerobic incubation Amendment with N, P, and carbon source	47-75%
[271]	Fuel oil	Fenton's Reagent	Aerobic incubation	100% in 3 step process with flushing with surfactant, ISCO, and bioremediation

2.3.1 Redox process conditions in pre-oxidized soils

Creating and maintaining aerobic or anaerobic environments for rapid removal of a particular contaminant is challenging. Aerobic conditions are generally conducive to biodegradation of petroleum-based hydrocarbons and less halogenated compounds, whereas anaerobic conditions are required for microbial mediation of highly halogenated compounds. Chemical oxidation and aerobic bioremediation can be combined relatively easily. Bioventing and aerobic flushing to encourage bioremediation of petroleum-derived hydrocarbons are practices common to the current subsurface remediation market and which in research have been mimicked in the lab by maintaining oxygen saturation in column or microcosm experiments [35, 103, 121, 189]. Often increased biological degradation has been attributed to aerobic conditions created by chemical oxidation [121, 188]. Studies have shown that low concentrations of hydrogen peroxide can be used instead of air flushing to meet biological oxygen demands for oil degradation in soils pretreated with Fenton's Reagent [271]. Other work associated an increase in hydrogen peroxide concentrations, from 100 mg/L to 900 mg/L, with TPH removal efficiencies improving from 47% to 69%, respectively [269]. In field applications, this has led to the development of a variety of commercially available oxygenating products that both mobilize and partially oxidize contaminants while simultaneously providing oxygen to stimulate aerobic biodegradation [15, 22, 133, 184].

Anaerobic *in situ* biodegradation of halogenated organic contaminants dissolved in groundwater by adding electron donors and nutrients has been proven and widely applied in the last decade [9, 80, 245]. However, the reduced bioavailability of pure product in the source zone and adsorption to the organic-rich fraction of the solid phase matrix impedes full bioremediation [230]. For these recalcitrant source areas, chemical oxidation pretreatment followed by anaerobic bioremediation may offer a solution. In contrast to aerobic bioremediation, rapid recreation of anaerobic conditions for dehalogenation in pre-oxidized soils can be challenging. Work in the field has confirmed the rebound of anaerobic dechlorination by molecular techniques in TCE-contaminated sites pre-oxidized with permanganate [100] and persulfate [249]. The regeneration of anoxic conditions amenable to dehalogenation generally requires input of electron donors. In the field, such an increase in available electron donors has been attributed to the mobilization of soil organic matter by chemical oxidation [294]. In soils and engineered lab systems with less organic matter content, addition of carbon substrates is often required (see Table 2.3; [87, 218]).

Table 2.3 Summary of investigations on the feasibility and optimal conditions for combined chemical oxidation and anaerobic bioremediation.

Reference	Contaminant Type	ISCO treatment	Conditions Set For Bioremediation	Removal Efficiency
[218]	Tetrachloroethene	Permanganate	Anaerobic incubation Amendment with nutrients, carbons source, trace elements	Overall efficiency in terms of ethene production not reported Degradation of PCE to <i>cis</i> -DCE observed
[87]	Trichloroethene	Permanganate	Anaerobic incubation Amendment with nutrients and carbon sources Bioaugmentation Addition of natural groundwater	Overall efficiency in terms of ethene production not reported Limited rebound of microbial dechlorination following ISCO treatment
[30]	Perchloroethylene	Fenton's Reagent	Anaerobic incubation Amendment with nutrients, trace elements and vitamins Bioaugmentation	17-74%

Although providing proper and ample carbon and nutrient sources is essential to microbial activity, the redox process conditions required for bioremediation must be considered when choosing an ISCO treatment. Thus, chemical oxidants that produce molecular oxygen are better suited to the aerobic biodegradation phases. In contrast, permanganate oxidation occurs via direct electron transfer without the generation of oxygen [295]; however, the byproduct of chemical oxidation, manganese oxide, can inhibit reductive dechlorination by acting as an alternative electron acceptor [84]. In spite of this, permanganate is often preferred for studies where anaerobic conditions must be regenerated [87, 100, 218, 270].

Addition of carbon substrates has been used to increase the rates of both aerobic and anaerobic bioremediation pathways (see Tables 2.2 and 2.3). In batch experiments with fuel oil-spiked soil, increased biodegradation was observed when microcosms were supplemented with cane molasses in the presence of excess oxygen [269]. Similarly, carbon sources, such as ethanol and acetate (each 100 mg/L, [87]) and methanol (2.0 mM, [218]) have been added as electron donors to increase the rate of anaerobic halorespiration. Whereas regeneration of anaerobic TCE dechlorination activity was successful in the former citation, multiple attempts by Hrapovic et al. to biostimulate with electron donors proved fruitless. As elaborated upon in section 3.3, biological degradation of TCE did occur upon flushing with natural groundwater, indicating that electron donor sources were sufficiently present and other factors were impeding halorespiration, such as nutrient availability and the presence of a microbial population acclimated to biodegradation.

2.3.2 Nutrients and biodegradation in pre-oxidized soils

Nutrient amendment is a common approach to stimulate bacterial growth and may be necessary in lab experiments with nutrient-poor samples without natural influx of nutrient-rich groundwater. Many batch experiments with ISCO have been performed in a phosphate buffer, which simultaneously controls the pH and provides phosphate to microbes [35, 140, 274]. Ammonium may be added for analytical reasons [35], or in conjunction with ample trace metals, minerals, and vitamins to create optimal conditions for biomass growth [218, 299]. In experiments in which ammonium and phosphate were either added to or absent from microcosms with fuel oil-spiked samples, TPH removal was 6-14% higher when nutrients were present [269].

Although excess nutrients may improve bioremediation, amendment may not always be necessary. It has been suggested that through oxidizing soil organic matter,

chemical oxidants actually release nutrients [240, 294]. Researchers investigating the impact of ISCO on subsequent plant growth found that phosphate concentrations increase in soils oxidized with either Fenton's Reagent or permanganate due to oxidation and release of soil organic matter [240]. Although ammonium concentrations increased up to 20 fold upon treatment with Fenton's Reagent, overall concentrations of nitrate and organic carbon decreased; such reductions in the availability of electron donors and acceptors as well as nutrients would have repercussions on biomass growth. Kulik et al. suggested that increased bioremediation in pre-oxidized peat, as compared to sand, was due to the high nutrient content in peat [121]. Further investigation is required to understand the relationships between soil type, nutrient availability, and dynamics thereof following chemical oxidation.

2.3.3 Bioaugmentation in pre-oxidized soils

Addition of bacterial cultures or enrichments frequently occurs in laboratory experiments. This occasionally takes place prior to chemical oxidation in order to ensure proper bacterial function. Microbial populations are added after ISCO to re-inoculate lab experiments and, arguably, to simulate the processes encountered in the field. However, work investigating bioremediation in pre-oxidized soils has not yet conclusively shown the necessity of bioaugmentation. In two recent studies, experimental setups were inoculated prior to ISCO treatment with either a dechlorinating microbial culture to guarantee PCE degradation ability [218] or a wastewater enrichment culture to ensure sufficient biomass was available [274]. Both investigations saw successful regeneration of bioremediation activity under some of the experimental conditions tested. Other researchers investigated the influence of cell number on PCE degradation [30]. Here, a culture of *Xanthobacter flavus*, a hydrogen peroxide resistant species known to degrade the major by-product of Fenton's Reagent oxidation of PCE (dichloroacetic acid), was grown under oxidative stress prior to experimentation. Although results did identify optimal cell numbers associated with specific Fenton's Reagent dosages, it would be nearly impossible to create such specific conditions in a field setting.

Enrichments of indigenous soil microorganisms have been used for re-inoculation following chemical oxidation. In lab experiments where microcosms were autoclaved prior to ISCO to ensure that all contaminant removal could be attributed to chemical oxidation, it was clear that re-inoculation was required to regenerate bioremediation [299]. The impact of bioaugmentation has been less conclusive in other studies testing this as a variable in coupled chemical and biological remediation. In one

case, microbial community dynamics with or without addition of an enriched mixed culture following ozonation of soil with an initial oil concentration of 56 mg/g were monitored on a microarray [140]. Although there was slightly less residual TPH following treatment, namely 17 mg/g in the setup with added biomass versus 18 mg/g in the blank, this could not be explained by a significant difference in the amount or variety of genes measured by the microarray. In permanganate-pre-oxidized TCE-spiked samples, no dechlorination was observed following a variety of bioaugmentation steps with an inoculation of a TCE-degrading *Dehalococcoides sp.* and other species able to respire on hydrogen and the carbon source provided [87]. However, once the column was flushed with site ground water, anaerobic conditions developed and *cis*-DCE was detected. Thus, it is not clear if bioremediation was initiated by (1) biostimulation with specific nutrients present in the site groundwater but not in synthetic groundwater, (2) the introduction of dehalogenating bacteria from the site, or (3) the addition of a groundwater bacterial consortium able to create anaerobic conditions necessary for the activity of cultured *Dehalococcoides* species. In work focused on investigating bioremediation parameters an enrichment culture or native sediment slurry was added to all experimental setups [132, 176]; thus, the impact of biomass addition could not be assessed. Clearly, future work is required to judge both the necessity of, and requirements for, successful bioaugmentation in pre-oxidized soils in the lab in order to ascertain the transferability of this technique to the field.

2.4 Conclusions and future directions

Coupled ISCO and bioremediation is not only feasible, but, when properly implemented, can provide more extensive, rapid and cost effective treatment than either chemical or biological techniques alone. Although preoxidation impacts microbial communities and may adversely alter redox conditions, chemical oxidation can stimulate bioremediation through improving bioavailability and biodegradability of contaminant substrates, providing oxygen, and in some cases releasing nutrients. In order to create a balanced and optimized treatment strategy, a variety of parameters within the chemical and biological remediation phases must be considered.

ISCO regimens should be chosen and performed in a manner that minimizes microbial interaction with concentrated chemical oxidants. When a variety of chemical oxidant types, concentrations, or injection strategies were tested, heightened chemical mass removal was not associated with overall increased

efficiency. This was due to the reduction in bioremediation potential from harsh chemical treatments. Chemical oxidation can also be manipulated to target the less biodegradable fraction of the contamination, leaving bioavailable oxidized by-products for microbial remediation. Future investigations should focus on further optimizing the chemical treatment to reduce microbial impact and improve the ease of substrate biodegradation. To this end, it is essential that experimentation include (1) more column setups which better recreate oxidant flow dynamics in the lab and (2) naturally aged samples with which the impact of field sorption of contaminants onto soil particles can be assessed.

Adjustment of redox conditions, electron donors and electron acceptors; amendment with nutrients; and addition of bacterial cultures are techniques used to stimulate the regeneration of microbial activity following ISCO. Redox conditions have been manipulated (in some cases with chemical oxidants) to encourage specific aerobic or anaerobic biodegradation pathways. Conclusive evidence for the necessity and/or success of bioaugmentation remains illusive. As laboratory work with enriched cultures under optimized conditions has proven to be inconclusive, field application would obviously be even more challenging; reliance on incomplete sterilization by ISCO and a natural or geohydrologically-enhanced re-inoculation by groundwater flow may be more feasible strategies. Through providing an excess of electron donors and acceptors, rebound of biodegrading communities may also be encouraged.

In view of the fact that often the time required for remediation or that the efficiency decreases when technologies are transferred from the laboratory to the field, a full understanding and optimization of the processes essential to sequential chemical and biological treatment are required to ensure success in field applications. Future work should focus on the implications of chemical oxidation on nutrient dynamics in a given soil matrix and on the bacterial requirements for regeneration. Through concentrating on the aforementioned directions, research-based, cost efficient and effective biphasic treatment strategies can be designed. Once tested in the lab, optimized coupled ISCO and bioremediation can then be further developed as an effective remediation technology in the field.

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Chapter 3

Biodegradation of aged diesel in diverse soil matrixes: impact of environmental conditions and bioavailability on microbial remediation capacity

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Abstract

3

While bioremediation of total petroleum hydrocarbons (TPH) is in general a robust technique, heterogeneity in terms of contaminant and environmental characteristics can impact the extent of biodegradation. The current study investigates the implications of different soil matrix types (anthropogenic fill layer, peat, clay, and sand) and bioavailability on bioremediation of an aged diesel contamination from a heterogeneous site. In addition to an uncontaminated sample for each soil type, samples representing two levels of contamination (high and low) were also used; initial TPH concentrations varied between 1.6-26.6 g TPH/kg and bioavailability between 36% and 100%. While significant biodegradation occurred during 100 days of incubation under biostimulating conditions (64.4-100% remediation efficiency), low bioavailability restricted full biodegradation, yielding a residual TPH concentration. Respiration levels, as well as the abundance of *alkB*, encoding mono-oxygenases pivotal for hydrocarbon metabolism, were positively correlated with TPH degradation, demonstrating their usefulness as a proxy for hydrocarbon biodegradation. However, absolute respiration and *alkB* presence were dependent on soil matrix type, indicating the sensitivity of results to initial environmental conditions. Through investigating biodegradation potential across a heterogeneous site, this research illuminates the interplay between soil matrix type, bioavailability, and bioremediation and the implications of these parameters for the effectiveness of an *in situ* treatment.

3.1 Introduction

Widespread anthropogenic subsurface contamination with recalcitrant hydrocarbons has led to the development of a variety of remediation techniques. Bioremediation, the exploitation of microbial metabolic ability to degrade contaminants, is often applied to remediate soils and sediments polluted with organic compounds. In the case of total petroleum hydrocarbons (TPH), microbial metabolic ability has been characterized [213, 276, 277], biodegradation has been shown experimentally [157], and the application of bioremediation has been proven in the field [161]. Experimental results indicate the necessity of certain environmental requirements, such as nutrient amendment under aerobic conditions [14, 46].

The bioavailability of a contaminant is essential for successful biodegradation. Bioavailability has a variety of definitions depending on the context; here focus is placed on how bioavailability affects bioremediation. [229] defines bioavailability as the “maximum quantity of a contaminant available for uptake by an organism within a given period of time”, indicating that low bioavailability can impede biodegradation. In the case of organic, hydrophobic compounds, reduced bioavailability is generally attributed to either (1) the presence of non-aqueous phase liquid (NAPL), indicative of low solubility where dissolution rates impede biodegradation, or (2) interaction or occlusion of the contaminant into the soil matrix, thereby hindering biodegradation [229, 230]. To improve the former, it has been shown that the application of surfactants can increase bioavailability and thus bioremediation [131]. Prolonged contaminant contact with the soil matrix enhances sorption to non-desorbable sites; this is termed aging and clearly contains a time component [78]. Low bioavailability can reduce the remediation efficiency of a bioremediation treatment, leaving residual concentrations above regulatory limits [43, 202, 208].

Soil characteristics determine the extent of bioavailability and consequently the potential for biodegradation. In the case of an aged contamination, reduction in bioavailability due to the sequestration of contaminants into soil particles is highly dependent on soil matrix characteristics. The quantity and type of organic matter (OM) impact the degree of aging [78, 204]. Additionally, during an *in situ* bioremediation project, the soil characteristics impact the environmental conditions experienced by the microbial community. The soil matrix offers an additional source of macro- and micro-nutrients, presents a carbon source alternative to the organic contaminant, and provides supplementary electron acceptors. Owing to these parameters, soil characteristics dictate what conditions are optimal for bioremediation [46] and yield the development of different biodegrading microbial populations [29, 198].

The aforementioned relationships among soil characteristics and bioavailability, nutrient pools, and microbial community development can have significant impacts on the success of bioremediation. It has been shown that a combined investigation of respiration, total and bioavailable contaminant concentrations, and quantities of microbial degraders in lab treatments is essential for adequate predictions of success in the field [52]. However, almost no scientific attention has been paid to the impacts of overall heterogeneity, and specifically the soil matrix, on the predictive value of the aforementioned lab-based tests. Previous work has often focused on spiked soils or recently contaminated locations, where the important impact of aging on bioavailability is disregarded [46, 160, 262]. Additionally, field studies commonly focus on the homogenous portion of a location. Although this allows clearer conclusions to be drawn from the site, the heterogeneity inherent to *in situ* remediation projects is disregarded.

In an effort to better understand, forecast and design *in situ* bioremediation, this research investigates biodegradation potential at a complex heterogeneous site, namely, a former railway yard contaminated with diesel. Four lithological layers are present in which contamination levels and extent of bioavailability are expected to vary significantly. The impact of soil matrix type, bioavailability, and the level of contamination on biodegradation and microbial respiration are considered. By assessing the interplay between environmental and contaminant characteristics and identifying the potential for, and limitations on, biodegradation on a microcosm scale, a spatial picture of the bioremediation potential across a heterogeneous site can be developed. This information is essential to predict and design successful remediation projects at such diverse locations.

3.2 Materials and Methods

3.2.1 Soil sampling

Soil sampling was performed at a railway site at Wegliniec, Poland in March 2010. At each location, hand augers were used to sample soil. The soil layers were spread across plastic bags in order to obtain a visual impression of the lithology at the sampling location. Samples were collected at various depths to obtain separate samples from the different soil matrix types (Table 3.1; Appendix 2). These soil matrices were collected in clean HDPE containers and stored the following day at 4 °C upon arrival in The Netherlands. All soils were sieved at 2 mm to homogenize the samples.

Table 3.1 Characterization of soil samples for biodegradation experiments. Four soil types and three contamination levels were identified as being representative of the heterogeneous conditions across the site. Soils used in multiple chapters are summarized in Appendix 2.

Sample Name	Soil Matrix	Contamination Level	Sampling Location	Depth (cm bgl ^a)	Groundwater level (cm bgl ^a)	pH	Organic Matter Content (%)	Total TPH (g TPH/ kg soil)	Bioavailable TPH (g TPH/ kg soil)	Bioavailable TPH (%)
FH	Fill	High	I	100-200	230	6.88	11.3	26.6 ± 0.9	23.0 ± 0.8	86.5
PH	Peat	High	II	170-260	170	6.80	9.9	11.6 ± 0.7	11.8 ± 2.9	102.1
CH	Clay	High	III	160-200	200	6.98	6.9	4.6 ± 0.9	4.0 ± 1.6	87.7
SH	Sand	High	III	230-250	200	6.22	3.5	13.3 ± 3.7	9.4 ± 2.6	71.0
FL	Fill	Low	I	50-100	230	7.10	10.5	4.9 ± 0.2	1.8 ± 0.1	36.0
PL	Peat	Low	III	200-230	200	6.18	54.0	8.0 ± 0.8	4.9 ± 0.4	61.8
CL	Clay	Low	II	80-150	170	5.15	4.7	2.0 ± 0.5	1.9 ± 0.5	95.9
SL	Sand	Low	IV	190-220	190	7.01	1.6	1.6 ± 0.3	0.7 ± 0.1	44.0
FU	Fill	Uncontaminated	V	50-100	200	7.55	8.9			
PU	Peat	Uncontaminated	V	100-160	200	7.37	6.3			
CU	Clay	Uncontaminated	III	130-160	200	7.17	7.6			
SU	Sand	Uncontaminated	IV	50-80	190	7.37	2.3			

^a below ground level

3.2.2 Experimental setup

Prior to starting the experiment, all soils were characterized for total and bioavailable TPH concentrations, dry weight, organic matter content and pH. A total of 12 soil types representing the four lithological layers (fill, peat, clay, sand) and three contamination levels (high, low, uncontaminated) were selected (Table 3.1). For each soil type, six identical microcosms were prepared in 125 mL serum bottles, three for total TPH extraction and three for determination of the bioavailable TPH fraction. Soil was weighed into 125 mL bottles and nutrient-rich medium was added to make a final ratio of 1 g soil: 2.5 mL liquid. The medium was a 100 mM phosphate buffer to which 10 mL of a macronutrient stock solution (containing 170 g/L NH_4Cl , 8 g/L CaCl_2 , and 9 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and 0.6 mL of micronutrient stock solution (containing 2 g/L $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$, 2 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 30 mg/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mg/L ZnCl_2 , 50 mg HBO_3 , 90 mg/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 100 mg/L $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, 50 mg/L $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 1 g/L EDTA, and 1 mL/L 36% HCl) were added. Bottles were sealed with Viton stoppers, crimped, and placed on a rotary shaker at 120 rpm at 30 °C for 100 days. At the end of the 100-day experiment, bottles were sacrificed for analysis of total and bioavailable TPH, both in triplicate.

Analysis of the headspace O_2 and CO_2 concentrations was performed by sampling the headspace with a 2 mL syringe at 13 time points during 100 days of incubation. Monitoring of the headspace gas concentrations was performed both to follow microbial respiration as well as to ensure fully aerobic conditions. Once headspace O_2 concentration dropped below 10%, the headspace was exchanged using air. Using headspace volume and gas composition measurements, calculations on cumulative CO_2 production and the rate per gram of soil were performed. Rates were calculated for specific time periods, as presented in Table 3.2.

3.2.3 Chemical analyses

Bioavailable and total TPH measurements were performed. Bioavailable TPH was determined in triplicate using 24 hour Tenax[®] extractions [43]. For every 1 g of dry soil, 1 g of Tenax[®], 10 mg NaN_3 , and 0.01 M CaCl_2 were added to the soil slurry to a final volume of 40 mL. After shaking at 20 °C for 24 hours, Tenax[®] was separated from soil particles and washed using 0.01 M CaCl_2 . TPH extraction was performed on the Tenax[®] granules. For total TPH measurements, extraction was performed on soil in triplicate.

Prior to TPH quantification with gas chromatography, the samples were extracted by solvent extraction. Solvent shake extraction with acetone and hexane

was performed according to NEN 5733 [178]. The hexane was dried with Na_2SO_4 and measured on a HP 5890 Gas Chromatograph (GC) with a SIM DIST column and Flame Ionization Detector with nitrogen as the carrier gas. Following 5 minutes at an initial temperature of 40 °C, the temperature was increased at 10 °C per minute to a final temperature of 300 °C. TPH was determined for samples portraying a diesel chromatogram pattern as the area between C10 and C40, with a detection limit of 0.60 g/kg. Carbon number fractions were determined using the retention times of a boiling point standard. Samples lacking a chromatogram indicative of diesel but rather typical for trace amounts of organic matter extracted with hexane, were considered uncontaminated for the purposes of this analysis.

Headspace analysis of O_2 and CO_2 was performed on a Shimadzu GC with Porabond and Molsieve 5a columns in parallel at 65 °C with helium as a carrier gas. Loop injection was at 120 °C and a thermal conductivity detector at 150 °C was used.

Dry weight was calculated by weight difference before and after 24 hours at 105 °C. Organic matter content was determined as the weight difference due to ignition at 550 °C. pH was measured in the supernatant of a 1 g soil:2.5 mL liquid ratio suspension of soil sample in 0.01 M CaCl_2 [191].

3.2.4 Molecular analyses

Sampling for DNA analysis was performed by collecting 2 mL of soil slurry with a sterile pipette in a sterile micro-centrifuge tube. Tubes were snap frozen in liquid nitrogen and stored at -20 °C. For DNA extraction, samples were thawed on ice and centrifuged at 14000 rpm for 10 minutes to pellet the soil particles and microbial cells. The starting weight of soil was noted prior to extraction of total microbial community DNA using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. The amount and quality of the extracted DNA was estimated with a Nanodrop spectrophotometer (Thermo Scientific). Extracted DNA was stored at -20 °C for downstream applications.

Quantitative PCR (qPCR) was performed using the iQ SYBR Green Supermix kit and the iQ5 iCycler (BioRad, Veenendaal, The Netherlands). All assays were done in triplicate in 25 μl reactions, and no-template controls were included. The primers used for Total Bacteria 16S rRNA gene were: 341F (5'-CCTACGGGAGGCAGCAG-3') and 534R (5'-ATTACCGCGGCTGCTGGC-3') [174]. For the *alkB* genes, primers were *alkB*Fd (5'-AAC TAC MTC GAR CAY TAC GG-3') and *alkB*Rd (5'-TGA MGA TGT GGT YRC TGT TCC-3') [199]. Primers were used at a final concentration of 200 nM. qPCR amplification parameters for the 16S rRNA gene assay were: 10 min at 95°C, followed

by 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C. Melt curve analysis was performed from 60°C-95°C in steps of 0.5°C and 10 sec at each step. As qPCR standards, cloned PCR-products were used that were carrying either the 16S rRNA gene or the *alkB* gene. Respective gene copy numbers were calculated as copies / g of sample. Significance of observed abundance differences were verified using the Student's t-test.

3.3 Results and Discussion

3

3.3.1 Site description and sample characterization

Soil from a contaminated railway site in Wegliniec, Poland with heterogeneous lithology was used to investigate biodegradation potential in diverse soil matrixes with different total and bioavailable contamination levels. The site was originally the location of a brook running from the south to the north of the site (Figure 3.1). A mixed peat and clay layer was deposited by the brook on top of sand. In order to level the site for the creation of the railroad, anthropogenic fill was added on top of the peat/clay layers. The composition of the fill is heterogeneous, containing coarse sand, gravel, and somewhat brown finer sand. Thus, four different soil matrix types were present: anthropogenic fill, peat, clay, and natural sand.

Contamination with diesel occurred due to refueling activities at the site, between 1970 and 2000. In 1997 and in 2001, monitoring wells were installed at 5-8 meters depth to sample the soil TPH concentrations and monitor the groundwater (Figure 3.1). During a remediation project in 2003, an additional 89 wells of 5 meters depth were sunk in a tight grid-like pattern. These wells were joined with submerged pipes lying around 0.30 m below ground level. The first 5 meters of subsurface, comprising the vadose zone, light non-aqueous phase liquid (LNAPL), and groundwater, were pumped and skimmed to remove free-phase oil. After 4 months of pumping, clogging of the filters resulted in closure of the remediation project.

The location of refueling and thus contamination with total petroleum hydrocarbons (TPH) is near monitoring wells B/01 and B/02, at the southwest corner of the site (Figure 3.1). During the sampling campaign in 2010, up to 70 cm of LNAPL were observed in piezometers, and solid phase concentrations were higher than 25 000 mg/kg dry matter in this source area. Higher TPH concentrations were found at or below the groundwater level. Contamination has spread across the site in the same direction as the groundwater flow, in a north-northeast direction, creating a contamination plume across the site.

A set of 12 samples representative of the different conditions across the site were investigated for biodegradation capacity (Table 3.1). Samples were selected from all four soil matrix types (fill, peat, clay, and sand) and three different contamination levels (high, low, and uncontaminated). Soil characterization revealed a large range of TPH concentrations (1.6-26.6 g TPH/kg in contaminated samples). Samples were assigned to the high or low contamination levels based on TPH concentration relative to other samples of a similar soil matrix type. Relative bioavailability varied significantly (36% - 100% of total TPH), and showed no relation with organic matter content (1.6% - 54% OM). Finally, soils had a wide-range of pH values (5.15-7.37), with lower pH observed in contaminated soils as compared to uncontaminated soils.

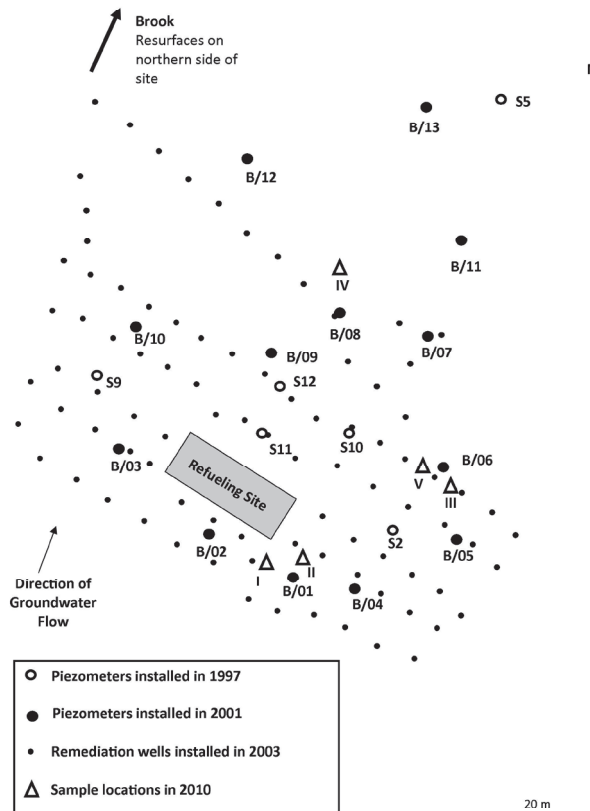


Figure 3.1 Site description for the railway refueling site. Piezometers from 1997 (open circles) and 2001 (large filled circles) as well as remediation wells (small filled circles) are given. The location of the five sampling sites from 2010 are indicated with triangles.

3.3.2 Bioavailability and biodegradation

Eight contaminated soil samples and four uncontaminated samples were incubated over the course of 100 days to investigate biological activity and biodegradation capacity. Significant biodegradation was observed in all microcosms, with remediation efficiencies between 65% - 100% (Figure 3.2). Complete remediation was observed in batches CL and SL, which had very low initial concentrations (Table 3.1). In nearly all cases, final concentrations were below the Dutch intervention value of 5 g TPH/kg [61], with only the most contaminated sample, FH, slightly above this regulatory limit.

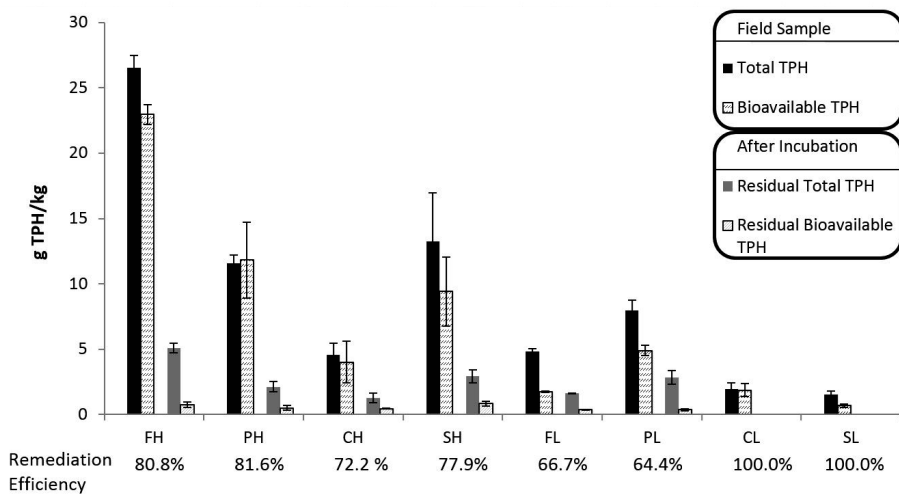


Figure 3.2 Total TPH (solid columns) and bioavailable TPH (patterned columns) prior to and following 100 days of incubation. Sample nomenclature is as in Table 1. Total and bioavailable TPH concentrations are the average of triplicate extractions; error bars are the standard deviations of triplicate extractions.

In many samples, degradation was incomplete indicating that bioavailability may have limited the extent of biodegradation possible. In the highly contaminated samples (FH, PH, CH, and SH), residual bioavailable TPH was below 1 g/kg at the end of the 100 day remediation period, which could have restricted further bioremediation. The lowest remediation efficiency was observed for samples FL and PL, which had relatively low TPH concentrations. Although high remediation efficiencies were observed for the other two low contaminated samples CL and SL, in the case of samples FL and PL, the initial bioavailability was also low (Table 3.1), indicating

that bioavailability is likely to have impeded biodegradation. This illustrates the importance of aging of a contaminant, in terms of reduction in bioavailability, on the endpoint of a bioremediation treatment and the residual concentrations expected in a field application.

Over the course of the degradation experiment, a shift in the composition of the TPH contamination, as assessed by carbon fraction number, was observed (Figure 3.3). Low molecular weight (LMW) compounds were preferentially degraded, thereby reducing the relative abundance of compounds within the C10-C12 and C12-C16 range. Conversely, although high molecular weight (HMW) compounds were degraded to some extent, the relative abundance of the C20-C24 and C24-C40 fractions increased. This effect was greatest in peat samples, where the C20-C40 fraction accounted for a larger portion of the residual TPH than in other soil matrix types. A similar biological preference for LMW components of TPH has been observed previously [36, 186], with linear alkanes being degraded preferentially over branched and cyclo-alkanes [156]. As low bioavailability was measured for the residual TPH present following biodegradation (Figure 3.2), it is unclear whether further biodegradation of these HMW compounds is impeded by bioavailability, by the physiological ability of microbes, or by a combined effect of these two factors.

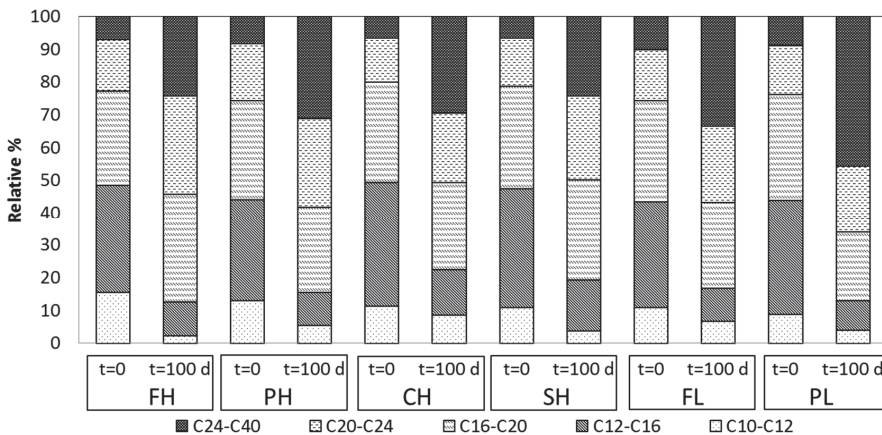


Figure 3.3 Shifts in distribution of carbon fractions in TPH due to biodegradation. Results are shown for field samples (t=0) and following biodegradation (t=100 d) for the six samples where residual TPH was measured at the end of the experiment. Carbon fractions are normalized to 100% to allow comparisons between samples.

This shift in TPH composition due to biodegradation further attenuates environmental risk in addition to the decrease in risk already obtained by reducing the overall TPH concentration. Owing to their higher volatility and solubility, LMW compounds are more mobile and pose more risk than HMW contaminants. Thus, by further reducing the concentrations of these lighter TPH components, the risk associated with soil vapor and groundwater transport is reduced. The low bioavailability observed following bioremediation for the remaining HMW compounds indicates a further reduction in the environmental risk for the residual TPH contamination.

3

3.3.3 Respiration

Throughout 100 days of incubation, respiration was followed using O₂ consumption and CO₂ production in order to assess microbial activity and biodegradation. Results indicated that the presence and degradation of TPH drove respiration (Table 3.2). Increased total respiration was observed for contaminated samples as compared to microcosms containing uncontaminated soils. In contaminated soils, higher respiration was seen at the beginning of the incubation period (data not shown). Up to 50% of the total CO₂ was produced by day 30. By the 50th day of incubation, at least 75% of total CO₂ production had already occurred. The phenomenon was more apparent for samples with low initial TPH concentrations, such as CL and SL, indicating a shift from easily degradable to less energetically favorable substrates.

Respiration has been commonly used to understand the microbial response to contamination and to monitor biodegradation [29, 158, 223]. In research assessing the extrapolation of laboratory results to predict field bioremediation efficiencies, Diplock et al. found that respiration rates greater than 0.1 mg CO₂/g soil/d were necessary in microcosm experiments to ensure bioremediation in the field [52]. In the current study, initial CO₂ production was between 0.33 and 1.75 mg CO₂/g soil/d in all contaminated soils, well above the threshold 0.1 mg CO₂/g soil/d rate. In contrast, initial respiration rates for uncontaminated soils were between 0.03-0.08 mg CO₂/g soil/d, showing a relationship between respiration rates and biodegradation of TPH. Final rates were slightly lower in samples CL and SL, where full remediation was observed. Nonetheless, based on the current results, significant remediation under biostimulating conditions can be expected at this field location.

Table 3.2 Absolute, relative, and rate data for respiration.

	Total Respiration ^a mg CO ₂ /g soil	Respiration relative to degradation mg CO ₂ /g TPH degraded	Maximum respiration rate ^b mg CO ₂ /g soil/d	Final respiration rate ^c mg CO ₂ /g soil/d
FH	57.4	2.68	1.51	0.39
PH	47.0	4.97	1.64	0.18
CH	31.5	9.55	1.55	0.10
SH	40.4	3.91	1.48	0.15
FL	19.6	6.05	0.61	0.12
PL	39.4	7.69	1.75	0.13
CL	9.5	4.88	0.58	0.03
SL	5.7	3.66	0.33	0.03
FU	6.0		0.08	0.09
PU	3.9		0.05	0.06
CU	3.2		0.08	0.02
SU	2.9		0.03	0.02

^a total respiration is cumulative for the 100 days of incubation

^b maximum respiration rates are from the initial 7 days of incubation

^c final respiration rates calculated from respiration in final 30 days of incubation.

Initial CO₂ production rates in general reflected the level of contamination, with greater rates in highly contaminated soils as compared to samples with low TPH concentrations (Table 3.2). However, within a contamination level, a similar order of respiration rates was observed. In both high and low groups, the peat samples had the highest initial respiration levels (1.64 and 1.75 mg CO₂/g soil/d for PH and PL, respectively). Thereafter, fill and clay samples show very similar rates. Finally, SH and SL had the lowest CO₂ production rates within the high contamination and low contamination groups (1.48 and 0.33 mg CO₂/g soil/d). As respiration rates were linked to TPH degradation, these results indicate that, in addition to contaminant concentration, microbial respiration rates were also related to soil matrix type.

In contaminated samples, a very consistent relationship between O₂ consumption and CO₂ production was observed. This ratio of O₂:CO₂ indicated that TPH degradation dominated respiration (Figure 3.4), regardless of the contamination level, bioavailability, or soil characteristics. Conversely, higher variability was measured in uncontaminated samples. This indicated the utilization of a homogeneous carbon source with consistent conversion efficiencies in contaminated soils. Although the composition of the remaining TPH shifted during the course of bioremediation towards more HMW compounds, this was not reflected in respiration data. Rather,

$O_2:CO_2$ ratios were consistent for TPH degradation and notably different when organic matter was utilized as an electron donor.

It has been shown that respiration correlates with contamination level [158], pointing towards a relationship between TPH degradation and CO_2 production. However, creating a mass balance between O_2 consumption, CO_2 production and TPH degradation is difficult. In addition to biological CO_2 production, abiotic processes such as the release of carbonate or chemical oxidation of OM can contribute to CO_2 production [225]. Although respiration was higher for contaminated soils in the current study, no clear relationship between CO_2 production and TPH mineralization was observed (Table 3.2). While in the cases of samples CH, FL, and PL the ratio was >6 mg CO_2/g TPH, in sample FH 2.7 mg CO_2/g TPH was observed. It is unclear whether the higher CO_2 production originated from abiotic processes which were not measured or was related to the utilization of other carbon sources such as OM. A biotic origin lies in contrast to the consistent $O_2:CO_2$ ratio seen in Figure 3.4.

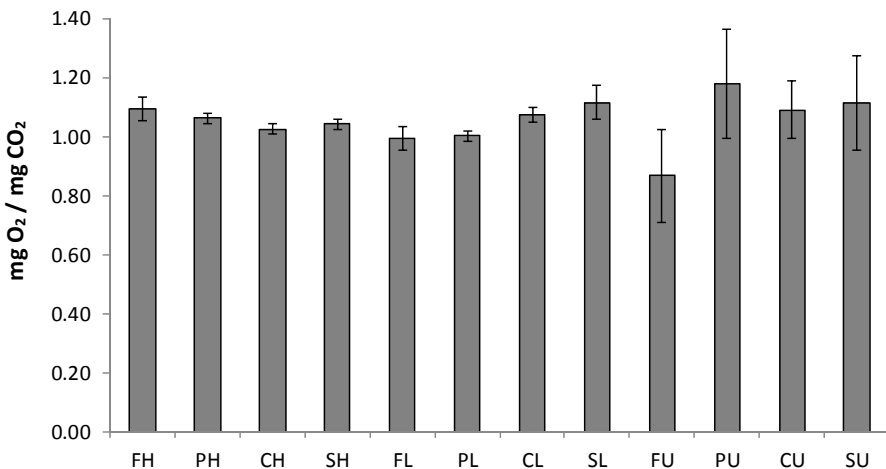


Figure 3.4 Ratio of O_2 consumption to CO_2 production in all microcosms. Values are the average of 13 measurement points over the 100 day incubation period and error bars are the standard deviation of these 13 data points.

3.3.4 Microbial community

In order to link observed degradation rates with the microbial capacity during the bioremediation of the different soil matrixes, a quantitative assessment of the microbial population and its degradation potential is necessary. Quantitative

polymerase chain reaction (qPCR) was performed to assess changes in bacterial biomass based on the 16S rRNA gene, and TPH degradation capacity based on the *alkB* gene encoding alkane mono-oxygenases, which catalyze the first oxidation step in the degradation of hydrocarbons [199]. The *alkB* gene has been used extensively for the molecular monitoring of biodegradation of alkanes [13, 116, 160, 199]. It was hypothesized that molecular results could also be used to better explain differences in respiration and mineralization rates.

There were no significant differences in bacterial biomass between the contaminated versus uncontaminated samples (Figure 3.5). In nearly all samples, at all time-points, between 10^7 and 10^8 bacterial 16S rRNA gene copies/g soil were measured. This was surprising, considering the fact that in contaminated samples respiration rates were higher and significant mineralization of TPH occurred (Table 3.2). In previous work, it was observed that respiration and degradation rates – as a measure of the population activity towards TPH biodegradation – were linked to the population size [52]. Although similar population sizes were present in contaminated and uncontaminated samples, a wide range of mineralization rates was observed in this study, suggesting that the average respiration rates per cell differed for microbial communities in contaminated versus uncontaminated soils.

In contrast to total bacterial biomass, presence of the *alkB* gene was higher in contaminated soils than in uncontaminated samples, both in terms of absolute numbers as well as relative to the bacterial community (Figures 3.5 and 3.6). Results are given for all samples except SH and PL, where reliable qPCR analysis for *alkB* was not possible due to inhibition of the qPCR reaction by matrix contamination in the DNA extract. In uncontaminated soils, less than 10^5 copies *alkB*/g soil were present and were two orders of magnitude lower than total bacterial 16S rRNA gene copies. Contaminated soils had consistently between 10^5 - 10^7 gene copies/g soil and between 0.02 and 0.12 copies *alkB*/ bacterial 16S rRNA gene. At $t=14$ days and $t=28$ days, the difference in *alkB* copy number between all contaminated and all uncontaminated setups was statistically significant ($p<0.05$). This suggested an enrichment of bacterial populations harboring the *alkB* genes over time in the microcosms. Contaminated clay, peat and sand samples all had higher *alkB* abundance than uncontaminated samples in the same soil matrix group. The largest difference was observed in sand, where the SL compared to SU *alkB* copy numbers differed by 2 orders of magnitude. SU, a very organic-poor soil (Table 3.1), also had the overall lowest abundance of *alkB*. As this gene can be involved in utilization of OM as a substrate, the absence of *alkB* at 28 and 100 days could indicate depletion of the small amount of OM present

in SU. There were no differences in *alkB* gene copy numbers between contaminated soils of different soil matrixes, indicating that when sufficient substrate was present, the contamination levels play the greater role in influencing the proliferation of hydrocarbon degrading populations.

A clear relation between increased *alkB* gene copy numbers and the presence and degradation of TPH was observed. *alkB* abundance was not only higher in contaminated soils, but also decreased as the available TPH was depleted. For CL and SL with the lowest TPH concentrations, a drop in *alkB* copy number was observed after 14 and 28 days, respectively. In both of these samples, by day seven over half of the total O₂ consumption and by day 18 over half of the CO₂ production, and thus TPH mineralization, had occurred, indicating that changes in *alkB* abundance were most likely due to substrate limitation. For other, more highly contaminated samples, a decrease in *alkB* at the end of the incubation period (t=100 d) also indicated population and/or activity shifts due to TPH substrate limitation (FL, FH, CH, and PH), as also concluded from final respiration rates (Table 3.2).

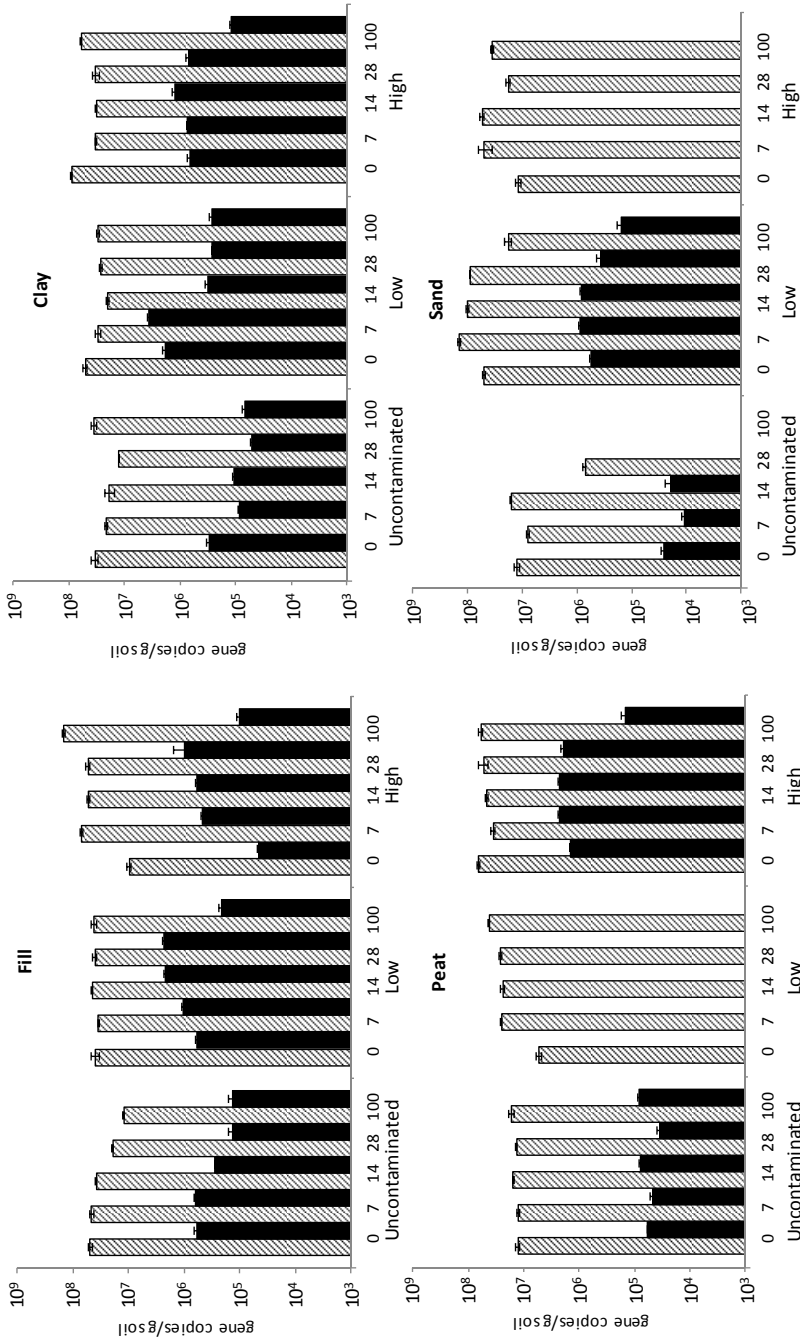


Figure 3.5 Quantitative PCR results for the total bacteria 16S rRNA gene (shaded bars) and *alkB* gene for hydrocarbon degradation (black bars) for five time points during incubation. Values are the average of triplicate qPCR reactions with standard deviations.

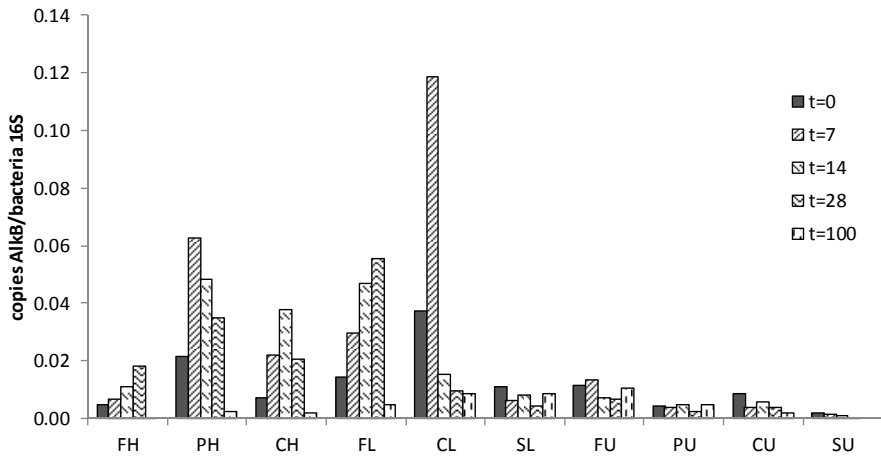


Figure 3.6 Abundance of *alkB* relative to total bacteria.

3.4 Conclusions

The current study showed the importance of a full understanding of the heterogeneous subsurface at a contaminated site. While bioremediation occurred under stimulating conditions in all contaminated soils, samples varied greatly in initial bioavailability and respiration rates during incubation of the different soil matrixes and contaminant concentrations. In many samples, reduced bioavailability due to aging of the contaminant impeded full biodegradation, yielding a residual concentration of TPH. There exists a clear relation between increased gene copy number of *alkB* and the presence and degradation of TPH. Shifts in *alkB* abundance over the course of incubation due to depletion of the TPH substrate indicated the usefulness of this molecular target as a line of evidence for hydrocarbon biodegradation.

Infield applications, heterogeneity can impact full remediation in terms of limitations during the treatment and the end-point concentration. While bioremediation is in general a robust technique, limitations to microbial ability due to environmental and contaminant characteristics do exist, which restrict the effectiveness of the treatment. Thus, prior to fully implementing *in situ* bioremediation, the implications of soil matrix type, bioavailability, and TPH degradation activity should be assessed to more accurately predict the success of the treatment and more appropriately design the remediation project.

Acknowledgements

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Chapter 4

Impact of long-term diesel contamination on soil microbial community structure

This chapter has been published:

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Abstract

Microbial community composition and diversity at a diesel contaminated railway site were investigated by pyrosequencing of bacterial and archaeal 16S rRNA gene fragments to understand the inter-relationships microbial community composition, pollution level, and soil geochemical and physical properties. To this end, 26 soil samples from four matrix types with varying geochemical characteristics and contaminant concentrations were investigated. The presence of diesel contamination significantly impacted microbial community composition and diversity, regardless of the soil matrix type. Clean samples showed higher diversity than contaminated samples ($p < 0.001$). Bacterial phyla with high relative abundances in all samples included *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, and *Chloroflexi*. High relative abundances of Archaea, specifically the phylum *Euryarchaeota*, were observed in contaminated samples. Redundancy analysis indicated increased relative abundance of the phyla *Chloroflexi*, *Firmicutes*, and *Euryarchaeota* correlated with the presence of contamination. Shifts in the chemical composition of diesel constituents across the site as well as the abundance of specific operational taxonomic units (OTUs, defined using a 97% sequence identity threshold) in contaminated samples together suggest that natural attenuation has occurred. OTUs with sequence similarity to strictly anaerobic *Anaerolinaea* within the *Chloroflexi*, as well as *Methanosaeta*, of the phylum *Euryarchaeota*, were detected. *Anaerolinaea* and *Methanosaeta* are known to be associated with anaerobic degradation of oil related compounds, therefore suggesting natural attenuation has occurred under anoxic conditions. This research underscores the usefulness of next generation sequencing techniques both to understand the ecological impact of contamination as well as to identify potential molecular proxies for detection of natural attenuation.

4.1 Introduction

Anthropogenic contamination with recalcitrant organic compounds has created a large industry for remediation of these polluted sites [239]. Among the variety of decontamination tools, *in situ* biodegradation of contaminants has emerged as a low cost, less invasive treatment compared to traditional *ex situ* techniques. The ubiquity of hydrocarbon utilizing microorganisms dictates that with time degradation will occur to some extent without human intervention, so-called natural attenuation [296]. Cultivation-independent molecular approaches have been used to assess the impact of pollution on microbial composition at contaminated sites, and phylogenetic and functional gene-targeted tools have been developed as one of the lines of evidence to monitor microbial populations at natural attenuation sites for degradation potential and activity [116, 199].

To this end, a number of studies have examined how the presence of total petroleum hydrocarbon (TPH) contamination impacts microbial community structure and function. In such studies, chemical analysis of TPH concentration is coupled to molecular analyses. Shifts in bacterial composition have been observed in microcosm experiments due to either the spiking of TPH at specific concentrations and degradation under fully oxic conditions [55, 157], or degradation of TPH coupled to a range of alternative electron acceptors [109]. Aerobic degradation of specifically the alkane fraction of TPH has been monitored with real time PCR targeting phylogenetic and functional gene markers [160, 199, 220, 303]. Results indicated that microbial community structure and ecological function are clearly impacted by the presence of TPH. However, conclusions from studies on short-term spiking of soils are difficult to translate to long-term *in situ* natural attenuation conditions.

In addition to changes in microbial populations due to the presence and concentration of contaminants, community composition and diversity are also dependent on geochemical properties of the subsurface. A number of studies have indicated that various factors dictate the composition and activity of microbes in uncontaminated locations, including geographic region [126], soil type [71], soil pH [126], and land use [175, 212]. Furthermore, it has been shown in TPH contaminated soil that soil type influences the makeup of biodegrading populations [29, 198]. This underscores the complexity of factors influencing soil microbial communities and the challenge of elucidating key populations in *in situ* bioremediation.

In this study, we have investigated microbial diversity and ecological significance in the natural attenuation process through identifying populations present *in situ* at

a diesel contaminated site. To this end, pyrosequencing of PCR-amplified bacterial and archaeal 16S rRNA gene fragments of both bacteria Bacteria and Archaea was utilized as a high throughput technique to examine diversity and microbial groups present in contaminated and corresponding clean samples. Whereas previous work examined community structure in diesel spiked sediments in microcosms [55, 157], this study investigated field collected samples representing various soil matrices and contamination levels. The interrelationships among community composition and diversity, contamination and soil geochemical properties were determined, giving an understanding of the ecological response to changing environmental conditions. Through investigating the impact of long term *in situ* diesel contamination, an understanding of natural attenuation characteristics and requirements is developed. This description of microbial community dynamics provides a basis for the development of more comprehensive theoretical models and molecular proxies for the monitoring of natural attenuation.

4.2 Materials and Methods

4.2.1 Site description

The railway refueling station in Wegliniec, Poland, was contaminated with diesel from 1970-2000 (Figure 4.1). Eighteen monitoring wells were installed in 1997 and in 2001 to characterize the soil diesel contamination, expressed as TPH concentrations, as well as to monitor contamination in the groundwater. During a previous remediation project in 2003, an additional 89 wells of five meters deep were placed in a tight grid like pattern for a pump-and-treat style approach. Use of these wells, however, was discontinued within six months due to clogging resulting from improper filtering of the wells.

The site was originally the location of a brook running from south to north across the site. This created a peat and clay layer on top of the base sand flanked by higher sandy dunes on the eastern side of the site. In order to level the site for the construction of the railroad, the brook was filled with a heterogeneous layer containing coarse sand, gravel, and fine brown sand. Thus, within the contaminated area 3-4 lithological layers were encountered: anthropogenic fill, followed by peat and/or clay, and finally natural sand. To the east of the site, essentially two layers were present, an upper more organic rich brown sand followed by very light sand.

The initial location of refueling was at the southwest corner of the site and was the source of contamination. In this area, up to 70 cm of light non-aqueous phase liquid

(LNAPL) of TPH was observed in piezometers in 2010, and solid phase concentrations were higher than 20 000 mg/kg dry matter. LNAPL has spread across the site in the same direction as the groundwater, which was in a north-northeast direction. The thicker layer of anthropogenic fill deposited on the northern area of the site was contaminated due to the groundwater transportation of dissolved diesel. The extent of weathering varied across the site, as observed from the color and texture of the contamination in piezometers.

4.2.2 Soil sampling

Sampling was performed in 2010 at nine locations across the site, including near the refueling station (sites A and B), just downstream thereof (sites C and D), and farther removed from the station (sites E, F, and G). Additionally, samples were taken where no anthropogenic fill was present either because the location was upstream and just outside of the rail yard (site H) or due to the natural lithology of the subsurface (site I). Hand augers were used to collect layers of sediment at various depths for each sampling location. A full description of the number of samples collected at each location and their depth is given in Table 4.1. The samples were collected and stored in large high-density polyethylene (HDPE) containers for further characterization. Additionally, the pure product LNAPL sample was collected and stored in a glass tube. All samples were stored at 4°C.

Sub-samples were taken directly from the sediment layers in the field for molecular analysis in sterile 50 mL tubes using sterile metal spatulas. These samples were immediately frozen in liquid nitrogen, stored on dry ice during transport (one day) and were stored at -80°C prior to further processing.

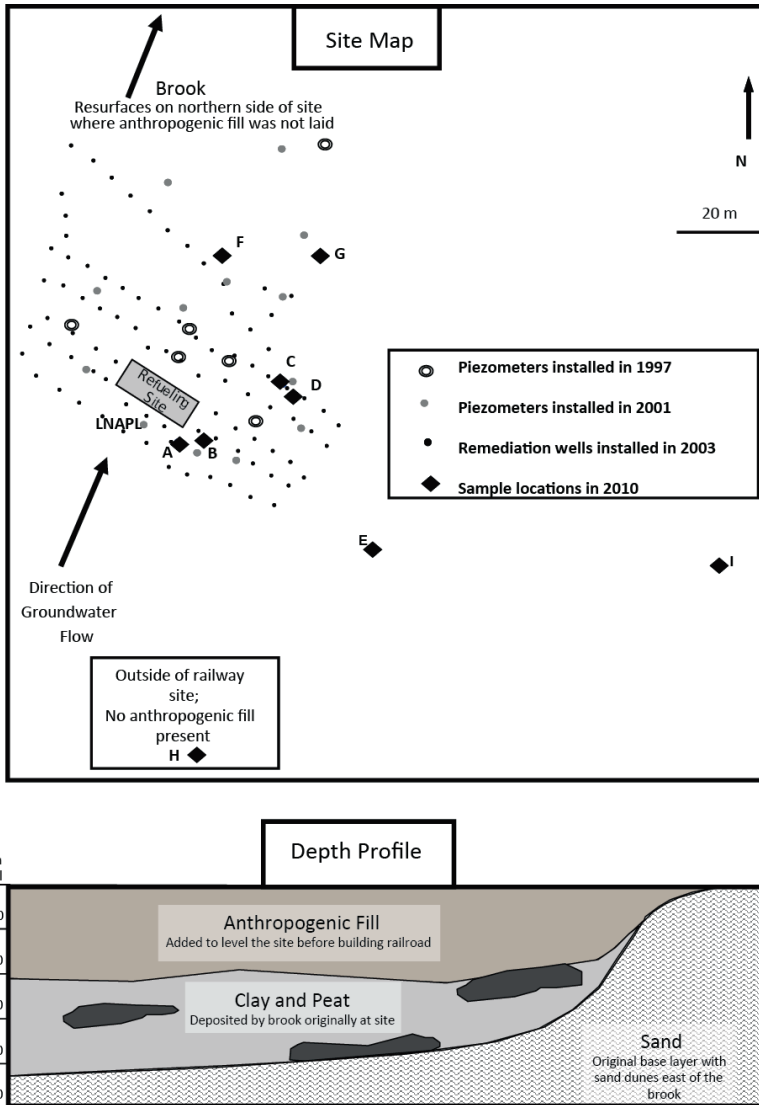


Figure 4.1 Site schematic (above) indicating the location of piezometers installed previously (open and filled large ovals), inoperable remediation wells (small ovals), and sampling locations for the current study (filled diamonds), as well subsurface lithology giving depth profile corresponding to the site map (below). The LNAPL sample was collected in a piezometer from 2001. Sample H was located upstream outside of the site in an area that neither encountered diesel contamination nor contains anthropogenic fill.

Table 4.1 Physical, geochemical, and contaminant characteristics of collected soil samples. In the TPH concentration column, clean samples without diesel contamination are empty. Soils used in multiple chapters are summarized in Appendix 2.

Sample Name	Depth cm bgl	Groundwater level cm bgl	Soil Matrix	Organic Matter (%)	pH	TPH concentration g TPH/kg
A1	0-100	230	Fill	10	7.10	4.26
A2	130-230		Fill	11	6.88	22.21
B1	0-70		Fill	9	7.41	
B2	80-150	170	Clay	5	5.15	2.88
B3	150-170		Peat	12	6.50	17.82
B4	150-250		Peat	10	6.80	16.31
C1	20-90	200	Fill	9	7.55	
C2	90-180		Peat	6	7.37	
C3	200-220		Peat	7	7.02	6.38
D1	0-130	200	Fill	13	7.35	
D2	130-160		Clay	8	7.17	
D3	170-200		Clay	7	6.98	9.99
D4	200-230	200	Peat	54	6.18	11.44
D5	230-250		Sand	3	6.22	13.65
E1	40-120		120	Fill	5	6.51
E2	120-130	Fill		35	7.11	8.47
F1	80-150	190	Sand	2	7.37	
F2	190-220		Sand	2	7.01	1.28
G1	50-120	unknown >180	Fill	4	7.17	
G2	120-160		Fill	5	7.33	
G3	160-180		Fill	4	7.31	
H1	10-40	40	Peat	29	6.55	
H2	40-70		Peat	56	5.48	
H3	70-100		Sand	5	5.82	
I1	10-40	240	Sand	2	4.42	
I2	70-240		Sand	1	4.23	

4.2.3 Chemical analyses

Soil samples were sieved at 2 mm and analyzed for a variety of parameters. Organic matter content was determined as the weight difference due to ignition at 550 °C. pH was measured in the supernatant of a 1:2.5 solid-to-liquid (g:mL) ratio suspension of soil sample in 0.01 M CaCl₂ [191]. Diesel concentrations, measured as TPH, were determined by extraction and quantification with gas chromatography. Extraction was performed according to NEN 5733 solvent shake extraction protocol with acetone and hexane [178]. For pure product samples, 0.2 g LNAPL was dissolved

in 25 mL hexane. Hexane was dried with Na_2SO_4 and TPH was measured on a HP 5890 Gas Chromatograph (GC) with a SIM DIST column and Flame Ionization Detector with nitrogen as the carrier gas. Following 5 min at an initial temperature of 40°C, the temperature was increased at 10°C per minute to a final temperature of 300°C. TPH was determined for samples portraying a diesel chromatogram pattern as the area between C10 and C40. Carbon number fractions were determined using the retention times of a boiling point standard. Samples lacking a chromatogram indicative of diesel but rather typical for trace amounts of organic matter extracted with hexane, were considered clean for the purposes of this analysis.

4.2.4 DNA extraction, amplification, and pyrosequencing of 16S rRNA gene

Total microbial community DNA was extracted from 0.5 g of each soil sample using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, USA) according to the manufacturer's instructions. The amount and quality of the extracted DNA (average molecular size and purity) were estimated on 1% agarose gels and using Nanodrop spectrophotometer readings (Thermo Scientific, Wilmington, USA). Extracted DNA was stored at -20 °C for downstream applications.

In this study, we used a two-step PCR protocol as previously described [1]. With this approach, tags and adapters were added in a second round of PCR amplification. This method facilitates DNA amplification from complex biological samples [93], increases reproducibility and recovers higher genetic diversity, avoiding the amplification bias that is introduced by using long fusion primers in one-step PCR amplification [20].

The first PCR amplification was performed using the modified primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) [93] to amplify a 466 bp fragment of 16S rRNA gene flanking the V3 and V4 regions. The PCR mix (40 µl) contained: 1× Phusion HF buffer containing 2.5 mM MgCl_2 (Finnzymes, Vantaa, Finland), 0.2 mM dNTPs mixture, 0.8 U Phusion Hot Start DNA Polymerase, 2 µM of each primer, 25 µg of BSA (molecular grade, stock 10 mg mL⁻¹) and 1 µL template. The PCR conditions were: an initial activation of the Hot Start polymerase at 98°C for 30s, followed by 30 cycles of 98°C for 5s, 56°C for 20s and 72°C for 20s, and final extension at 72°C for 5 min. Analysis of PCR products was done on 1% agarose gel stained with ethidium bromide, and bands of the correct size were cut and purified using the Montage Gel Extraction Kit (Millipore, Billerica, USA). The quality and concentration of the purified DNA was determined through analysis on 1% agarose gel stained with ethidium bromide (BioRad Gel Doc, Hercules, USA) and using Nanodrop. A second round of PCR was performed as described above, except that the primers

with an adapter and barcodes of 10 nucleotides length were used. Furthermore, the number of PCR cycles was reduced to 15. The PCR products were analyzed on 1% (w/v) agarose gel, DNA was extracted from agarose gel as mentioned above, quantified using Quant-iTdsDNA High-Sensitivity Assay Kit and the Qubit fluorometer (Invitrogen, Grand Island, USA), and mixed in approximately equal concentration (4×10^6 copies μL^{-1}) to ensure equal representation of each sample. These samples were pooled with other samples (total 72 samples), and were sequenced on one of the two regions of a 70_75 GS Pico TiterPlate (PTP) by using a Titanium kit and GS FLX System according to manufacturer's instructions (Roche, Branford, USA).

4.2.5 Analysis of the pyrosequencing dataset

Pyrosequencing data were analyzed using the QIIME pipeline (<http://qiime.sourceforge.net/>) [32]. Low quality sequences were removed that did not comply with the following default quality parameters: (a) include a perfect match to the sequence tag and the 16S rRNA gene primer; (b) be at least 200 bp in length; (c) have no ambiguous bases; and (d) have no homopolymers longer than 6 nucleotides. Once trimmed and assigned to samples, data were processed using the QIIME's UCLUST method in order to cluster the sequences into operational taxonomic units (OTUs) at the 97% similarity level. A set of representative sequences (the most abundant sequence in each OTU) were aligned using PyNAST [49] against the Greengenes core set [50]. Possible chimeric sequences were identified using QIIME's ChimeraSlayer and subtracted from the previously generated OTU list, producing a non-chimeric non-redundant OTU list. The taxonomic affiliation of each OTU was determined using the RDP Classifier at a confidence threshold of 80% [290]. Aligned sequences (OTU representatives) were used to generate a phylogenetic tree with FastTree [200]. The complete pyrosequencing data set is available at the European Bioinformatics Institute under ERP 001944.

4.2.6 Statistical analyses

In order to relate the changes in microbial community composition to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands). Presence/absence and relative abundances of OTUs obtained from the pyrosequencing data set were used. The environmental variables tested were presence/absence of contamination, TPH concentration, pH, organic matter and ground water level. All of the environmental data were transformed as $\log(1 + x)$. A Monte Carlo permutation

test based on 999 random permutations was used to determine whether or not and which of the experimental variables significantly contributed to explaining the observed variance in composition of microbial communities. The community structure was visualized via ordination triplots with scaling focused on inter-sample differences. For all statistical analyses, correlations were considered highly significant at $P < 0.001$ and significant at $P < 0.05$. The significance of the observed differences for carbon fraction distribution and Shannon and Chao1 diversity indexes were verified using the Student's *t*-test.

4.3 Results and Discussion

4.3.1 General characteristics of the soil samples

Owing to both the lithological and contamination patterns at the site, characterization of the 26 samples indicated considerable variation in the physical and geochemical properties of the soils collected (Table 4.1). Organic matter content correlated well with soil type, with fill and sand samples generally below 10 % and 2%, respectively, and peat samples above 10 % organic matter in most cases. pH varied across samples from 4.2 to 7.5; sandy soils showed a slightly lower pH. TPH concentrations of the 11 contaminated samples varied from 1.3 g/kg to 22.2 g/kg. The highest levels of contamination were encountered at sampling sites near the refueling location and at or below the groundwater level.

The distribution and composition of the TPH contamination were indicative of natural *in situ* degradation processes. Diesel concentrations were notably reduced in samples above the groundwater level in the source area where contamination originated; this indicates that degradation may occur in the vadose zone. TPH carbon number fraction distribution also points towards degradation. An LNAPL sample collected in the source zone at the location of refueling shows that the original diesel contamination contained mainly low molecular weight (LMW) hydrocarbons, with 63% of the mass from compounds in the C10-C16 range (Figure 4.2). Soil samples were overall depleted in LMW compounds when compared to the LNAPL. Samples well above the groundwater level (A1) or farther downstream (E2 and F2) showed notably less LMW, with up to 50% of the C10-C16 fraction depleted relative to the LNAPL signature. Reduction in the amount of LMW compounds was also observed across the site; the difference in relative carbon fraction abundance was significant for fractions C10-C12 and C12-C16 when comparing source samples A2 and B3 to plume samples E2 and F2 ($p=0.02$).

This shift in contaminant composition can most likely be attributed to biological degradation. As the LNAPL source spreads across the site, microbial degradation occurs, thereby degrading LMW compounds. Microbial preference for low molecular weight compounds has been observed previously [36, 186]. These results suggest that natural attenuation potential is present at this location and that biodegradation processes are ongoing, which is further corroborated to observed changes in microbial community composition as described below.

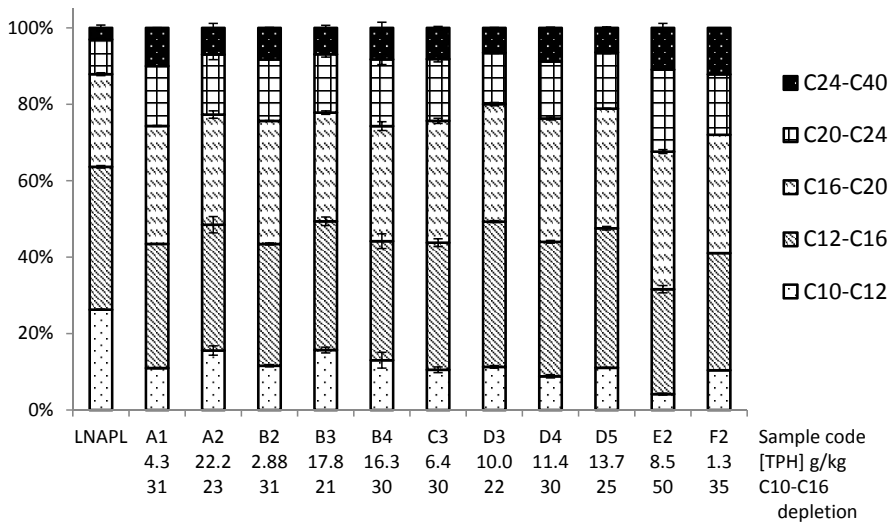


Figure 4.2 TPH carbon fraction distribution in LNAPL and contaminated samples. Carbon fraction percentages are the relative distribution within each sample to allow comparison irrespective of TPH concentration. C10-C16 depletion is calculated using the composition of the LNAPL as the starting material. Absolute TPH concentrations are given below each sample name. Error bars on soil samples are the standard deviation of the average of each fraction for triplicate TPH extractions.

4.3.2 General analysis of the pyrosequencing dataset

Phylogenetic composition and diversity of the microbial communities present in the soil samples collected throughout the diesel contaminated railway site were profiled using pyrosequencing of PCR-amplified bacterial and archaeal 16S rRNA gene fragments. After trimming and quality filtering the raw reads, the sequences were clustered into operational taxonomic units (OTUs) using a similarity threshold of 97%, yielding a total of 40912 high quality non-chimeric OTUs. These OTUs represented

a total of 201585 reads, distributed by 7753 ± 1032 sequences per sample with an average length of 401.8 bp. Representative sequences of each OTU were classified into the domains *Bacteria* (91.7% of the total data set) and *Archaea* (7.7%), using the RDP classifier with a confidence threshold of 80%. A small proportion of sequences (0.6%) could not be classified at the domain level.

A relatively high proportion of sequences, ranging from 2-46% (average 21%), could not be classified below the phylum level. This considerable proportion of unclassified bacterial and archaeal sequences falls in the range of values obtained in other recent studies applying tag-pyrosequencing to different soil and sediment ecosystems, where, in principle, a high microbial diversity is expected. For example, Inceoglu et al. found 15-45% of unclassified bacteria in a pyrosequencing data set obtained from potato field soil samples [93], and a similar range of unclassified bacterial reads (11-24%) was found in a collection of forest and grassland soils [175]. A possible interpretation of these high proportions of unclassified sequences can be that they belong to as-yet uncultured, unrecognized, or novel bacteria or archaea species.

The OTUs were classified into the domains *Bacteria* (27 phyla) and *Archaea* (2 phyla). On average for the complete data set, the major microbial phyla were *Proteobacteria* ($36.6 \pm 14.5\%$ of the reads), *Firmicutes* ($9.0 \pm 10.2\%$), *Actinobacteria* ($9.3 \pm 5.1\%$), *Acidobacteria* ($6.6 \pm 5.5\%$), *Euryarchaeota* ($6.1 \pm 11.3\%$), *Chloroflexi* ($2.8 \pm 3.0\%$), *Bacteroidetes* ($1.8 \pm 1.7\%$) and *Verruomicrobia* ($1.1 \pm 1.3\%$) (Figure 4.3).

Initial examination of the phylogenetic distribution identified sample I2 as an outlier. Whereas relative abundance of individual phyla does not surpass 54.4% in any other sample, 83.6% of sequences were classified as *Proteobacteria* in sample I2 (Figure 4.3). Additionally, 88% of *Proteobacteria* were from the *Betaproteobacteria* (Figure 4.3). Sampling was performed at location I to provide a clean sand sample as a control for comparison to contaminated sand samples. However, visual comparison of I2, a coarse light-colored sand, to other clean sand samples (F1, H3, I1), which were notably darker and finer, showed that I2 was dissimilar. This was further confirmed by geochemical parameters (Table 4.1), phylogenetic distribution (Figure 4.3), and alpha-diversity (Table 4.2 and Figure 4.4). Redundancy analysis (Figure 4.5) indicated that along with I2, I1 is also not representative of clean samples.

The majority of the most abundant bacterial OTUs were affiliated with *Proteobacteria*, which were observed at relative abundances of 10.4-82.9% and all five major classes were represented (Figure 4.3). *Proteobacteria* has been identified in many studies as the predominant phylum in soil samples [55, 168, 175, 212],

playing an integral role in nutrient cycling [113]. For example, the two most abundant bacterial OTUs were classified within the genera *Variovorax* (OTU 7163, 4.1% of the total number of sequences; family *Comamonadaceae*, *Betaproteobacteria*), and *Methylocystis* (OTU 64060, 1.4%, family *Methylocystaceae*, *Alphaproteobacteria*). The ability of *Alpha*-, *Beta*-, and *Gammaproteobacteria* to utilize aliphatic and aromatic compounds has been previously established [73, 192, 276], and shifts in their abundance are often noted upon contamination with TPH or during bioremediation [14, 197, 284].

OTUs with sequences similar to *Methanosaeta* in the *Methanosaetaceae* family in the archaeal phylum *Euryarchaeota* showed remarkably high abundance. These OTUs were observed in many contaminated samples and represented more than 30% of all 16S rRNA gene sequences in samples A1 and D5 (Figure 4.3). A thorough analysis of archaeal abundance and diversity is given below.

The phyla *Firmicutes*, *Actinobacteria*, and *Acidobacteria*, found in this study in high abundances, have been noted previously in work examining uncontaminated soil samples [101, 175, 212]. Furthermore, these phyla have been observed in soils contaminated with aliphatic or aromatic compounds, in both the polluted samples as well as the clean reference soil [6, 109, 168, 222]. *Bacteroidetes*, which were not dominant in this study, have been found in clean and contaminated samples at similar relative abundances (1.9% and 1.8%, respectively). In a previous work using pyrosequencing to study an uncontaminated soils identified *Bacteroidetes* to be quite prevalent with relative abundances of 15-25% [212]; a different study, where pyrosequencing was also utilized to compare community structure in forest versus grasslands, placed *Bacteroidetes* into the rare phyla group with <1% overall relative abundance [175].

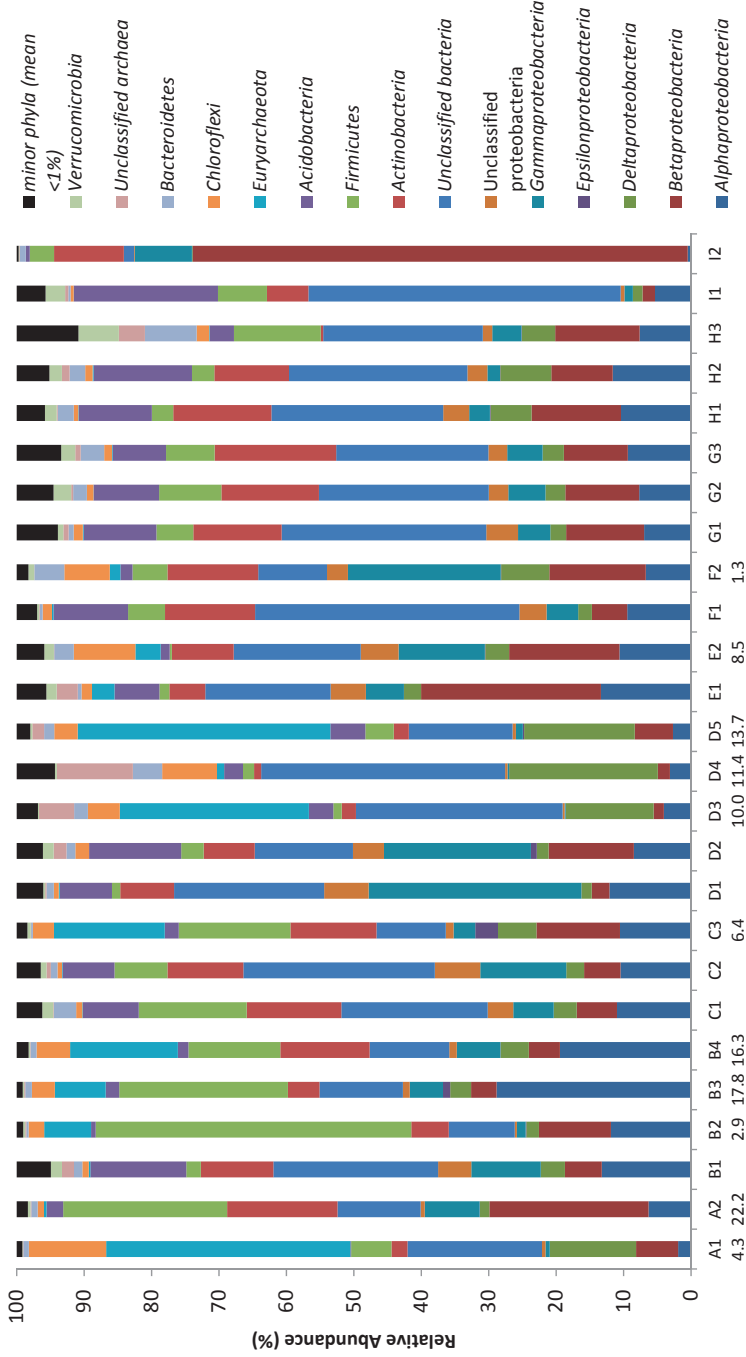


Figure 4.3 Relative abundance of dominant microbial phyla and *Proteobacteria* classes (>1% of average abundance for the total pyrosequencing data set). Unclassified bacteria and archaea corresponds to sequences that could only be classified to the domain level. Unclassified *Proteobacteria* corresponds to sequences that could only be classified to the phylum level. Groups that were observed at less than 1% of average abundance were grouped in minor phyla. Values below sample names indicate contamination as g TPH/kg soil.

4.3.3 Microbial diversity in pristine and contaminated samples

Alpha-diversity estimations, based on OTUs defined at a genetic distance of 3% (i.e. sequence identity of 97%), showed that there was a significant reduction in microbial richness and diversity in the contaminated samples. Differences in the Chao1 richness estimator and Shannon diversity indexes were highly significant when comparing clean (uncontaminated) versus contaminated samples ($p=0.00003$ and $p=0.0002$, respectively; Table 4.2). When each soil matrix type (fill, peat, clay and sand) was considered separately, only fill and peat samples showed a significant difference in microbial diversity between clean and contaminated samples ($p<0.05$). The clay group was too small to assess the significance of observed differences in diversity. Within the sand group, diversity indices in clean and contaminated samples were not significantly different, most likely due to the low TPH concentration in sample F2 leading to a non-significant microbial response to the contamination. The peat and fill groups contained samples with much higher TPH concentrations in the polluted samples.

Finally, rarefaction curves estimating OTU richness confirmed the difference between clean and contaminated samples (Figure 4.4), where clean samples were predicted to harbor higher microbial species richness than contaminated samples when excluding the outlier sample I2. Since no similar correlations between the other soil properties and richness were observed, the noted change in richness could be attributed to the presence of TPH contamination. No notable correlation between TPH concentration and diversity was observed, indicating that the presence of contamination, rather than its concentration, dictates changes in diversity. Apparently, a threshold concentration of TPH is required to elicit changes in diversity, above which no correlations of concentration and diversity would be observed, at least for the range of concentrations observed at this field site.

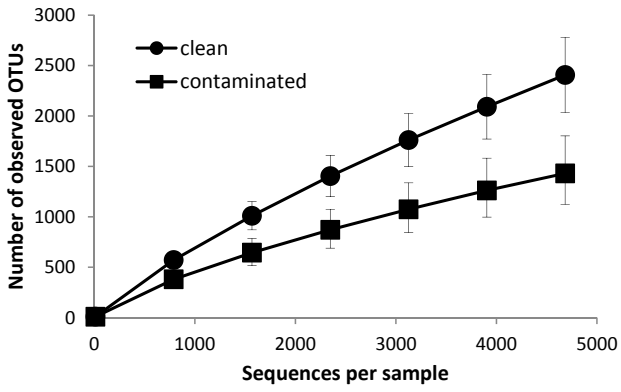


Figure 4.4 Rarefaction curves indicating the average observed number of operational taxonomic units (OTUs) for clean samples (circles) and contaminated samples (squares) without the outlier I2 of the clean group. Error bars indicate \pm standard deviation.

Table 4.2 Microbial richness and diversity as determined by Chao1 estimator and Shannon Index.

Sample	Contamination	Estimated OTU Richness		Shannon Index (H')	Number of high quality sequences
		Chao1	95% confidence interval		
A1	Contaminated	3127	2804; 3520	6.49	8462
A2	Contaminated	3259	2994;3575	8.59	5259
B1	Clean	13024	11963; 14222	10.69	8285
B2	Contaminated	4493	4183; 4853	9.02	7876
B3	Contaminated	8348	7599; 9211	8.77	8054
B4	Contaminated	6431	5847; 7112	8.81	7636
C1	Clean	11783	10891; 12785	10.8	7781
C2	Clean	9226	8499; 10053	10.25	7458
C3	Contaminated	5315	4848; 5860	8.25	7527
D1	Clean	6794	6235; 7437	8.86	8200
D2	Clean	7811	7184; 8528	9.44	8158
D3	Contaminated	4227	3783; 4761	7.18	9474
D4	Contaminated	3849	3472; 4302	7.43	8258
D5	Contaminated	4587	4155; 5099	7.52	8192
E1	Clean	6948	6346; 7646	9.17	7691
E2	Contaminated	6529	5983; 7159	9.54	7639
F1	Clean	14683	13506; 16005	10.94	7798
F2	Contaminated	5439	4932; 6032	9.08	5619
G1	Clean	9820	8941; 10826	10.44	5663
G2	Clean	11929	11075; 12884	11.06	7711
G3	Clean	14447	13512; 15483	11.16	10332
H1	Clean	12824	11878; 13885	11.19	7637
H2	Clean	12637	11767; 13609	11.27	8294
H3	Clean	7716	7227; 8269	10.69	7821
I1	Clean	4382	3987; 4851	8.54	7867
I2	Clean	521	463; 612	3.75	6893

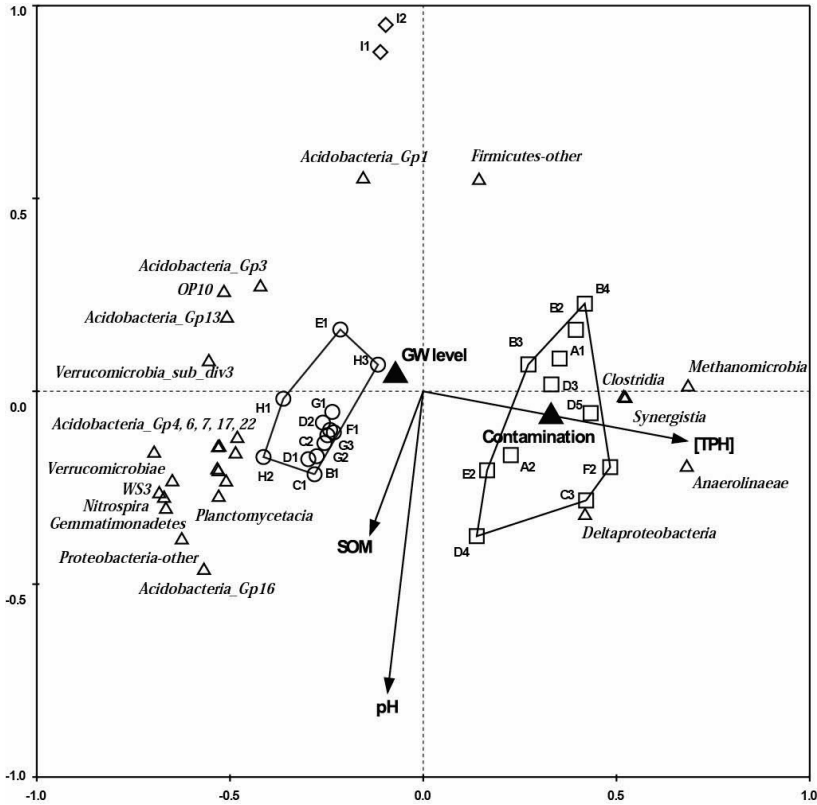


Figure 4.5 Redundancy Analysis Triplot showing relationship between microbial composition at class level and soil geochemical and physical properties as described in Table 1. Clean samples with open circles cluster on the left quadrant; contaminated samples with open squares cluster on the right quadrant. Outliers I1 and I2 are open diamonds. Classes are indicated with triangles, environmental parameters with arrows, and nominal environmental variables in filled triangles. The eigenvalues of the first (x) and second (y) canonical axes are 0.265 and 0.073, respectively. The plot can be interpreted qualitatively by following the direction of arrows for environmental parameters. The arrow length corresponds to variance that can be explained by the environmental variable. The direction of an arrow indicates an increasing magnitude of the environmental variable. The perpendicular distance between classes and environmental variable axes in the plot reflects their correlations. The smaller the distance, the stronger the correlation. SOM: soil organic matter; GW level: above groundwater level; [TPH]: concentration of TPH

4.3.4 Correlations between soil properties including contamination and microbial community composition

Differences in microbial community composition have been attributed to soil properties, including vegetation and land use [175], soil matrix type [71, 198], and soil pH [101]. We performed multivariate analysis in order to determine the impact of environmental parameters on the community structure. Here, geochemical soil characteristics including organic matter content, pH, sample depth relative to the groundwater level, and soil matrix type were considered. There was no significant correlation between overall community composition with either organic matter content or sample depth. However, redundancy analysis (RDA) indicated that the relative abundance of some microbial phyla such as *Acidobacteria* subgroup 16 and unclassified *Proteobacteria* was correlated with organic matter content (Figure 4.5).

pH was found to significantly contribute to explaining the observed variation in community composition ($p=0.008$). *Acidobacteria* subgroup 1 was correlated with low pH, while *Acidobacteria* subgroup 16 was somewhat correlated with increasing pH values (Figure 4.5). High abundances of *Acidobacteria* subgroup 1 have previously been observed in samples with pH below 5.5 and high abundance of *Acidobacteria* subgroup 16 in samples above pH 6 [100]. Additionally, all *Acidobacteria* subgroups were negatively correlated with presence of TPH (Figure 4.5), which has been observed previously [304]. A similarly deleterious effect on the abundance and diversity of *Acidobacteria* was observed due to both the short-term and long-term presence of 2,4,6-trinitrotoluene [70], further supporting the conclusion that *Acidobacteria* are particularly sensitive to changes in soil conditions including the presence of organic contamination.

RDA was also performed in order to analyze the impact of diesel contamination in general as well as more specifically the TPH concentration on community composition (Figure 4.5). Presence of diesel in soil samples significantly contributed to explaining the variation in microbial community structure ($p=0.002$), indicating that contamination, above all other geochemical characteristics, dictates microbial diversity. Contaminated samples were positively correlated with TPH contamination (Figure 4.5 right cluster), whereas the clean samples were negatively correlated with TPH contamination (Figure 4.5 left cluster). However, the clean samples I1 and I2 show a divergent pattern. The concentration of TPH present was found to correlate with the relative abundance of a number of phyla (Figure 4.5 and 4.6), including *Chloroflexi*, *Firmicutes*, and *Euryarchaeota*, as described in the following sections.

4.3.5 Impact of TPH contamination on soil microbial communities

A highly significant reduction in OTU richness and microbial diversity was observed to be correlated with TPH contamination, as described above. Previous work investigating microbial diversity have found that contamination may yield an increase [102, 108, 307] or a decrease [25, 284] in diversity. The reduction in diversity observed in our dataset lies in stark contrast to the only other pyrosequencing based study investigating the impact of diesel contamination on microbial community structure [55]. In that study, an increase in diversity and richness was observed 23 days after spiking and was attributed to the addition of hydrocarbon substrates with diverse structures.

Whereas similar short term effects are possible in the field, our study investigated microbial diversity under natural *in situ* conditions in soil contaminated for more than forty years with diesel. Reduced diversity is most probably caused by the selective ecological pressures of TPH contamination, including: i) the introduction of toxic TPH concentrations and biodegradation products [122, 127, 248]; ii) the dominance of a pool of less diverse, easily degradable diesel-originating hydrocarbon substrates as compared to complex soil organic matter compounds [36, 156, 186]; iii) presence of imbalanced C:N:P ratios due to the influx of carbon rich diesel leading to competition for nutrients and reduced nutrient availability for the microbial community originating from the pristine system [157, 306, 308]; and, iv) possible electron acceptor limitation for the native microbial community in the subsurface due to microbial TPH mineralization [127, 306].

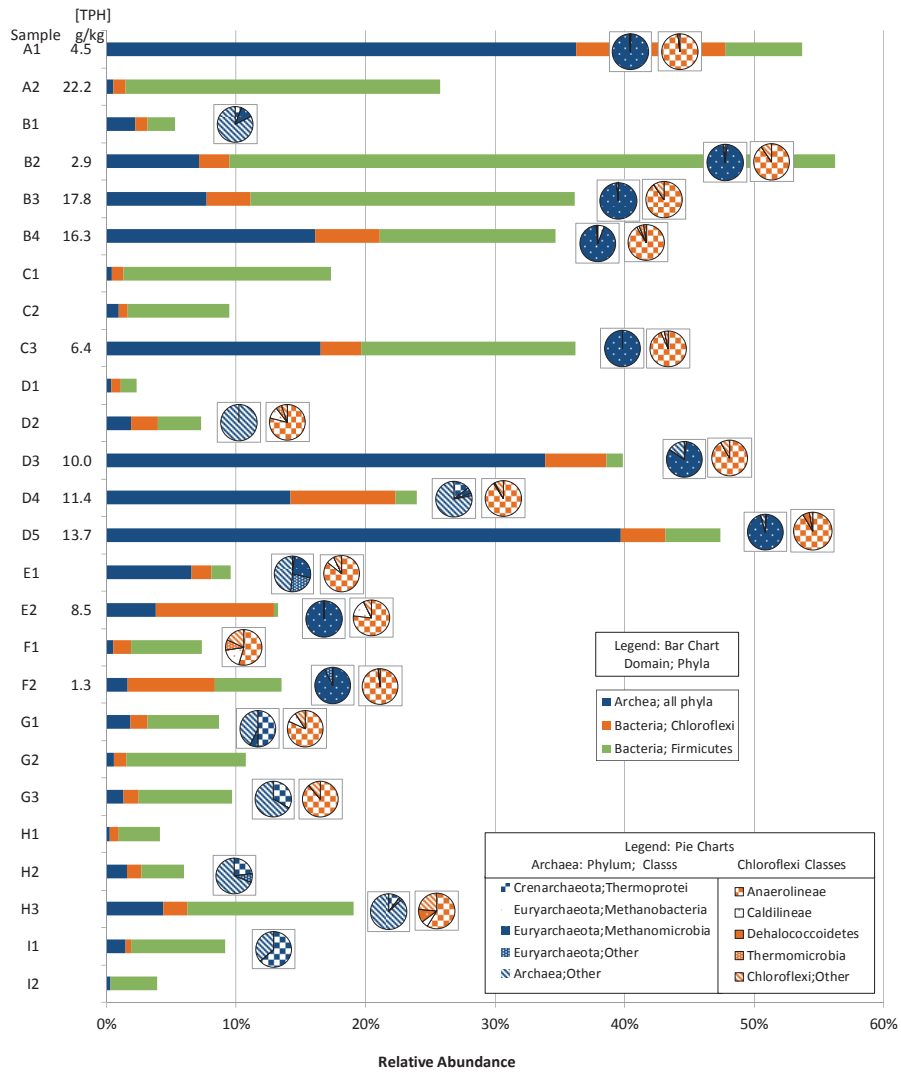


Figure 4.6 Comparison of relative abundances within total data set of archaeal and bacterial phyla and classes associated with contamination. TPH concentrations are given next to sample names. Abundances of *Archaea* (blue), *Chloroflexi* (orange), and *Firmicutes* (green) and given in the bar graph using the x-axis. For samples in which the relative abundance of total *Archaea* or *Chloroflexi* exceeds 1%, pie charts are included for class level distribution.

We further explored the specific microbial groups within the phyla *Chloroflexi*, *Firmicutes*, and *Euryarchaeota* identified to be associated with contamination. Although these phyla were found in nearly all samples, combined relative abundances in contaminated samples were on average 33% (as compared to 7% in clean samples) and reached more than 50% in contaminated samples A1 and B3 (bar graph, Figure 4.6). Additionally, the high relative abundance in contaminated samples of certain classes in the aforementioned phyla explains the reduced richness noted above (pie graphs, Figure 4.6). This difference was reflected in the apparent class composition of *Chloroflexi* and *Euryarchaeota* in contaminated samples. Whereas a number of classes were represented in clean samples, in the presence of TPH, OTUs classified as *Euryarchaeota* were almost exclusively from the class *Methanomicrobia* and *Chloroflexi* from the class *Anaerolineae*. The increased abundance of the phyla *Euryarchaeota*, *Chloroflexi*, and *Firmicutes* in association with hydrocarbon impacted samples, as found here, is in line with observations made in previous studies [55, 141, 195, 196, 297].

Euryarchaeota have been observed to be the dominant archaeal phylum in heavily oil contaminated environments [141]. The most dominant OTU classified in the class *Methanomicrobia* showed 98% identity to the 16S rRNA genes of members of the *Methanosaeta*, microorganisms that have been previously observed in oil sands [196] and have been observed in consortiums able to perform anaerobic mineralization of long alkane chains [125, 305]. This genus is known to solely perform acetate fermentation [115], under methanogenic conditions, indicating that methanogenic processes could prevail at the contaminated locations.

The presence and high abundance of *Euryarchaeota* in contaminated samples in the current study lies in contrast to previous pyrosequencing studies that reported on archaeal abundance and diversity in clean soil samples. A study on Archaeal diversity from 146 uncontaminated soil samples collected across 6 continents showed that the relative abundance of *Archaea* averaged 2% and was less than 16% in all samples; the main phylum observed was *Crenarchaeota* [16]. The observed differences in Archaeal phyla diversity and abundance between the current study on TPH contaminated samples and clean soils investigated previously point towards the role of contamination in Archaeal community composition.

A major OTU in contaminated samples showed 99% sequence identity to members of the family *Anaerolineae* within the *Chloroflexi*. *Anaerolineae* comprise obligate anaerobes, often found under sulfate reducing conditions [297]. This *Anaerolineae* OTU showed 100% sequence identity to a 16S rRNA gene sequence of an uncultured

Anaerolineae phylotype that was also observed at a tar oil contaminated site with toluene degradation [297]. Although present at higher relative abundances in contaminated samples, OTUs related to this class were also observed in a variety of clean samples (E1, G1, H3), indicating the subsurface was most likely naturally anoxic. Finally, members of the *Firmicutes* have been identified in diesel contaminated samples either within a bacterial consortium [55, 195] or as an isolate selected for biosurfactant production to improve hydrocarbon bioavailability [19].

Current knowledge regarding the physiology of characterized phylotypes showing sequence similarity to members of the microbial phyla identified to be predominantly associated with contaminated soils in this study indicates the presence of anoxic conditions. anoxic subsurface conditions have implications for the potential for and especially the rate of natural attenuation. Although biodegradation of aromatics and alkanes can occur at low redox potentials, rates are severely reduced as compared to oxic environments [250].

4.3.6 Implications of community structure in contaminated samples for natural attenuation

In this study we explored the long term effect of TPH contamination on the microbial community at a site with a diesel contamination history of forty years, giving microorganisms the ability to adapt and eventually utilize the present aliphatic and aromatic hydrocarbons. As a result, the impact of diesel on the microbial community structure is illuminated beyond the initial shifts in composition associated with the addition of carbon substrates and/or toxicants in the short term.

The increasing prevalence of natural attenuation projects due to risk based remediation regulations requires improved understanding of the microbial populations present and active in contaminated soils. Regulations generally require multiple lines of evidence, including: i) historical data showing a stabilization or reduction in plume size; ii) hydrogeological or geochemical indications of the type of natural attenuation processes; and, iii) proof of biological degradation potential through the use of microcosm studies or molecular diagnostics [18, 206]. The current study is a step towards the further development of molecular tools to determine microbial degradation potential in support of natural attenuation of TPH contaminated locations.

We identified a number of OTUs, showing high similarity to microbes known to be involved in TPH degradation. These were mainly anaerobic microbes, including members of the phyla *Euryarchaeota*, *Chloroflexi* and *Firmicutes*. This finding

correlates with the degradation potential that was found at the site, based on the shifts in diesel carbon number distribution (Figure 4.2). Soil samples, especially those from the vadose zone or in the plume, had notably fewer LMW compounds, with up to 50% of the C10-C16 fraction having been depleted as compared to the composition of the LNAPL source. Microbial preference for degradation of the more mobile, and thus high risk, LMW compounds fits well into risk-based natural attenuation approaches. Such microbial degradation potential is another line of evidence in addition to traditional chemical analyses of soil and groundwater, indicating the usefulness of a full understanding of the microbial community in order to substantiate the choice for natural attenuation a site.

The redox conditions noted here also allow more specific direct molecular analyses of bioremediation under such natural attenuation conditions. Typical investigations into TPH degradation include utilization of qPCR assays targeting genes encoding enzymes involved in aerobic TPH degradation, such as the alkane monooxygenases *AlkB* and *P450* [276, 283]. In the case of this site, any response to such alkane monooxygenase primers with a DNA template would indicate whether or not there is a potential for aerobic degradation. However, the potential for continued natural attenuation under the prevailing anoxic conditions would not be illuminated, and the exploitation of any existing aerobic degradation potential would require extensive manipulation of *in situ* circumstances.

This work highlights a number of potential molecular proxies, which at this site were indicative of long term diesel contamination and natural attenuation. The reduced diversity noted in samples with TPH, in addition to being ecologically relevant, means that a large portion of the microbial community could be targeted with relatively few molecular assays. Thus, microbial characterization of such a site could be simplified, as fewer microbial species need to be detected to identify the potential for degradation. OTUs related to *Archaea*, specifically of the class *Methanomicrobia* in the phylum *Euryarchaeota*, are particularly associated with TPH contamination in this study and have been observed in previous work to be present in microbial consortiums performing alkane degradation [125, 141, 196]. This class dominates archaeal sequences in most contaminated samples, where *Archaea* relative abundance reaches up to 35%. Additionally, sequences identified as belonging to the phyla *Chloroflexi*, specifically *Anaerolineae*, as well as *Firmicutes* show higher abundances in diesel contaminated samples compared to clean samples. Previous work identifying these phyla in TPH contaminated location and describing their role in hydrocarbon degradation supports the choice of these phyla

for consideration as potential targets for molecular based techniques [19, 55, 195, 297]. Additional studies investigating the microbial community composition at TPH contaminated locations where natural attenuation has been observed are required to further develop and validate this approach.

The results presented here indicate that a number of taxa can be identified at a contaminated site that are correlated with TPH contamination, providing a first insight into the potential for degradation of the TPH contamination present. This knowledge can be further used to examine the microbial population in terms of ecological relevance in order to understand the underlying conditions that are conducive to degradation processes. Finally, with the increasing prevalence of natural attenuation projects due to risk based remediation regulations, an understanding of the microbial populations present in contaminated soils is needed for the acceptance of monitored natural attenuation as a remediation strategy.

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Part Two

Chapter 5

Impact of organic carbon and nutrients mobilized during chemical oxidation on subsequent bioremediation of a diesel contaminated soil

Chapter 6

Recovery of microbial diversity and activity during bioremediation following chemical oxidation of diesel contaminated soils

Chapter 7

Geochemical and microbiological characteristics during *in situ* chemical oxidation and *in situ* bioremediation at a diesel contaminated site

Chapter 5

Impact of organic carbon and nutrients mobilized during chemical oxidation on subsequent bioremediation of a diesel contaminated soil

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Abstract

Remediation with *in situ* chemical oxidation (ISCO) impacts soil organic matter (SOM) and the microbial community, with deleterious effects on the latter being a major hurdle to coupling ISCO with *in situ* bioremediation (ISB). We investigate treatment of a diesel-contaminated soil with Fenton's reagent and modified Fenton's reagent coupled with a subsequent bioremediation phase of 187 d, both with and without nutrient amendment. Chemical oxidation mobilized SOM into the liquid phase, producing dissolved organic carbon (DOC) concentrations 8-16 times higher than the untreated field sample. Higher aqueous concentrations of nitrogen and phosphorous species were also observed following oxidation; NH_4^+ increased 14-172 times. During the bioremediation phase, dissolved carbon and nutrient species were utilized for microbial growth-yielding DOC concentrations similar to field sample levels within 56 d of incubation. In the absence of nutrient amendment, the highest microbial respiration rates were correlated with higher availability of nitrogen and phosphorus species mobilized by oxidation. Significant diesel degradation was only observed following nutrient amendment, implying that nutrients mobilized by chemical oxidation can increase microbial activity but are insufficient for bioremediation. While all bioremediation occurred in the first 28 d of incubation in the biotic control microcosm with nutrient amendment, biodegradation continued throughout 187 d of incubation following chemical oxidation, suggesting that chemical treatment also affects the desorption of organic contaminants from SOM. Overall, results indicate that biodegradation of DOC, as an alternative substrate to diesel, and biological utilization of mobilized nutrients have implications for the success of coupled ISCO and ISB treatments.

5.1 Introduction

The application of *in situ* technologies, including *in situ* chemical oxidation (ISCO) and *in situ* bioremediation (ISB), for the remediation of soils contaminated with organic compounds is expanding. ISCO treatments, such as permanganate, persulfate, catalyzed hydrogen peroxide, and ozone have shown success in the oxidation and thus remediation of a variety of contaminants [233]. ISB, whereby microorganisms are used to degrade contaminants, has also proved to be a robust, cost-effective remediation technology. While each of these treatments can stand alone, increasing interest focuses on the development of a biphasic technology, whereby ISCO is followed by an ISB step [35, 217, 255, 274].

The oxidative and pH stress associated with chemical oxidation treatments are known to effect soil microbial activity. Thus, research has investigated the regeneration of biodegradation capacity following chemical oxidation [103, 140, 218, 255]. In general, results indicate that, when properly implemented, chemical oxidation only has a minor impact on the biodegradation capacity of soils, with full regeneration occurring rapidly with minimal lag times of 7-21 d [103, 274]. This fast regeneration compared to the time frame of *in situ* remediation offers opportunities for cost-effective soil clean-up approaches based on chemical pretreatment followed by a biological polishing step [255]. Once optimized, biphasic treatments are expected to achieve higher remediation efficiencies than approaches based on either chemical or biological methods alone.

Further improvements of these biphasic technologies should focus on tailoring ISCO treatments to biological requirements and ensuring necessary amendments, including nutrients, electron donors, and electron acceptors. In addition to investigating microbial community composition and biodegradation capacity, it is important to assess the impact of oxidation on soil organic matter (SOM) quantity and quality. Non-selective chemical oxidants react with a range of organic compounds and SOM may, depending on the quantity and reactivity, consume more oxidants than the contaminant of interest. Previous investigations have focused on the effect of oxidants on soil structure and quality. A comparison of seed germination and root elongation in soils treated with either Fenton's reagent (Fe^{2+} catalyzed H_2O_2) or permanganate showed that Fenton's reagent, although unfavorable, was more compatible with re-vegetation [240]. Ozone treatment also degrades the soil matrix, with a reduction in overall SOM and an increase in dissolved organic carbon (DOC) noted [185, 289]. Jung and Choi (2003) found an increase in the biodegradability of

SOM following ozone treatment due to an increase in the hydrophilic content upon oxidation. Changes in the relative abundance of soil fractions such as humic and fulvic acids (HA and FA, respectively) and the humin fraction have been reported [104, 185, 289]. Wang et al. (2012) found DOM, HA and FA content increased during short ozonation treatments (4-10 h), but these decreased after 15 h of oxidation. Finally, a reduction in SOM and HA content and an increase in FA was observed following Fenton's reagent treatment [251].

To date, research has focused on the impact of ISCO on either biodegradation or SOM characteristics, with the goal of understanding the deleterious effect of chemical oxidants on microbial communities or soil structure. However, SOM oxidation during chemical oxidation can also have consequences for a subsequent bioremediation phase once microbial biomass has regenerated, thus impacting the overall remediation efficiency. We investigated how changes in aqueous constituents due to oxidation of SOM with Fenton's reagent and modified Fenton's reagent impact the remediation of diesel-contaminated soils. By identifying changes in the quantity of biodegradable organic carbon and nutrients upon chemical treatment, and linking these changes to biodegradation activity under different nutrient conditions, this research seeks to improve the application of coupled ISCO and ISB.

5.2 Materials and Methods

5.2.1 Soil sampling

A field-contaminated soil was sampled at a railroad station in Węglińiec, Poland in March 2010. Refueling activities between 1970 and 2000 caused diesel contamination with up to 70 cm of non-aqueous phase liquids present in some areas. Sampling was performed directly adjacent to the refueling location in the vadose zone of an anthropogenic fill layer at a depth of up to 100 cm (Table 5.1). Soil, collected with hand-augers, was immediately placed in clean HDPE vessels (CurTec, The Netherlands) and stored at 4 °C upon arrival in our laboratory in The Netherlands. The soil was sieved at 2 mm to homogenize the sample prior to use.

Table 5.1 Properties of field-collected soil. Values and standard errors are given for triplicate analyses. Soils used in multiple chapters are summarized in Appendix 2.

Parameter	Value
Sample Depth (cm bgl ^a)	0-100
Total TPH (g TPH ^b kg ⁻¹)	5.24 ± 0.14
Bioavailable TPH (g TPH kg ⁻¹)	1.75 ± 0.00
TOC ^c (%)	10.49 ± 0.32
pH	7.1

^a below ground level

^b total petroleum hydrocarbons

^c total organic carbon

5.2.2 Experimental setup

Chemical oxidation was performed with catalyzed hydrogen peroxide as either traditional Fenton's reagent or modified Fenton's reagent. In each case, H₂O₂ was added either at once (1X), or was portioned into three aliquots added sequentially over 3 d (3X). In the four chemical oxidation treatments, the same dosage of Fe²⁺ and H₂O₂ was used. Additionally, biotic and abiotic control treatments were included. In the biotic control without chemical oxidation, all constituents of Fenton's reagent were added; however H₂O₂ was replaced with water. Abiotic control microcosms underwent 1X modified Fenton's reagent treatment; thereafter, NaN₃ (Merck, Germany) was added to a final concentration of 2mM after chemical oxidation and at days 56 and 124 of incubation.

Microcosms in 125 mL serum bottles contained 4 g of sieved soil and a final volume of 10 mL of liquid. The liquid contained a total concentration of 150 mg L⁻¹ Fe²⁺ as Fe₂SO₄•7H₂O (Merck, Germany), 5% H₂O₂ (30%; Merck, Germany), de-ionized-H₂O, and either 23 mM of H₂SO₄ (for Fenton's Reagent; pH of less than 3) or 1.2 g L⁻¹ citrate (monohydrate; Merck, Germany) as a chelator (for modified Fenton's reagent; pH between 6.5 and 7). For sequential addition microcosms, one third of the H₂O₂ was added each day for 3 d. The reaction was performed in open bottles until no residual hydrogen peroxide was measured with testing strips (Quantofix, Germany).

After chemical oxidation, bioremediation was stimulated under oxic conditions. No bioaugmentation was performed in order to understand the natural regeneration of microbial communities that had been exposed to chemical oxidation. Microcosms that did not receive nutrient amendment were immediately closed with Viton stoppers (Rubber bv, The Netherlands) and crimped. For nutrient amendment, 1 mL of a concentrated mineral medium in 100 mM phosphate buffer was added prior to

stoppering. The mineral media contained 10.2 g L⁻¹ NH₄Cl, 0.48 g L⁻¹ CaCl₂, 0.54 g L⁻¹ MgSO₄•7H₂O, 12 mg L⁻¹ FeCl₃•4H₂O, 12 mg L⁻¹ CoCl₂•6H₂O, 3 mg L⁻¹ MnCl₂•4H₂O, 0.18 mg L⁻¹ CuCl₂•2H₂O, 0.3 mg L⁻¹ ZnCl₂, 0.3 mg HBO₃, 0.5 mg L⁻¹ (NH₄)₆Mo₇O₂₄•4H₂O, 0.6 mg L⁻¹ Na₂SeO₃•5H₂O, 0.3 mg L⁻¹ NiCl₂•6H₂O, 6 mg L⁻¹ EDTA, and 1 mL L⁻¹ 36% HCl. Prepared microcosms were incubated at 20 °C on a shaker at 120 rpm in the dark.

A number of parameters were monitored throughout the experiment (Table 5.2). pH measurements indicated that the soil buffering capacity was not significantly impacted by treatment and that soil pH remained near neutral. Total petroleum hydrocarbons (TPH) were measured in all microcosms before and after chemical oxidation, and on days 28, 56, 124, and 187 of incubation; microcosms with nutrient amendment were also measured on day 14. Remediation efficiency was calculated relative to the abiotic control in order to account for any abiotic losses related to the long incubation periods. Bioavailable TPH was measured before and after chemical oxidation and on days 56, 124, and 187 of incubation. In the filtrate, total carbon (TC), inorganic carbon (IC), chemical oxidant demand (COD), ammonium (NH₄⁺), nitrate and nitrite (NO₃⁻ + NO₂⁻) total nitrogen (TN), and phosphate (PO₄) were measured before and after chemical oxidation and on days 56, 124, and 187 in all microcosms. Additionally, throughout incubation, respiration was monitored by headspace analysis of O₂ consumption and CO₂ production. The headspace was periodically flushed with atmospheric gas to maintain O₂ levels above 10% to ensure fully aerobic conditions. Similar gas exchange in abiotic control microcosms showed no measurable losses in low molecular weight TPH components due to volatilization.

5.2.3 Extraction procedures

Total and bioavailable TPH were measured by sacrificing triplicate microcosms for each analysis. Bioavailable TPH was determined using 24 h Tenax extraction as described previously [259]. Briefly, to every 1 g of dry soil, 1 g of Tenax, 10 mg NaN₃ and 40 mL of 0.01 M CaCl₂ were added to the soil slurry. After shaking for 24 h, the Tenax and soil were separated by washing with 0.01 M CaCl₂. TPH extraction was performed on the Tenax granules. An additional three microcosms were extracted for TPH. In both cases, solvent shake extraction according to the Dutch standard procedure was performed with acetone and hexane [178].

For filtrate analyses (TC, IC, COD, NH₄⁺, (NO₃⁻+NO₂⁻), TN, and PO₄), triplicate microcosms were sacrificed. The field sample was prepared by incubating soil and water together on a shaker for 3 d prior to analysis; other microcosms were prepared as described in section 2.1. The entire soil slurry was filtered through a 0.45 μm

mixed cellulose ester filter (Whatman, United States). Analysis on the filtrate was performed within 24 h of sacrificing the microcosm.

5.2.4 Chemical analyses

O₂ and CO₂ were measured on a gas sample collected from the microcosm headspace on a Shimadzu Gas Chromatograph (GC) with Porabond and Molsieve 5a columns in parallel at 65 °C. Loop injection was at 120 °C, a thermal conductivity detector at 150 °C was used, and the carrier gas was helium. TPH samples were measured on a HP 5890 GC with a SIM DIST column and Flame Ionization Detector with nitrogen as the carrier gas. After an initial 5 min at 40 °C, ramping at 10 °C min⁻¹ was performed to a final temperature of 300 °C. TPH was determined as the area between C10 and C40 using a boiling point standard to determine the retention times.

COD was measured spectrophotometrically according to the manufacturer's recommendations (Hach Lange, The Netherlands). Other components in the filtrate were analyzed by the Chemical Biological Laboratory Soil Center (CBLB, Wageningen, The Netherlands). TC and IC were determined on a Shimadzu analyzer; dissolved organic carbon (DOC) was calculated as the difference between TC and IC. NH₄⁺, (NO₃⁻ + NO₂⁻), TN, and PO₄ were measured on a segmented flow analyzer (SFA). For TN, destructions with first K₂S₂O₈ at pH 4 and then UV with Na₂B₄O₇•10H₂O were performed prior to measurement on the SFA. Filtrate measurements, given as aqueous concentrations, were recalculated to express these values as soil concentrations based on the quantity of soil and liquid in microcosms, in order to allow comparison with other soil parameters. Organic nitrogen (N_{organic}) was determined as the difference between TN and the sum of NH₄⁺ and (NO₃⁻+NO₂⁻). pH was measured in the supernatant of 1 g soil in 2.5 mL 0.01 M CaCl₂ [191]. Total organic carbon (TOC) was measured gravimetrically upon ignition at 550 °C.

5.3 Results and Discussion

5.3.1 Organic carbon and nutrient mobilization during chemical oxidation

Following chemical oxidation with Fenton's reagent and modified Fenton's reagent, the concentration of DOC and dissolved N and P species increased relative to untreated soil (Table 5.2). DOC was 8 and 16 times higher after treatment with modified Fenton's reagent and Fenton's reagent, respectively, than in the field sample. Previous studies have also investigated the effect of non-selective oxidants

on SOM, with both decreases in SOM and increases in DOC being observed [104, 185, 240, 289]. Modified Fenton's reagent, performed at soil neutral pH with a chelator, produced a milder reaction than traditional Fenton's reagent, as observed in DOC concentrations (Table 5.2). While no significant remediation was observed with modified Fenton's reagent, a decrease in TPH concentrations occurred upon treatment with Fenton's reagent, indicating this to be the stronger and more effective reagent.

Treatment type showed more influence on DOC concentrations than the timing of dosage. Previous work has indicated that higher remediation efficiencies can be achieved with sequential addition of H_2O_2 [300], most likely due to the increased contact time between unstable H_2O_2 and contaminants. A similar result is observed in the current study, where higher TPH removal efficiencies were achieved with sequential application of Fenton's reagent (18%) versus a single dosage (8%; Table 5.2). In contrast, DOC concentrations showed no significant difference between single and sequential oxidant dosage, indicating SOM and TPH react differently upon longer and repeated incubation with H_2O_2 .

Oxidant application also led to an increase in the concentration of dissolved nutrient species (Table 5.2). After chemical oxidation, higher nitrogen and phosphorous concentrations were measured as compared to the field sample. Following Fenton's reagent treatment, increased PO_4^{3-} -P concentrations were measured and some nitrogen species increased by 1-2 orders of magnitude, as indicated by NH_4^+ (14-172 times) and TN (11-62 times). NH_4^+ release due to oxidation with Fenton's reagent has been documented previously at concentrations of 12-104 mg NH_4^+ kg^{-1} , which are similar to those observed here (Table 5.2; [240]). The mobilization of nitrogen and phosphorous suggests that observed DOC can be attributed to SOM oxidation. A linear relationship between DOC and $N_{organic}$ indicates that the former is not a product of increased TPH solubility upon partial oxidation, but rather originates from SOM (Table 5.2). The ratio between dissolved C and N of approximately 10:1 (g C: g N) fits well into the results of a previous survey of Eastern European soils, where C:N ratios of 6.0-20.7 were found [17]. Thus, chemical oxidation appears to non-selectively degrade all organics present in soil, including SOM, which results in the release of carbon, nitrogen, and phosphorous species into the aqueous phase, all of which have implications for microbial biodegradation activity following an ISCO treatment.

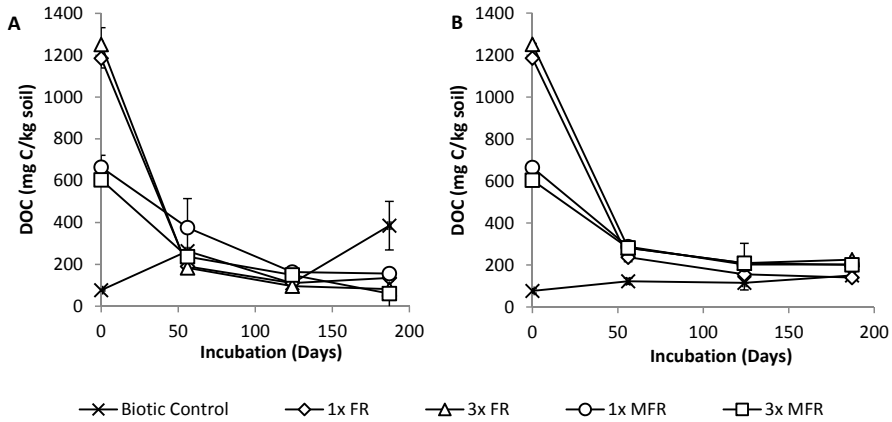


Figure 5.1 Dissolved organic carbon dynamics during incubation either without nutrient amendment (A) or with nutrient addition (B). Results are presented for the period of incubation starting directly after chemical oxidation (time point “0”) and ending at 187 d. Treatments are with Fenton’s reagent (FR) and modified Fenton’s reagent (MFR) where the oxidant is added at once (1X) or sequentially over three days (3X). Values are recalculated from the filtrate concentration to soil amounts. Data points are the average of triplicate extractions and error bars are the standard error.

Table 5.2 Summary of analytical data for dissolved constituents (DOC, COD, NH₄-N, (NO₃+NO₂)-N, TN, and PO₄-P) and headspace O₂ consumption and CO₂ production. Treatments are from before chemical oxidation (CO; Field Sample) and following CO, as in Figure 5.1. Given are the averages and standard errors of triplicate measurements. Dissolved constituents, measured in the leachate following filtration of the soil slurry, are recalculated to the quantity of soil in the microcosm. Headspace gas concentrations are cumulative for that time point.

Treatment	Time	Nutrient Amendment	DOC ^a		COD ^b		NH ₄ ⁺ -N		
			[mg kg ⁻¹]	± ^c	[mg kg ⁻¹]	± ^c	[mg kg ⁻¹]	± ^c	
Field Sample	Before CO		77	4	760	3	0.5	0.0	
1X FR	After CO		1185	81	6378	21	58.3	2.8	
3X FR			1251	46	5364	18	86.2	3.6	
1X MFR			664	57	4442	51	5.7	0.4	
3X MFR			604	5	4129	6	6.9	0.2	
Biotic Control	56	-	263	6	2462	52	1.3	0.1	
1X FR				190	20	1532	34	66.1	15.4
3X FR				183	8	1521	29	7.8	6.9
1X MFR				376	139	2012	99	1.6	0.3
3X MFR				237	5	3582	759	1.9	0.7
Biotic Control	124	-	110	1	1098	14	1.2	0.1	
1X FR				110	1	963	40	86.0	5.5
3X FR				96	9	776	52	137.2	14.3
1X MFR				163	11	1455	61	2.1	0.6
3X MFR				149	25	1371	203	1.0	0.1
Biotic Control	187	-	385	116	1967	958	0.6	0.1	
1X FR				135	38	835	390	6.1	1.7
3X FR				82	8	321	73	25.0	16.1
1X MFR				156	8	943	73	3.3	1.6
3X MFR				60	107	1043	176	0.9	0.2
Biotic Control	56	+	122	10	1212	60	255.3	26.9	
1X FR				237	12	2040	230	459.6	39.9
3X FR				227	4	1693	33	523.8	24.1
1X MFR				287	11	2202	105	280.6	24.5
3X MFR				282	17	2164	133	392.8	16.9
Biotic Control	124	+	115	15	1312	254	507.5	141.3	
1X FR				156	21	1054	222	935.7	21.4
3X FR				163	10	1260	70	1107.8	43.9
1X MFR				202	26	1693	203	678.4	60.6
3X MFR				209	14	1739	121	736.1	4.0
Biotic Control	187	+	150	34	1035	221	1150.8	255.2	
1X FR				140	5	712	44	1423.9	33.6
3X FR				225	94	1342	625	1306.5	57.2
1X MFR				202	14	1241	46	1068.9	66.5
3X MFR				201	15	1253	98	871.6	98.5

(NO ₃ ⁻ +NO ₂ ⁻)-N		TN		PO ₄ ³⁻ -P		O ₂ consumption		CO ₂ production	
[mg kg ⁻¹]	± ^c	[mg kg ⁻¹]	± ^c	[mg kg ⁻¹]	± ^c	[mol kg ⁻¹]	± ^c	[mol kg ⁻¹]	± ^c
0.2	0.1	3.3	0.2	0.00	0.00				
1.6	0.1	175.5	6.9	0.15	0.02				
1.9	0.1	202.7	6.4	0.20	0.00				
1.8	0.7	37.7	3.5	0.00	0.01				
0.9	0.0	41.5	0.7	0.00	0.00				
0.9	0.2	5.3	0.5	0.06	0.03	0.10	0.01	0.12	0.01
1.0	0.4	91.0	20.3	0.17	0.17	0.07	0.01	0.07	0.01
0.4	0.1	19.8	7.4	0.04	0.02	0.13	0.03	0.10	0.02
0.6	0.1	11.7	0.7	0.03	0.02	0.19	0.01	0.19	0.01
0.5	0.1	10.7	1.5	0.03	0.02	0.05	0.00	0.05	0.01
0.6	0.1	3.4	0.3	0.00	0.00	0.14	0.02	0.16	0.04
1.9	0.2	111.1	5.5	0.11	0.04	0.12	0.00	0.08	0.00
1.9	0.2	162.8	17.2	0.00	0.02	0.17	0.00	0.11	0.00
0.8	0.2	9.6	2.3	0.15	0.10	0.27	0.01	0.30	0.01
0.5	0.1	5.5	0.4	0.00	0.01	0.09	0.00	0.07	0.00
0.5	0.1	2.5	0.3	0.06	0.02	0.21	0.05	0.20	0.03
1.7	0.5	14.4	4.0	0.66	0.16	0.15	0.03	0.10	0.00
30.2	28.1	62.8	21.0	0.20	0.06	0.20	0.00	0.13	0.00
0.8	0.1	7.1	1.6	0.50	0.31	0.38	0.02	0.37	0.02
0.8	0.1	5.5	0.3	0.11	0.02	0.11	0.00	0.08	0.00
70.7	34.7	331.6	8.8	0.63	0.06	0.18	0.01	0.18	0.01
0.7	0.1	477.3	41.6	0.47	0.03	0.17	0.04	0.14	0.02
1.3	0.5	542.3	24.9	0.84	0.09	0.15	0.00	0.12	0.00
0.7	0.1	292.0	24.1	2.21	0.27	0.20	0.01	0.18	0.01
0.8	0.1	407.2	15.0	3.33	0.25	0.30	0.01	0.21	0.01
415.4	145.8	952.8	20.2	15.25	1.33	0.22	0.00	0.20	0.00
2.5	0.3	962.2	10.4	11.43	1.48	0.23	0.00	0.16	0.00
1.9	0.2	1173.1	46.7	12.88	1.26	0.21	0.01	0.15	0.00
2.4	0.8	707.3	57.0	27.91	3.49	0.27	0.03	0.26	0.05
1.7	0.1	797.5	6.2	42.21	2.76	0.35	0.00	0.24	0.00
502.5	147.8	1630.0	389.1	92.98	13.35	0.31	0.03	0.26	0.01
2.5	0.1	1387.6	18.8	63.19	5.32	0.31	0.04	0.21	0.02
1.5	0.6	1301.7	59.5	73.64	1.11	0.34	0.07	0.23	0.05
2.2	0.1	1051.9	72.7	122.66	17.89	0.36	0.02	0.31	0.01
1.9	0.5	878.5	99.0	99.83	21.75	0.41	0.04	0.26	0.02

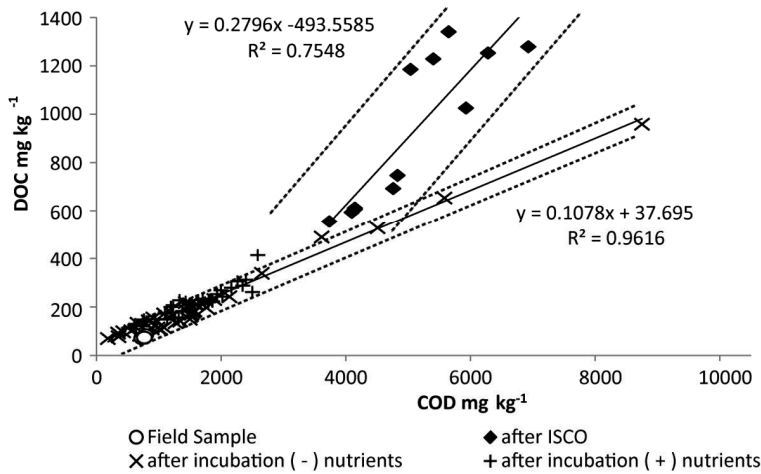


Figure 5.2 Shift in DOC-COD signature due to chemical oxidation (Field Sample → after CO) and bioremediation (after CO → after incubation), either without (-) or with (+) nutrient amendment. After ISCO data points are given for individual microcosms, with linear fit (solid line) and 95% confidence interval (dashed lines). After incubation data points are given for individual microcosms at days 56, 124, and 187. Linear fit (solid line) and 95% confidence intervals (dotted lines) are given for all after incubation data points together, regardless of nutrient amendment. The ratio between DOC and COD indicates the extent of oxidation of dissolved organic species. A high ratio of DOC:COD, as in samples following chemical oxidation, indicates relatively oxidized species. A lower ratio, as in field and post bioremediation samples, indicates more reduced organic species.

5.3.2 Organic carbon dynamics during bioremediation

Following chemical oxidation, microcosms were incubated for 187 d either with or without nutrient amendment in order to measure the effects of aqueous organic carbon and macronutrient concentrations on bioremediation. Significant reduction in DOC concentrations occurred in the first 56 d of incubation in all chemically treated microcosms, regardless of nutrient amendment (Figure.5.1). At day 56, DOC concentrations were between 190 and 376 mg kg⁻¹, 2-6 times less than the values measured after chemical oxidation treatment (Table 5.2). DOC continued to decrease, reaching at day 187 concentrations similar to those observed in the field sample. Thus, temporary perturbations in DOC concentrations due to chemical oxidation can be restored by incubation.

In addition to a decrease in the concentration of DOC, a shift in the organic carbon signature occurred (Figure 5.2). After chemical oxidation, a higher ratio between DOC and COD is observed, indicative of more oxidized compounds that contain less COD per gram of carbon. Following incubation for 56, 124 and 187 d, the DOC:COD ratio decreased; this implies that carbon species are generally more reduced, with a higher oxidation demand. This change in the organic carbon signature indicates microbial degradation of DOC with a preference for more oxidized species. Interestingly, following incubation either with or without nutrient amendment, the DOC signature returns to that observed in the field sample, indicating the role of microbial activity in regulating DOC properties (Figure 5.2).

Biodegradation of DOC is also observed in the relationship between respiration and TPH degradation in nutrient-amended microcosms. When DOC is used as an alternative substrate to TPH for microbial growth, CO_2 production occurs without TPH removal, changing the ratio of g TPH degraded to mol CO_2 produced (R_{TPH}). Additionally, as TPH generally contains more reduced compounds than DOC, the ratio of O_2 consumption to CO_2 production (mol mol^{-1} ; $R_{\text{Respiration}}$) can also be used to determine which substrate is utilized [259]. When TPH is the main substrate for biodegradation, values for both R_{TPH} and $R_{\text{Respiration}}$ would increase. Conversely, both parameters would decrease when DOC is utilized as the main substrate for biological growth.

A comparison of R_{TPH} and $R_{\text{Respiration}}$ following chemical oxidation with the biotic controls indicates biodegradation of DOC (Figure 5.3). In the biotic control, $R_{\text{Respiration}}$ was consistently at $1.35\text{-}1.41 \text{ mol O}_2 \text{ mol}^{-1} \text{ CO}_2$ and corresponded to R_{TPH} of $10\text{-}14 \text{ g TPH mol}^{-1} \text{ CO}_2$ (Figure 5.3A). In contrast, in the first 56 d of incubation following chemical oxidation, $R_{\text{Respiration}}$ values are on average lower, $1.07 \pm 0.15 \text{ mol O}_2 \text{ mol}^{-1} \text{ CO}_2$ with more variability ($0.75\text{-}1.34 \text{ mol O}_2 \text{ mol}^{-1} \text{ CO}_2$; Figure 5.3B). This decrease in $R_{\text{Respiration}}$ indicates biodegradation of DOC, as reflected in the overall lower R_{TPH} values during incubation following chemical oxidation (Figure 5.3B) as compared to the biotic controls (Figure 5.3A). Differences in R_{TPH} and $R_{\text{Respiration}}$ are particularly evident for Fenton's reagent-treated microcosms, where the highest DOC concentrations were noted following chemical oxidation (Table 5.2). In the period between days 56 and 187, results indicate a shift in substrate from DOC to TPH (Figure 5.3C). $R_{\text{Respiration}}$ increases ($0.96\text{-}1.53 \text{ mol O}_2 \text{ mol}^{-1} \text{ CO}_2$; average 1.26 ± 0.19), indicating the degradation of more reduced substrates, namely TPH. However, R_{TPH} values remain lower than those observed in the biotic control microcosms, implying DOC bioconversion continues throughout the incubation period.

Previous work has noted biological DOC utilization following chemical oxidation. The improvement in biodegradability has been attributed to an increase in hydrophilic structures upon oxidation [104], with one study suggesting that biodegradation of DOC is one pathway by which ISCO may actually stimulate microbial activity [185]. Although easily biodegradable substrates may encourage the regeneration of indigenous microbial communities following chemical oxidation, DOC utilization as an alternative substrate can significantly reduce the efficiency of biphasic treatment. The degradation of an additional 1 g TPH kg⁻¹ rather than 1 g DOC kg⁻¹, as observed in the first 56 d of incubation following Fenton's treatment, would contribute strikingly to the remediation efficiency.

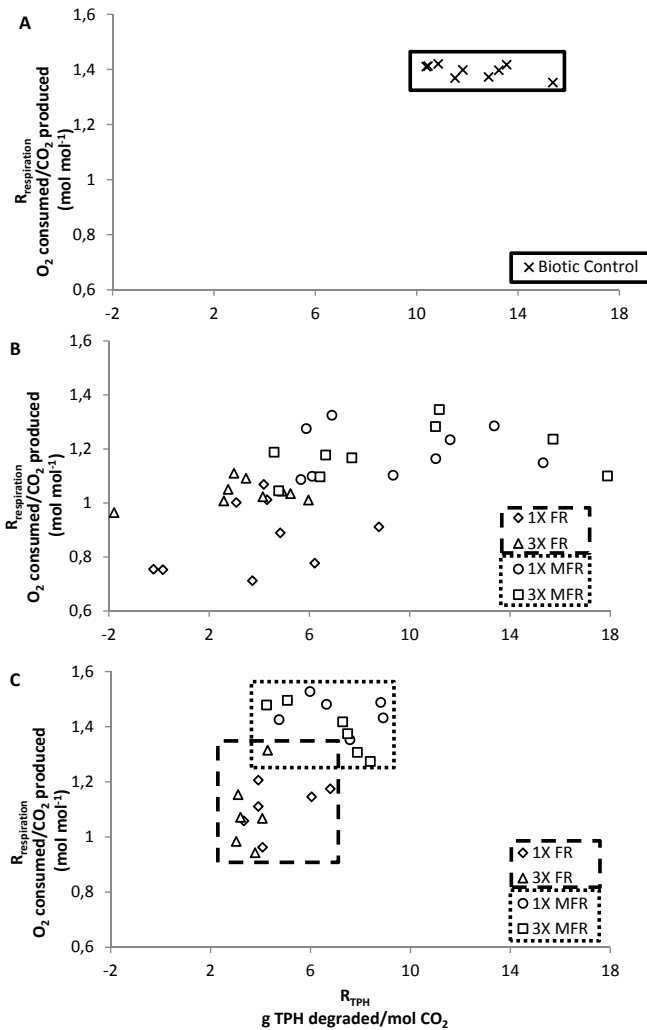


Figure 5.3 Relationship between TPH degradation and CO_2 production (R_{TPH} ; x-axis) and the molar ratio of O_2 consumed and CO_2 produced ($R_{Respiration}$; y-axis). Values are given for individual nutrient-amended microcosms in the first 56 days of incubation (A: biotic control, B: chemically treated microcosms) or for the period day 56-187 (C). Solid box surrounds biotic control data; dashed boxes indicate Fenton's reagent data points; dotted line boxes indicate modified Fenton's reagent data points. The biotic control was not included in (C), as no TPH degradation was observed in this period. Higher values for $R_{Respiration}$ indicate utilization of the relatively reduced aliphatic components of TPH. Similarly, higher values for R_{TPH} indicate that microbial respiration utilizes TPH. In contrast, lower values for each of these ratios shows the influence of respiration using organic matter substrates.

5.2.3 Nutrient dynamics during bioremediation

Although natural soils do contain some macronutrients, amendment improves bioremediation efficiency of hydrocarbons [67, 157]. This was confirmed when comparing microcosms that did not receive amendment with those that did (Figure 5.4). Both TPH degradation and CO₂ production were higher with nutrient addition, indicating the essential role that nitrogen and phosphorous play in microbial activity and biodegradation. Although little bioremediation occurred without nutrient supplements, higher CO₂ production (Figure 5.4) following Fenton's reagent treatment could be attributed to the increase in bioavailable nutrients upon treatment with a stronger oxidant (Table 5.2). Apparently nutrient amendment is not required for DOC utilization (Figure 5.1a), either because nutrients mobilized during chemical treatment were sufficient for DOC degradation or because trace quantities of nitrogen and phosphorous present in DOC serve as a nutrient source.

In nutrient amended microcosms, NH₄⁺ was consumed rapidly in all treatments, regardless of whether or not incubation was preceded by chemical oxidation (Table 5.3). Although similar rates of 21-31 mg N kg⁻¹ soil d⁻¹ were observed across all microcosms, initial rates (day 1-56) were lower following chemical treatment with Fenton's reagent, most likely indicating the biological utilization of nitrogen naturally present in DOC. Biological nitrogen consumption following chemical oxidation differed from the biotic control. Between days 57-124, lower nitrogen removal rates were observed in chemically treated microcosms, which coincide with a shift from rapid DOC utilization (Figure 5.1) to long-term TPH degradation (Figure 5.4). As microbial community structure is related to the carbon substrate signature, this lag period in nitrogen removal may relate to a shift in the microbial community [134].

Table 5.3 Rate of NH₄⁺ consumption (mg N kg⁻¹ soil d⁻¹) in nutrient amended microcosms during three addition periods. Average and standard errors of triplicate extractions are given. Treatments as in Figure 5.1.

	Day 1-56	Day 57-124	Day 125-187
Biotic control	31.3 ± 0.5	26.2 ± 2.1	22.0 ± 4.1
1X FR	28.7 ± 0.7	22.9 ± 0.3	24.5 ± 0.5
3X FR	28.1 ± 0.4	21.3 ± 0.7	29.2 ± 0.9
1X MFR	31.0 ± 0.4	24.1 ± 0.9	26.1 ± 1.1
3X MFR	29.0 ± 0.3	24.9 ± 0.1	30.2 ± 1.6

In contrast, a consistent decrease in NH_4^+ consumption rate of 30% between the first and final measurement periods is noted in nutrient-amended biotic control microcosms (Table 5.3). Additionally, an accumulation of nitrogen as NO_3^- and NO_2^- is also observed, representing between 4 and 20% of NH_4^+ consumption (Figure 5.5). The accumulation of oxidized nitrogen species points towards nitrification, whereby NH_4^+ is oxidized to NO_2^- and then NO_3^- in a two-step process via a heterotrophic or autotrophic pathway. The latter, where CO_2 is used as a carbon source, has been shown to be the dominant nitrification pathway in un-forested soils [194]. As nitrification was only observed in the biotic control microcosms, these results indicate that chemical oxidation selects for a different microbial community, either (1) directly, by impacting ammonium-oxidizing Bacteria or Archaea through oxidative stress, or producing toxic intermediates, thereby reducing the starting concentration of these slow-growing micro-organisms or (2) indirectly, by changing conditions in a manner that suppresses growth of nitrifiers.

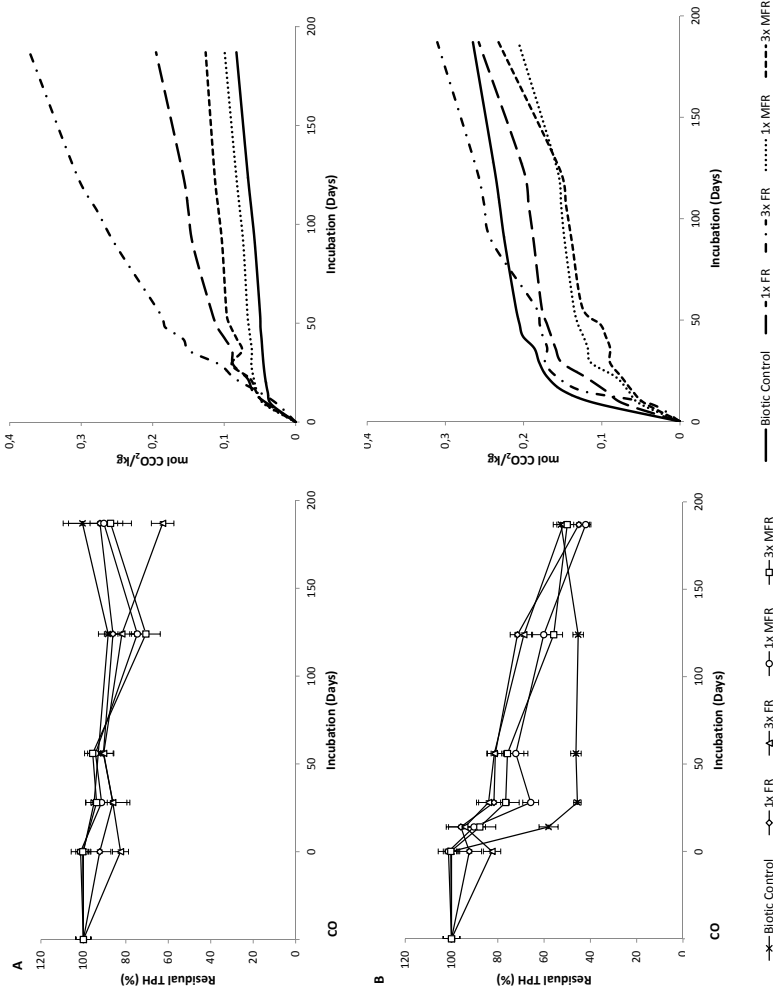


Figure 5.4 Residual TPH concentration (left, %) after chemical oxidation (CO) and during incubation. Cumulative CO₂ production (right, mol CO₂ kg⁻¹ soil) for microcosms without nutrient amendment (A) and with nutrient addition (B). TPH values and standard errors are calculated from triplicate microcosms. CO₂ production is cumulative over the period and is the average of triplicate microcosm measurements. Treatment names are as given in Figure 5.1.

5.2.4 Implications for TPH remediation

In this study, the highest TPH remediation efficiency of 58% was attained upon coupling single dosage modified Fenton's reagent, the mildest oxidant treatment tested, with a nutrient-amended bioremediation treatment (Figure 5.4). Only limited biodegradation was observed in the absence of amendment. When nutrients were added, 47 -58% of TPH was removed in all microcosms. However, a simple calculation of TPH removal does not accurately describe the impact that chemical oxidation has on treatment efficiency.

The release and subsequent biodegradation of DOC due to SOM oxidation reduces the efficiency of the overall system. The full extent of remediation was achieved within 28 d of incubation in the biotic control; however, in chemically treated microcosms, TPH concentrations continue to slowly decrease until day 187 of incubation (Figure 5.4). This reduction in the rate of TPH degradation translates into longer treatment periods in the field, reducing both the cost-effectiveness and swiftness generally associated with biphasic treatment. Additionally, biological DOC utilization as an alternative substrate to TPH in chemically treated soils requires the use of additional amendments to achieve results comparable to exclusively biological treatment. In the first 56 d of incubation, 65% less TPH was oxidized in microcosms that had been treated sequentially with Fenton's reagent than in the biotic control (Figure 5.4); however, O_2 and NH_4^+ consumption were only 17 and 10% lower, respectively (Table 5.2). Therefore, DOC biodegradation utilizes additional electron acceptors and nutrients that, in a field system, would have to be added at an additional cost.

On the other hand, chemical treatment may change the structure of SOM in a manner that improves long term remediation beyond temporary reductions in efficiency due to increased availability of DOC. Chemical oxidation with Fenton's reagent improves the bioavailability of TPH (Table 5.4). Increased bioavailability may be related to partial oxidation and thus higher solubility of residual TPH. Additionally, partial oxidation of the soil matrix may change the tight association between TPH and SOM. Bioavailability, a measurement of the amount of contaminant available for uptake by organisms at a single time point, is a dynamic parameter, with desorption of hydrophobic organic compounds often the rate-limiting step in biodegradation [229]. Although bioavailability at days 124 and 187 was below the limit of quantification, continued TPH degradation and a shift in the relative abundance of low molecular weight versus high molecular weight TPH compounds was observed in chemically-oxidized, nutrient-amended microcosms (Table 5.4, Figure 5.4). This implies a slow, sustained mobilization and degradation. Such long term TPH release and degradation

was not observed in biotic control microcosms, suggesting that chemical oxidation altered the interaction between TPH and SOM. Changes in contaminant binding upon chemical oxidation have been noted previously [190, 251, 289]. Wang et al. suggested an oxidation-desorption-oxidation mechanisms between SOM, oil, and ozone [289]; a similar pathway, including biological oxidation in addition to chemical oxidation, may explain the long-term biodegradation observed in our study.

Table 5.4 Bioavailability prior to and following chemical oxidation (after CO) and bioremediation given as percentage of residual TPH (%). On days 124 and 187, bioavailable TPH was below the limit of quantification. Given are the average and standard deviation of triplicate total and bioavailable TPH extractions. Treatment acronyms are as in Table 5.2.

	Field Sample	After CO	Day 56	
			(-) nutrients	(+) nutrients
	33.4 ± 0.9			
Biotic control			28.0 ± 1.1	19.4 ± 1.3
FR 1x		35.0 ± 1.9	25.3 ± 1.5	23.2 ± 1.0
FR 3x		44.7 ± 2.2	18.9 ± 1.4	18.4 ± 1.3
MFR 1x		28.6 ± 1.1	24.4 ± 1.4	20.7 ± 2.1
MFR 3x		32.9 ± 0.3	20.8 ± 2.0	14.0 ± 2.2

Chemical oxidation treatment also appears to change the bioavailability of SOM-derived nutrients, which can improve biological activity under nutrient-limitation. A comparison between amended and most non-amended microcosms clearly indicates the impact of nutrient limitation on biological respiration and TPH biodegradation. However, microcosms treated with sequential addition of Fenton's reagent showed the highest CO₂ production and some TPH biodegradation at day 187 (Figure 5.4). While initial respiration could be attributed to NH₄⁺ abundance upon oxidation, sustained CO₂ production indicates consumption of nutrient reserves in SOM. Thus, in addition to improving the bioavailability and the overall remediation efficiency of TPH, chemical oxidation may also improve the bioavailability of naturally present nutrients, thereby reducing the potential costs of amendments in field applications.

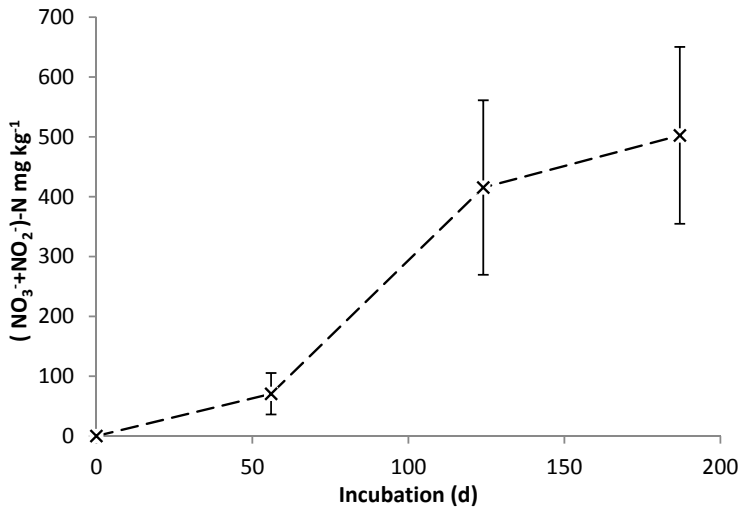


Figure 5.5 $\text{NO}_3^- + \text{NO}_2^-$ accumulation, which was only observed in biotic control microcosms (Table 5.2). Values and standard errors are given for triplicate microcosm extractions and recalculated from the filtrate concentration to per kg soil based on the starting soils and liquid quantity in the microcosm.

5.2.5 Conclusions

The implications of chemical oxidation for subsequent bioremediation go beyond temporary disruptions in subsurface conditions and microbial activity. Mobilization of aqueous carbon, nitrogen and phosphorus species upon oxidation with Fenton's reagent and modified Fenton's reagent are utilized during a following bioremediation phase, thereby influencing the efficiency of the overall treatment. Biodegradation of DOC as a preferential substrate to TPH occurs regardless of nutrient amendment and results in a delay in diesel bioremediation. In nutrient amended microcosms, more prolonged bioremediation was observed following chemical oxidation than in biotic control microcosms, implying that chemical oxidation changes the dynamics of TPH desorption from SOM. While results indicate that coupling of ISCO with ISB can be an effective biphasic treatment, mobilization and biodegradation of mobilized carbon and macronutrient species have implications for the timing and cost of a remediation project. Therefore, prior to field application, an investigation of changes in SOM, DOC, nutrient and contaminant concentrations and characteristics following chemical oxidation should be included in order to better predict the efficiency of a subsequent bioremediation phase.

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Chapter 6

Recovery of microbial diversity and activity during bioremediation following chemical oxidation of diesel contaminated soils

This chapter has been published:

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Abstract

To improve the coupling of *in situ* chemical oxidation and *in situ* bioremediation, a systematic analysis was performed of the impact of chemical oxidation with Fenton's reagent, modified Fenton's reagent, permanganate or persulfate, on microbial diversity and activity during 8 weeks of incubation in two diesel-contaminated soils (peat and fill). Chemical oxidant and soil type impacted the microbial community diversity and biodegradation activity; however, this was only observed following treatment with Fenton's reagent and modified Fenton's reagent, and in the biotic control without oxidation. Differences in the highest overall removal efficiencies of 69% for peat (biotic control) and 59% for fill (Fenton's reagent) were partially explained by changes in contaminant soil properties upon oxidation. Molecular analysis of 16S rRNA and alkane monooxygenase (*alkB*) gene abundances indicated that oxidation with Fenton's reagent and modified Fenton's reagent negatively affected microbial abundance. However, regeneration occurred, and final relative *alkB* abundances were 1-2 orders of magnitude higher in chemically treated microcosms than in the biotic control. 16S rRNA gene fragment fingerprinting with DGGE and prominent band sequencing illuminated microbial community composition and diversity differences between treatments, and identified a variety of phylotypes within *Alpha*-, *Beta*-, and *Gammaproteobacteria*. Understanding microbial community dynamics during coupled chemical oxidation and bioremediation is integral to improved biphasic field application.

6.1 Introduction

A growing awareness of the health and environmental risks associated with toxic pollutants in the environment has spurred the development of remediation technologies. While a number of innovative technologies are in use for remediation of contaminated soils, increasing focus is being placed on less invasive and more cost-effective *in situ* remediation technologies, including *in situ* chemical oxidation (ISCO) and *in situ* bioremediation (ISB). These treatments can be efficient and are adaptable to a variety of subsurface conditions and contaminant types [68, 210, 231, 239, 255, 292].

During an ISCO treatment, chemical oxidants are distributed in the subsurface to oxidize organic contaminants. Typical treatments include Fenton's reagent (ferrous iron-catalyzed hydrogen peroxide at pH 2-4), modified Fenton's reagent (ferrous iron catalyzed hydrogen peroxide at neutral pH with a chelator), permanganate, persulfate (alone or activated with metals, heat, or alkaline conditions), or ozone injection [233]. In addition to dissolved contaminants, these aggressive treatments remove compounds that are sorbed to soil organic matter (SOM) or present as pure product in the form of light or dense non-aqueous phase liquid (LNAPL or DNAPL). Chemical oxidation has been demonstrated to rapidly remediate soil and groundwater contaminated with a variety of solvents and organic contaminants, including oil-derived compounds, also referred to as total petroleum hydrocarbons (TPH) [53, 103, 129, 285]. Nevertheless, despite the advantages of ISCO, application can also result in undesirable changes in the subsurface, including mobilization of metals [40], structural and quantitative changes to SOM [190], increase or decrease in pH [96] and reduction in soil and groundwater microbial communities [207, 217].

In situ bioremediation relies on natural microbial metabolic processes for the remediation of organic pollutants. Degradation can occur by indigenous bacterial populations under natural conditions as part of natural attenuation processes, or by technologies that increase the rate of biodegradation (enhanced natural attenuation), by, for example, amending with nutrients, adjusting redox conditions, or bioaugmenting. Aerobic biological TPH degradation is well described [213, 243]; biodegradation of aliphatic and aromatic hydrocarbons has been demonstrated in the laboratory, and the application of ISB for TPH contaminated sites has been proven in various field studies [120, 171, 280].

Although chemical oxidation can be aggressive, recent studies have shown that sequential application of ISCO followed by ISB is not only feasible, but can also

provide a more efficient and extensive cleanup of contaminated subsurfaces [30, 87, 176, 177, 217, 255]. When properly implemented, chemical oxidation reduces high concentrations of a contaminant; thereafter, biological polishing treats the residual concentration, resulting in improved remediation efficiencies compared to ISCO alone. For example, when coupling ozone treatment with bioremediation of crude oil, 22% degradation was observed during ozonation, with a further removal of 12-20% during the bioremediation phase [140]. In another study investigating the impact of ozonation time on subsequent bioremediation of diesel-contaminated soil, it was found that, while longer ozone application (900 min) oxidized more TPH (48%), biodegradation was only observed following shorter treatment times (180 min) [103]. In the latter case, the combination of chemical (37% removal) and biodegradation (17% removal) resulted in overall increased remediation efficiency (54%). Finally, in a triphasic treatment of fuel oil-contaminated soil with surfactant flushing, Fenton's reagent oxidation and bioremediation, removal efficiencies of 80%, 15%, and 5% of the initial contaminant concentration were observed for each step, respectively, leading to complete remediation [271].

6

Although sequential coupling of ISCO and ISB for TPH removal can be highly effective, relatively little is known about the impact of the chemical treatment on composition and activity of the microbial community, and how these influence the effectiveness of the subsequent biological treatment. Molecular biological tools are available to both elucidate the potential for bioremediation and quantify the presence of genes integral to biodegradation processes [55, 153, 198, 199, 258]. However, to date only one study has used molecular tools to investigate the impact of chemical oxidation on TPH-degrading microbial communities. In that study, an analysis of microbial community dynamics, using a functional gene microarray throughout a coupled chemical and biological treatment, showed an overall decrease in genetic diversity after ozonation [140].

Each chemical oxidant can have various impacts on soil geochemistry and contaminant characteristics in different soil types. Therefore, a thorough understanding of the overall system is required to optimize conditions for a subsequent biodegradation step. While previous work has often investigated the remediation efficiencies of one chemical oxidant treatment in a single soil type, the present study takes a more systematic approach. Here, the impact of four chemical oxidants (modified Fenton's reagent, Fenton's reagent, permanganate and persulfate) on TPH removal, bioavailability, and microbial activity and diversity under biostimulating conditions in two different diesel-contaminated soils was investigated

using respiration tests, 16S ribosomal RNA gene-targeted community profiling by denaturing gradient gel electrophoresis (DGGE), and quantitative polymerase chain reaction (qPCR) of 16S rRNA and *alkB* genes, the latter of which encode monooxygenases involved in TPH degradation [199]. By linking chemical oxidation treatments and biodegradation activity to changes in the microbial community, this study aims to provide a better understanding of the overall processes that determine the outcome of coupling ISCO with ISB, thereby allowing further optimization of an integrated approach to subsurface remediation.

6.2 Materials and Methods

6.2.1 Soil samples

In this study, aged field samples were taken from a diesel-contaminated railway site at Węglińiec, Poland. A variety of lithological layers were present, allowing for the sampling of both peat and an anthropogenic fill layer at different locations on the site [259]. In addition to different soil matrix types, these samples also had different original TPH concentrations, 11.1 and 25.4 g TPH/kg, respectively (Table 6.1). Further details about the site and the sampling can be found elsewhere [259]. After collection, storage at 4°C (1 year) and sieving at 2 mm, the <2 mm fraction was used to study the natural degradation capacity of the soil, the effect of chemical oxidation on both contaminant degradation and the microbial community, and subsequent biological degradation in batch experiments.

Table 6.1 Physical and chemical characteristics of soils used in this study. Organic matter and TPH concentrations are the average and standard error for triplicate measurements. Soils used in multiple chapters are summarized in Appendix 2.

Parameter	Peat	Fill
Sample Depth (cm bg ^a)	150-250	130-230
Organic Matter (%)	11.4 ± 0.3	11.3 ± 0.5
pH	6.8	6.9
Initial TPH (g TPH/kg)	11.1 ± 0.4	25.4 ± 0.5

^a below ground level

6.2.2 Microcosm setup

In order to accommodate the measurement of a number of parameters throughout the experiment, multiple replicate microcosms were setup for each treatment and sacrificed at different time points. For measurement prior to treatment (field sample), 10 microcosms were prepared for each soil for the measurement of total TPH (3 microcosms), bioavailable TPH (3), DOC (3), and for DNA extraction (1). Each chemical oxidation treatment and the biological control were performed on 32 replicate microcosms for each soil. Total TPH extraction was performed in triplicate at time points after chemical oxidation, and after 1, 2, 4, and 8 weeks of incubation (15 microcosms). Bioavailable TPH and DOC were measured in triplicate after chemical oxidation and after 8 weeks of incubation (12 microcosms total). Additionally, DNA extraction was performed by sacrificing separate microcosms after chemical oxidation, and after 1, 2, 4 and 8 weeks of incubation (5 microcosms total), for molecular analysis of microbial abundance (bacterial 16S rRNA gene), TPH degradation capacity (alkane monooxygenase gene *alkB*), and diversity based on the 16S rRNA gene using denaturing gradient gel electrophoresis (DGGE).

Microcosms in 125 mL serum bottles contained 4 g of soil and 10 mL of liquid. To the soil, chemical oxidants permanganate and persulfate were added at final equimolar (0.58 M) concentrations of 92 g/L KMnO_4 and 139 g/L $\text{Na}_2\text{S}_2\text{O}_8$, respectively, similar to concentrations common in field application (10% wt/wt; [114, 232, 272]). In persulfate microcosms, NaHCO_3 buffer (0.5 M final concentration) was added to maintain near-neutral pH. While such amendments are not typical in an open field system where influx of groundwater can help to neutralize the pH, in a closed microcosm system buffering was required to ensure that the regeneration of activity was not pH limited following persulfate treatment. Bottles were closed with viton rubber stoppers (Rubber bv, the Netherlands), capped, and shaken (100 rpm) at 20°C until the chemical reaction was complete as measured spectrophotometrically. For Fenton's and modified Fenton's reagent, final concentrations of 150 mg/L Fe^{2+} as $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ and 5% H_2O_2 were added based on previous studies [300]. In the case of Fenton's reagent, 15% H_2SO_4 was added to reduce the pH to below 3. For modified Fenton's reagent, 1.2 g/L citrate was added as a chelator. The oxidation reaction was allowed to occur in open bottles over the course of 2 days until no residual hydrogen peroxide was measured with testing strips (Quantofix, Germany). Biotic control microcosms, which were also incubated 2 days and received Fe^{2+} and H_2SO_4 (as for Fenton's reagent) without the addition of H_2O_2 , showed no volatile losses.

Following chemical oxidation, bioremediation was stimulated through oxic incubation with nutrient amendment. To assess the ability of indigenous microorganisms to regenerate after chemical oxidation, no bioaugmentation was performed. To each microcosm, 1 mL of ten times concentrated mineral medium in 100 mM phosphate buffer (pH 7) was added to the slurry. The mineral media contained final concentrations of 10.2 g/L NH_4Cl , 0.48 g/L CaCl_2 , and 0.54 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 12 mg/L $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$, 12 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 3 mg/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.18 mg/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 mg/L ZnCl_2 , 0.3 mg HBO_3 , 0.5 mg/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.6 mg/L $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, 0.3 mg/L $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 6 mg/L EDTA, and 1 mL/L 36% HCl. Additionally, the biotic and abiotic control microcosms, the latter of which contained 130 mg/L NaN_3 and was used to correct for abiotic TPH losses, received identical biostimulation treatments. Stoppered bottles were incubated at 20°C on a shaker at 120 rpm. Throughout 8 weeks of incubation, headspace analysis of the concentrations of O_2 and CO_2 was performed at least weekly both to follow microbial activity and to monitor headspace O_2 concentrations. Only O_2 consumption was measured in persulfate microcosms, as the bicarbonate buffer also contributed to headspace CO_2 concentrations. Once O_2 levels were below 10%, microcosms were flushed with air to attain atmospheric gas composition. Periodic flushing of abiotic control microcosms indicated limited loss of volatile TPH components due to headspace exchange. pH was periodically measured with testing strips (Quantofix, Germany) and remained close to neutral throughout incubation.

6.2.3 Chemical analyses

Bioavailable TPH was determined using 24 hour Tenax[®] extractions [43]. For every 1 g of dry soil, 1 g of Tenax[®], 10 mg NaN_3 , and 0.01 M CaCl_2 was added to the soil slurry to a final volume of 40 mL. After shaking at 20°C for 24 hours, Tenax[®] was separated from soil particles and washed using 0.01 M CaCl_2 . TPH extraction was performed on the Tenax[®] granules.

Prior to TPH quantification with gas chromatography, the samples were extracted by solvent extraction. Solvent shake extraction with acetone and hexane was performed according to a Dutch standard procedure NEN 5733 [178]. The hexane was dried with Na_2SO_4 and measured on an HP 5890 Gas Chromatograph (GC) with a SIM DIST column and Flame Ionization Detector with nitrogen as the carrier gas. Following 5 min at an initial temperature of 40°C, the temperature was increased at 10°C per min to a final temperature of 300°C. TPH was determined as the area between C10 and C40. Carbon number fractions were determined using the retention times of a boiling point standard.

Headspace analysis of O₂ and CO₂ was performed on a Shimadzu GC with Porabond and Molsieve 5a columns in parallel at 65°C with helium as a carrier gas. Loop injection was at 120°C and a thermal conductivity detector at 150°C was used. Dry weight was calculated by weight difference before and after 24 hours at 105°C. Organic matter content was determined as the weight difference due to ignition at 550°C. The pH was measured in the supernatant of a 1 g soil:2.5 mL liquid ratio suspension of soil sample in 0.01 M CaCl₂ [191]. Dissolved organic carbon (DOC) was measured before and after chemical oxidation and following incubation. The starting sample was prepared by incubating soil and water together on a shaker for 3 days prior to processing. All other microcosms were prepared as described above. Samples were prepared by filtering the entire microcosm contents through a 0.45 µm mixed cellulose ester filter (Whatman, United States). The filtrate was analyzed for total carbon (TC) and total inorganic carbon (TIC) on a Shimadzu analyzer by the Chemical Biological Laboratory Soil Center (Wageningen, The Netherlands). DOC was calculated as the difference between TC and TIC.

6.2.4 Biomolecular analyses

DNA extraction. The soil slurry was transferred from the microcosm to a sterile tube and centrifuged at 10000 rpm, 22°C for 17 min. After the liquid was discarded, 0.5 gram of soil was transferred to a sterile microcentrifuge tube and preserved at -20°C for further applications. DNA extraction was performed with the FastDNA Spin Kit for Soil (Mp Biomedicals, USA) according to most of the manufacturer's recommendations. In order to improve DNA recovery, step 10 was disregarded and step 11 was modified transferring all the binding mixture to a SPIN™ filter. DNA was eluted with 80µL of DES (DNase/Pyrogen-Free Water) and stored at -20°C. DNA quantity and quality were estimated on a Nanodrop spectrophotometer (Thermo Scientific).

Quantitative polymerase chain reaction. Quantitative PCR (qPCR) was used to quantify total bacteria by 16S rRNA gene quantification (341F and 534R), and the *alkB* gene was used to estimate TPH-degradation potential using the iQ SYBR Green Supermix kit and the iQ5 iCycler (BioRad, Veenendaal, The Netherlands), as described previously [259]. All DNA extracts were diluted 100-fold and assayed in triplicate, including a no-template control. Respective gene copy numbers were calculated as gene copies / g of soil sample.

Denaturing gradient gel electrophoresis and sequencing. For DGGE analysis, the 16S rRNA gene was partially amplified using primers 968f-GC (5'-

CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAGGGG AACGCGAAGAACCTTAC-3') and 1401r (5'-CGGTGTGTACAAGACCC-3') [182]. PCR reactions were performed using the GoTaq DNA Polymerase kit (Promega, USA) and consisted of 10 µL of Buffer, 1 µL of 10 mM dNTPs, 0.25 µL of 5 U/µL Taq polymerase, 1 µL of a 10 µM stock solution of each primer, 1 µL of DNA template, and 35.75 µL nuclease free water for a total volume of 50 µL. The PCR consisted of 2 min initial denaturation followed by 35 cycles of 30 s denaturation at 94 °C, 20 s annealing at 56°C, and 40 s extension at 72 °C; final extension was 7 min at 72 °C. PCR products were visualized by electrophoresis on 1% agarose gel using SYBR® Safe dye (Invitrogen, USA).

In cases where poor PCR products were obtained, probably due to the interference of DNA extraction impurities such as TPH or soil components, slight modifications to the PCR reaction were made. Either a 10x dilution of template was used to reduce the quantity of impurities, or 0.25 µL of bovine serum albumin (BSA; molecular grade, 10 mg/mL) were added to temper the impact of impurities on the PCR reaction.

DGGE was performed as described previously [174], using the DCode device (BioRad). Ten µL of PCR product were run on a 8% polyacrylamide gel with a 30%-60% denaturant gradient (100 % denaturant solution corresponding to 7 M urea and 40% (v/v) formamide). Gels were run in 0.5xTAE buffer at 60°C for 10 min at 200 V and for 16 h at 85 V. For bionumeric analysis, gels were stained with a silver staining solution, sealed with a cellophane sheet and visualized [221]. For band excision, gels were visualized with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, USA) on a Gel Doc system (BioRad, USA).

Two rounds of DGGE were performed. First, samples from before and after chemical oxidation and following 1, 2, 4, and 8 weeks of incubation were profiled. In a second round, selected samples that were representative of the composition for a given treatment were run in order to excise bands for sequencing. The brightest DGGE bands were excised from the gel with a sterile razorblade and incubated in 50 µL nuclease-free water overnight. This was used as a template for re-amplification using primers 968f and 1401r according to the PCR procedure described above. PCR amplicons were sequenced commercially (Eurofins, MWG-Biotech, Germany).

Microbial diversity analyses. BioNumerics 4.0 (AppliedMaths, Belgium) was used to further analyse the DGGE fingerprints. Dendograms and band pattern similitude were determined using the Jaccard Index. Additionally, after performing bandmatching (optimization = 0.5, position tolerance = 1%), band heights were exported for calculation of Shannon Diversity Indexes, as described previously [60].

Sequence analysis. Excised band sequences were aligned with reference sequences using the online alignment tool SINA available at <http://www.arb-silva.de> [201]. The aligned sequences were manually refined using tools available in the ARB software package [145]. A phylogenetic tree of the clones with most closely related reference sequences (full-length, when available) was constructed using the Neighbor-Joining method as implemented in ARB. Sequences were submitted to GenBank with accession numbers KC841278-KC841310.

6.3 Results

6.3.1 Contaminant and soil properties before and after chemical oxidation

Prior to and following chemical oxidation with Fenton's reagent, modified Fenton's reagent, permanganate and persulfate, the total concentration and bioavailable fraction of TPH were measured to determine the impact of chemical oxidation on contaminant characteristics (Table 2). Results indicated that the effectiveness of each chemical oxidant on the total TPH concentration as well as the bioavailability differed for the two soil types. In the peat sample, initial bioavailability was 121.9% of the total TPH concentration of 11.1 ± 0.4 g TPH/kg, which is most likely related to increased TPH extraction efficiency using a hydrophobic solid phase extractant which better supports desorption of TPH from SOM (Table 6.2). Whereas the measured total TPH concentration increased following oxidation with Fenton's reagent and modified Fenton's reagent to 14.0 ± 0.3 and 13.8 ± 0.2 g TPH/kg, respectively, the bioavailable fraction of the TPH decreased to only 72.5% and 67.9%, respectively (Table 6.2). Chemical treatment with permanganate decreased the total TPH concentration of peat by 2.8 g TPH/kg (25.2%) and the relative bioavailable fraction to 98.7%, indicating removal of mainly bioavailable TPH. Total TPH concentration was not changed by persulfate treatment; however, the bioavailable fraction decreased strongly.

Table 6.2 Total and bioavailable TPH concentrations prior to treatment (untreated) and following chemical oxidation with modified Fenton's reagent (MFR), Fenton's reagent (FR), permanganate (PM), and persulfate (PS). Mean values and standard errors are given for triplicate extractions. ^δ Percentage is the bioavailable fraction relative to the residual TPH concentration following chemical oxidation.

		Peat			Fill		
		TPH g TPH/kg	Bioavailable TPH g TPH/kg	% ^δ	TPH g TPH/kg	Bioavailable TPH g TPH/kg	% ^δ
Untreated		11.1 ± 0.4	13.5 ± 0.1	121.9 ± 4.0	25.4 ± 0.5	23.0 ± 0.5	90.3 ± 2.5
ISCO	MFR	13.8 ± 0.2	9.4 ± 1.4	67.9 ± 9.8	24.0 ± 1.1	16.2 ± 0.5	67.5 ± 3.4
	FR	14.0 ± 0.3	10.2 ± 0.3	72.5 ± 2.5	25.2 ± 1.6	17.1 ± 0.7	67.9 ± 5.1
	PM	8.3 ± 1.0	8.2 ± 0.1	98.7 ± 11.6	22.0 ± 0.9	15.2 ± 1.0	69.1 ± 5.6
	PS	11.0 ± 0.3	7.6 ± 0.1	69.1 ± 2.2	21.6 ± 0.5	20.9 ± 1.9	96.9 ± 9.3

The fill soil sample had an initial TPH concentration of 25.4 ± 0.4 g TPH/kg, of which 90.3% was bioavailable before chemical treatment (Table 6.2). While essentially no degradation was observed due to treatment with Fenton's and modified Fenton's reagent, oxidation with permanganate and persulfate reduced the TPH concentration by 13.4% and 15.0%, respectively. The bioavailable fraction decreased to 67.9% and 67.5% following oxidation with Fenton's and modified Fenton's reagent, respectively, which is comparable to the post-oxidation bioavailable fractions observed in the peat samples. A similar decrease in the bioavailable fraction was observed after oxidation with permanganate. Bioavailability was essentially unchanged following persulfate treatment.

Chemical oxidation caused not only changes in measured contaminant concentration and bioavailability, but also impacted the soil matrix. Increases in dissolved organic carbon (DOC), especially in the more organic-rich peat soil (Table 6.3), can be attributed to degradation of SOM upon oxidation. Absolute DOC concentrations in peat samples were in general higher than for fill, with the average post-chemical oxidation DOC value 10.6 times higher than that observed prior to chemical treatment. Fill DOC values were lower than for peat, with permanganate treatment being a notable exception.

Table 6.3 Dissolved organic carbon (DOC) concentrations before (untreated) and after chemical oxidation as well as after biological incubation. Values are the mean and standard error of triplicate microcosms. BC is biotic control. All other acronyms are as in Table 6.2.

DOC (mg C/kg)	Peat		Fill	
	before/after ISCO	after incubation	before/after ISCO	after incubation
Untreated	340 ± 10		70 ± 5	
BC		500 ± 20		480 ± 30
MFR	2870 ± 100	1680 ± 260	580 ± 20	1080 ± 130
FR	3750 ± 120	780 ± 150	580 ± 10	1120 ± 260
PM	4870 ± 140	4020 ± 230	4640 ± 350	3400 ± 80
PS	2370 ± 50	5450 ± 690	590 ± 30	750 ± 40

6.3.2 Diesel biodegradation following chemical oxidation

Recovery of biodegradation activity following chemical treatment as well as of the overall remediation efficiency were dependent upon not only the oxidant used, but also the soil matrix type (Figure 6.1). Biodegradation was observed following treatment with Fenton's reagent and modified Fenton's reagent in both soils. In contrast, no biodegradation was observed following treatment with permanganate and persulfate. The highest removal efficiency of 69% in the peat sample was attained in the biotic control. The fill samples showed a positive response to treatment with both Fenton's reagent and modified Fenton's reagent coupled to bioremediation, with overall remediation efficiencies of 59% and 57%, respectively, compared to 43% in the biotic control (Figure 6.1).

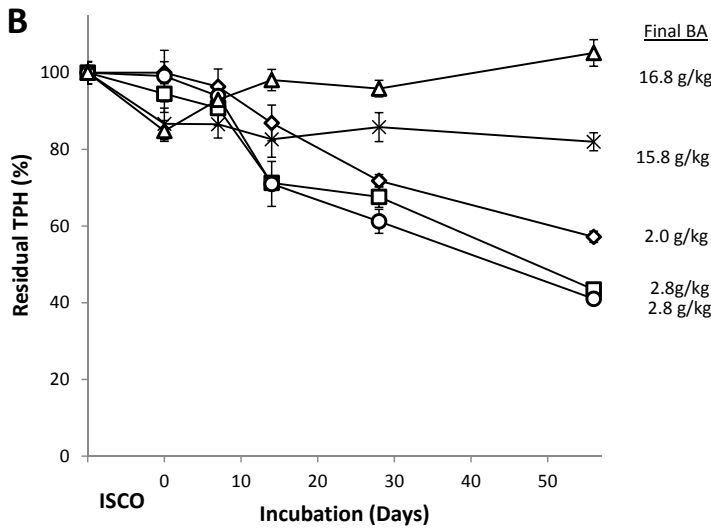
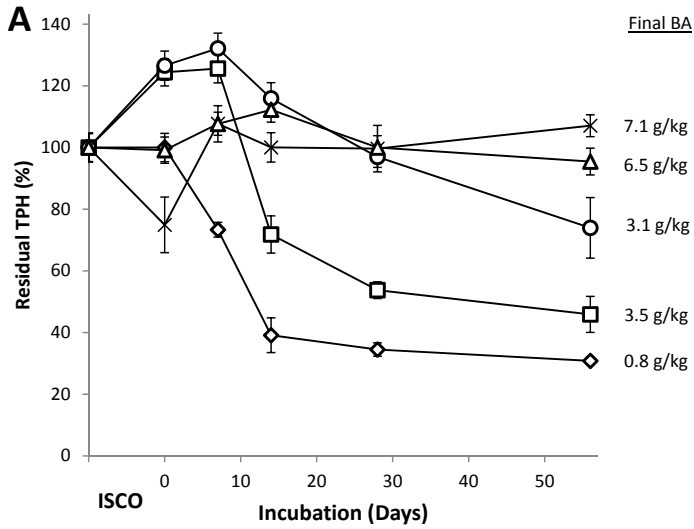
Initial rates of biodegradation following chemical oxidation differed greatly between chemical treatments and soil matrix types. Rapid biodegradation was observed in the peat biotic control sample without chemical treatment, with 2.9 g/kg degraded in the first week. Following chemical oxidation with Fenton's reagent and modified Fenton's reagent, biodegradation was observed only after a lag period of 1-2 weeks in peat microcosms. Thereafter, biological activity increased substantially, with biodegradation of 1.8 g TPH/kg and 6.0 g TPH/kg observed in the second week for Fenton's reagent- and modified Fenton's reagent-treated microcosms, respectively. Lower biodegradation rates towards the end of treatment could be related to low bioavailability of the residual contaminant (Figure 6.1).

In contrast to peat microcosms, in fill soils no large differences in the recovery of biodegradation activity were observed between biotic control microcosms and those treated with Fenton's reagent and modified Fenton's reagent. In the first week of incubation, 0.9 g TPH/kg was biodegraded in the biotic control and modified

Fenton's reagent treated microcosms; 1.3 g/kg were biodegraded following Fenton's reagent treatment. Rates of biodegradation remained similar throughout the 8 week incubation; however, bioremediation could have become bioavailability limited upon prolonged incubation, as observed previously [259].

In general, respiration (O_2 consumption and CO_2 production) matched the trends observed for degradation of TPH (Figure 6.2). Relatively constant rates were observed in fill soils. In microcosms with peat, initial respiration in the first week of incubation was higher in the biotic control (0.38 mol O_2 /kg soil; 0.24 mol CO_2 /kg soil) than in Fenton's reagent (0.02 mol O_2 /kg soil and 0.03 mol CO_2 /kg soil) and modified Fenton's reagent (0.10 mol O_2 /kg soil and 0.01 mol CO_2 /kg soil) treated microcosms. Limited total O_2 consumption was observed for microcosms treated with permanganate (0.15 and 0.17 mol/kg soil for peat and fill, respectively) and persulfate (0.29 and 0.11 mol/kg soil for peat and fill, respectively, data not shown), as compared to the biotic control (0.90 and 1.23 mol/kg soil for peat and fill, respectively).

Finally, a reduction in the concentration of DOC mobilized by chemical oxidation in peat soils was observed during the bioremediation phase (Table 6.3). This reduction can most likely be attributed to microbial degradation, indicating that mobilized DOC may have in some instances taken the place of TPH as a substrate for microbial growth.



- ◆ biotic control
- Fenton's reagent
- ▲ persulfate
- modified Fenton's reagent
- × permanganate

Figure 6.1 Relative residual TPH concentration during chemical and biological treatment in diesel contaminated peat (A) and fill samples (B). Values are the mean of triplicate extractions for each time point; error bars are the standard error. Values on the right hand side give final absolute bioavailability (BA) for each of the treatments after 8 weeks of incubation.

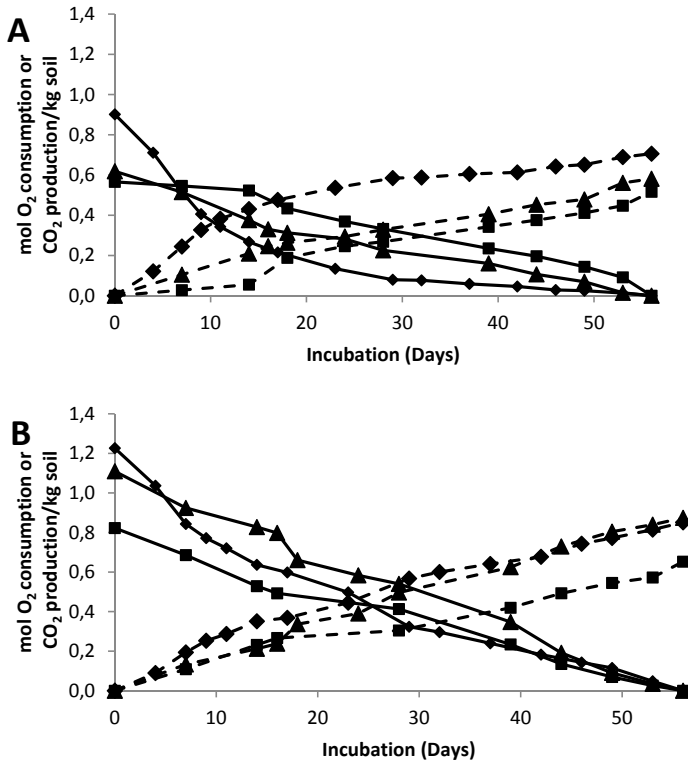


Figure 6.2 Cumulative O₂ consumption (solid line, decreasing) and cumulative CO₂ production (dotted line, increasing) for peat (A) and fill (B) for the biotic control (diamonds) or following treatment with either Fenton's reagent (squares) or modified Fenton's reagent (triangles).

6.3.3 Recovery of microbial community and degradation ability

The abundance of the 16S rRNA gene and the monooxygenase gene (*alkB*) involved in alkane degradation were used to quantify the microbial community prior to and following chemical oxidation, as well as throughout incubation [13, 116, 160]. Initial 16S rRNA gene and *alkB* abundance varied between the two soils, with both targets being higher in the peat soil than in the fill soil (Figure 6.3). Especially low *alkB* abundance was measured in the fill soil (10^3 copies/ g soil), indicating that biodegradation capacity was limited prior to chemical treatment.

The bacterial community was impacted by most chemical treatments. Following chemical oxidation with Fenton's reagent and modified Fenton's reagent, total bacterial 16S rRNA gene abundance was reduced approximately one order of

magnitude in both soils, confirming the impact of chemical oxidation on indigenous soil micro-organisms (Figure 6.2). Thereafter, regeneration of bacterial abundance was observed after 1-2 weeks, which agrees with the recovery of TPH biodegradation (Figure 6.1). Final total bacterial 16S rRNA gene abundances in soils treated with Fenton's reagent and modified Fenton's reagent were varying within one order of magnitude around the values of the biotic control microcosms, indicating full recovery of microbial biomass.

In contrast to 16S rRNA gene measurements, no reduction in *alkB* abundance was observed following chemical oxidation in either of the soils (Figure 6.3). Rapid enrichment of the *alkB* gene was observed after 1 week of incubation, especially in fill. After 8 weeks of incubation, *alkB* abundance in Fenton's and modified Fenton's reagent treated microcosms was within one order of magnitude of the biotic control in both soils. In general, the relative abundance of *alkB* copies (*alkB*/16S rRNA gene) during the first 2 weeks of incubation was higher for soils that had been chemically treated than for the biotic control.

After persulfate treatment, copy numbers of the bacterial 16S rRNA gene and *alkB* were reduced to levels at or below the detection limit (data not shown). Surprisingly, qPCR analyses indicated that permanganate treatment did not negatively impact the abundance of bacteria and the *alkB* gene, even though TPH biodegradation was not observed in either persulfate- or in permanganate-treated soils. The absence of microbial degradation capacity following persulfate oxidation was in line with *alkB* measurements. It is noteworthy that the measured abundance was not sharply affected by permanganate treatment itself, but rather slowly declined over the incubation period.

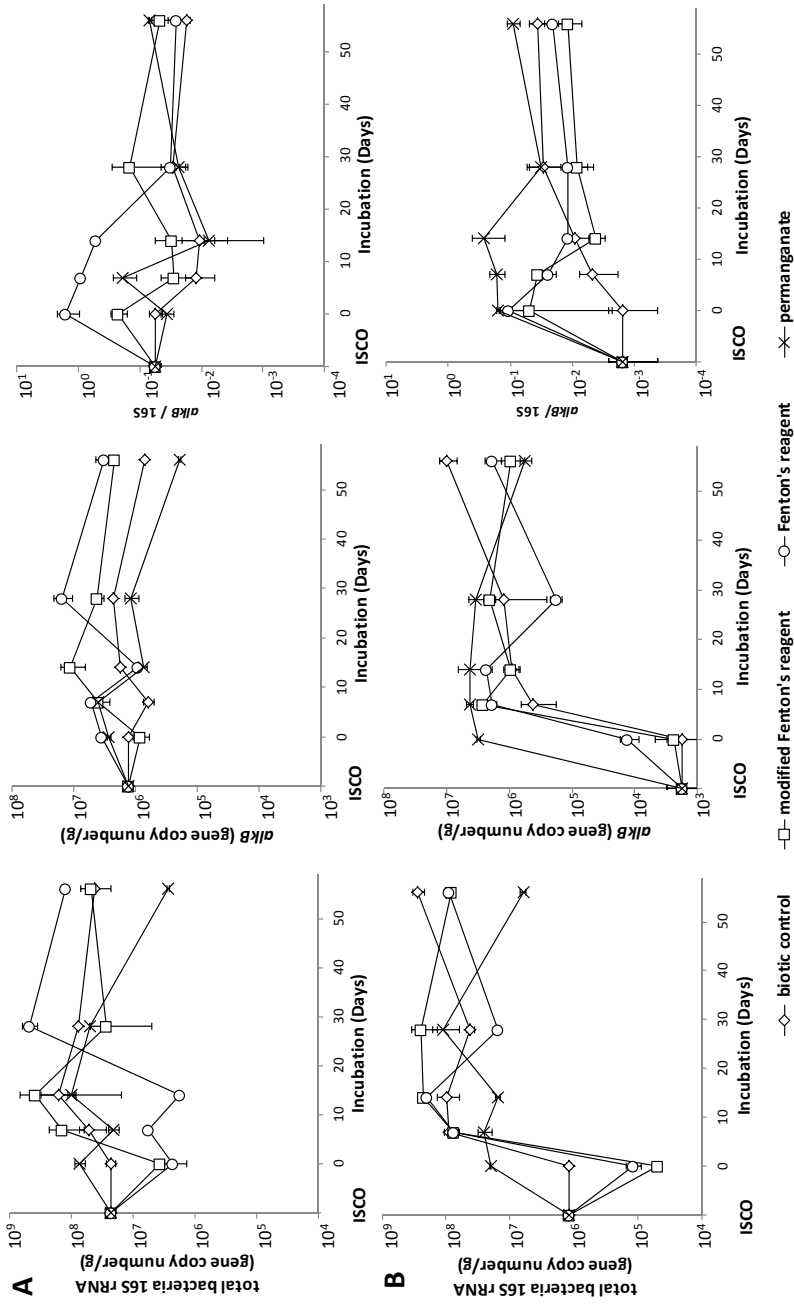


Figure 6.3 Absolute abundance of total bacteria by 16S rRNA gene (left) and *alkB* gene (center), and relative abundance of *alkB* gene (*alkB*/16S ratio; right) for peat (A) and fill (B) soil samples treated with selected chemical oxidants. Results are the average of triplicate qPCR measurements; error bars are the standard deviation of those measurements.

6.3.4 Regeneration of phylogenetic diversity

Bacterial community composition was analyzed using DGGE fingerprinting of 16S rRNA gene fragments PCR-amplified from samples taken before and after chemical oxidation, and following 1, 2, 4 and 8 weeks of incubation (Figure 6.4). DGGE profiles were analyzed with the goal of understanding how chemical treatment impacted bacterial diversity and composition. Additionally, samples with patterns representative of a certain treatment were used for sequencing of excised DGGE bands for identification of important phylotypes (Figure 6.5).

Community differences were observed following chemical oxidation in microcosms. Jaccard analysis of DGGE band profiles showed the development of dissimilar communities in microcosms which had received different treatments (Figure 6.4). In peat microcosms, samples treated with Fenton's and modified Fenton's reagent cluster together in a branch separate from the branch with biotic control, permanganate, and persulfate treated samples. Results are similar for the fill microcosms, where samples treated with Fenton's and modified Fenton's reagent form one branch and permanganate samples cluster separately.

Shannon diversity estimates from DGGE profiles indicated that fill samples generally harbored a higher bacterial diversity than peat samples, regardless of the treatment (Table 6.4). In both biotic control setups, diversity increased within 1-2 weeks of incubation and declined by week 8. Following chemical oxidation with Fenton's reagent, modified Fenton's reagent, and permanganate, a similar temporary increase and subsequent decrease in diversity was observed in peat soils. In fill microcosms, diversity after 1 and 8 weeks of incubation was similar for Fenton's and modified Fenton's reagent.

In biotic control microcosms, bacterial composition differed between peat and fill microcosms as based on sequence analysis of selected bands. Incubated peat soils contained 16S rRNA gene sequences belonging to a variety of phyla, including *Bacteroidetes*, *Verrucomicrobia*, and *Cyanobacteria* (Figure 6.5). In contrast, exclusively *Proteobacteria* sequences were found for the fill biotic control microcosms, with all sequences either belonging to *Betaproteobacteria* or the *Xanthomonadales* order of *Gammaproteobacteria*. Although biodegradation was observed in Fenton's and modified Fenton's reagent treated soils as well as in biotic control microcosms, different phyla were observed following chemical oxidation. In both peat and fill microcosms, *Proteobacteria* were found following Fenton's and modified Fenton's treatment, with a high prevalence of sequences belonging to the order *Pseudomonadales* of the *Gammaproteobacteria*. This is in contrast to the composition in biotic control microcosms, as described above.

Finally, sequences found in inactive permanganate-treated microcosms were closely related to degraders of alkanes and aromatics. Following permanganate treatment, sequences similar to those of the aliphatic degraders *Microbacterium hydrocarbonoxydans* and *Microbacterium oleivorans* (P-PM-2-a and P-PM-2-c) and well characterized naphthalene degrader *Pseudomonas putida* (P-PM-4-b) were found [97, 226]. This supports *alkB*-targeted qPCR data indicating that microbial biodegradation capacity was measured in permanganate treated microcosms. The observed inactivity of microbes, in terms of biodegradation activity and respiration, could be ascribed to inhibition by manganese oxides, or could indicate that measured microbial DNA targets belonged to damage cells.

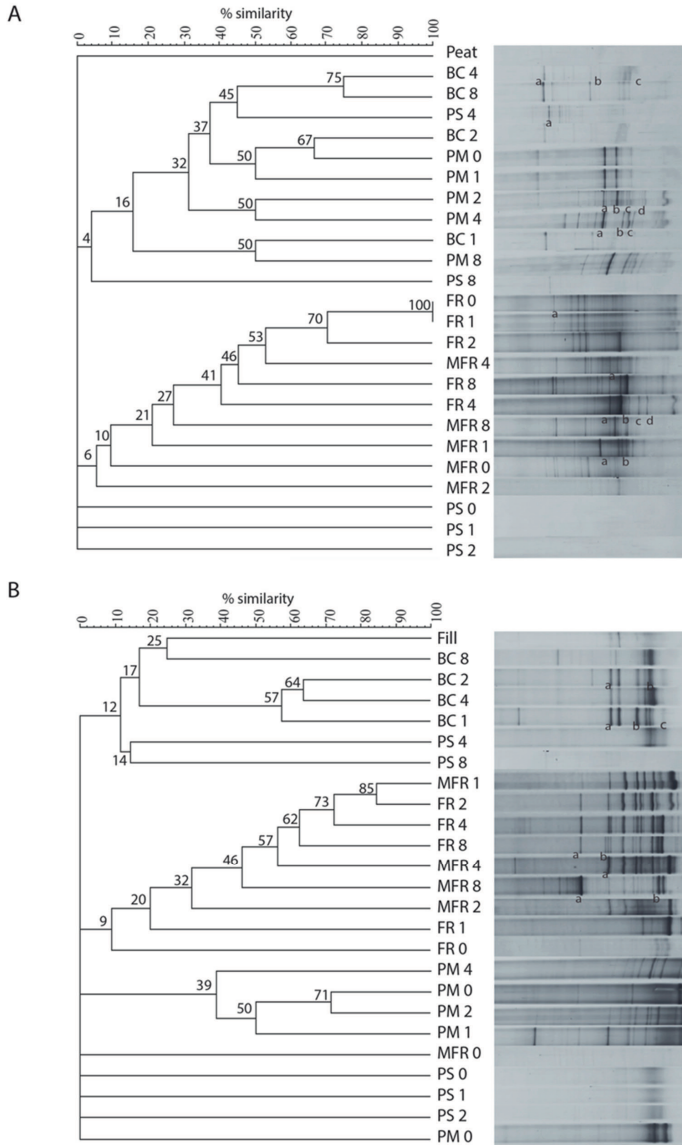


Figure 6.4 Dendograms of DGGE profiles using Jaccard index (left) and DGGE profiles (right) for peat (A) and fill (B) prior to treatment (peat or fill, respectively) and following chemical oxidation. Treatment abbreviations are as in Tables 2 and 4. Numbers after each treatment indicate sampling point following ISCO (0) or during incubation (1, 2, 4, or 8 weeks). Similarity values are given for each branch. Excised bands are marked with letters below them.

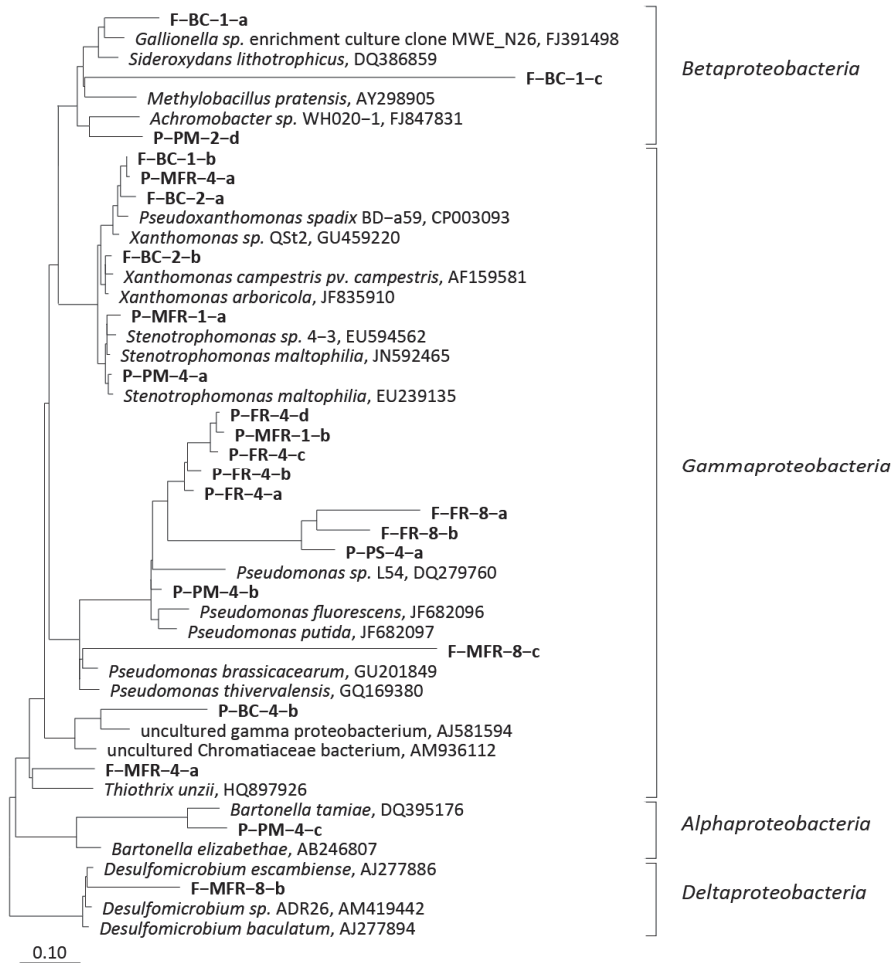


Figure 6.5 Phylogenetic tree of sequenced 16S rRNA genes (bold) excised from DGGE (Figure 3) and belonging to *Proteobacteria*. Acronyms for soil type, treatment, and timing of the sample are as in Tables 2 and 3 and Figure 3. A fully summary of sequences including the GenBank accession number are given in [257].

Table 6.4 Shannon diversity indices of DGGE band profiles based on bands identified with automated bandmatching (optimization = 0.5; position tolerance = 1%). Treatment acronyms are as in Tables 2 and 3; values given for after ISCO (0 weeks of incubation) or for 1, 2, 4, or 8 weeks of incubation.

		Incubation (weeks)				
		0	1	2	4	8
Peat	BC	0	0.41	0.67	0.67	0.57
	MFR	0	0.1	0	0.27	0.25
	FR	0.56	0.56	0.6	0.22	0.3
	PM	0.62	0.46	0.72	0.68	0.14
	PS	0	0	0	0.56	0
Fill	BC	0	1.03	0.94	0.82	0.49
	MFR	0	1	0.66	0.99	1.04
	FR	0.45	0.75	1.09	1.07	1.05
	PM	0.35	0.51	0.27	0.39	0
	PS	0	0	0	0	0.83

6.4 Discussion

6.4.1 Factors contributing to overall remediation efficiency

While overall remediation efficiencies were similar, absolute TPH biodegradation differed for each treatment for the two soils studied here (Figure 6.1; Figure S3). The highest remediation efficiency was observed in the biotic control microcosm for peat at 69%. For the fill soil, remediation efficiency was highest following Fenton's reagent treatment at 59%. However, in absolute values, nearly twice as much TPH was degraded in the fill treated with Fenton's reagent (14.8 g/kg) than in the peat biotic control microcosm (7.6 g/kg).

The choice of chemical oxidant has direct implications for the disturbance and subsequent recovery of biodegradation activity and thus the overall remediation efficiency (Figure 6.1). Following chemical oxidation with Fenton's reagent and modified Fenton's reagent, microbial activity was demonstrated by TPH degradation, reduction in DOC concentrations (in peat), and O₂ consumption and CO₂ production. In contrast, no significant biological activity was measured in permanganate- and persulfate- treated microcosms.

Biodegradation rates differed between soil types following Fenton's reagent and modified Fenton's reagent treatment. Traditional Fenton's reagent, applied under acidic conditions (pH of 2-4), is a stronger treatment than modified Fenton's reagent, the latter of which, due to the use of a chelator for the Fe²⁺ catalyst, is performed at

natural pH. Thus, the milder conditions during modified Fenton's reagent treatment should support more rapid recovery of biological activity. This was observed to some extent for peat, where, following Fenton's reagent treatment, recovery of biodegradation was delayed a week (Figure 6.1) and overall biodegradation was lower (0.10 ± 0.01 g TPH/kg/d) as compared to modified Fenton's reagent (0.16 ± 0.02 g/kg/d). However, in fill, rates were not significantly different for modified Fenton's reagent and traditional Fenton's reagent, 0.23 ± 0.03 and 0.26 ± 0.03 g TPH/d respectively. While it has often been hypothesized that modified Fenton's reagent is more conducive to a subsequent bioremediation step [300], this result indicates that either treatment can be combined with biodegradation.

While biodegradation was observed following Fenton's reagent and modified Fenton's reagent treatment, most probably other factors than solely the chemical oxidant contributed to the overall remediation efficiency. Specifically, we observed changes in contaminant characteristics including mobilization and bioavailability as well as differences in DOC concentrations, and it can be assumed that these factors also impacted the efficiency of the treatment.

Previous work has suggested that chemical oxidation can improve biodegradation by reducing contaminant concentrations to less toxic levels and by improving bioavailability [37, 121, 169]. However, in the current study, bioavailability was not considerably improved by chemical oxidation (Table 6.2), which could limit further biodegradation (Figure 6.1). Additionally, in peat soils, Fenton's reagent and modified Fenton's reagent treatment mobilized TPH, as observed in the increase in concentrations measured by traditional solvent extraction. In the field, such mobilization could result in increased environmental risk and reduced remediation efficiencies, requiring treatment strategies to include the fraction of immobile contaminants strongly bound to SOM.

Increased DOC concentrations following chemical oxidation indicated degradation of the soil matrix in addition to increased solubility of TPH upon oxidation (Table 6.3). Additionally, in the case of peat, DOC mobilized during chemical oxidation was removed during incubation most likely due to biological processes (Table 6.3), which may contribute to lower absolute TPH biodegradation. For example, while 2.5 times more TPH was degraded in Fenton's reagent-treated fill than in Fenton's reagent-treated peat, CO₂ production was only 25% higher in fill (Figure 6.2), indicating that sources of carbon other than TPH, namely DOC, may have been mineralized. Previous work also found that chemical oxidation changed the structure of the soil matrix, where an increase in the fraction of water-soluble, biodegradable SOM was observed

following ozone treatment due to an increase in the hydrophilic content of SOM upon oxidation [104]. While DOC mobilization was observed in fill, no removal of DOC was noted. These results imply that the quality and physical properties of organic compounds present in each soil differ, especially with regard to biodegradability, demonstrating that soil type influences the effectiveness of coupled chemical and biological treatment.

6.4.2 Implications of microbial community

Quantitative PCR was utilized to understand the impact of chemical treatment and incubation on the bacterial community size and biodegradation capacity. This was accomplished by targeting a gene which encodes an alkane degrading enzyme (*alkB*) as a proxy for diesel degradation capacity [13, 79, 116, 228]. Results indicated that chemical treatment can profoundly impact the bacterial community in terms of size and biodegradation capacity (Figure 6.3). For example, persulfate treatment strongly disrupted biological functions. In addition, although *alkB* was measured in permanganate treated microcosms, no biotic activity was observed. This result indicates that microbial DNA, potentially damaged during chemical oxidation, may not be a suitable proxy in this instance, as the presence of specific genes can only indicate the potential for a certain function, but cannot be taken as direct proof for actual activity. Our results suggest that neither permanganate nor persulfate is compatible with efficient biodegradation, although groundwater flow may to some extent mediate the impact of these treatments in the field through inoculation with indigenous bacteria.

The absolute abundance of *alkB* was not impacted by chemical oxidation, with enrichment of this gene being observed in all the active microcosms. In the case of Fenton's reagent and modified Fenton's reagent, the relative abundance of the *alkB* gene was initially higher than in the biotic control, indicating a strong shift towards TPH degraders. Previous work has also found that treatment with Fenton's reagent can to some extent stimulate bioremediation [114]. These findings indicate that either: (1) TPH degrading bacteria are especially resilient towards chemical oxidation, as observed in the *alkB* data, or (2) that chemical treatment somehow gives TPH degraders a competitive advantage through either creating additional carbon substrates such as DOC or by mobilizing partially oxidized TPH. In either case, our results indicate that sequential treatment with either Fenton's reagent or modified Fenton's reagent followed by bioremediation is a feasible, efficient technology.

Microbial community analysis using DGGE and sequence analysis of key populations supports qPCR results indicating the presence of communities associated with TPH degradation. Previous work has shown the dominance of *Proteobacteria* in hydrocarbon degradation, especially *Gammaproteobacteria* upon nutrient amendment [73, 109, 170, 284]. *Betaproteobacteria* have been identified as copiotrophs or r-strategists, able to rapidly, although inefficiently, utilize resources under conditions of carbon and nutrient abundance [64]. In our study, 70% of sequences were categorized as *Proteobacteria*, with 91% of those sequences classified as either *Betaproteobacteria* or *Gammaproteobacteria* (Figure 6.5). Additionally, a number of sequences showed high similarity to known TPH-degraders, underscoring the prevalence of a biodegrading population under optimized bioremediation conditions.

While TPH degraders were present, Jaccard analysis and Shannon Diversity estimates of DGGE profiles indicated the development of dissimilar microbial communities, both in terms of diversity and composition. Differences in Shannon Diversity Indices were noted across all treatments, with Fenton's reagent and modified Fenton's reagent promoting diversity in fill as compared to the biotic control; the opposite was observed for peat. In both peat and fill soils, the microbial community observed following treatment with Fenton's reagent and modified Fenton's reagent differed from that observed in the biotic control microcosms (Figure 6.4 and 6.5), showing a longer term impact of chemical treatment on microbial composition beyond the oxidation period. In addition to oxidative stress from chemical oxidation, the parameters investigated in this study, such as (1) changes in the soil geochemistry, including SOM degradation and DOC mobilization, and (2) contaminant characteristics, including mobilization and bioavailability, can clearly have an impact on the composition of the microbial community. Additionally, chemical oxidation can cause a reduction in the soil buffering capacity, the deposition of manganese oxides, and the release of sulfate, all of which can have long-term implications for soil and groundwater parameters. Even in open systems where groundwater flow would re-inoculate the soil with indigenous microorganisms, changes to subsurface characteristics and thus microbial communities, are lasting. The effect of re-inoculation with indigenous microorganisms after chemical oxidation can only be investigated in field scale experiments.

Overall, our results indicate that coupling certain chemical oxidants with bioremediation can be an effective treatment. Although microbial communities may initially be adversely affected in the first 2 weeks by Fenton's reagent or modified

Fenton's reagent application, a rebound of microbial abundance and biodegradation activity can be expected in the longer term (1-4 weeks). Following chemical oxidation, dissimilar microbial communities developed, with an increase in the relative abundance of *alkB* as compared to biotic control microcosms, indicating that chemical oxidation may support a shift towards TPH-degraders. Overall remediation efficiency was dependent upon the soil and contaminant characteristics, highlighting the need to characterize and understand the system in terms of microbiology, pollutant chemistry, and geochemistry prior to application in the field. This study shows that this is only valid under the condition that the treatment is properly applied on the basis of an adequate soil system characterization.

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Chapter 7

Geochemical and microbiological characteristics during *in situ* chemical oxidation and *in situ* bioremediation at a diesel contaminated site

This chapter has been published:

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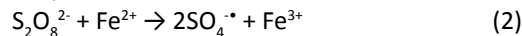
Abstract

While *in situ* chemical oxidation with persulfate has seen wide commercial application, investigations into the impacts on groundwater characteristics, microbial communities and soil structure are limited. To better understand the interactions of persulfate with the subsurface and to determine the compatibility with further bioremediation, a pilot scale treatment at a diesel-contaminated location was performed consisting of two persulfate injection events followed by a single nutrient amendment. Groundwater parameters measured throughout the 225 day experiment showed a significant decrease in pH and an increase in dissolved diesel and organic carbon within the treatment area. Molecular analysis of the microbial community size (16S rRNA gene) and alkane degradation capacity (*alkB* gene) by qPCR indicated a significant, yet temporary impact; while gene copy numbers initially decreased 1-2 orders of magnitude, they returned to baseline levels within 3 months of the first injection for both targets. Analysis of soil samples with sequential extraction showed irreversible oxidation of metal sulfides, thereby changing subsurface mineralogy and potentially mobilizing Fe, Cu, Pb, and Zn. Together, these results give insight into persulfate application in terms of risks and effective coupling with bioremediation.

7.1 Introduction

Anthropogenic contamination of soil requires remediation of such locations to both mediate environmental risk and allow redevelopment. To this end, *in situ* remediation techniques, including *in situ* chemical oxidation (ISCO) and *in situ* bioremediation (ISB), are gaining acceptance as being often more cost-effective and sustainable than more traditional *ex situ* technologies [233]. As *in situ* remediation becomes more commonplace, emphasis has been placed on understanding the subsurface processes that occur during treatment and the coupling of different technologies, with the goal of improving overall remediation efficiencies.

During ISCO treatment, chemical oxidants, such as permanganate, persulfate, ozone, or Fenton's reagent (iron-catalyzed hydrogen peroxide), are injected into the soil to rapidly degrade organic contaminants [62, 96, 233]. Of the treatments available, commercial application of sodium persulfate for contaminant removal was more recently introduced, with limited scientific investigations into field application [246, 272]. Oxidation with persulfate occurs either through direct electron transfer (Equation 1), or by activation to produce the sulfate radical (Equation 2; [86, 96, 272]).



Persulfate activation has been reported with heat, alkaline conditions, addition of hydrogen peroxide, or use of transition metals (Equation 2), with the efficiency of each treatment dependent upon the contaminant type and subsurface conditions [41, 89, 265, 272, 273]. Additionally, it has been shown that naturally occurring aquifer minerals such as birnessite, goethite, and pyrite can catalyze the production of sulfate radicals [4, 138, 264]. Persulfate has been shown to degrade a variety of compounds, including chlorinated solvents, mono-aromatics including benzene, toluene, ethylbenzene and xylene (BTEX), total petroleum hydrocarbons (TPH), methyl tert-butyl ether (MTBE), polycyclic aromatic hydrocarbons (PAHs), and chlorinated benzenes [41, 89, 136, 139, 207, 302].

Although ISCO treatment can effectively and efficiently remediate a location, application has implications for soil and groundwater characteristics. As with all chemical oxidants, non-specific reactions between persulfate and naturally occurring soil organic matter (SOM) not only reduce the treatment efficiency, but also degrade SOM [89, 135, 190]. ISCO treatment reduces the quantity of SOM, causing an increase in dissolved organic carbon (DOC), and causes structural changes to residual SOM [42, 185, 289]. Changes in the relative abundance of humic acid and fulvic acid

soil fractions as well as a decrease in the amorphous fraction of SOM have been observed following chemical oxidation [42, 104, 289]. Additionally, the oxidation of reduced minerals, although a suggested activator of persulfate, irreversibly changes the subsurface [4, 135]. Finally, persulfate can cause groundwater acidification, with pH drops of up to 6 units being reported in laboratory studies [89, 137, 207, 273]. Groundwater acidification as well as alkaline activation can have similarly irreversible impacts on soil buffering capacity, heavy metal-mobilization, and subsurface geochemistry.

While it was previously thought that conditions created during chemical oxidation treatment preclude subsequent bioremediation, recent research has shown that coupling ISCO and ISB is not only possible, but can be quite advantageous [35, 207, 255, 274, 299]. The inclusion of a so-called bio-polishing step reduces costs associated with chemical oxidants while improving the final soil quality by removing residual contaminants and encouraging regeneration of soil function. Although pH changes due to alkaline activation or acidification can severely impair biological function, persulfate has nonetheless been successfully coupled with bioremediation. In a column experiment, phenanthrene degradation activity was temporarily interrupted following persulfate application, but regenerated within 100-500 days [207]. In a batch test for the degradation of di-nitrotoluene, persulfate oxidation was less detrimental to bioremediation than ozone or modified Fenton's reagent, with some evidence of simultaneous chemical and biological degradation [35]. Finally, a laboratory investigation into the impact of heat-activated persulfate also concluded that it was less harmful to indigenous microorganisms than Fenton's reagent [273].

While persulfate has shown increased commercial viability, there remains a lack of scientific literature into the subsurface processes occurring during *in situ* application. Therefore, a pilot treatment was performed at a diesel contaminated location, consisting of two persulfate injections followed by a single nutrient amendment at the end to stimulate ISB. Extensive monitoring of contaminant characteristics, groundwater constituents, soil characteristics, and microbial TPH degradation capacity identified temporary perturbations and permanent changes to the subsurface. Through better understanding the processes associated with persulfate oxidation, this research aims to improve its application as an ISCO treatment and its coupling with ISB.

7.2 Materials and Methods

7.2.1 Site description

The field test was conducted at a former railway refueling station in Węglińiec, Poland. Operation activities between 1970 – 2000 led to significant diesel contamination; light non-aqueous phase liquid (LNAPL) of up to 70 cm in the source area feeds a plume that spreads across the site in a north-easterly direction. The field test was situated in the downstream plume area, where mainly residual phase and dissolved TPH were present at or below the groundwater level (1.5 m bgl). Very heterogeneous TPH distribution was observed in the soil profiles analyzed (Table 7.1). The soil profile consists of approximately 2.5 to 3 meter of anthropogenic fill confined by an authigenic peat layer. In total, 15 monitoring wells were sunk within the 5 m pilot location (Figure 7.1). Each monitoring well, including the injection well (C1), was placed with the bottom of its 1 m filter on the confining peat layer.

Prior to injection, calculations were made based on the injection volume, porosity, and filter length to determine the radius of influence (ROI; Figure 7.1). However, this should not be viewed as a definitive determination of persulfate spreading, but rather as an estimate of the wells directly impacted by high persulfate concentrations. Due to the small scale of the pilot, significant heterogeneity in soil structure and porosity, and different injection volumes, it is difficult to determine the exact spreading of persulfate. Rather, ROI is given in order to differentiate wells in direct contact with persulfate from those downstream or upstream from the treatment area.

Table 7.1 Soil total petroleum hydrocarbon (TPH) concentrations profiles and total organic carbon (TOC). Samples were taken prior to treatment (C1, C2, C3, C4, C11), following the first persulfate injection (P1) and at the end of the treatment period (P2, P3).

Depth m bg ^a	C1	C2	C3	C4	C11	P1	P2	P3
TPH (mg/kg)								
1.3-1.4	11	96	201	271	68	bd ^b		
1.5-1.6	141	95	254	999	631	608	152	450
1.7-1.8	932	3756	131	2441	1177	52	78	2226
1.9-2.0	1405	4659	90	2914	12912	2215	740	5245
2.1-2.2	5311	3488	26	6121	9165	1370		20910
2.3-2.4	8890		29	1924	5834	3722	1863 ^c	
2.5-2.6	9950 ^c	5911	227		7192	3680 ^c		
2.7-2.8		4784		1303	4498	3001		
2.9-3.0			89	2960		930		
TOC (%)								
1.3-1.4	0.1	7.8	1.8	1.0	1.3	0.8		
1.5-1.6	0.1	6.1	1.9	0.4	0.5	0.8		
1.7-1.8	3.8	1.3	2.2	1.7	0.3	1.2		3.1
1.9-2.0	0.5	0.8	1.4	0.5	6.7	1.1	2.7	1.9
2.1-2.2	0.4	0.9	0.4	0.7	5.6	1.4		2.1
2.3-2.4	0.5		0.2	1.6	1.3	0.9	2.0 ^c	
2.5-2.6	1.1 ^c	0.3	1.1		5.5	1.1 ^c		
2.7-2.8		1.5		1.2	1.3			
2.9-3.0			26.0	39.0		1.2		

^a below ground level

^b below detection level

^c soil samples also underwent TGA, XRD, and on-line sequential extraction analysis.

7.2.2 Injection and monitoring

In May 2012, approximately 0.55 m³ 10% (m/m) sodium persulfate solution was injected in C1 (day 0). A second injection with a volume of approximately 0.77 m³ 10% (m/m) sodium persulfate solution was performed on day 43. The commercially available persulfate (Termopasty AG, Poland) was not activated. Once the pH had risen to levels suitable for the injection of nutrients (pH>5), approximately 0.20 m³ water containing a solution of a commercially available fertilizer (N-NH₄ 0.5 g·l⁻¹; N-NO₃ 0.3 g·l⁻¹; P-PO₄ 0.3 g·l⁻¹; pH 6.7) was injected on day 93. Regular analysis of groundwater and soil samples with field and laboratory measurements was performed prior to injection and throughout the experiment, as described below.

7.2.3 Chemical analyses

Field measurements. The propagation of the injected chemical oxidant was followed using electrode measurements of pH (SenTix41 pH electrode; WTW GmbH, Germany), electrical conductivity (EC; Professional Plus 1700/1725; YSI, USA) and oxidation reduction potential (ORP; ORP200; HM Digital, USA). Calibration of the field equipment was performed regularly.

Groundwater analyses. Groundwater samples were collected using a peristaltic pump. For analysis of dissolved organic carbon (DOC), Na^+ , K^+ , Ca^{2+} , Cl^- , SO_4^{2-} , NO_2^- , and NO_3^- , samples were filtered through a 0.45 μm filter. Additionally, samples for total organic carbon (TOC), dissolved TPH, NH_4^+ , and PO_4^{3-} were collected without filtering. Samples were collected in dark bottles and stored at 4°C prior to analysis.

DOC was measured on an automatic carbon analyzer HiPerTOC (Thermo Scientific, Germany). TPH was measured by solvent extraction and analysis on a gas chromatograph (GC) with a flame ionization detector (FID), as described below. Na^+ and K^+ were determined on a flame atomic absorption spectrometer (AAAnalyst 400, Perkin Elmer, USA). Ca^{2+} was measured on a IP-OES Liberty 220 (Varian, USA). Anions were determined using ion chromatography on a ICS 3000 (Dionex, USA). NH_4^+ and PO_4^- were measured using UV-Vis spectrophotometry.

Soil analyses. Soil profiles were sampled during the sinking of monitoring wells (C1-C4 and C11), after the first persulfate injection (P1), and at the end of treatment (P2 and P3). Total organic carbon (TOC) was measured gravimetrically. TPH-content of the soil samples was determined after extraction using acetone and hexane in accordance with the Dutch NEN5733-standard [178]. The TPH in the extracted solution was measured on a Hewlett Packard HP5890 GC with a SIM DIST column and a FID with nitrogen as the carrier gas. Following 5 minutes at an initial temperature of 40°C, GC temperature was increased at 10°C·min⁻¹ to a final temperature of 300°C.

Additionally, thermal gravimetric analysis (TGA), x-ray diffractometry (XRD), and on-line extraction coupled to ICP-OES were performed on three soil samples: (a) from prior to treatment (C1, 2.5-2.6 m), (b) 40 days after the first injection (P1, 2.5-2.6 m), and (c) a final sample (P2, 2.3-2.4 m). TGA, in accordance with the Dutch NEN 5748, was applied to determine water, carbonate and organic matter content of the samples [179]. A temperature ramp of 2°C·min⁻¹ was performed to determine different organic matter fractions and changes therein as a result of the injection of persulfate [42].

XRD was performed on the total soil samples, and the clay size fraction (<2 μm) to determine soil mineralogy. Prior to analysis, samples were dried at 60°C for 24 hours,

an internal standard was added (ZnO), and the samples were ground in ethanol. XRD-diffractograms were obtained using CuK α -radiation between 5-80°2 θ at a rate of 0.01°-s⁻¹. Quantification was performed using the Rietveld method.

On-line sequential extractions were performed to measure changes in soil mineralogy and determine the presence of iron sulfides [69, 236]. For each on-line extraction, 5 g of soil sample was mixed in a 1 to 2.5 ratio with acid washed glass beads and transferred to a glass tube on-line with an ICP-OES (Spectro Arcos, Germany). The sample was flushed sequentially with 0.01 M CaCl₂, 800 s with a 2% H₂O₂, 600 s with a 0.45 M HNO₃, and finally 600 s with a 3 M HCl-solution (Table 7.2). Each extraction fluid was diluted on-line in a 1:1 ratio with an internal standard solution containing all major cations and metals and measured directly on the ICP-OES.

Table 7.2 On-line extraction steps.

Step	Duration Period	Extraction solution	Targeted Soil Matrix
1	500 s 1-500 s	0.01 M CaCl ₂	Reversibly bound cations on clays and organic matter
2	800 s 500-1300 s	2% H ₂ O ₂	Organic matter
3	600 s 1300-1900 s	0.45 M HNO ₃	Easily extractable sulfides, oxides and carbonates Minerals such as feldspars, clay minerals and dolomite dissolved after prolonged extraction times [21, 39]
4	600 s 1900-2500 s	3 M HCl	Amorphous and crystalline Fe and Mn hydroxides, clay minerals, feldspars and pyrite [90]

7.2.4 Biomolecular analyses

For analysis of microbial abundance, groundwater was collected in sterile plastic containers, 500 mL was vacuum-filtered over a 0.2 μ m membrane filter (Millipore, USA), and filters were stored at -20°C in sterile microcentrifuge tubes. For DNA extraction, filters were cut into small strips prior to bead-beating with the FastDNA Spin Kit for Soil (MP Biomedicals, USA). DNA quantity and quality were estimated on a Nanodrop spectrophotometer (Thermo Scientific) prior to storage at -20°C. Quantitative PCR (qPCR) was performed using an iQ5 SYBR Green Supermix kit on the iQ5 iCycler (Biorad, The Netherlands). Hundred-fold dilutions were assayed in triplicate and no-template controls were included. Total bacterial 16S rRNA genes (341F and 534R) [174] and the *alkB* gene encoding for an alkane monooxygenase involved in TPH degradation were assayed [199]. The qPCR protocols were performed

as described previously [259]. Gene copy numbers were calculated as copies/mL sample.

7.3 Results and Discussion

7.3.1 Oxidant delivery

Following each persulfate injection event (on days 0 and 43), changes in groundwater parameters confirmed the delivery of oxidant within the radius of influence (ROI; Figure 7.1). An immediate rise in EC and ORP in the injection well (C1) and nearby wells (C6-C9) was observed (Figure 7.2). EC increased 20-30 fold and ORP rose from approximately -100 mV to between 450 and 550 mV following each injection. Groundwater acidification from circumneutral pH to values below 4 occurred 2-6 days after each injection, indicating spreading of the oxidant and reaction with the subsurface. Downstream, groundwater mixing caused a slight rise in EC in C5 3 days after the first injection and C10 on day 13; C3, further downstream, was not affected. During the second persulfate injection, EC and ORP values in C5 and C10 were similar to C1, indicating direct oxidant delivery to these downstream wells. Slight fluctuations in groundwater parameters in C11 upstream from the injector following the second injection suggest mixing of impacted water during the second injection event.

Perturbations in EC, ORP, and pH returned more rapidly to baseline values following the first injection than the second. This phenomenon was to some extent attributable to the difference in mass injected on day 0 (0.55 m³) versus day 43 (0.77 m³), as supported by the higher EC peak and by direct delivery of oxidant to downstream wells C5 and C10. Depletion of soil oxidant demand and soil buffering capacity during the first injection also contributed to the delayed attenuation of ORP and pH following the second injection. Whereas groundwater parameters within the ROI started to approach pre-injection levels within 21 days of the first injection, following the second injection, ORP remained above 300 mV and pH below 4 for more than 24 days (through day 77; Figure 7.2). Long-term disturbance of groundwater pH was observed in C1, where the pH remained below 5 at the final sampling point 205 days after the first injection, and in downstream C5 and C10, due to continued groundwater flow of impacted water from the ROI. Interestingly, no substantial influence of groundwater flow was observed in wells C3 or T3. The injection of nutrient rich water on day 93 did not mediate groundwater acidification in C1. Decrease in pH due to persulfate treatment has been observed previously [89, 137, 207, 273].

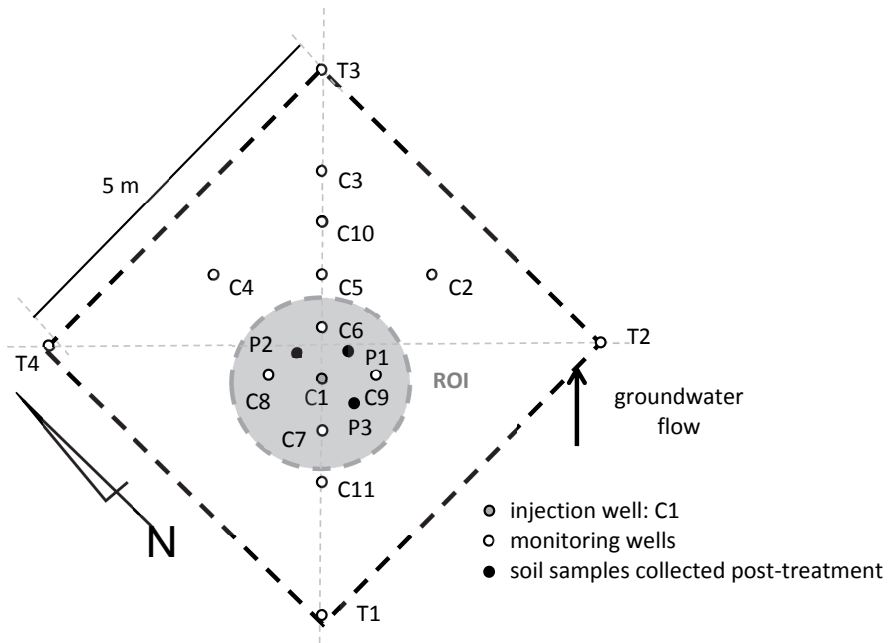


Figure 7.1 Schematic layout of pilot location. The radius of influence (ROI) is shaded.

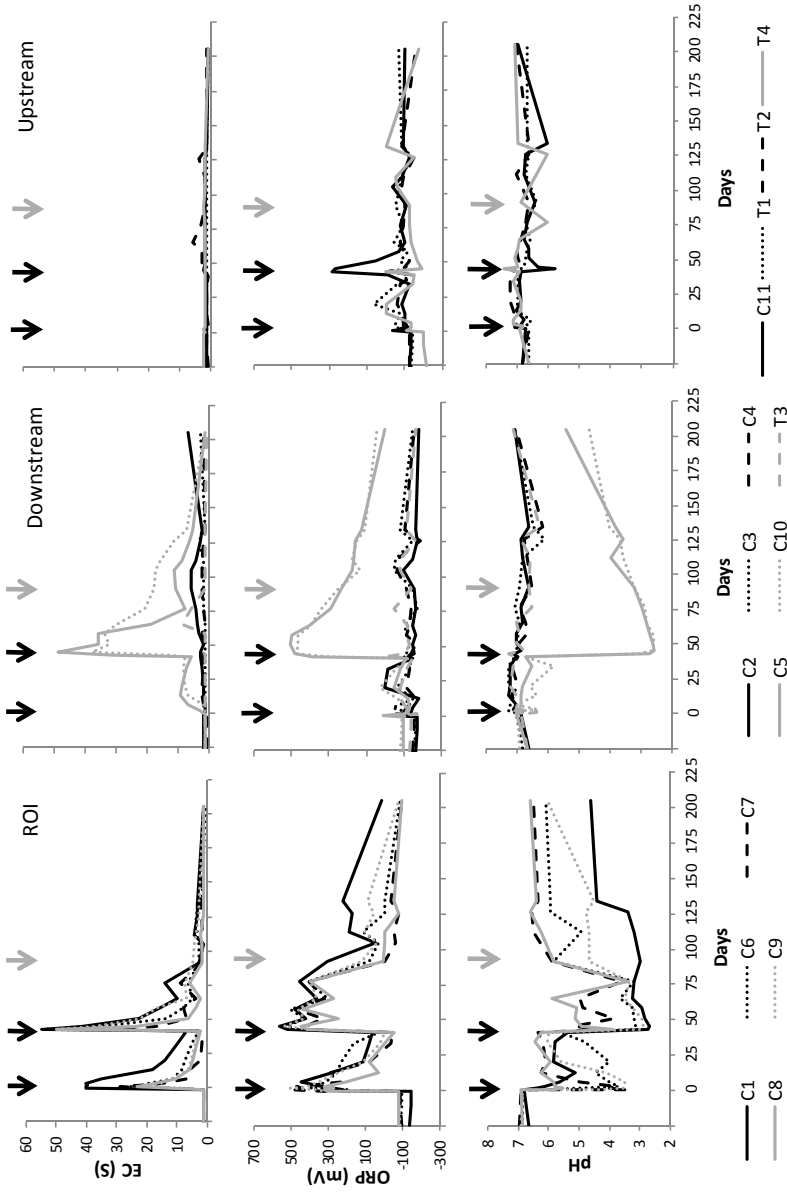


Figure 7.2 Electrical conductivity (EC; top panel), oxidation reduction potential (ORP; middle), pH (bottom) during 205 days of monitoring. Results are given for the radius of influence (ROI; left, wells C1, C6, C7, C8, C9), downstream (middle, wells C2, C3, C4, C5, C10, T3) and upstream (right, wells C11, T1, T2, T4). Black arrows indicate the timing of persulfate injection on day 0 and day 43, gray arrow indicates nutrient amendment on day 93.

7.3.2 Microbial community abundance and degradation capacity

The impact of ISCO on microbial communities was estimated by measuring the abundance of i) total bacteria based on the 16S rRNA gene, and ii) the *alkB* gene encoding for an alkane monooxygenase for hydrocarbon degradation [199]. Whereas gene copy numbers showed relatively little temporal variation in most downstream and upstream wells, significant fluctuations were noted in wells receiving persulfate (Figure 7.3). 16S rRNA and *alkB* gene abundance were reduced 1-2 orders of magnitude in wells within the ROI (C6, C7, C8). It is noteworthy that in general total bacterial abundance showed a greater absolute decrease than *alkB* gene copy number, indicating a relative enrichment of microorganisms able to degrade alkanes, as has been observed previously [257].

Wells where the largest impacts on microbial communities were observed correlated with those having more significant or prolonged decreases in pH (C1, C6, C9; Figures 7.2 and 7.3). The effect on the microbial community was moderated in wells with less pronounced pH decrease, such as C7. Some downstream effects of acidic groundwater were observed in C5 (*alkB*) and C10 (16S rRNA), however, the impact was minimal compared to the prolonged drop in pH.

Regardless of the magnitude of disruption, rapid regeneration was observed after each persulfate injection event. Just prior to nutrient injection on day 92, 16S rRNA and *alkB* gene measurements were within one order of magnitude of starting conditions in nearly all wells. A notable exception was C1, where microbial abundance remained low. As C1 was not sampled on days 5, 13, and 51, it is difficult to estimate how significant the impact was on microbial community and thus the extent of regeneration. Full regeneration occurred following the nutrient amendment on day 93, which is notable considering that groundwater pH remained low. This suggests that biostimulation can ease the environmental pressures associated with chemical oxidation allowing regeneration of the microbial community.

The deleterious effects of persulfate-mediated oxidation on indigenous microorganisms have been described in the literature [207, 273]. Batch experiments with heat-activated persulfate showed inhibition at concentrations above 10 g l^{-1} , an order of magnitude lower than the concentrations injected in our pilot experiment [273]. That said, oxidant concentrations cannot be directly compared in batch experiments and field injections, as the soil-to-liquid ratios and extent of mixing differ significantly. Column experiments investigating persulfate treatment (20 g l^{-1}) of polycyclic aromatic hydrocarbon (PAH)-contaminated soil also showed an impact on microbial abundance and degradation capacity following oxidation [207].

In that study, regeneration of phenanthrene degradation activity and specific PAH degraders required 100-500 days. Higher threshold persulfate concentrations and lower rebound times for the gene targets measured in our study were most likely due to (1) the natural heterogeneity of the subsurface which reduces the efficiency of oxidant spreading and thus contact with microorganisms and/or (ii) transport of microorganisms by groundwater flow from upstream areas. These results highlight the differences between field and lab scale experiments and underscore the need for field investigations to understand subsurface processes.

7.3.3 Contaminant and organic carbon mobilization

Changes in groundwater concentrations of DOC and dissolved TPH were observed following each persulfate injection event. DOC concentrations increased 2- to 4-fold in impacted wells as compared to baseline concentrations of around 50 mg C l⁻¹, found in both ROI wells prior to oxidation and in upstream wells (C11, T1, T2, T4; Figure 7.4). Similarly high concentrations in downstream C5 and C10 directly after the second persulfate injection suggested oxidant delivery, as noted in EC and ORP measurements (Figure 7.2). Prolonged perturbations in DOC through day 125 and especially the delayed increase in DOC in downstream C4 starting around day 90 most probably indicate spreading of DOC-enriched groundwater due to natural groundwater flow (Figure 7.4).

Similar changes in dissolved TPH concentrations were observed (Figure 7.4). Initially, TPH concentrations within the ROI decreased slightly due to displacement of groundwater with the injection liquid. Thereafter, concentrations increased, with peak dissolved TPH concentrations occurring 13-20 days after each injection. Changes in measurements in downstream wells were indicative of oxidant delivery, as seen in the drop in dissolved TPH during the second persulfate injection (C5 and C10). Groundwater flow was most likely responsible for further fluctuations noted between days 50-125; however, the impact of heterogeneous TPH distribution in the soil matrix cannot be excluded (Table 7.1). As this area was contaminated by groundwater transport of dissolved diesel, varied TPH concentrations are expected due to heterogeneous hydraulic conductivity or different partitioning of diesel to different lithological layers.

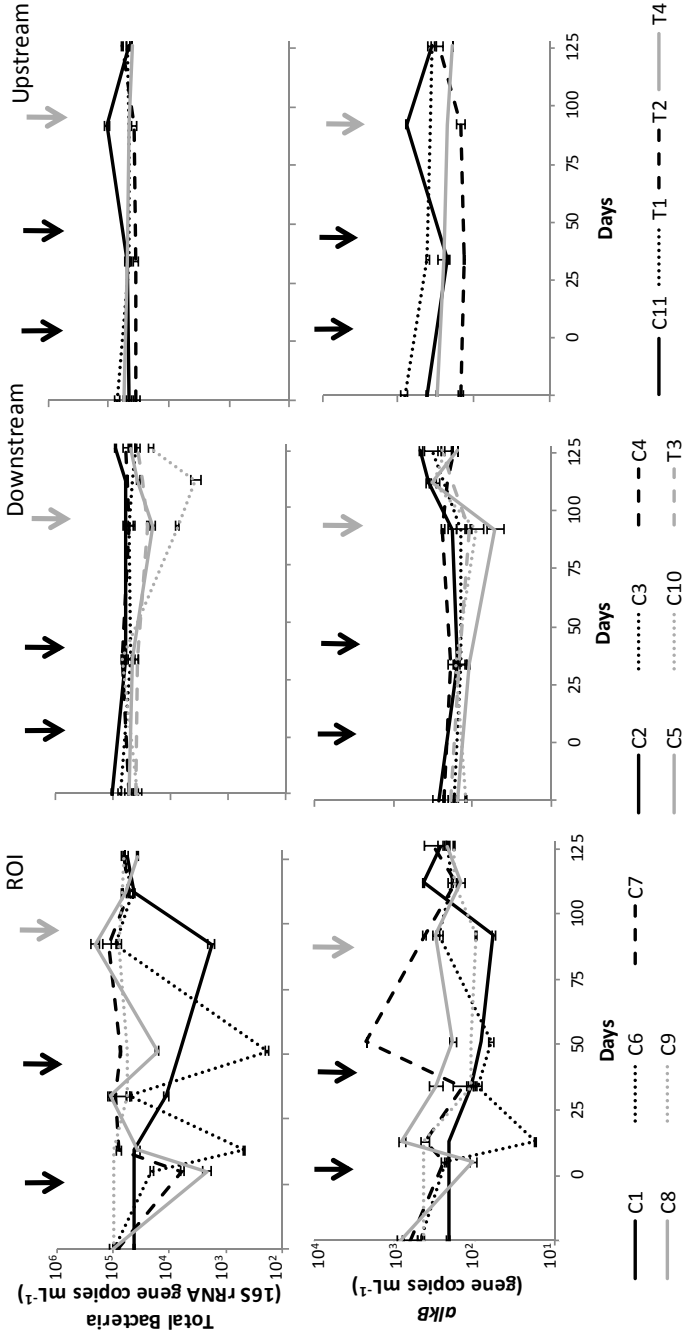


Figure 7.3 Groundwater total bacterial 16S rRNA gene (above) and *alkB* gene abundance (below). Division of wells into radius of influence (ROI), downstream, and upstream is as in Figure 7.2. Black arrows indicate the timing of persulfate injection on day 0 and day 43, gray arrow indicates nutrient amendment on day 93.

Increased DOC concentrations after persulfate injection can be attributed to partial oxidation of the soil matrix. Non-specific oxidation of SOM releases DOC and nutrient species [185, 240, 256, 289]. Increased concentrations of nitrogen species observed in our study showed a relationship with DOC measurements after ISCO (Figure 7.5), which is in line with previous work showing NH_4^+ release from SOM upon chemical oxidation [240, 256]. Improved nutrient bioavailability as well as the abundance of readily biodegradable DOC could positively influence the regeneration of microbial communities following chemical treatment.

In addition to SOM-derived DOC, organic contaminants can also be mobilized by ISCO. This occurs either (1) from degradation of SOM to allow desorption of bound TPH, [23, 121] or (2) through increased contaminant solubility due to partial oxidation of TPH [140]. Both pathways result in increased contaminant bioavailability, and the latter improves biodegradability, thereby underscoring the potential for ISCO treatment to stimulate bioremediation. The combination of improved nutrient and TPH bioavailability could support the enrichment of microbial degradation capacity observed especially within the ROI (Figure 7.3).

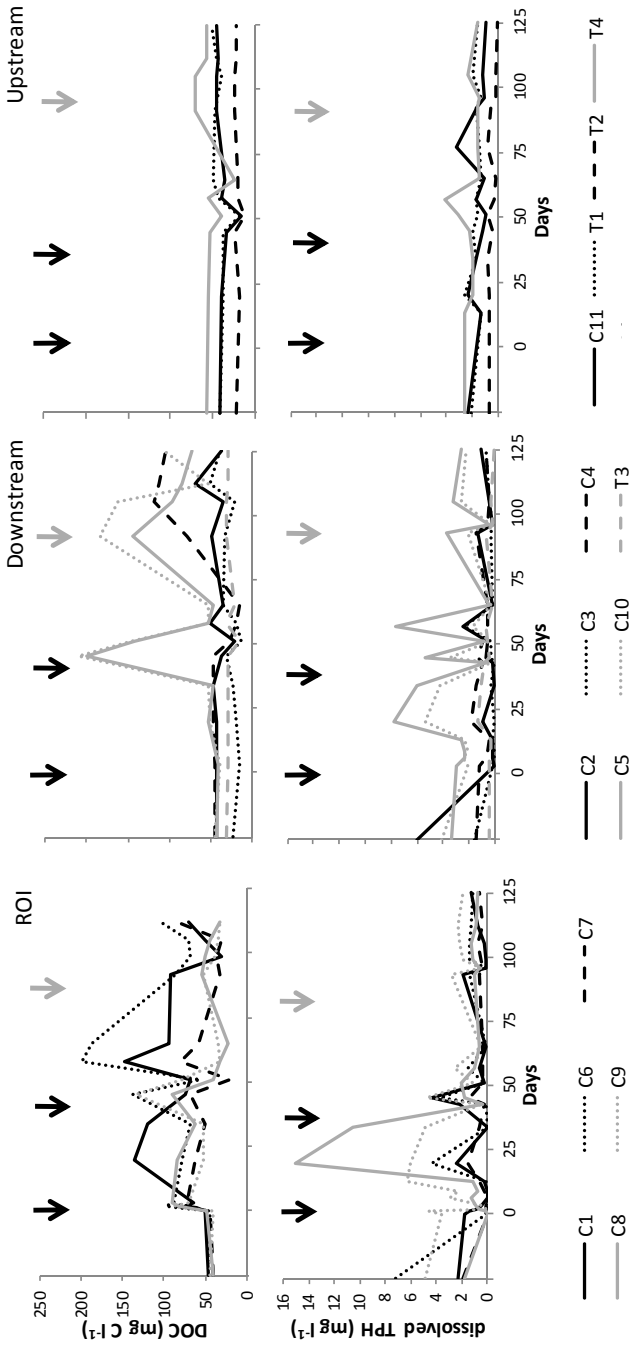


Figure 7.4 Groundwater dissolved organic carbon (DOC; above) and aqueous total petroleum hydrocarbon (TPH; below). Division of wells into radius of influence (ROI), downstream, and upstream is as in Figure 7.2. Black arrows indicate the timing of persulfate injection on day 0 and day 43, gray arrow indicates nutrient amendment on day 93.

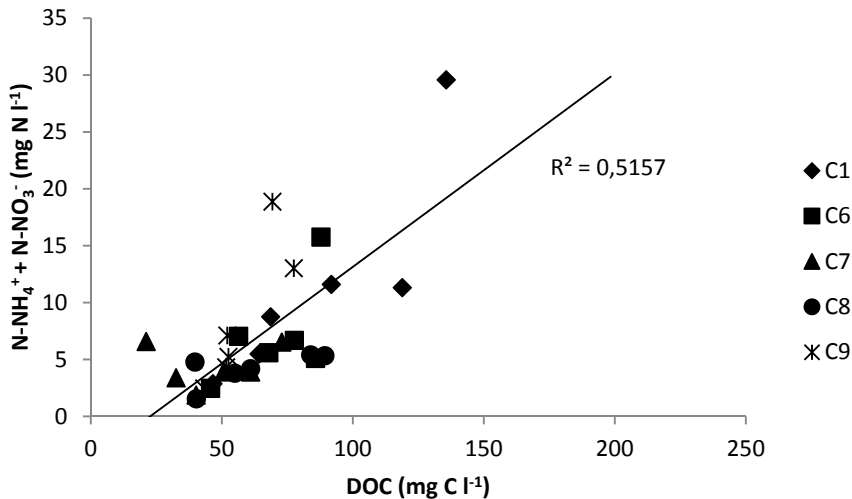


Figure 7.5 Relationship between DOC and nitrogen species NH_4^+ and NO_3^- . Degradation of SOM upon oxidation with persulfate releases carbon and nitrogen species into the aqueous phase.

7.3.4 Irreversible changes to soil matrix

While most disturbances to groundwater and microbial community parameters were mitigated within 60 days, changes to the soil matrix due to persulfate oxidation were irreversible. Impacts on the subsurface were noted indirectly in the stronger impact of the second injection on pH, ORP, and DOC (Figure 7.2 and 7.4). The longer attenuation time required to stabilize these parameters indicates a reduction in the soil oxidant demand and soil buffering capacity, implying permanent changes to soil structure, SOM, and mineralogy. In order to understand changes to the soil matrix, soil samples from before treatment (C1, 2.5-2.6 m bgl), after the first injection (P1, 2.5-2.6 m bgl) and at the end of the experiment (P2, 2.3-2.4 m bgl) were analyzed (Table 7.1).

Oxidation of SOM was reflected in increased DOC concentrations following each persulfate injection (Figure 7.4), but was not directly observed in soil TOC measurements (Table 7.1). However, TGA analysis did reveal changes in SOM composition due to ISCO treatment (Figure 7.6). Prior to treatment, more thermo-unstable organic compounds were present, as observed in the chromatogram peak between 150-200°C. In the final sample, the contribution of more thermo-stable, recalcitrant SOM with a peak between 600-700°C increased. Such changes in SOM composition have been noted previously, where a decrease in amorphous,

bioavailable organic matter was observed upon oxidation with heat-activated persulfate [42]. Degradation of bioavailable SOM can have long-term impacts on microbial communities and thus soil biological functioning beyond the temporary stimulation related to the availability of biodegradable DOC, as observed in our results (Figures 7.3 and 7.4).

Soil mineralogy, analyzed by sequential extraction procedures measured on-line with ICP-OES (Table 7.2), showed changes in reduced minerals due to persulfate injection (Figure 7.7). In the initial sample, concurrent release of Fe and S was mainly observed during extraction with HCl (Figure 7.7a), which suggests the presence of iron sulfides [39, 90]. Following the first persulfate injection, the contribution of easily extractable, amorphous S in the HNO_3 step increased, while that of HCl decreased (Figure 7.7b), indicating a decrease in the presence of iron sulfides and increase in amorphous mineral phases. In the final sample, essentially no concomitant Fe and S extraction was observed, pointing towards the complete degradation of amorphous iron sulfides (Figure 7.7c). Instead, S was found to be almost exclusively present as reversibly bound anion (CaCl_2 extractable), most likely as residual sulfate. The minor peak observed during HNO_3 extraction may be related to gypsum precipitation due to saturation of sulfate following persulfate oxidation, as supported by XRD measurements (Table 7.3) and high groundwater concentrations (Table 7.4). In contrast, Fe was associated with organic matter (H_2O_2 extractable), as well as with amorphous and crystalline mineral phases (HNO_3 and HCl extractable). XRD analyses noted the appearance of hematite in the final sample, indicating the precipitation of Fe oxides following oxidation with persulfate (Table 7.3). Similar decreases in the concurrent extraction of Pb, Cu, and Zn with S were observed, although these species were found in lower concentrations than Fe (Figure 7.8).

Overall, results indicate that during ISCO, metal sulfides were oxidized, causing the precipitation of metal oxides. Alterations to mineralogy can be attributed to oxidation of the subsurface. While degradation of iron sulfides does irreversibly alter the soil matrix, it has been suggested that pyrite activates persulfate, therefore improving oxidant reactivity [138, 264]. Such a pathway may be of interest in a laboratory setting where the interaction between oxidant, activator and contaminant can be controlled; however, it is unclear whether activation can contribute to remediation in the field. Additionally, degradation of otherwise stable species such as sulfide-associated Pb, Cu, and Zn could be a source of further environmental risk. Mobilization of heavy metals due to ISCO treatment has been observed both in laboratory and field experiments [5, 40, 266].

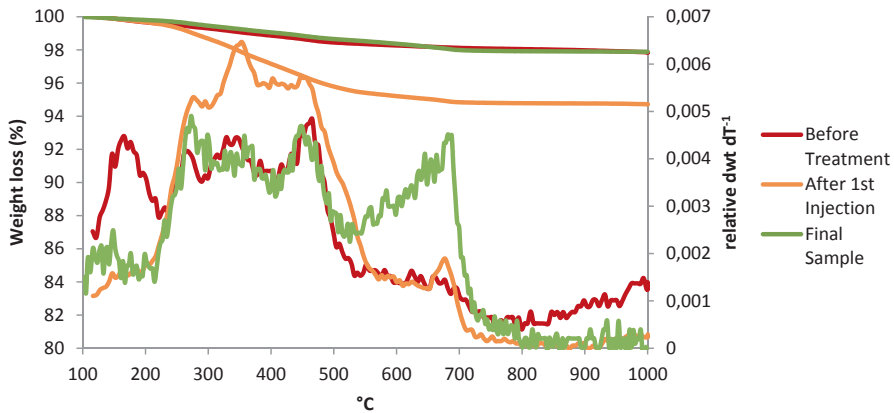


Figure 7.6 Changes in SOM as measured by TGA analysis. Total weight loss is given on the left axis. On the right axis the derivative of weight loss with respect to temperature relative to the total weight loss is given to allow comparison of the relative contribution of different soil components [42].

Table 7.3 XRD and TGA mineral data. XRD results are the relative concentration (%) of each component. TGA results give the percent of weight loss upon ignition relative to the dry weight.

Sample Location (as in Table 7.2 and Figure 7.1)		Before treatment	After 1 st injection	Final
		C1	P1	P2
Depth (m bgl)		2.5-2.6	2.5-2.6	2.3-2.4
Quartz	SiO ₂	84.0	85.9	78.0
Alkali feldspars	(K,Na)AlSi ₃ O ₈	9.4	8.7	9.2
Plagioclase	(Na,Ca)(Al,Si) ₄ O ₈	2.5	1.3	4.0
Calcite	CaCO ₃	0.4	0.1	0.6
Hematite	Fe ₂ O ₃	n.d. ^a	n.d. ^a	0.7
Gypsum	CaSO ₄	n.d. ^a	0.7	0.4
2:1 Al silicates	(K,Na,Ca,Mg) _x (Al,Mg,Fe) ₂ (Si,Al) ₄ O ₁₀ (OH,F) ₂ •n(H ₂ O)	2.2	0.9	5.3
Kaolinite	Al ₂ Si ₂ O ₅ (OH) ₄	1.1	1.2	1.4
Chlorites	(Mg,Al,Fe) _x Si ₄ O ₁₀ (OH) ₂ (Mg,Al,Fe) _x (OH) ₆ (2<x<3)	n.d. ⁵	n.d. ⁶	0.3
LOI ^b	(105-1000°C)	2.3	5.4	2.1

^a not detected

^b loss on Ignition

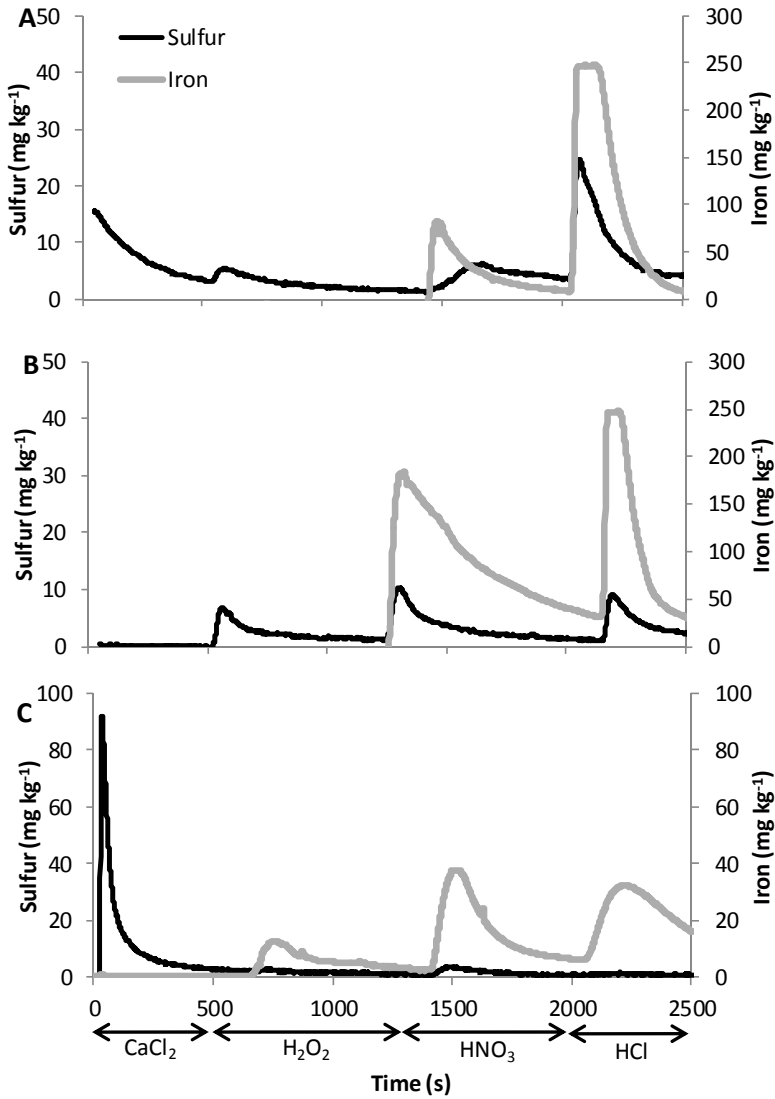


Figure 7.7 On-line sequential extraction of soil samples from before treatment (A), after the first persulfate injection (B), and at the end of the experiment period (C) for sulfur (black line, left axis) and iron (gray line, right axis). Additional data on other sulfide-associated metals (copper, zinc, and lead) is available in Figure 7.8.

Table 7.4 (A) SO₄²⁻ concentrations (mg l⁻¹) and **(B)** Ca²⁺ concentration (mg l⁻¹) in monitoring wells throughout the experiment.

SO ₄ -Day	ROI								Downstream								Upstream													
	C1	C6	C7	C8	C9	C2	C3	C4	C5	C10	T3	C11	T1	T2	T4	C1	C6	C7	C8	C9	C2	C3	C4	C5	C10	T3	C11	T1	T2	T4
-10	4	7	5	4	3	96	97	17	4	19	175	5	4	27	5	4	7	5	4	3	96	97	17	4	19	175	5	4	27	5
0		1815	2389	2956	3548																									
1		5681	4618	4116	8099																									
2		6826	4648	4224	8272																									
3	4236	6981	7049	3824	7290																									
6	7873	6302	2519	3480	6528	163	136	471	5123	529	79	319	14	42	13															
13	9230	5082	2320	4411		387	299	608	6195	4361	545	319	299																	
20	7749	2904	925	2392	3142	467	144	560	5022	4697	104	108																		
33	117	4573	3499	1017		6972	687	353	176	2835																				
45	9809	10214	4826	4267	11614	747	168	809	20719	24269	193	889	122	955	91															
51	10581	6901	3571	2848	13561	1226	358	1091	25162	27940	161	1104	67	1182																
65	6005	2333	1192	954	3704	1155	214	816	15074	22740	1033	218	109	2008	50															
77	6487	3550	5996	4123	4956	1834	203	462	10018	16234	2330	287	27	1407																
92	4777	1851	1016	803	3073	2346	147	551	8576	14377	82	180	17	1179	1609															
112	3854	2433	853	1235	2157	2444			6115	11572																				
127	4168	1346	526	614	34640	1218	320	276	4051	7454	56	56	15	1961	2															

Ca ²⁺ -Day	ROI								Downstream								Upstream													
	C1	C6	C7	C8	C9	C2	C3	C4	C5	C10	T3	C11	T1	T2	T4	C1	C6	C7	C8	C9	C2	C3	C4	C5	C10	T3	C11	T1	T2	T4
-10	252	260	267	273	252	257	241	227	232	270	289	264	268	170	310	252	260	267	273	252	257	241	227	232	270	289	264	268	170	310
0		623	1052	864	754																									
1		958	1245	1009	693																									
2		801	1108	853	621																									
3	821	770	585	702	494																									
6	925	508	297	405	423	307	242	348	1024	349	259	255																		
13	435	350	226	234	405	281	262	361	872	892	243	221	240																	
20	445	270	229	278	448	358	242	383	665	883	255	267	259																	
33	418	378	282	317	522	441	271	327	560	776	282	281	257																	
42		864	1015	1126	810	426	267	301	634	545																				
45	632	803	394	454	685	417	277	366	629	573	300	421	266																	
51	427	370	226	154	466	463	270	412	481	477	292	415	268																	
65	299	173	189	145	353	463	272	402	397	407	262	277	235																	
77	270	182	265	208	420	493	298	338	375	360	264	257	259																	
92	247	202	193	205	447	535	310	427	365	362	255	268	279																	
112	289	291	246		421	528			419	367																				
127	377	209	161	173	316	459	377	388	440	353	250	257	243																	

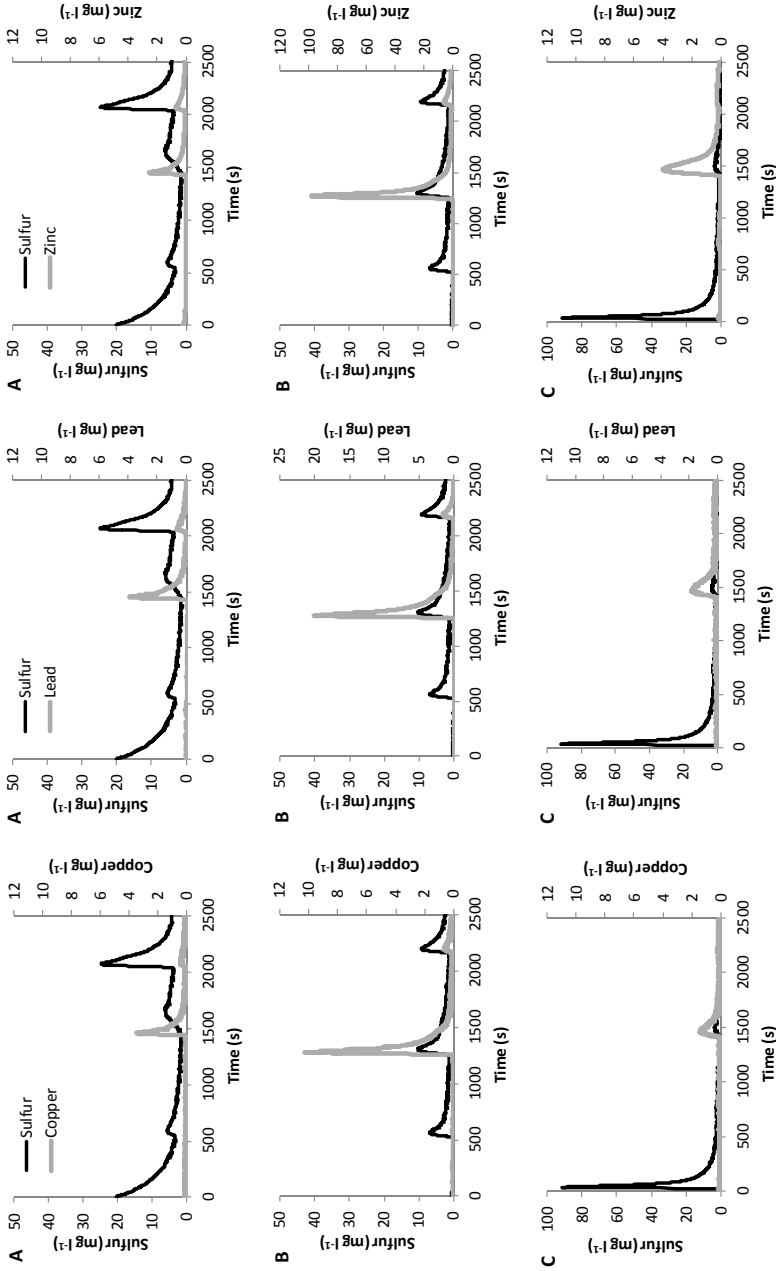


Figure 7.8 On-line sequential extraction comparison of sulfur and copper (left), lead (middle) and zinc (right) in samples taken prior to injection (A), following the first persulfate application (B) and at the end of the field experiment (C). In the initial sample, metals were associated with more amorphous and mineral phase sulfides, as seen in the simultaneous release of sulfur with the heavy metal. A shift away from concomitant sulfur and metal extraction in (B) and (C) indicates the oxidation of sulfides due to persulfate injection.

7.3.5 Implications for full-scale field application

Results presented here provide insight into the impact of persulfate oxidation on geochemical and microbiological parameters. Temporary perturbation of groundwater characteristics and disruption of the microbial community were mediated in most cases within 30-60 days of injection; however, irreversible changes to mineralogy and SOM were measured, which could have long-term impacts on microbial activity and diversity. Full regeneration of bacterial abundance and degradation capacity occurred, indicating the feasibility of coupling *in situ* chemical and biological treatments. Fluctuations in ORP and pH closely matched other parameters, implying that simple electrode measurements are quite representative of the entire system. This suggests that relatively straightforward techniques could be used to monitor the distribution of persulfate, to determine the timing of sampling events, and to gauge the potential effectiveness of further biostimulation additions.

This research also highlighted the risk of contaminant mobilization during chemical treatment. In addition to increased aqueous TPH concentrations, persulfate injection also oxidized otherwise stable metal sulfides, thereby mobilizing some heavy metals. While risks were quite insignificant within our small treatment area, contaminant mobility in combination with groundwater flow could jeopardize receptors in a full scale field application. Therefore, proper risk assessment and mitigation measures must be in place prior to ISCO treatment.

Acknowledgements

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Part Three

Chapter 8

Microbial community dynamics
of an organohalide respiring enrichment culture
during and after chemical oxidation

Chapter 9

Microbial dynamics during and after
in situ chemical oxidation of chlorinated solvents

Chapter 8

Microbial community dynamics of an organohalide respiring enrichment culture during and after chemical oxidation

This chapter is in preparation for publication:
Sutton, N.B.; Atashgahi, S.; Grotenhuis, T.; Smidt, H.; Rijnaarts, H.H.M.,
Microbial community dynamics of an organohalide respiring enrichment
culture during and after chemical oxidation.

Abstract

While *in situ* chemical oxidation (ISCO) is often used to remediate tetrachloroethene (PCE) contaminated locations, very little is known about the influence of oxidation on organohalide respiration (OHR) activity, and microbial community structure in particular. Here, we investigate the impact of oxidation with permanganate on OHR rates, the abundance of organohalide respiring bacteria (OHRB) and reductive dehalogenase (*rdh*) genes using quantitative PCR, and microbial community composition and dynamics based on pyrosequencing of 16S rRNA gene fragments. During 21-day experiments, microcosms with a PCE degrading enrichment culture received multiple oxidant doses of one of the following: 25 μmol , 50 μmol , or 100 μmol permanganate, or no oxidant treatment (biotic control). Results indicate that under mild permanganate treatments (25 μmol or 50 μmol), chemical oxidation slightly stimulated biodegradation leading to higher OHR rates as compared to the biotic control. OHRB *Geobacter* was more abundant in microcosms receiving mild permanganate treatments. In contrast, 100 μmol permanganate doses disrupted biodegradation activity and caused at least a 2-4 orders of magnitude reduction in the abundance of all measured OHRB and *rdh* genes, as compared to the biotic control. Chemical oxidation appeared to select for *Deltaproteobacteria*, especially *Geobacter*, and *Epsilonproteobacteria*, especially *Sulfurospirillum*, in 100 μmol permanganate microcosms. This detailed investigation into microbial community structure changes due to chemical oxidation contributes to our fundamental understanding of dynamics of OHR upon oxidation, and eventually our capacity to stimulate *in situ* bioremediation following ISCO treatment of PCE.

8.1 Introduction

Anthropogenic contamination of soil and groundwater with organic pollutants such as tetrachloroethene (PCE) requires innovative, efficient, and cost-effective remediation technologies. To this end, there is increased emphasis on *in situ* technologies that are less invasive and less expensive than more traditional *ex situ* treatments. Various approaches, including *in situ* bioremediation (ISB) and *in situ* chemical oxidation (ISCO), can effectively and efficiently remediate sites without significant disruption to above ground activities [111, 233].

Microbial capacity to biodegrade PCE has been exploited in the development of ISB technologies for treatment of chlorinated solvent contaminated locations. In an energy conserving process known as organohalide respiration (OHR), sequential substitution of chlorine atoms for hydrogen atoms produces ethene under strictly anaerobic conditions [241]. Full-scale ISB treatment of PCE contaminated locations often includes amendment with electron donor and nutrients. In some cases bioaugmentation with organohalide respiring bacteria (OHRB) able to perform OHR of the target compound is required to ensure the presence of biodegradation capacity. A limited number of very specific organisms have the capacity to reductively dechlorinate PCE to trichloroethene (TCE), *cis*-dichloroethene (DCE), vinyl chloride (VC) and finally ethene. *Dehalococcoides mccartyi* is the only organism known to perform full transformation of PCE to ethene [143]. A variety of molecular techniques have been developed to specifically monitor OHRB and their reductive dehalogenase (*rdh*) genes. These tools can be used to qualitatively verify biodegradation capacity by monitoring changes in target abundance, thereby assisting in contaminated site management, for example, by indicating whether or not bioaugmentation may be necessary. Additionally, recent advances in next-generation sequencing have yielded valuable insight on microbial community dynamics during PCE degradation in the lab [45] and in the field [130].

Furthermore, chemical treatment of chlorinated solvents *in situ* is often performed to rapidly oxidize high concentrations of contaminants [233]. Chemical oxidants such as permanganate, persulfate, hydrogen peroxide, and ozone can efficiently decrease contaminant mass more rapidly than, for example, biological treatment. However, once the oxidant has finished reacting, rebound of aqueous contaminant concentrations may occur, either through dissolution of residual pure product or desorption of solvents sorbed to the soil matrix [121, 172, 173]. In such instances, a bio-polishing step following ISCO could be advantageous by removing residual contaminants and ensuring complete remediation.

Whereas ISB has been successfully implemented following ISCO for the remediation of aliphatic and aromatic hydrocarbons [257, 274, 299], coupling chemical oxidation with an anaerobic bioremediation step for the treatment of chlorinated solvents is more challenging. Increased oxidation-reduction potential associated with ISCO and subsequent oxidative stress may adversely impact the OHR or non-dechlorinating populations that provide essential nutrients to OHRB. A number of studies have examined the regeneration of OHR capacity following chemical oxidation at batch [54], column [87, 218], and field scales [232]. These investigations successfully illuminated threshold oxidant concentration [54] and necessary amendments, such as electron donor and bioaugmentation for biodegradation [87, 218]. In some instances, the abundances of OHRB and *rdh* genes were measured to quantify the impact of chemical oxidation on and subsequent regeneration of OHR capacity [54, 218].

However, these investigations were limited to quantification of OHRB during and after chemical oxidation, and do not consider the roles played by the rest of the microbial community following chemical treatment. It has recently been shown that the non-dechlorinating community members provide OHRB with requisite fermentation intermediates such as electron donors (for example, acetate and hydrogen), essential co-factors (such as corrinoids), and oxygen scavenging enzymes [91, 165, 301]. Additionally, following chemical oxidation the entire microbial community plays a role in attenuation of oxidation-reduction potential to ensure regeneration of OHR activity. Here we investigate the impact of chemical oxidation with permanganate on a PCE degrading consortium using both quantitative PCR (qPCR) and pyrosequencing of 16S rRNA gene fragments. By determining changes in OHRB and *rdh* gene abundance as well as microbial community structure due to chemical oxidation, this study provides valuable insight into the impact of ISCO on microbial communities and their subsequent regeneration. To our knowledge, the results presented here are a new step towards improved understanding, and thus application, of bio-polishing following chemical oxidation.

8.2 Materials and Methods

8.2.1 Microcosm setup

Thirty-eight replicate microcosms were prepared in 125 mL glass serum bottles with Viton stoppers (Rubber b.v., The Netherlands) using a PCE dechlorinating enriched culture, maintained on lactate and PCE at 25 °C prior to this study. Prior

to inoculation, a final concentration of 10 mM sodium lactate and 2.0 μmol of PCE, both dissolved in anoxic water, were added to the medium [83]. Inoculation was performed by anoxic transfer of 5 mL of the enriched culture to each microcosm in order to reach a final volume of 50 mL. Microcosms were incubated at 25 °C shaking at 120 rpm. Prior to treatment with chemical oxidation or incubation, chlorinated ethenes were measured in a number of microcosms to ensure OHR activity. Two microcosms were sacrificed for DNA sampling for the initial microbial community, as described below.

8.2.2 Treatment and sampling

Nine replicate microcosms were prepared for each of the four different conditions tested: three permanganate treatments at 25 μmol , 50 μmol , and 100 μmol and a biotic control that did not receive chemical oxidation. Permanganate doses are relatively low compared to previous field application experiments [232], but were similar to those applied in column [218] and batch [54] experiments. Because the majority of permanganate was consumed by other reduced constituents, such as sulfides, the low oxidant concentrations resulted in very little PCE oxidation. As the goal of this research was to determine the impact of permanganate treatment on the microbial community, the low chemical oxidation efficiency of PCE was not relevant.

All microcosms received PCE spiking of 0.86 μmol . Permanganate treatment coincided with a PCE addition, although interim PCE spikes were also performed between chemical oxidation treatments (Table 8.1). Sampling for molecular analyses was always performed in duplicate by sacrificing two entire microcosms to ensure that sufficient biomass was collected for DNA extraction, especially in the case of high permanganate treatments. Headspace analysis of PCE, TCE, *cis*-DCE, VC, and ethene concentrations was performed in triplicate almost daily, and was always performed 24 hours after each PCE spiking. In order to properly link the chemical and molecular analyses, chemical analyses were shuffled between replicate microcosms of each treatment. Microcosms were initially used for headspace analyses of ethenes and thereafter sacrificed for DNA extraction. Thus, the chemical results presented here are a conglomeration of chemical data from a total of 9 microcosms.

The timing of PCE additions, oxidation treatments, and microcosm sacrifice for DNA extraction are shown in Table 8.1. Biotic controls were treated similarly to microcosms with mild permanganate treatments (25 μmol and 50 μmol), except that the latter two received two doses of chemical oxidant between each DNA sampling. Accordingly, for these microcosms (biotic control and mild permanganate

treatments), days 7, 13, and 21 were considered as the end of cycle 1, 2, and 3, respectively, at which time molecular analyses were performed. To ensure analysis was performed on the regenerated community, each DNA sampling was performed 3-4 days after the last chemical oxidation treatment (Table 8.1 A).

Microcosms receiving 100 μmol permanganate treatments showed a larger disruption in microbial OHR activity after the initial permanganate addition (Table 8.1B). Hence, only one permanganate treatment was performed between DNA sampling events, and additional incubation time was considered to allow regeneration of the microbial community. Sampling for molecular analyses was performed when all PCE had been degraded to *cis*-DCE (day 13, Table 8.1 B). Following the second permanganate treatment in the 100 μmol microcosms, the second and final DNA sampling was performed at the final stage of the experiment (day 21) during which some PCE biodegradation activity was observed.

Occasional lactate measurements in all microcosms receiving permanganate treatment indicated when amendment was required to maintain lactate concentrations around 10 mM. Additional vitamins were added twice during the 3-week experiment.

Table 8.1 Treatment scheme of (A) biotic control, 25 μmol and 50 μmol permanganate microcosms, and (B) 100 μmol microcosms. DNA samples taken in duplicate (2x), PCE spiking (0.86 μmol), and KMnO_4 (25, 50 or 100 μmol) dosing are indicated (x).

	A																					
	Day																					
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
DNA	2x							2x						2x								2x
KMnO_4 (25 or 50 μmol)	x				x			x		x				x				x				
PCE (0.86 μmol)	x			x	x			x		x	x			x				x	x			

	B																					
	Day																					
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
DNA	2x													2x								2x
KMnO_4 (100 μmol)	x													x								
PCE (0.86 μmol)	x				x									x								

8.2.3 Chemical Analyses

Lactate was measured in liquid samples with HPLC with an organic acids column (Ion 300) and refractive index detector. PCE, TCE, *cis*-DCE, VC, and ethene were measured in the headspace of triplicate microcosms. PCE, TCE, and *cis*-DCE were extracted for 2 minutes with a 100 μm polydimethylsiloxane (PDMS) coated fiber. The compounds were injected at 250 $^{\circ}\text{C}$ on a Fisons 8000 series GC with a CP-Sil8 column (25m x 0.53mm x 5.0 μm) using helium as the carrier gas and detected using a Flame Ionization Detector (FID). The program started at 50 $^{\circ}\text{C}$, ramped at 20 $^{\circ}\text{C}/\text{min}$ to 140 $^{\circ}\text{C}$, and remained at 140 $^{\circ}\text{C}$ for the final 1.5 min. For VC and ethene, 100 μL of headspace was sampled with a glass syringe and directly injected on a HP6890 series GC with a CP PoraBond Q column (25m x 0.53mm x 10 μm). The temperature program was isothermal at 60 $^{\circ}\text{C}$ and detection was with a FID at 300 $^{\circ}\text{C}$.

Due to regular spiking, the total mass of the PCE and degradation products increased throughout the experiment. Hence, molar relative abundance of each compound is given. At a given time point, the relative contribution of each chlorinated ethene is given as a percentage of all chlorinated ethenes (Figure 8.1). Rates of PCE and TCE conversion were calculated by comparing the total mass of either PCE or TCE as measured one day after each PCE spike to the sum of those measured and spiked the previous day (Figure 8.2).

8.2.4 DNA sampling and extraction

Samples for molecular analyses of total DNA were collected by sacrificing two replicate microcosms of each condition. The total volume of each microcosm was vacuum filtered over a separate sterile 0.22 μm membrane filter (Milipore, USA). Each filter was folded and placed in a sterile microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at -20 $^{\circ}\text{C}$ until DNA extraction. The filtered liquid volume was measured using a graduated cylinder to allow for calculation of gene copy numbers/mL. DNA extraction was performed using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) according to manufacturer's instructions; the filter was sliced into pieces to aid extraction prior to placement in the bead beater. DNA was extruded with DNase/Pyrogen-Free water, controlled for quantity and quality using a Nanodrop spectrophotometer (Thermo Scientific, Germany), and stored at -20 $^{\circ}\text{C}$.

8.2.5 Quantitative PCR

Quantitative PCR (qPCR) was performed as described previously to measure total bacteria and specific OHRB based on the 16S rRNA gene and *rdh* genes [254]. Assayed OHRB were *D. mccartyi*, *Geobacter*, *Desulfitobacterium*, *Dehalobacter*, and assayed *rdh* genes were *tceA*, *bvcA*, and *vcrA*, encoding TCE and VC reductive dehalogenases in *D. mccartyi*, respectively. Ten-fold dilutions of total DNA extracts were assayed in triplicate using an iQ5 SYBR Green Supermix kit on the iQ5 iCycler (Biorad, The Netherlands). Assayed targets and primer sequences have been described previously [254] and are summarized in the supplementary material (Appendix 3). Gene copy numbers are expressed as copy/mL culture. *Dehalobacter* was not detected in any of microcosms, both with and without permanganate treatment.

8.2.6 Bacterial 16S rRNA gene amplicon pyrosequencing

Bacterial 16S rRNA gene fragments were amplified using barcoded primers covering the V1-V2 region of the bacterial 16S rRNA gene. The primers were generated using the 27F-DegS primer (5'- GTTYGATYMTGGCTCAG- 3') [278] appended with the titanium sequencing adaptor A (5'- CCATCTCATCCCTGCGTGTCTCCGACTCAG- 3') and an 8 nucleotide sample specific barcode [77] at the 5' end as the forward primer, and an equimolar mix of the two reverse primers i.e. 338R I (5'- GCWGCCTCCGTAGGAGT- 3') and 338R II (5'- GCWGCCACCCGTAGGTGT- 3') [44] that carried the titanium adaptor B (5'- CCTATCCCCTGTGTGCCCTTGGCAGTCTCAG- 3') at the 5' end. The sequences of both titanium adaptors were kindly provided by GATC Biotech (Konstanz, Germany). The PCR mix (100 µl final volume) contained 20 µl of 5× HF buffer (Finnzymes, Vantaa, Finland), 2µl PCR Grade Nucleotide Mix (Roche Diagnostic GmbH, Mannheim, Germany), 1µl of Phusion hot start II High-Fidelity DNA polymerase (2U/µl) (Finnzymes), 500 nM of the reverse primer mix and the forward primer (Biolegio BV, Nijmegen, The Netherlands), 2 µl (40 ng) template, and 65 µl nuclease free water. PCR was performed using the following conditions: 98 °C for 30 s to activate the polymerase, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 20 s, elongation at 72 °C for 20 s, and a final extension at 72 °C for 10 min. Five µl of the PCR reactions were analyzed by 1% (w/v) agarose gel electrophoresis, containing 1× SYBR® Safe (Invitrogen, Carlsbad, CA, USA) to verify the right length of the amplicons (approximately 450 bp). PCR products were purified using the GeneJET PCR purification kit (ThermoScientific, Germany) according to the manufacturer's instructions. The DNA concentration of the purified amplicons was measured using NanoDrop, and amplicons were mixed

in equimolar amounts and rerun on an agarose gel prior to excision and purification by using a DNA gel extraction kit (Millipore, Billerica, MA, USA). Amplicons obtained from the 24 samples were analyzed simultaneously with 30 unrelated samples by pyrosequencing on half a plate using an FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany).

8.2.7 Analysis of the pyrosequencing data

Pyrosequencing data was analyzed using the QIIME 1.7.0 pipeline [32]. Low quality sequences were removed using default parameters, and operational taxonomic units (OTUs) were defined at a 97% identity level. A representative sequence from each OTU was aligned using PyNAST [49]. The taxonomic affiliation of each OTU was determined at a confidence threshold of 80% using the BLAST algorithm [7]. Possible chimeric OTUs were identified using QIIME's Chimera Slayer [76] and subtracted from the previously generated OTU list, producing a non-chimeric OTU list.

8.3 Results

8.3.1 Regeneration of OHR activity following permanganate treatment

The impact of chemical oxidation on OHR activity was investigated by comparing microcosms receiving permanganate treatment with the biotic control without chemical treatment. During the 21 days of treatment, dechlorination was not severely interrupted in microcosms receiving mild permanganate treatments of 25 μmol or 50 μmol (Figure 8.1). Rather, biodegradation rates were higher in microcosms receiving mild permanganate treatments as compared to the biotic control.

Higher OHR activity was, for example, observed in the rate of PCE and TCE degradation in the first 24 hours following PCE spiking (Figure 8.2). In the biotic control microcosms, residual PCE or TCE were often observed the day after a PCE spike (Figure 8.1A), which is in line with slower degradation rates in these microcosms (Figure 8.2A). This trend intensified over the course of the experiment, with the PCE spikes on days 16 and 17 remaining in the system until the end of incubation on day 21. In contrast, in nearly all instances complete conversion of spiked PCE to *cis*-DCE occurred within 24 hours after spiking in microcosms treated with 50 μmol permanganate (Figure 8.1C), yielding consistent degradation rates (Figure 8.2C). Considering that all other amendments and timing of addition were identical, this difference in OHR rate can only be attributed to the impact of chemical oxidation.

Improved degradation in microcosms receiving mild permanganate treatments was not limited to the more chlorinated ethenes PCE and TCE. Higher VC and ethene degradation and production rates were also observed (Figure 8.1). For example, on day 8 more than twice as much VC had been produced in microcosms receiving 50 μmol permanganate as compared to the biotic control (80% VC versus 37% VC, respectively). In the biotic control, the 80% threshold was not crossed until day 14, a delay of over a week as compared to the 50 μmol permanganate treatment (Figure 8.1). Similarly, higher final ethene production was observed in permanganate treated microcosms as compared to the biotic control. On day 21, 31% of all PCE had been converted to ethene in microcosms receiving 50 μmol permanganate treatments, whereas only 20% ethene was observed in the biotic control. These results are noteworthy, as they indicate that chemical oxidation does not disrupt OHR; rather, mild treatments appear to slightly stimulate biodegradation activity.

In contrast to the more mild permanganate treatments, significant disruption of microbial OHR activity was observed in microcosms receiving 100 μmol permanganate (Figure 8.1D). Following the first treatment on day 0, *cis*-DCE production in the 100 μmol permanganate microcosms (93%; Figure 8.1D) was similar to that of all other treatments (91-95%; Figure 8.1A-C). However, the influence of higher permanganate concentrations was observed following the second PCE spike on day 3. Whereas in microcosms receiving 50 μmol permanganate full conversion of PCE to *cis*-DCE occurred within 24 hours following nearly every PCE spike (Figure 8.1C), this was delayed by a week in microcosms receiving 100 μmol permanganate (Figure 8.1D). The second permanganate treatment on day 13 oxidized all *cis*-DCE, VC, and ethene present (1.72 μmol) as well as 0.26 μmol PCE spiked on day 13. Following this second treatment on day 13, OHR was more significantly disrupted (Figure 8.1D). Regeneration of PCE biodegradation coupled to TCE and *cis*-DCE accumulation resumed 4 days after the second permanganate addition on day 13. Throughout the entirety of the experiment, no VC or ethene production was observed at any point in microcosms receiving 100 μmol permanganate (Figure 8.1D). These results indicate that stronger chemical oxidant treatments can severely disrupt microbial OHR activity.

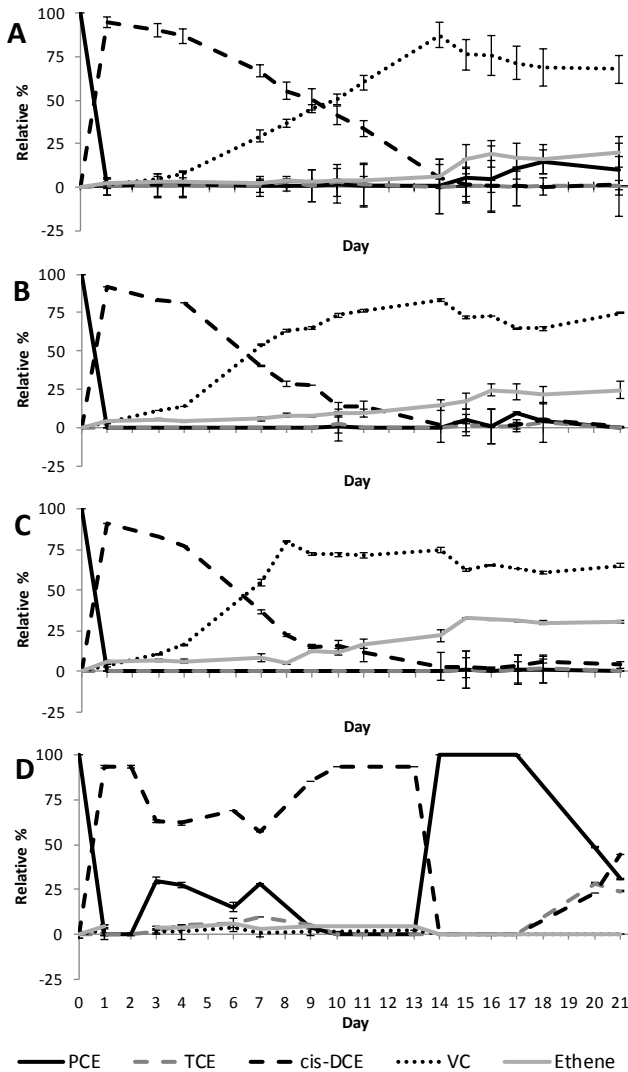


Figure 8.1 Molar percentage of chlorinated solvent PCE and degradation products TCE, *cis*-DCE, VC, and ethene for (A) the biotic control, (B) 25 μmol permanganate treatment, (C) 50 μmol permanganate treatment, and (D) 100 μmol permanganate treatment. As the absolute mass increased over the course of the experiment due to PCE spiking (see Table 8.1), values are given as the molar percentage of all chlorinated compounds and ethene present at any time point.

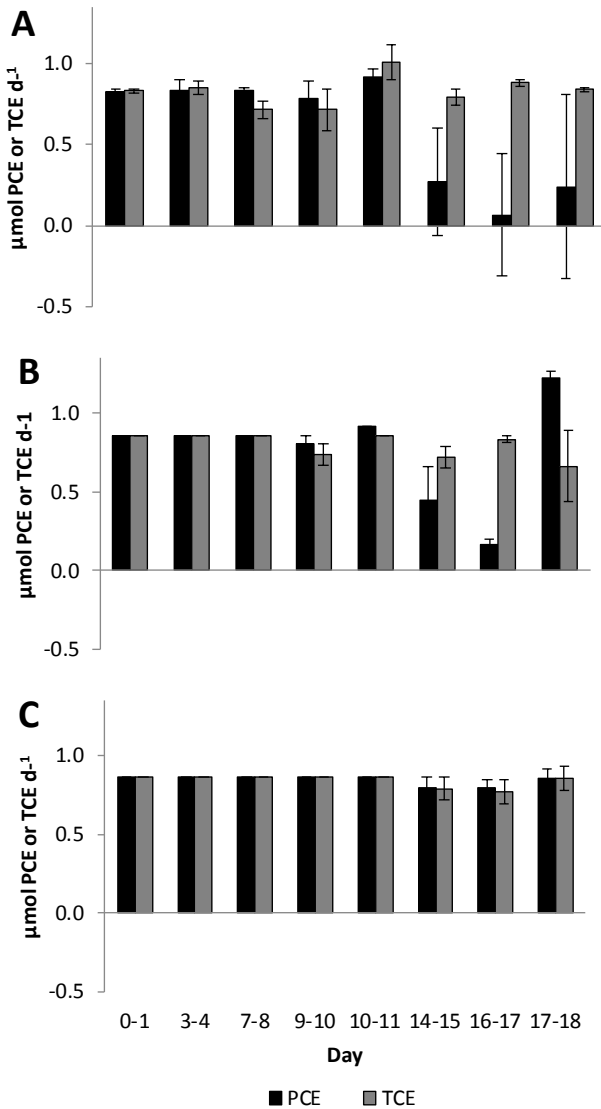


Figure 8.2 PCE (black) and TCE (grey) degradation rates in the first day after spiking in biotic control (A), 25 μmol permanganate (B), and 50 μmol permanganate (C) microcosms. The x-axis indicates the period for which degradation was measured (for example, spiking on day 9 and measurement on day 10). Rates of 0.86 $\mu\text{mol/day}$ indicate full degradation of the 0.86 μmol PCE spike within one day.

8.3.2 Influence of permanganate treatment on OHRB and *rdh* gene abundance

To understand the impact of chemical oxidation on microbial populations associated with OHR, the abundance of a variety of relevant OHRB and *rdh* genes were quantified using qPCR. Total bacteria, *D. mccartyi*, *Geobacter*, and *Desulfitobacterium* were measured based on their 16S rRNA gene sequence, and primers for specific *rdh* were used to detect *tceA*, *bvcA*, and *vcrA*, encoding TCE and VC reductive dehalogenases in *D. mccartyi*, respectively (Figure 8.3). In biotic controls and microcosms receiving a mild permanganate treatment (25 or 50 μmol), an increase in the absolute abundance of total bacteria, *D. mccartyi*, and *Geobacter* was observed as compared to the initial measurement on day 0, indicating their further growth over the course of the experiment (Figure 8.3). In contrast, the abundance of all targets decreased in microcosms receiving 100 μmol permanganate, showing the detrimental effect of stronger chemical oxidant treatments on the microbial community. For example, neither *D. mccartyi* nor *Desulfitobacterium* were detected on day 21, with the latter also absent on day 13 (Figure 8.3).

To account for natural shifts in target abundance over the course of the experiment, at each time point the enrichment or depletion of each target gene abundance observed in microcosms receiving permanganate were normalized to the corresponding target gene in the biotic controls (Figure 8.4). Overall, higher total bacteria and *Geobacter* normalized abundances were observed in microcosms receiving mild (25 μmol or 50 μmol) permanganate treatments. In the case of *Geobacter*, 3-6 times higher abundances were measured in 50 μmol permanganate microcosms at days 7, 13, and 21 than in the biotic control, indicating a significant enrichment of this species (Figure 8.4A). During the first two weeks, a slight enrichment of *D. mccartyi* and *vcrA* was detected in microcosms receiving mild permanganate treatment. However, at the last sampling time point on day 21, the abundances of both targets were lower than those of the biotic control. *Desulfitobacterium*, *bvcA*, and *tceA* were consistently lower in permanganate treated microcosms than in the biotic controls (Figure 8.4A). In the case of *tceA*, this result was particularly noteworthy considering the higher degradation rate observed in permanganate treated microcosms (Figure 8.2).

Whereas slight enrichment in normalized abundance of certain targets was associated with mild chemical oxidation, treatment with 100 μmol permanganate caused a 2-4 orders of magnitude decrease in the normalized abundance of all targets (Figure 8.4B). Together these results indicate that stronger oxidation treatments have a significant negative effect on the capacity for OHR.

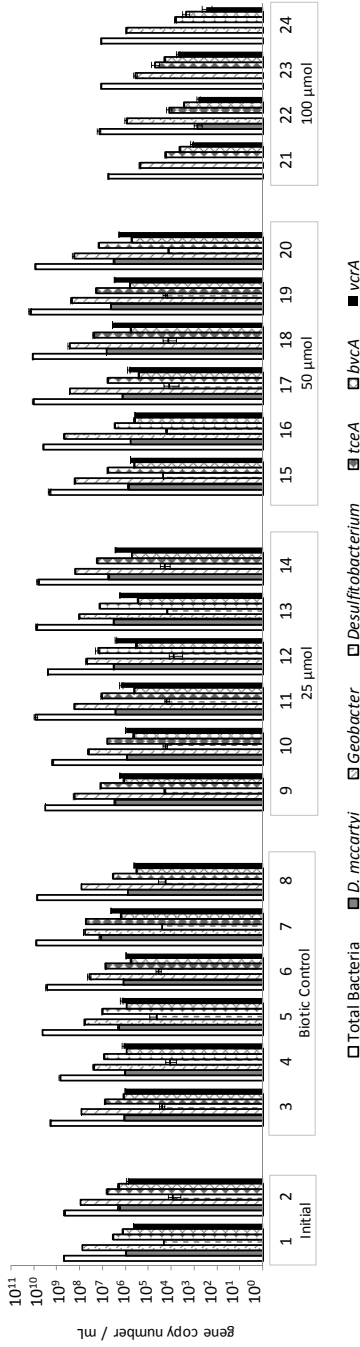


Figure 8.3 OHRB and *rdh* gene abundance for individual microcosms. Individual microcosm numbers are given in Table 8.2.

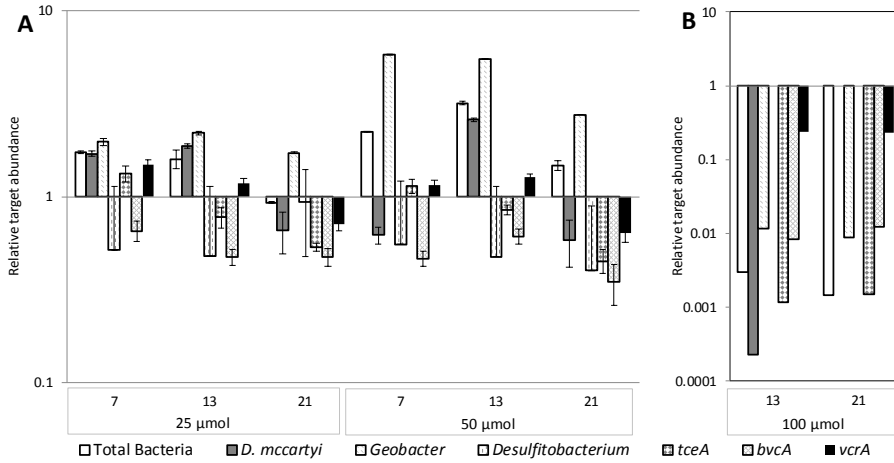


Figure 8.4 Relative target abundance of OHRB (based on 16S rRNA gene) and *rdh* genes measured by qPCR. Labels indicate time point in the experiment in days. Values indicate the enrichment or reduction of a target relative to that measured in the biotic control at the same time point for (A) mild permanganate treatments (25 µmol and 50 µmol), and (B) high permanganate treatment (100 µmol). Note that different concentration scales are used for Y axes of (A) and (B) panels.

Table 8.2 Summary of sample treatment and timing, microbial richness and diversity as estimated by Chao1 and Shannon (H') Diversity Index, and relative abundance of OHRB genera as based on pyrosequencing data. Diversity indexes calculated for randomly chosen subsets of 5326 reads per sample.

Sample	Treatment	Time	Seqs	OTUs	Chao1	Shannon Index (H')	Relative Abundance OHRB (%)				
							<i>D. mccartyi</i>	<i>Dehalogenimonas</i>	<i>Geobacter</i>	<i>Sulfurospirillum</i>	Sum
1	Initial	0	6689	5667	301	3.81	4.38	0.00	14.00	9.57	27.95
2		0	8111	7277	307	4.25	7.44	0.00	15.22	11.00	33.65
3	Biotic	7	10585	10044	253	3.8	0.74	0.00	3.02	0.23	3.99
4		7	7957	7835	207	3.02	1.56	0.00	3.02	0.00	4.58
5	Control	13	8311	7185	217	3.18	0.25	0.00	1.01	0.06	1.31
6		13	10247	9951	179	2.33	0.28	0.00	0.30	0.12	0.70
7	21	6791	5825	214	2.86	3.22	0.00	0.31	0.00	3.53	
8		21	10059	9005	163	2.82	0.14	0.00	0.92	0.00	1.06
9	7	9451	8478	213	3.47	0.33	0.00	11.79	1.86	13.97	
10		7	9194	8731	184	3.49	0.46	0.00	8.04	1.16	9.66
11	25 µmol	13	9837	8810	187	2.6	0.32	0.00	6.94	1.42	8.68
12		13	11578	10729	216	2.92	0.64	0.00	3.73	0.20	4.56
13	21	10678	9895	213	2.22	0.44	0.00	3.61	0.32	4.37	
14		21	11513	10359	229	3.13	0.66	0.00	6.83	0.28	7.77
15	7	11831	10695	258	4.01	0.35	0.02	15.11	0.88	16.36	
16		7	10311	9897	190	3.45	0.03	0.00	37.92	3.78	41.73
17	50 µmol	13	12062	10105	234	3.83	0.18	0.00	23.89	3.45	27.52
18		13	10763	8476	303	4.07	1.33	0.00	22.97	2.26	26.56
19	21	9147	8862	187	3.02	0.22	0.00	7.17	1.01	8.40	
20		21	9130	8432	162	2.69	0.11	0.00	8.07	0.00	8.18
21	13	9059	8759	110	3.02	0.18	0.00	11.77	44.27	56.22	
22		13	24380	24026	169	2.96	0.06	0.00	4.81	45.72	50.58
23	100 µmol	21	10190	10062	139	2.9	0.02	0.00	8.70	30.05	38.77
24		21	7875	7727	116	1.93	0.05	0.00	28.59	58.44	87.08

8.3.3 Microbial community diversity and structure

To understand the influence of chemical oxidation on microbial community dynamics, PCR amplified 16S rRNA gene fragments were sequenced in samples from the four different treatments at time points of the experiment. After filtering and trimming, between 6689 and 24380 (average of 10239 ± 3356) high quality sequences were found per sample and clustered into 5667-24026 operational taxonomic units (OTUs; average 9451 ± 3400) per sample (Table 8.2). Alpha-diversity indexes revealed an overall decrease in microbial diversity upon incubation in all treatments as compared to the initial sample (Table 8.2). The highest diversity was observed in the initial samples taken prior to the start of the experiment (Figure 8.5), with, in most cases, the Chao1 and Shannon Diversity indexes showing an overall decrease over the course of experiment (Table 8.2). Mild permanganate treatments at 25 μmol and 50 μmol did not significantly impact diversity indices as compared to the biotic control (Table 8.2). However, rarefaction curves of microcosms receiving 100 μmol permanganate treatments show a significant reduction in bacterial diversity (Figure 8.5), reinforcing the detrimental effects of chemical oxidation on the microbial community that were previously observed when focusing on OHRB.

OTUs were classified into 29 bacterial phyla, with 99.6% of the OTUs belonging to 5 major phyla: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Chlorobi*, and *Chloroflexi* (Figure 8.6A). The most abundant OTUs were found to belong to the genus *Acetobacterium* in the class *Clostridia* (*Firmicutes*), the genus *Sulfurospirillum* in the class *Epsilonproteobacteria*, and *Geobacter lovleyi* of the class *Deltaproteobacteria*. Chemical oxidation had a noted effect on microbial community structure, with shifts in the relative abundance of a number of classes observed between treatments. For example, *Clostridia* was highly prevalent in the in the initial sample, the biotic control and the 25 μmol permanganate treatment, with between 23 and 79% of OTUs observed belonging to this class (Figure 8.6B). In contrast, *Clostridia* were less abundant in most microcosms treated with 50 μmol or 100 μmol permanganate. Day 21 samples from the 50 μmol permanganate treatment were notable exceptions to this trend, and appear to confirm the gradual increase in the relative abundance of *Clostridia* in repeated mild permanganate treatments, as was also observed in reaction to 25 μmol permanganate treatments (Figure 8.6B).

Permanganate treatment was associated with higher relative abundances of *Proteobacteria* (Figure 8.6A). Under mild permanganate treatments of 25 μmol or 50 μmol , a shift towards *Deltaproteobacteria* was observed, while *Betaproteobacteria* and *Epsilonproteobacteria* were also abundant in microcosms treated with 100 μmol

permanganate (Figure 8.6B). *Deltaproteobacteria* were dominated by the family *Geobacteraceae* (Figure 8.6C). *Geobacter lovleyi* was particularly abundant, with 33-81% of *Deltaproteobacteria* classified as this species when 50 μmol permanganate treatments were applied and 78-96% in the 100 μmol permanganate microcosms. *Epsilonproteobacteria* were dominated by the family *Campylobacterales*, more specifically the genus *Sulfurospirillum*, with nearly all *Epsilonproteobacteria* belonging to this genus in microcosms treated with 100 μmol permanganate (Figure 8.6C). In contrast, the relative abundance of the family *Dehalococcoidaceae* decreased with increasing permanganate treatments, in line with the reduction in the abundance of this family of OHRB as observed by specific qPCR analysis described above.

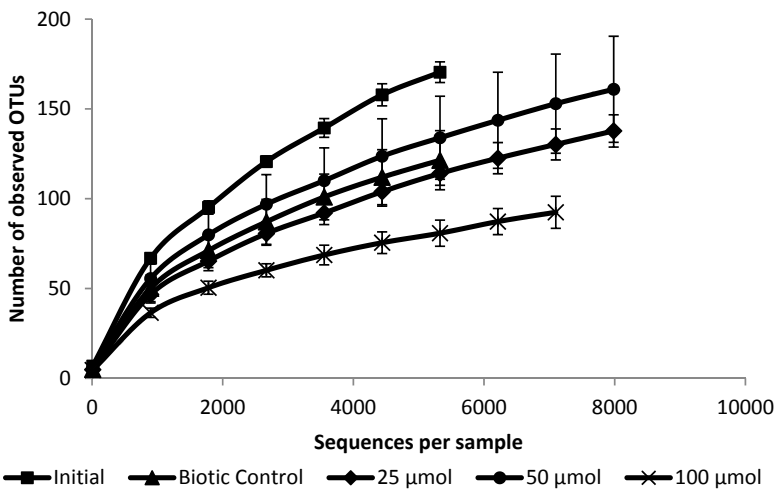


Figure 8.5 Rarefaction curves of average number of OTUs for each treatment. Error bars indicate standard deviation.

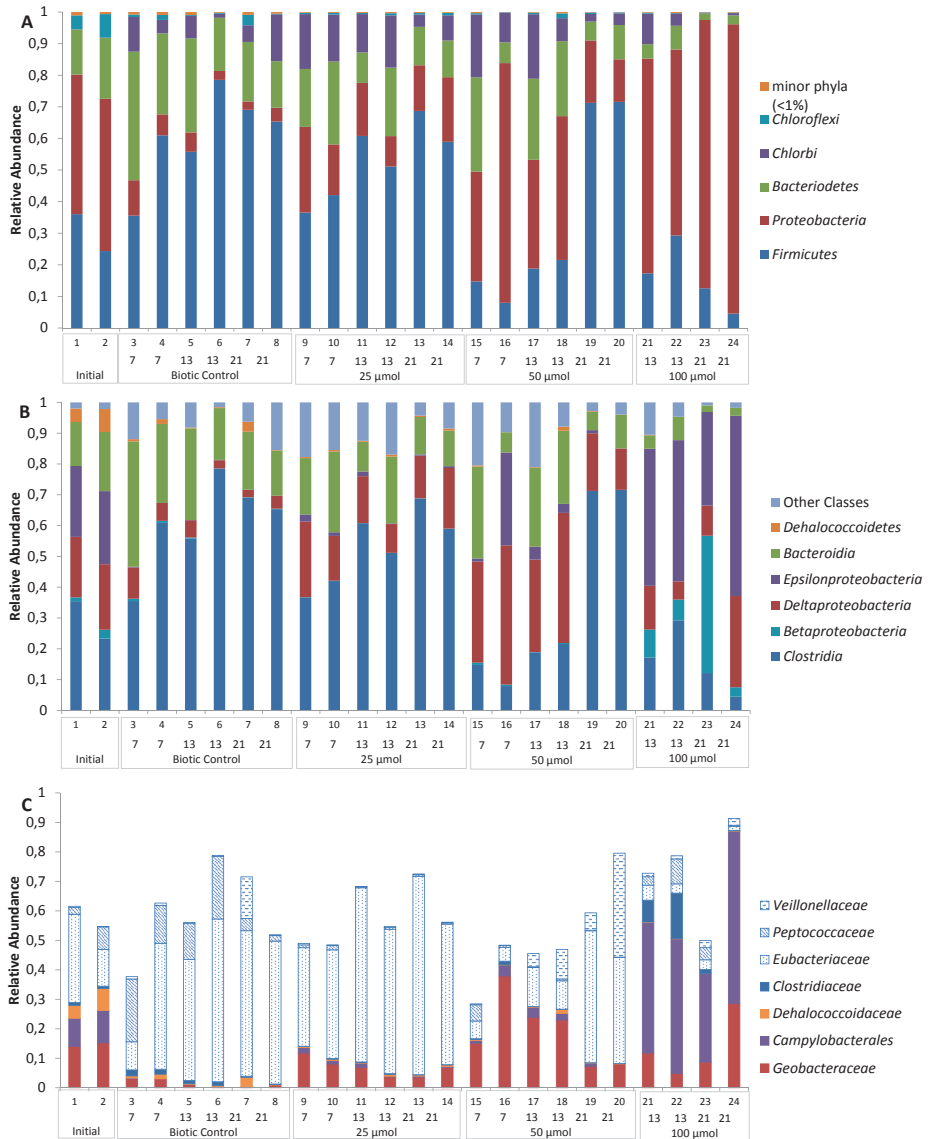


Figure 8.6 Relative abundance of dominant bacteria (A) phyla, (B) classes, and (C) important families from classes given in (A). Treatments and sample numbers are as given in Table 8.2.

8.4 Discussion

8.4.1 Impact of chemical oxidation on OHR activity and microbial community structure

Permanganate treatment induced a marked change in microbial OHR activity and community dynamics. Trends in certain microbial parameters such as biodegradation rates (Figures 8.1 and 8.2), OHRB and *rdh* gene abundance (Figure 8.3 and 8.4), and bacterial alpha-diversity (Figure 8.5) were similar in 25 μmol and 50 μmol permanganate treated microcosms, but notably different upon application of 100 μmol permanganate. However, general trends in microbial community structure could be observed across all permanganate treatments and were related to increased chemical oxidant doses (Figure 8.6).

Under mild chemical oxidation treatments, biodegradation rates were higher, indicating a slight stimulation of OHR activity (Figures 8.1 and 8.2). Microcosms receiving 25 μmol or 50 μmol permanganate treatments showed more rapid conversion overall of PCE and TCE to *cis*-DCE (Figure 8.2). The capacity of manganese reducing bacteria to catalyze the first two steps in OHR from PCE to *cis*-DCE is well known [252], with bacteria such as *Geobacter lovleyi* and *Sulfurospirillum* able to use, among other compounds, either Mn(VI) or higher chlorinated ethenes as electron acceptors [144, 147]. The increased relative abundance of these populations observed in permanganate treated microcosms (Figures 8.3 and 8.6; Table 8.2) thus indicates shifts in the microbial community structure that support higher OHR rates.

Nevertheless, improved OHR activity was not limited to degradation of higher chlorinated ethenes; increased rates of VC and ethene production were also observed in microcosms receiving mild permanganate treatments, as compared to the biotic control (Figure 8.1). While improved PCE and TCE degradation is somewhat attributable to the presence of *G. lovleyi* and *Sulfurospirillum*, faster degradation of *cis*-DCE and further did not directly correlate with observed shifts in the microbial community. Rather, the absolute abundance of *D. mccartyi* and vinyl chloride reductive dehalogenase encoding genes *bvcA* and *vcrA*, as assessed with qPCR, decreased in many instances as compared to the biotic control (Figure 8.4). Together, these results appear to indicate the development of a microbial community less adept to degrade *cis*-DCE and VC to ethene.

This interpretation, however, disregards the importance of symbiotic relationships in supporting full transformation of *cis*-DCE to ethene. For example, *D. mccartyi* lacks the capacity to synthesize corrinoid species, an integral component of reductive

dehalogenase enzymes, which necessitates the amendment of vitamin B12 in cultures. *G. lovleyi*, identified as the predominant *Deltaproteobacteria* in microcosms treated with 50 μmol permanganate (Table 8.2), is able to produce cobamide in synthetic media and research suggests that interspecies corrinoid transfer supports OHR by *D. mccartyi* [301]. This is one example of the complementary relationships between species that support biodegradation. Although our experiments were performed in media, which included vitamin B12, supplemental production by *G. lovleyi* may explain the higher VC and ethene production rates observed in microcosms treated with 50 μmol permanganate.

In contrast to the mild treatments described above, significant disruption of OHR (Figure 8.1), the abundance of OHRB and *rdh* genes (Figure 8.4), and microbial community diversity (Figure 8.5) were observed in microcosms treated with 100 μmol permanganate. Interruption of biodegradation beyond *cis*-DCE, and reductions in the abundance of OHRB and *rdh* genes of 2-4 orders of magnitude, indicate the severe effect of strong permanganate treatments on OHR activity and capacity. More notably, the abundance of 16S rRNA genes decreased 2 orders of magnitude, indicating that 100 μmol permanganate dosing directly attacks the microbial community (Figure 8.4). As this result was not observed in milder permanganate treatments, this points toward a qualitative difference in the impact of permanganate on the system. At low doses, oxidant concentrations are only sufficient to react with reduced mineral and electron donor in the microcosms. Between permanganate treatments, the microbial community appears to have sufficient time to bring the system back towards reduced conditions while possibly performing dissimilatory Mn(VI) reduction. In contrast, 100 μmol permanganate appears to exceed the oxidant demand of readily oxidized media components, thereby leaving sufficient chemical oxidant to react directly on the microbial community. Thus, significantly more regeneration of the microbial community is required to regain normal function, including OHR activity, following stronger chemical oxidation treatments.

8.4.2 Implications for field treatment

The results presented here are a critical first step in better understanding the impact of chemical oxidation on OHR, and thus on designing improved biphasic treatments. Whereas previous work has either examined the composition of reductive dechlorinating consortia in microcosms [45] and in contaminated locations [130], or investigated the abundance of OHRB and *rdh* genes following chemical oxidation [54, 218, 254], this is the first study to investigate microbial community

dynamics following chemical oxidation. Although determining the presence of OHRB does indicate the capacity for OHR, previous studies on consortia community structure repeatedly indicated the importance of non-dechlorinating populations on the activity of OHRB [91, 165]. Our study provides the initial framework for further insight into the impact of chemical oxidation on microbial OHR.

The importance of the chemical oxidant concentration in dictating the result of ISCO treatment on OHR activity has implications for field applications. Our results indicate that mild chemical oxidation treatments have a slight stimulatory impact on biodegradation activity, while stronger permanganate doses of 100 μmol are detrimental to the microbial community. These results demonstrate the necessity for a balancing act in field applications between stimulation and disruption of biodegradation. The difference between positive and negative outcomes of chemical oxidation on microbial OHR will surely be site specific, thus requiring laboratory experiments prior to field application to determine appropriate permanganate doses

Biomolecular tools to determine microbial community structure and quantify OHRB will be integral to the development of a full-fledged, robust field application of chemical oxidation coupled to OHR. Our results indicated that with mild chemical oxidation treatments, especially at 50 μmol permanganate doses, higher biodegradation rates may have been related to an optimal balance between manganese reducing bacteria, such as *G. lovleyi* and *Sulfurospirillum*, and fermenters within the class *Clostridia*. With higher permanganate treatments, both the abundance of *Clostridia* and OHRB decreased, which correlates well with the lower OHR rates observed in these microcosms. The ability to link microbial diversity to the eventual response of the microbial community towards chemical oxidation will prove essential to translating laboratory investigations into field applications.

The results presented here are an important new step towards a more complete body of knowledge on the impact of chemical oxidation, not only on OHR directly, but also the entire community structure. In field application, remediation goals, soil heterogeneity, natural oxidant demand, and groundwater composition will influence the effects of chemical oxidation on microbial community structure and biodegradation activity. Therefore, further research should focus on illuminating the influence of the aforementioned factors on microbial community structure and resilience towards chemical oxidation, especially under field conditions where the combined impact of a variety of parameters can be monitored. This will lead to both an enhanced capacity to design biphasic treatments and an improved understanding of the microbial ecology of OHR under oxidative stress.

Acknowledgments

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Chapter 9

Microbial dynamics during and after *in situ* chemical oxidation of chlorinated solvents

This chapter has been accepted as:

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Abstract

In situ chemical oxidation (ISCO) followed by a bioremediation step is increasingly being considered as an effective biphasic technology. Information on the impact of chemical oxidants on organohalide respiring bacteria (OHRB), however, is largely lacking. Therefore, we used quantitative PCR (qPCR) to monitor the abundance of OHRB (*Dehalococcoides mccartyi*, *Dehalobacter*, *Geobacter*, and *Desulfitobacterium*) and reductive dehalogenase genes (*rdh*; *tceA*, *vcrA*, and *bvcA*) at a field location contaminated with chlorinated solvents prior to and following treatment with sodium persulfate. Natural attenuation (NA) of the contaminants tetrachloroethene (PCE) and trichloroethene (TCE) observed prior to ISCO was confirmed by the distribution of OHRB and *rdh* genes. In wells impacted by persulfate treatment, a 1-3 order of magnitude reduction in the abundances of OHRB and complete absence of *rdh* genes was observed 21 days after ISCO. Groundwater acidification (pH<3) and increase in the oxidation reduction potential (>500 mV) due to persulfate treatment were significant and contributed to disruption of the microbial community. In wells only mildly impacted by persulfate, a slight stimulation of the microbial community was observed, with more than 1 order of magnitude increase in the abundance of *Geobacter* and *Desulfitobacterium* 36 days after ISCO. After six months, regeneration of the OHRB community occurred, however, neither *D. mccartyi* nor any *rdh* genes were observed, indicating extended disruption of biological NA capacity following persulfate treatment. For full restoration of biological NA activity, additional time may prove sufficient; otherwise addition electron donor amendment or bioaugmentation may be required.

9.1 Introduction

Soil and groundwater contamination with chlorinated solvents such as tetrachloroethene (PCE) and trichloroethene (TCE) is common in urban and industrial areas. At contaminated locations, the high density and low solubility of these solvents causes pure product to sink through aquifers and accumulate as pools on less-permeable layers. These dense non-aqueous phase liquid (DNAPL) pools slowly dissolve with time to feed large plumes of contaminated groundwater. The application of *in situ* technologies to remediate both DNAPL source areas and plumes is increasing, owing to their cost-effectiveness, improved sustainability, and reduced above ground disturbance as compared to many *ex situ* techniques. These *in situ* technologies mainly rely on biological or chemical conversion of contaminants to harmless by-products and include *in situ* bioremediation (ISB) and *in situ* chemical oxidation (ISCO).

Microbes able to perform organohalide respiration (OHR) of PCE to daughter products TCE, *cis*-dichloroethene (*cis*-DCE), vinyl chloride (VC) and finally ethene have been identified and utilized for ISB. These organohalide respiring bacteria (OHRB) dechlorinate chlorinated compounds via metabolic and co-metabolic pathways, with *Dehalococcoides mccartyi* being the only bacteria known to perform full dechlorination from PCE to ethene [33, 56, 142, 241]. In field application, biostimulation by providing electron donor, nutrients, and in some cases bioaugmentation with commercial cultures is performed to improve the rate of degradation and ensure full dechlorination to ethene [151, 155].

ISB can to some extent be monitored through measuring the shift from more chlorinated parent compounds to less chlorinated degradation products. However, molecular tools are increasingly being utilized to ensure microbial degradation capacity and activity. Quantitative assays confirm the presence of OHRB based on 16S rRNA sequences and measure genes encoding for reductive dehalogenases (*rdh*) using both DNA and RNA templates [8, 132, 153, 154]. While planktonic and biofilm microbial communities do differ to some extent in structure [65, 232], field investigations have shown that culture-independent molecular tools targeting specific degraders or degradation genes in groundwater samples can be used to monitor and confirm degradation capacity [24]. Such molecular tools have been integrated into routine monitoring campaigns at locations contaminated with chlorinated compounds.

Although ISB can be quite effective, at sites with large pure product source zones or quick remediation goals, biodegradation may be too slow or inefficient. In such situations, ISCO treatments such as permanganate, persulfate, and Fenton's reagent (iron-activated hydrogen peroxide) can very effectively and rapidly decrease DNAPL mass and reduce groundwater concentrations [164, 267]. Oxidation with persulfate, the treatment investigated here, can occur through direct electron transfer or by activation with transition metals to produce the sulfate radical [272]. Although effective for mass removal, some residual contaminant often remains following ISCO, that can lead to a rebound of groundwater concentrations due to desorption from soil [173]. Coupling ISCO with a subsequent ISB phase to remediate all residual contaminants is therefore a promising option. Efficient application of a two phase remediation approach would reduce the quantity of chemical oxidants required while improving the overall remediation efficiency. The effectiveness and advantages of such a bio-polishing step has been confirmed for non-chlorinated aliphatic and aromatic hydrocarbon contamination [103, 257, 274].

There is less information on the coupling of ISCO treatments with OHR of chlorinated compounds. Whereas chemical oxidants can improve groundwater oxidation reduction potential (ORP) for aerobic conversion of non-chlorinated hydrocarbons, ISCO invariably interrupts OHR. In microcosm studies, regeneration of reductive TCE dechlorination activity was not observed following MnO_4^- and $\text{S}_2\text{O}_8^{2-}$ treatments above 0.5 g l^{-1} and 1 g l^{-1} , respectively [54]. Similarly, Fenton's reagent treatment irreversibly disrupted biodegradation of hexachloroethane and PCE in microcosm experiments [98]. In two column experiments, some OHR was observed following KMnO_4 treatment of PCE contaminated soils [87, 218]. In both experiments, disruption of microbial activity following chemical oxidation occurred, however, some dechlorination capacity was observed following variable lag periods and amendments. Whereas Hrapovic and colleagues performed bioaugmentation steps [87], Sahl and co-workers observed regeneration without bioaugmentation [218]. Their success is most likely attributable to the fact that they utilized a commercially available culture (KB1), designed to be robust during field application; this may explain the community's resilience to chemical oxidation and persistence under changing environmental conditions.

Field studies in which microbial community diversity and OHR capacity were monitored during ISCO treatment of chlorinated compounds are also sparse. In conference proceedings from one field location, some regeneration of *D. mccartyi* was observed following KMnO_4 treatment of PCE after more than a year [148, 149].

A more recent investigation used terminal restriction fragment length polymorphism (TRFLP) and microarray assays to monitor subsurface microbial communities during NaMnO_4 treatment of a TCE contamination [232]. Temporary changes in population size and diversity due to ISCO were measured, with regeneration occurring shortly after the oxidant finished reacting. Although 16S rRNA genes of a number of OHRB were measured, OHR was not observed and was deemed improbable due to the geochemistry of the location.

In order to gain insight into the coupling of ISCO with ISB for the remediation of chlorinated solvents, we investigated a PCE contaminated location during and after sodium persulfate ($\text{Na}_2\text{S}_2\text{O}_8$) treatment. Whereas in a previously published field study no OHR was measured before ISCO [232], at our location significant natural attenuation (NA) activity had been observed prior to chemical treatment. This gave the unique opportunity to investigate changes in the abundance of OHRB and *rdh* genes upon chemical oxidation in a location with NA activity. This study therefore aims to determine temporary and extended impacts from ISCO treatment on the presence of OHRB and *rdh* genes and gives insight into the potential for bio-polishing following chemical oxidation.

9.2 Materials and Methods

9.2.1 Site description

A site in the eastern portion of The Netherlands with historical chlorinated solvent contamination was investigated. The subsurface consists of Holocene deposits with a mixture of sand, clay, peat, and loam, with a higher concentration of organic matter present at depths of 2-6 m (Table 9.1). Below 8 m, sand is coarse, which precluded further sampling of soil for analysis. The groundwater level is between 2.5 and 2.8 m below ground level and flows in a southeasterly direction (Figure 9.1).

Industrial activities at the site were first officially documented in 1895, however, they probably preceded this. Previous purposes include furniture maker, tire center, window maker, and automobile garage in addition to carpet beating, textile dyeing, and dry cleaning. Cleaning services were performed with gasoline, PCE, and TCE, with the first reported use of chlorinated solvents in 1942. The main washer was located above present day well W and was one of the main sources for contamination (Figure 9.1). Between 1965-1975, containers of solvents were stored just south of the main washer in structures with dirt floors, which may be a second point source.

Table 9.1 Description of subsurface lithology at the site. Information is an average of N3, N4, N11, M1, and S1.

Depth (m bgl)	Soil matrix
0-2	Fine sand, some silt and gravel
2-3	Sandy loam, heterogeneous distribution
3-6	Very fine sand, high silt content
6-8	Fine sand, some silt
8-8.4	Coarse sand, some silt

Monitoring of soil and groundwater contamination has occurred over the past 15 years in order to track plume size and estimate risk. Initial characterization of the site in 1997 indicated two areas of high contaminant concentrations, a source area on the north side of the site around well N5, and a second source area on the south end near well S1 (Table 9.2). In accordance with the groundwater flow, the plume was found almost exclusively between these two wells with some slight eastern spreading (wells N7 and N9). The highest contaminant concentrations were found in shallower wells at depths of 2-4 m. The more organic rich silt layers may have retarded sinking of contaminants (Table 9.1).

9.2.2 ISCO

ISCO was performed in September 2011 in two separate areas with high contaminant concentrations around well N5 and well S1. At each location, iron-activated persulfate was injected at a concentration of 10 kg $\text{Na}_2\text{S}_2\text{O}_8/\text{m}^3$. Separate stock solutions of FeSO_4 (chelated with citrate) and $\text{Na}_2\text{S}_2\text{O}_8$ were made in drinking water and mixed at a ratio of 1 mol Fe^{2+} : 78 mol $\text{S}_2\text{O}_8^{2-}$. Around well N5, 1000 kg of $\text{Na}_2\text{S}_2\text{O}_8$ was injected using twelve wells within a radius of 5 m and at a depth of 2-6 m. Two injection periods of two days each were performed. Owing to the reduced concentration and distribution of contaminants around well S1, only six wells were used to deliver 100 kg of $\text{Na}_2\text{S}_2\text{O}_8$ at a depth of 2-4 m during a one day injection. In both cases, the injection speed was 2.3 m^3/hour . An indication of the anticipated persulfate treatment area is given in Figure 9.1.

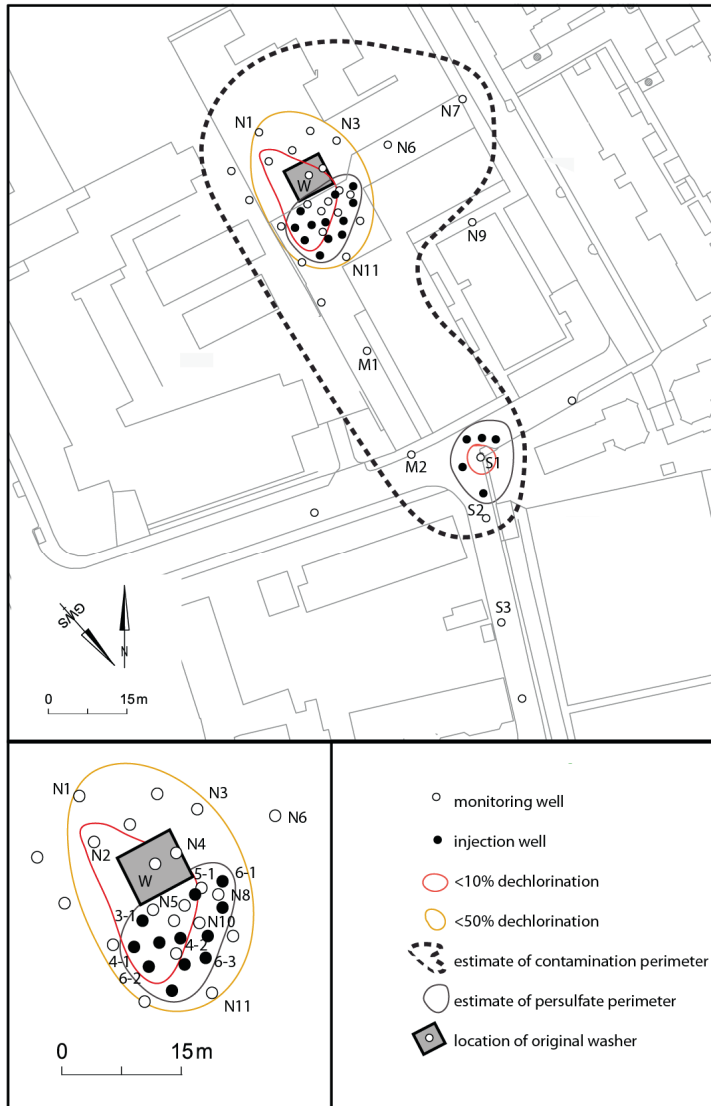


Figure 9.1 Schematic of monitoring wells (open circles) and injection wells used for persulfate delivery (closed circles) at site. The location of the historic washing machine (at monitoring well W) is given with a shaded square. The northern source area around well N5 is given in more detail below. Dechlorination contour lines indicate changes in the aqueous phase distribution of contaminants and are estimates based on a combination of monitoring in 2009 (Table 9.2) and in 2012 (Table 9.4, before ISCO). The estimate of contamination perimeter is based on historic monitoring of the area which included wells not indicated on this map.

Table 9.2 Concentrations of contamination in groundwater obtained from previous monitoring campaigns. Depths indicate the filtered portion of the well from which groundwater was collected. Wells are ordered from northwest to southeast.

Well	Depth (m bgl ^a)	Year	PCE	TCE	<i>cis</i> -DCE (µg/L)	VC	Total	BTEX (µg/L)
N1	2.5-3.5	2003	69	100	9.2	na ^b	178	
		2009	1.1	6.1	2.6	0.33	10	
N3	3.5-4.5	2009	370	210	580	320	1480	<11
N4	6.2-7.2	2009	11	11	200	540	762	
		1997	36000	260	18	na	36278	
N5	2.5-3.5	2003	11000	170	100	na	11270	
		2005	20000	12	860	na	20872	
		2009	34000	1500	8900	200	44600	<320
N5	7.0-8.0	2003	12	3.7	48	na	64	
		2005	110	4.4	2.3	na	117	
		2009	30	4.6	38	1.1	74	<1
N6	2.5-5	1997	44	20	25	na	89	
		2003	85	31	43	na	159	
N7	2.5-5	1997	15	2.7	bdl ^c	na	18	
		2003	50	13	10	na	73	
N9	2.5-5	2003	0.7	0.4	bdl	na	1.0	
		2005	0.7	bdl	bdl	na	0.7	
N10	2.5-5	2009	58	280	300	6.3	644	
N11	4-5	2009	1.5	7.5	440	0.5	450	
N11	9-10	2009	0.5	bdl	0.3	bdl	0.8	
		2005	110	230	bdl	na	340	
M1	2.5-5	2009	0.4	bdl	bdl	bdl	0.4	
		2005	750	10	7.7	na	768	
S1	2.5-5	2009	1300	130	45	2.4	1477	
		2005	bdl	bdl	bdl	na	bdl	

^a below ground level

^b not analyzed

^c below detection limit

9.2.3 Sampling and chemical analyses

Soil and groundwater samples were collected in order to determine the distribution of contaminants and the impact of ISCO. The chosen location showed significant NA activity and was nonetheless scheduled for persulfate treatment, therefore presenting an ideal site for investigations into the impact of ISCO on microbial biodegradation capacity. However, as this site was undergoing full scale remediation, in some instances scientific aims were compromised due to remediation

goals. Every effort was taken to coordinate routine sampling rounds with sampling for molecular analyses, however, full integration was not possible. Additionally, due to other activities at the site, not all of the wells focused on in the initial sampling rounds could be monitored at the 6 month time period. Unfortunately, incomplete or incongruent sampling restricts interpretation of the results to only a partial description of the result of chemical oxidation on microbial NA capacity. Incongruent timing or location of sampling has been noted in the text.

Groundwater and soil sampling for analysis of contaminant concentration were performed according to standardized field procedures[94]. Wells were flushed by pumping prior to collection of groundwater in completely filled and sealed glass bottles. Soil samples dug with augers were similarly collected in filled, sealed glass containers. Samples were stored at 4 °C prior to analysis. Both groundwater and methanol extracted soil samples were measured using headspace GC-MS according to standardized procedures [95]. Groundwater pH and ORP were measured using a WTW pH325 handset (Germany) with qi electrodes QR400x and QP108x.

9.2.4 Molecular analyses

For quantitative PCR (qPCR) analysis of microbial 16S rRNA and *rdh* gene abundance, groundwater was collected in sterile glass bottles before ISCO, and 21, 36, and 180 days after persulfate injection. 1000 mL groundwater was vacuum-filtered over a 0.2 µm membrane filter (Millipore, USA) and filters were stored at -20°C in sterile microcentrifuge tubes. For DNA extraction, filters were cut into small strips prior to extraction with the FastDNA Spin Kit for Soil (MP Biomedicals, USA). DNA quantity and quality were estimated on a Nanodrop spectrophotometer (Thermo Scientific) prior to storage at -20°C. QPCR was performed using a iQ5 SYBR Green Supermix kit on the iQ5 iCycler (Biorad, The Netherlands). Ten-fold dilutions were assayed in triplicate and no-template controls were included. Assayed 16S rRNA genes were total bacterial (341F and 534R; [174]), *D. mccartyi* (728F and 944R; [244]), *Dehalobacter* (441F and 645R; [244]), *Geobacter* (577F and 822R; [11], and *Desulfitobacterium* (406F and 619R; [244]). Assayed *rdh* genes were *tceA* (1270F and 1336R; [99]), *vcrA* (Vcr1022F and Vcr1093R; [209]), and *bvcA* (Bvc925F and Bvc1017R; [209]). A full list of targets and primers is available as supplementary material (Appendix 3). Gene copy numbers were calculated as copies/mL sample.

9.3 Results

9.3.1 NA capacity before ISCO

The site is characterized by two source areas, one on the northern side around well N5 and a second to the south around well S1. Based on *cis*-DCE and VC concentrations, it was concluded that NA has occurred at the site (wells N5 and S1, Table 9.2). Additionally, some decrease in concentrations in wells on the edge of the site supports this conclusion (wells M1 and N1; Table 9.2). Previous sampling campaigns have found contamination with gasoline, especially xylene (Table 9.2), which may serve as an electron donor for OHR of contaminants. An indication of the extent of dechlorination in the areas around the historic washer and around well S1 are given (Figure 9.1).

Soil and groundwater analysis prior to ISCO found the highest concentration of PCE present at close proximity to well N5 (Tables 9.3 and 9.4). Groundwater in well 6-3 and in soil samples from wells 6-1 and 6-2 indicate spreading of contaminants to the south and east (Table 9.4; Figure 9.1). Relatively lower contaminant concentrations were measured in soil and groundwater from well N2, just to the north and thus upstream of source area one.

Table 9.3 Concentrations of contamination in soil from before ISCO.

Location	Depth (m bgl ^a)	PCE (mg/kg)	TCE	<i>cis</i> -DCE	VC
3-1	2.5-3.0	0.27	bdl	bdl	bdl
N2	1.5-2.0	0.66	bdl	bdl	bdl
N2	3.0-3.5	2.00	0.42	0.2	bdl
6-1	4.0-5.0	52	2.2	0.54	0.037
6-2	5.5-6.0	15	0.33	0.61	0.028

^a below ground level

Table 9.4 Concentrations of contamination in groundwater from before ISCO and at two time points after ISCO with groundwater direction.

	Well	Depth (m bg ^a)	PCE (µg/L)	TCE	<i>cis</i> -DCE	VC	Total
Before ISCO	N2	2-4	2100	920	770	9.2	3809
	5-1	4-5	32000	1800	4700	900	39400
	6-3	5-6	13000	1700	1800	380	16880
2 months after ISCO	N2	2-4	420	770	390	18	1598
	N5	7-8	20000	1300	6100	310	27710
	5-1	4-5	40000	1900	4600	1800	48300
	6-3	5-6	8800	790	860	210	10660
	S1	2.5-5	2.3	bdl	0.68	0.20	3
	N8	3-4	29	24	150	5.8	209
4 months after ISCO	N2	2-4	180	50	15	1.1	246
	4-1	3-4	81	23	59	39	202
	4-2	3-4	2.4	3.0	71	33	109
	M2	3-4	bdl ^b	bdl	bdl	bdl	0
	S1	2.5-5	340	92	330	27	799
	S2	3-4	1.4	bdl	0.45	bdl	2.9

^a below ground level^b below detection limit

Prior to ISCO treatment, qPCR assays were used to track the presence of OHRB and *rdh* genes, which had developed at the site over 70 years of contamination due to industrial use. In line with contaminant measurements, molecular analysis of groundwater samples confirmed the presence of a variety of OHRB and *rdh* genes in contaminated areas (Figure 9.1). All assayed OHRB targets, *D. mccartyi*, *Dehalobacter*, *Geobacter*, and *Desulfitobacterium* 16S rRNA genes were found in the first source area around well N5, and in the second by well S1 (Figures 9.2). Genes *bvcA* and *vcrA*, encoding reductive dehalogenases for the conversion of VC to ethene, were found in the shallower filter in well N5 (2.5-3.5 m) and in S1, but not at the deeper filter of well N5 (7.0-8.0 m). Additionally, *D. mccartyi* 16S rRNA gene was found to be 2 orders of magnitude higher in the shallower filter of N5 than in the deeper. Together, this indicates presence of higher NA capacity in shallower wells. This may have been supported by the higher contaminant concentrations observed; additionally, the proximity of shallower wells to the surface may have meant more temporal continuity in the delivery of PCE and TCE from aboveground activities and longer contamination period, thereby supporting further NA capacity development. Moreover, hydrocarbons from spilled gasoline may have provided additional electron donors to stimulate full dehalogenation (Table 9.2).

Between the two source areas, downstream from well N5 and upstream from well S1, the abundance of OHRB and *rdh* genes was lower than in the source areas. *D. mccartyi* was not found in well N10 or at either depth of well N11, and the only functional gene detected in well N10 was *tceA* (Figures 9.2). Interestingly, other 16S rRNA targets such as *Geobacter* and *Desulfitobacterium* were detected in wells N10 and N11 at similar concentrations to those found in more source area wells of N5 and S1. Thus, in these areas, partial degradation of PCE and TCE to *cis*-DCE and VC may occur at similar rates, however, the capacity for full conversion to ethene is absent, in line with the absence of the functional genes *vcrA* and *bvcA*. Further downstream from source area one at well M1, none of the qPCR targets were found, indicating an interruption in NA capacity between the source zones around wells N5 and S1.

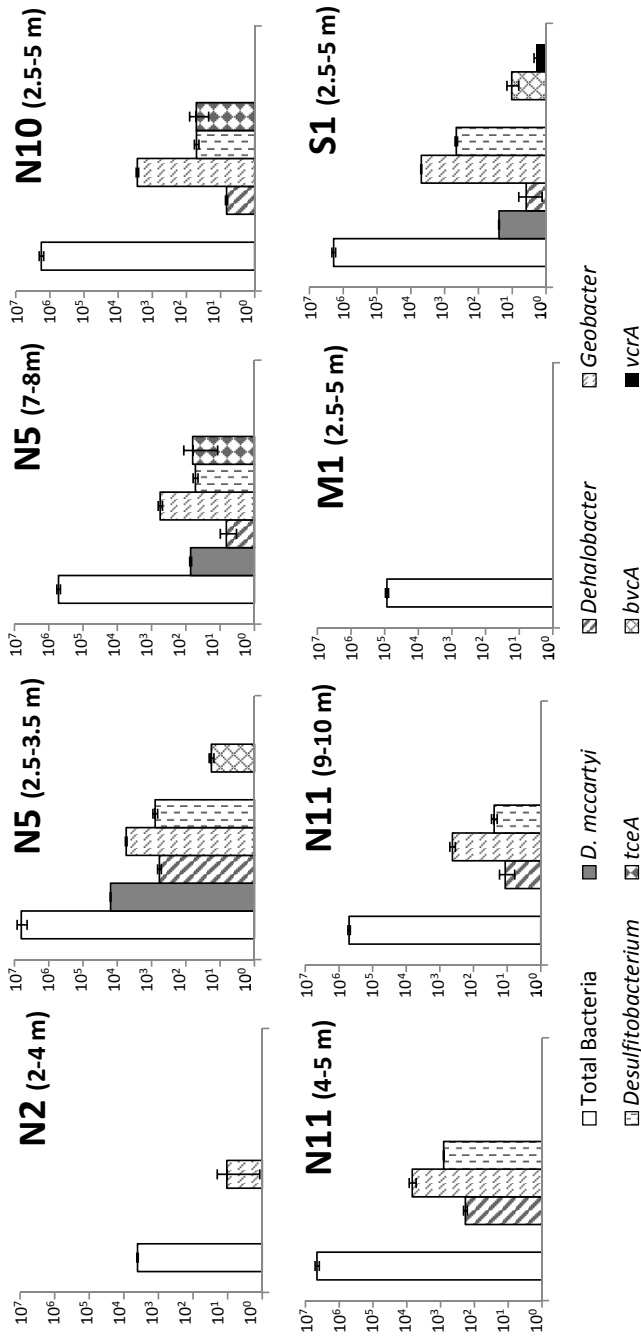


Figure 9.2 OHRB 16S rRNA and *rdh* gene abundance (copies/mL) prior to chemical oxidation. High gene abundance coincides with the presence of chlorinated solvents and degradation products in the two source areas around wells N5 and S1. In locations with less contamination (Table 9.2), for example further upstream at N2 and between the two source areas at M1, only *Geobacter* was detected and no *rdh* genes were observed.

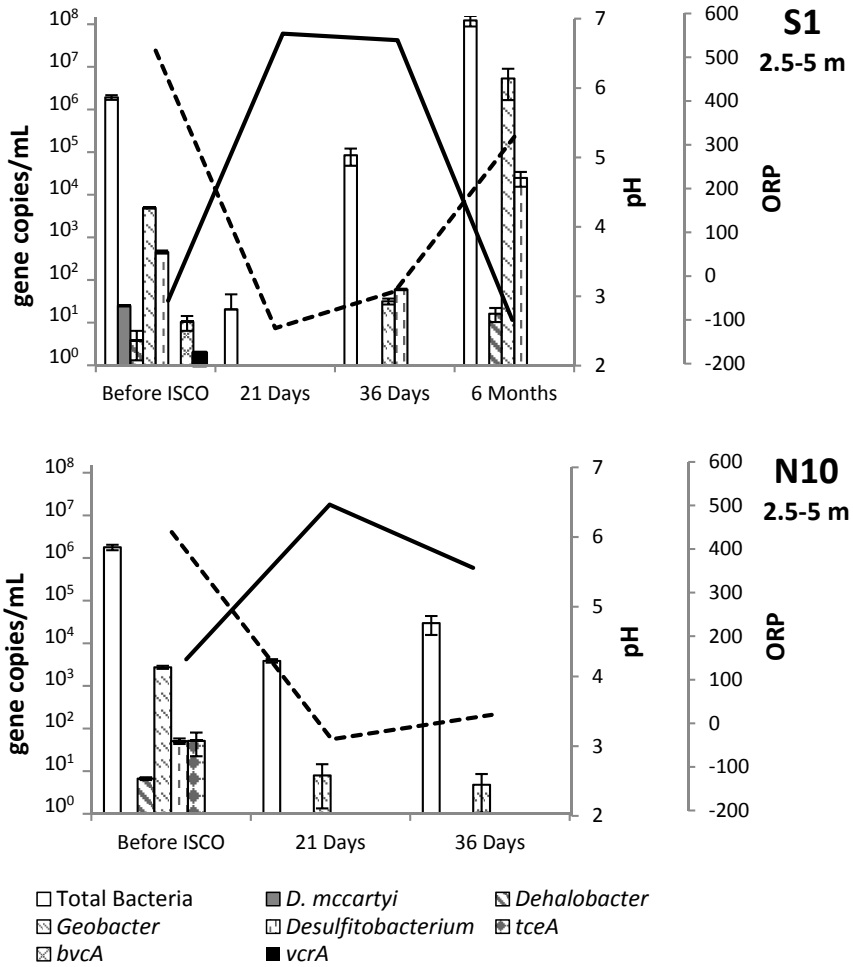


Figure 9.3 16S rRNA gene copy numbers of bacteria, *D. mccartyi*, *Dehalobacter*, *Geobacter*, *Desulfitobacterium* and *rdh* genes *tceA*, *bvcA*, and *vcrA*. qPCR results are average of triplicate assays and error bars the standard deviation. pH (dashed line) and ORP (solid line) are given on the near and far right axis, respectively.

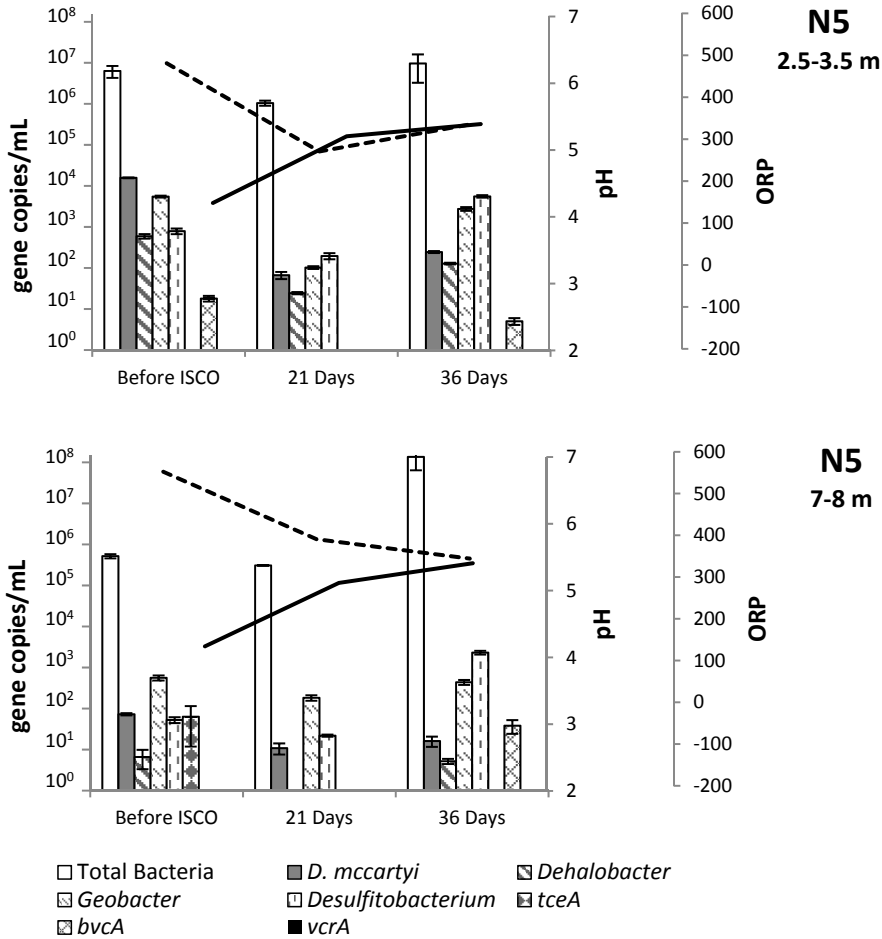


Figure 9.4 16S rRNA gene copy numbers of bacteria, *D. mccartyi*, *Dehalobacter*, *Geobacter*, *Desulfitobacterium* and *rdh* genes *tceA*, *bvcA*, and *vcrA*. qPCR results are average of triplicate assays and error bars the standard deviation. pH (dashed line) and ORP (solid line) are given on the near and far right axis, respectively.

9.3.2 Impact of ISCO treatment

ISCO with persulfate was performed around each of the source areas at wells N5 and S1 (section 2.2). Following ISCO, an increase in ORP and decrease in pH of the groundwater confirmed delivery of persulfate to the various wells (Figures 9.3

and 9.4). Additionally, measurements of contaminant concentrations two months after ISCO indicated lower overall contaminant concentrations (Table 9.4). After two months, reductions of groundwater contaminant concentrations of 89% and 48% were observed for wells N2 and 6-3, respectively (Table 9.4). Contaminant concentrations in well S1 were also significantly reduced relative to the 2009 measurements (Tables 9.2 and 9.4); however, persulfate was most likely still active in well S1 two months after ISCO, as seen in the rebound in concentrations four months after injection (Table 9.4). No notable change in contaminant concentrations was measured in well 5-1 (Table 9.4).

A notable decrease in the abundance of OHRB and *rdh* genes was observed following ISCO treatment in wells S1 and N10 (Figure 9.3). Analysis with qPCR showed a significant impact on the microbial community 21 days after injection as total bacterial 16S rRNA gene abundance was 3-5 orders of magnitude lower than prior to treatment and only low concentrations of *Geobacter* 16S rRNA gene were found (Figure 9.3). The reduction in target gene abundance correlates well with the low pH of 2.54 and 3.10 and high ORP of 554 and 502 mV in groundwater in wells S1 and N10, respectively. Some regeneration of total bacteria and particularly of *Geobacter* and *Desulfitobacterium* in well S1 could be observed 36 days after treatment which is not expected considering that groundwater pH (3.07) and ORP (539) remained inhospitable for these microorganisms.

Although both depths of well N5 were within the treatment area, ISCO appeared to have less impact on groundwater parameters and the microbial community (Figure 9.4). While *D. mccartyi* 16S rRNA gene abundance did drop 1-3 orders of magnitude, total bacterial 16S rRNA gene copy number as well as the abundance of other OHRB did not decrease significantly in the first 21 days after injection (Figure 9.4). Thirty-six days after injection, certain targets were higher than prior to treatment, including total bacterial and *Desulfitobacterium* 16S rRNA genes. Additionally, 36 days after injection, *bvcA* was detected in well N5 at a depth of 7.0-8.0 m where it had not been previously found. While some changes in pH and ORP were measured, a muted effect was observed, with pH remaining at or above 5 and ORP below 333 mV. Together, these results indicate that in contrast to wells N10 and S1, the less aggressive ISCO conditions at well N5 appeared to slightly stimulate the microbial community. It is unclear whether this difference is due to heterogeneous chemical oxidant distribution or differences in soil reactivity and geochemistry. Although mild oxidation does reduce contaminant degradation (as seen for well 5-1, at close proximity to well N5), these results indicate that under the proper conditions, ISCO can have a slight stimulatory effect on the microbial community.

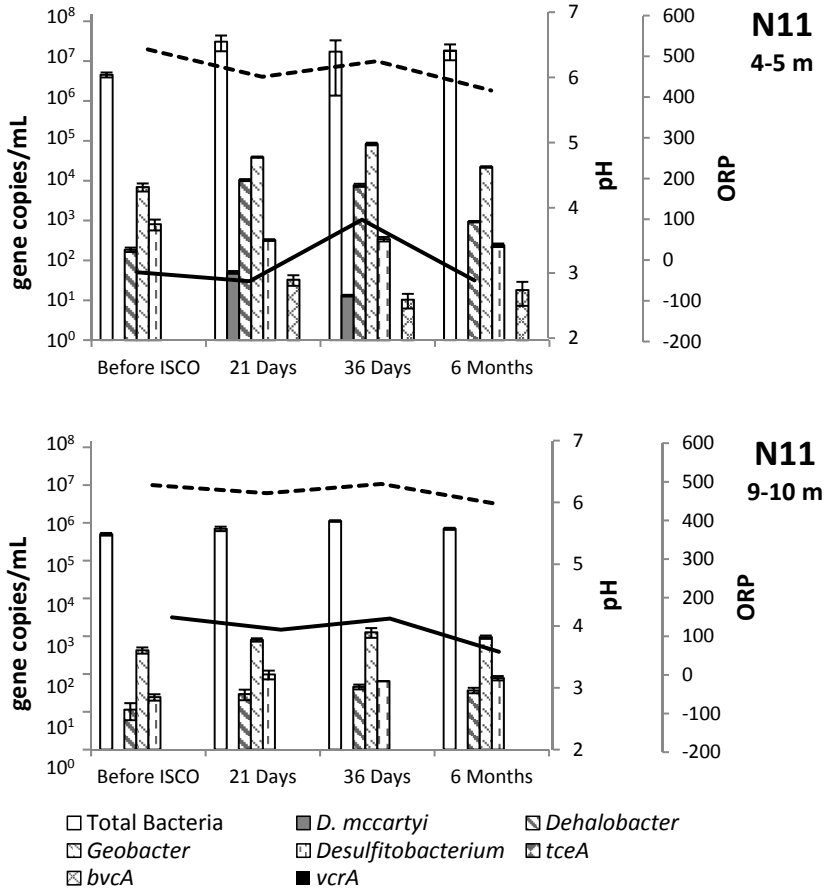


Figure 9.5 16S rRNA gene copy numbers of bacteria, *D. mccartyi*, *Dehalobacter*, *Geobacter*, *Desulfitobacterium* and *rdh* genes *tceA*, *bvCA*, and *vcrA*. qPCR results are average of triplicate assays and error bars the standard deviation. pH (dashed line) and ORP (solid line) are given on the near and far right axis, respectively.

9.3.3 Long-term impacts of ISCO

Within six months of injection, regeneration of microbial community size and OHRB abundance was observed in well S1 (Figure 9.3). Whereas the microbial community was significantly diminished 21 days after ISCO, six months later, 16S rRNA gene copies of total bacteria, *Geobacter*, and *Desulfitobacterium*, were each 2 orders of magnitude higher than prior to chemical oxidation. This suggests a somewhat stimulating effect of ISCO on microbial growth and the potential for bio-

polishing. Subsurface conditions were to some extent conducive to microbial growth. Groundwater ORP and pH did return to values close to those observed during pre-oxidation measurements, although six months after treatment pH remained below 6. Some rebound of contaminant concentrations to total of 799 $\mu\text{g/l}$ was observed four months after ISCO once persulfate finished reacting (Table 9.4), which most likely also supported regeneration of OHRB community and highlights the necessity of a bio-polishing step.

On the other hand, neither *D. mccartyi* nor any *rdh* genes were detected during the final monitoring (Figure 9.3). This is in contrast to the pre-ISCO results, where *D. mccartyi*, *bvca* and *vcrA* were present. Although some VC was measured four months after ISCO, the absence of these *rdh* genes indicates at least a temporary disruption in the NA capacity of the microbial community. This may be a direct result of oxidation on the microbial community, or the presence of electron acceptors such as Fe^{3+} (activator of persulfate) or SO_4^{2-} (from persulfate), which might interfere with complete OHR. Diminished electron donor abundance following chemical oxidation may also inhibit full regeneration. While similar rebound is expected for wells N10 and N5, due to other activities at the site, only a limited number of wells could be sampled at the six month time point.

Six months after ISCO, N11 well at depths of 5 and 10 m were monitored to determine if any long-term or delayed influence of chemical oxidation could be observed. These wells were not within the treatment area for ISCO, as seen in stable pH and ORP measurements (Figure 9.5). Nonetheless, an increase in *D. mccartyi* abundance 21 and 36 days after ISCO in the shallower N11 well (5 m) was observed, most likely due to groundwater mixing during injection. Although these wells lay directly down gradient from the northern ISCO site around well N5 and in close proximity to well N10 which was significantly impacted, microbial community size and structure did not drastically change between groundwater sampled before ISCO and six months after ISCO (Figure 9.5). This indicates that upstream impacts of ISCO were not directly transferred to downstream wells by groundwater flow.

9.4 Discussion

9.4.1 Impact of ISCO on microbial community

Through bio-molecular monitoring of microbial communities during an ISCO treatment at a location with NA activity, this study gave unique insight into the impact of persulfate on microbial OHR capacity. In general, characterization of NA capacity

using molecular-based assays often focuses on discerning the heterogeneity of OHRB distribution under varying geochemical and contaminant conditions [33, 56], rather than looking at the impact of chemical oxidation. As ISCO is infrequently applied at locations where OHR has been confirmed, there exists very limited research into the impact of ISCO on OHRB, their recovery and subsequent remediation potential. This knowledge gap precludes the development of techniques that support a biopolishing step following ISCO at locations contaminated with chlorinated solvents.

In our work, we were able to identify changes in the endogenous NA capacity due to chemical oxidation and the associated changes in groundwater pH and ORP. While significant reduction in microbial community size and OHRB abundance was observed in wells impacted by persulfate oxidation (Figure 9.3), disruption was temporary, and regeneration of many target biomarkers was observed (Figure 9.4). Such rebound of OHR capacity has been observed previously in microcosm [54] and column studies [218] with permanganate oxidation of PCE;. In the column experiment, a similar pH drop was seen, in line with the groundwater acidification in our field study [218]; however, with an oxidation potential of 1.7V, permanganate is a less aggressive oxidant than either the sulfate radical (2.5V) or persulfate (2.0V; [96]). In contrast to our study, Sahl and colleagues (2007) required media amendments to stimulate microbial growth. In the field, amendments maybe less necessary, as re-inoculation can occur naturally in such an open system and chemical oxidant delivery is most likely more heterogeneous, thereby providing niches in which the microbial community remains intact.

Our study revealed that certain OHRB were more resilient to chemical oxidation. For example, *Geobacter* and *Desulfitobacterium* abundances were less impacted by persulfate (Figure 9.3). *Geobacter* remained present during treatment in well N10 and *Desulfitobacterium* abundance showed some rebound within 36 days in well S1. After six months, both of these targets had regrown to concentrations up to 3 orders of magnitude higher than those present prior to treatment in well S1 (Figure 9.4). The resilience of *Geobacter* and *Desulfitobacterium* may be related to their versatile metabolism as these bacteria are not restricted to organohalide respiration [153]. In addition to a higher growth rate, their ability to utilize a more extensive list of electron donors and acceptors than, for example *D. mccartyi* as a dedicated OHRB, including Fe(III) generated during ISCO treatment, may provide an advantage following a disruptive event, such as ISCO. Chemical oxidation degrades soil organic matter, thereby changing the availability of substrates [104, 256]; electron donor and nutrient abundance can also influence the ability of microbes to regenerate.

Thus, the absence of *D. mccartyi* may be attributable to the oxidation of adequate electron donor, implying that regeneration could be expected upon amendment with substrate.

While regeneration of *Geobacter* and *Desulfitobacterium* is promising, all currently available isolates from these genera lack the ability to perform full dechlorination of PCE or TCE to ethene. Thus, the absence of *D. mccartyi* in well S1 at the six month time point indicates that disruption of full NA capacity is ongoing (Figure 9.3). On the other hand, regeneration of *Dehalobacter*, which is also a slow grower, was observed, which could be promising for continued regeneration in the future (Figure 9.3; [153]).

Well S1 was an example of revival of the microbial community and the potentially stimulating effect of ISCO on certain OHRB. Additionally, under situations where less aggressive changes in groundwater parameters occurred, a slight increase in microbial population size and OHRB abundance was observed (well N5, Figure 9.4). Together, these results highlight the potential for bio-polishing following ISCO. For such a treatment, chemical oxidation should be applied sparingly, in order to ensure sufficient survival of the microbial community. Both the injection rate and the concentration of oxidants have been found to have an impact on regeneration of endogenous OHRB in column studies [218]. Additionally, in the field, the extent of oxidant distribution determines whether some areas are left unaffected, thereby providing a source for re-inoculation, as seen in both N11 wells in our study (Figure 9.5). Overall, our results indicate that ISCO treatments should be designed in a manner that accounts for a subsequent bio-polishing step in order to optimize a biphasic approach at a location.

9.4.2 Importance of molecular tools

In addition to illuminating the impacts of ISCO on the microbial community, our results also highlighted the usefulness of molecular tools. Due to heterogeneous contaminant distribution, groundwater flow, and concentration rebound following ISCO, it can be difficult to confirm ISB potential solely based on routine physical-chemical monitoring. Molecular tools are especially applicable to locations contaminated with chlorinated solvents, which in general require more specialized microorganisms and more specific geochemical conditions for biodegradation. In our study, qPCR was used to determine the presence of degradation capacity prior to treatment, identify wells impacted by ISCO, confirm the regeneration of biomass and degradation capacity, and identify wells in which the microbial community was not

impacted by ISCO, which could serve as point sources for re-inoculation during a subsequent ISB step.

The application of molecular tools is limited by the sampling techniques available. Groundwater samples, which only include planktonic microbial communities, do not give an indication of the soil-associated biofilm portion of the population. Nonetheless, groundwater sampling has become a standard practice in the field, as it allows for detailed and non-invasive sampling at individual locations over a long period of time. Additionally, studies have suggested that *Dehalococcoides* presence as planktonic microorganisms is similar if not higher than those observed associated in the solid phase [209, 215]. This indicates that groundwater sampling provides a sufficient indication of the capacity for OHR.

Molecular-based assays may prove essential to the effective design and application of biphasic treatments coupling ISCO and ISB. Our results not only indicate that regeneration can occur following ISCO, but that less aggressive treatments may even be somewhat stimulating for biological activity. An explanation of this may be that ISCO oxidizes soil organic matter into more readily degradable substrates, as found in other studies [104, 256]. Molecular monitoring could be integrated into ISCO projects to give a more real-time indication of the impact of ISCO on the microbial community and the extent of disruption and regeneration. Combining such data from a variety of locations would give more general knowledge on microbial regeneration capacity, provide quantitative limits to regeneration, and identify microorganisms with higher resilience to harsh ISCO treatment. In the future, molecular data could be used to guide field application of ISCO in a manner that optimizes a subsequent bio-polishing phase.

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Chapter 10

General Discussion:

**Towards understanding subsurface processes
during soil remediation**

10.1 Introduction

With rising awareness of the costs and environmental impacts associated with soil remediation, increasing focus has been placed on the development of cost-effective and efficient remediation technologies. Current efforts aim to further improve *in situ* remediation practices, expanding their versatility through the application of treatment trains in which multiple techniques are used sequentially. This dissertation investigated one such treatment train, the coupling of *in situ* chemical oxidation (ISCO) with *in situ* bioremediation (ISB), for the remediation of organic contaminants in subsurface systems. Experimental setups considered remediation efficiency as an essential factor in further optimizing the application of *in situ* remediation technologies. To this end, conclusions were drawn on the advantages of applying chemical oxidation prior to bioremediation, and recommendations are given for further improvements.

However, rather than solely focusing on the quantity of contaminant degraded, this dissertation especially aimed at understanding the key factors and processes behind remediation. By approaching research into soil contamination and remediation from a more fundamental framework, this dissertation drew conclusions on subsurface processes and microbial community dynamics. The impacts of contamination and remediation treatments on each of these were investigated in order to develop a better understanding of the subsurface, thereby identifying the underlying processes or factors responsible for improved or reduced remediation efficiencies. The latter portion of this discussion integrates the outcomes of each chapter to provide conclusions on subsurface processes and give recommendations on utilizing contaminated areas and remediation projects as systems for fundamental scientific investigations.

10.2 Outcomes

The research chapters in this dissertation included a wide range of experimental conditions, with each chapter focused on investigating a specific system. Two classes of contaminants, TPH and chlorinated ethenes, were investigated, which necessitated either aerobic or anaerobic bioremediation conditions, respectively. Laboratory experiments focused on microcosms containing soil, as well as on more defined and homogeneous liquid cultures. Field studies encompassed both pilot and full-scale treatments, with each allowing for different levels of monitoring. The individual

investigations described in this dissertation focused on elucidating the influence of a variety of parameters on the system, including treatment and soil type, soil and groundwater geochemistry, contaminant characteristics, and microbial community diversity and function (Table 10.1).

While the systems investigated, and thus experimental designs, differed between chapters, integrating these results leads to a number of outcomes. These outcomes, as described in the following sections, support the application of coupled chemical oxidation with bioremediation. Additionally, the work presented in this dissertation gives insights into the subsurface ecosystem, including microbial resilience and subsurface processes occurring during and after chemical treatment. Finally, the application of molecular tools, a mainstay in this dissertation, is essential to understanding microbial response to chemical oxidation, which provides insights for both the fields of soil remediation and microbiology.

Table 10.1 Summary of the diverse parameters investigated in the various chapters in this dissertation.

Parameter	Chapter							
	3	4	5	6	7	8	9	
soil type	X	X		X				
soil characteristics ^a					X			
groundwater geochemistry ^b			X	X	X		X	
contaminant bioavailability	X		X	X				
chemical oxidant type				X				
nutrient amendment			X		X			
microbial community	X	X		X	X	X	X	

^a soil characteristics are limited to specific analysis of soil organic matter and mineralogy

^b groundwater geochemistry includes parameters such as pH, oxidation-reduction potential, dissolved organic carbon, aqueous nutrient species

10.2.1 The advantages of coupling ISCO with ISB

With the increasing application of *in situ* remediation has come greater interest in the improved application of these technologies [111, 239]. The combination of chemical oxidation with bioremediation has been gaining acceptance as a robust technology that can reduce the treatment costs while delivering improved remediation efficiencies [217, 255]. In this dissertation, different aspects of the system were investigated (Table 10.1), with the results validating the application of coupled ISCO and ISB for the remediation of organic contaminants.

The major conclusions of this dissertation support the implementation of a bioremediation step following chemical oxidation. Both laboratory and field experiments indicated that in many instances a chemical treatment is either required prior to bioremediation to ensure degradation occurs, or enhances natural attenuation capacity. Previous studies often focus on chemical treatments with high mass removals followed by some bio-polishing [35, 103, 274, 299]. In this dissertation, the chemical oxidation treatments were in most cases moderate, with the experimental design focused on utilizing ISCO as a true pretreatment and balancing this with bioremediation for the rest of mass removal. This design gave insight into subsurface processes occurring after chemical oxidation (section 10.2.3), while simultaneously allowing for improved design of biphasic treatments.

In **Part One**, the importance of pretreatment with chemical oxidants was illuminated in investigations of a TPH-contaminated site in the laboratory ([Chapter 3](#)) and in the field ([Chapter 4](#)). It was determined that bioremediation was inhibited by contaminant and subsurface characteristics. Biodegradation experiments performed in [Chapter 3](#) with field-collected soils showed that, even under very ideal conditions, full remediation would most likely be inhibited by the bioavailability of the contaminant. Bioavailability is an important parameter that can prevent timely full remediation of a location [52]; residual contaminant can prevent release of the location for non-restricted use and may pose a risk due to its gradual desorption from soil into the aqueous phase [230]. In [Chapter 4](#), a survey of the microbial diversity *in situ* indicated that the low oxidation-reduction potential also hindered biodegradation of TPH. While other amendments such as surfactants or bioventing could improve conditions for bioremediation, both treatments would be required for remediation at this location. Alternatively, chemical oxidation simultaneously improves bioavailability ([Chapter 5](#)) and raises the oxidation-reduction potential (through the addition of electron acceptors). Together, the results of [Chapters 3 and 4](#) indicate that the application of chemical oxidation will improve a number of conditions for bioremediation and thus support the application of biphasic treatment.

The coupling of chemical oxidation with bioremediation was also verified by the overall remediation efficiencies achieved in work described in this dissertation (Figure 10.1). In **Part Two** [Chapters 5 and 6](#), higher remediation efficiencies were often achieved when bioremediation was preceded by chemical oxidation as compared to the biotic control without chemical treatment. In [Chapter 5](#), the highest overall remediation efficiency was with Fenton's reagent treatment followed by nutrient amendment; when nutrients were not added, notably higher remediation

efficiencies and higher respiration rates were observed following Fenton's reagent treatment. In [Chapter 6](#), overall remediation efficiencies were higher for fill when Fenton's reagent or Modified Fenton's reagent was used as compared to the biotic control. Similar results for peat may have been achieved if longer bioremediation periods had been applied to compensate for TPH mobilized during the chemical treatment. Previous investigations have also found that improved remediation efficiencies can be achieved when coupling chemical oxidation with bioremediation [103, 274, 299]. Together, this supports the application of biphasic treatment while simultaneously spurring interest into the geochemical and biological processes behind these improved remediation efficiencies.

This dissertation showed that not only can biphasic treatment result in more contaminant degradation, but also that a bio-polishing step is often required following chemical oxidation to mitigate risks associated with mobilization and rebound. Contaminant mobilization occurs due to either (1) partial oxidation of contaminants, causing increased solubility, or (2) when chemical oxidants degrade soil organic matter (SOM) resulting in desorption of hydrophobic contaminants previously tightly bound to the soil matrix [121, 210]. Additionally, once oxidants have been fully consumed, further desorption of contaminants from the soil matrix causes rebound of aqueous phase contaminant concentrations [172, 173]. In this dissertation, both contaminant mobilization ([Chapter 6](#)) and rebound ([Chapters 7 and 9](#)) were observed. Moreover, increased aqueous phase contaminant concentrations were effectively mitigated with bioremediation. In the case of TPH contaminated soil, the improved biodegradability and bioavailability of hydrocarbons were advantageous to the bioremediation step ([Chapter 6](#)). Additionally, as microbes preferably consume low molecular weight compounds, which are the most mobile and thus present the highest risk [36, 186], biodegradation directly targets the most risky fraction of TPH ([Chapter 5](#)). Finally, biomolecular tests in [Chapters 7 and 9](#) indicated that following ISCO the microbial community in the field was able to degrade rebound contaminant concentrations, which suggests that continued biodegradation could occur and would thus mitigate risks associated with rebound.

Finally, **Part Three** of this dissertation supports the application of chemical oxidation coupled to bioremediation for treatment of locations contaminated with chlorinated solvents. Whereas applying ISCO followed by aerobic bioremediation of various hydrocarbon contaminants has been well investigated, very limited research has been performed to determine the feasibility of anaerobic bioremediation following ISCO [87, 218, 232]. Studies in the lab ([Chapter 8](#)) and the field ([Chapter 9](#))

indicated the resilience of organohalide respiring bacteria (OHRB) and their capacity for regeneration of organohalide respiration (OHR) following chemical treatment. As acceptability of biphasic treatment of chlorinated solvents increases, research should focus on further optimizing application of anaerobic bioremediation following chemical oxidation.

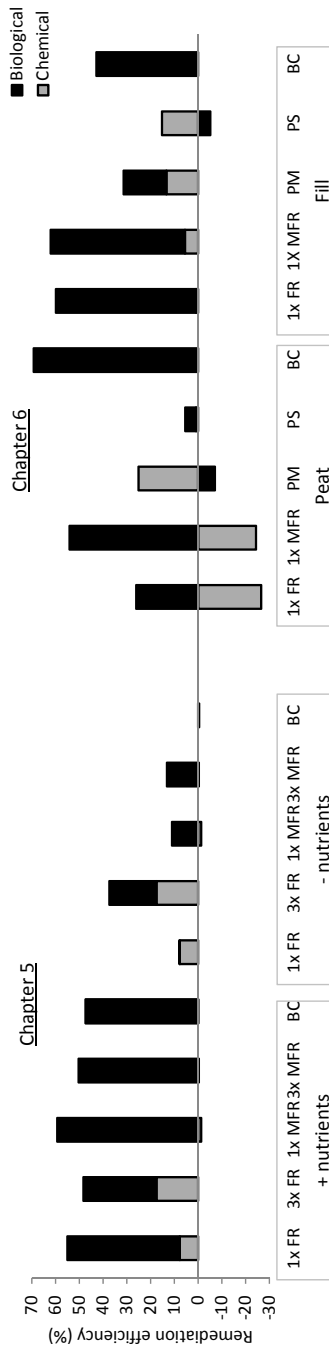


Figure 10.1 Remediation efficiencies achieved in this dissertation through chemical oxidation (gray) and bioremediation (black). Results are presented for research performed within Chapter 5 (left) and Chapter 6 (right). Positive remediation efficiencies indicate contaminant removal, while negative remediation efficiencies indicate mobilization. Chemical treatments investigated are Fenton's reagent (FR), Modified Fenton's Reagent (MFR), permanganate (PM), persulfate (PS), and biotic control (BC). The impact of nutrient amendment was studied in Chapter 5; soil type was investigated in Chapter 6.

10.2.2 Microbial resilience

The deleterious effects of chemical oxidants on the microbial community have often been assumed to fully prohibit bioremediation following ISCO. Oxidative and pH stress in particular can disturb microbial communities and disrupt biodegradation [31, 149, 169, 207]. In this dissertation, the resilience and regenerative capacity of the microbial community following chemical oxidation were tested in a variety of closed- and open-system experimental setups. Not only was regeneration observed under nearly all treatments, but in some cases chemical oxidation appeared to have a stimulatory effect, especially on degraders. Together these factors contributed significantly to the higher remediation efficiencies observed in biphasic treatments (Section 10.2.1).

In this dissertation, focus was placed on investigating the natural capacity for microbial communities to regenerate following chemical oxidation. Thus, no bioaugmentation was performed in microcosm experiments. Nonetheless, rebound of microbial activity was observed in laboratory experiments following a variety of chemical oxidation treatments, as described in Chapters 5, 6, and 8. Regeneration occurred after application of Fenton's reagent, modified Fenton's reagent, and permanganate for the treatment of TPH and chlorinated solvents. While biomolecular tools were often used to investigate microbial community diversity and bioremediation capacity, multiple lines of evidence are required to definitively determine biodegradation activity. In this dissertation, variations in contaminant concentration and composition, changes in aqueous phase components (i.e. dissolved organic carbon [DOC] and nutrients), and measurements of respiration levels were used to confirm degradation activity and gain better insight into the limits of regeneration (Table 10.1).

Similarly, regeneration of the microbial community was also observed in open field systems. In Chapters 7 and 9, biomolecular assays confirm the regeneration of biodegradation capacity for remediation of TPH (Chapter 7) and chlorinated solvents (Chapter 9) following persulfate treatment. While no microbial activity was seen following microcosm-scale persulfate treatment (Chapter 6), significant regeneration of TPH biodegradation capacity was observed following a pilot injection in the field (Chapter 7). Although the heterogeneity and groundwater flow in open systems are more conducive to restoration of the biological activity, it is nonetheless noteworthy that microbial community size and degradation capacity rebound even in acidic groundwater (pH<5; Chapter 7). Likewise, regeneration of OHRB and reductive dehalogenase gene (*rdh*) abundance was observed following persulfate treatment

without electron donor amendment or bioaugmentation ([Chapter 9](#)). While lab-scale investigations into coupling chemical oxidation and anaerobic bioremediation have been reported [54, 87, 218], the field-scale investigation in [Chapter 9](#) is novel in the scientific literature. These results support the coupling of chemical oxidation with reductive dehalogenation, thereby opening a new chapter in the remediation of chlorinated solvents and investigations into the resilience and regeneration capacity of OHRB.

In some instances, biomolecular assays specific for bioremediation capacity indicated an enrichment of biodegraders following chemical oxidation. While deleterious effects of ISCO on microbial communities are well known, this work displays the stimulatory effects of chemical oxidation. Although the positive impact of ISCO on bioremediation has been reported in the literature [37, 114, 121], the studies in this dissertation provided quantitative data on the stimulatory effects of chemical oxidation on the microbial community. Seven days after treatment with Fenton's reagent, modified Fenton's reagent, and permanganate, an increase was observed in nearly all cases in the relative abundance of the *alkB* gene, a monooxygenase involved in hydrocarbon degradation ([Chapter 6](#); Figure 10.2). While no change was observed in the biotic control fill microcosm that did not undergo chemical treatment, in peat the relative abundance of the *alkB* gene decreased in biotic control microcosms. These results support the conclusion that chemical oxidation can have a stimulatory effect on the biodegrading portion of the microbial population. Similar enrichment of the *alkB* genes was measured in the field following each persulfate treatment ([Chapter 7](#); Figure 10.2). In wells within the radius of influence, enrichment of *alkB* relative to the initial situation prior to treatment was observed five days after the first persulfate injection, and eight days following the second ISCO application (performed on day 43). The rise in *alkB* abundance is most likely to some extent related to the increase in readily degradable organic substrates such as DOC and mobilized TPH following chemical oxidation, as this gene encodes a monooxygenase for degradation of any alkanes or alkenes [13, 116]. That said, the enrichment of *alkB* nonetheless points towards an enhancement of the biodegradation capacity following chemical oxidation.

An small augmentation in the capacity to degrade chlorinated ethenes was also observed following chemical oxidation with permanganate in microcosms ([Chapter 8](#)) and with persulfate in the field ([Chapter 9](#)). In [Chapter 8](#), following mild-dose sequential permanganate treatments, a slight initial enrichment was observed in certain OHRB in chemically treated microcosms, as compared to the biotic control

(Figure 10.3). Similarly, in [Chapter 9](#), some enrichment of OHRB and *rdh* genes was observed in wells receiving lower persulfate doses (Figure 10.3). However, in those microcosms (100 μmol permanganate) and locations (well S1) receiving the strongest oxidant treatments, the full capacity to transform PCE into ethene did not regenerate ([Chapters 8 and 9](#)). These results are novel, as thus far very few investigations have been oriented at the combination of chemical oxidation and OHR for the treatment of chlorinated solvents [232]. These results indicate some promise for expanding the application of chemical oxidation and bioremediation at locations contaminated with chlorinated ethenes. Additional research is required to establish threshold oxidant concentrations for regeneration and to determine ideal conditions for stimulation of biodegradation with ISCO treatments.

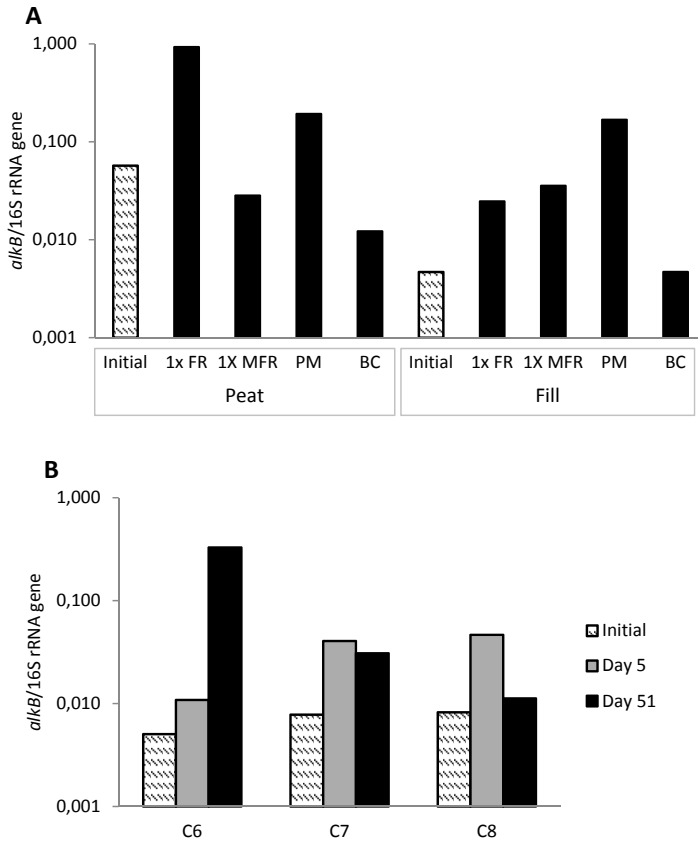


Figure 10.2 Enrichment of *alkB* gene following chemical oxidation in (A) laboratory experiments in [Chapter 6](#), and (B) in pilot-scale field experiments in [Chapter 7](#). Initial ratios prior to chemical treatment are given as patterned bars. (A) Black bars indicate gene ratios measured seven days after chemical oxidation. Treatments are Fenton’s reagent (FR), modified Fenton’s reagent (MFR), permanganate (PM) and biotic control (BC). (B) Gray bars indicate gene ratios measured five days after the first persulfate injection (on day 0) and black bars are ratios measured eight days after the second persulfate application (on day 43). Results are presented for wells C6, C7, and C8, which were within the radius of influence of the ISCO treatment and were monitored more often than other wells.

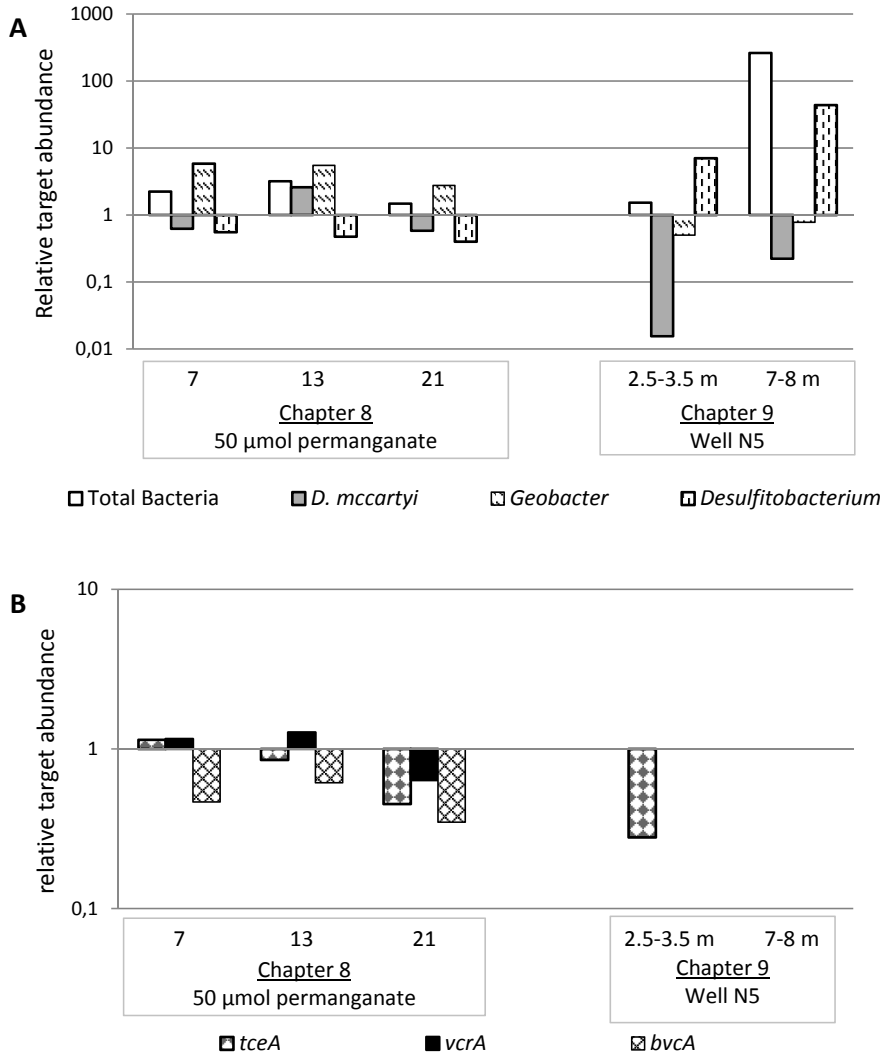


Figure 10.3 Relative abundance of total bacteria and OHRB (A) and *rdh* genes (B) found in Chapters 8 and 9. Values for Chapter 8 are calculated by dividing the target abundance for a certain time point by that measured in the biotic control for the same time point. Labels indicate the time point in the experiment in days. Values for Chapter 9 are calculated by dividing the target abundance for the 36 day time point by the before ISCO measurements. Results are given for the two depths at well N5

10.2.3 Insights into impact of ISCO on subsurface processes

The research aims and experimental approaches of this dissertation focused on not just improving the efficiency of remediation, but also understanding the implications of treatment for the subsurface. The most important results went beyond the field of soil remediation to give insights on temporary and permanent changes to the subsurface. The results provide new information concerning subsurface processes and how chemical oxidation impacts soil function, with some of these impacts found to affect treatment efficiency. As described below, both reversible and irreversible impacts of chemical oxidation have implications for microbial community diversity and function.

Chemical oxidation causes some changes to groundwater; although often reversible, these changes do temporarily affect subsurface processes and function (Figure 10.4). For example, groundwater acidification ([Chapters 7 and 9](#)) due to chemical oxidation interrupts microbial communities and impedes regeneration of biodegradation activity. Similarly, in the case of chlorinated solvents, an increase in oxidation-reduction potential also disrupts bioremediation ([Chapter 9](#)). While microbial resilience was in general able to overcome temporary changes in pH and oxidation-reduction potential, some disruption of normal subsurface function, as well as delay in biodegradation were incurred. Similarly, mobilization of DOC and TPH due to chemical oxidation also delayed the bioremediation phase. DOC from SOM oxidation was preferentially degraded over TPH ([Chapter 5](#)). While microbial activity did reduce DOC concentrations towards pre-oxidation concentrations, additional time and amendments were required for bioremediation of TPH to occur. TPH mobilized by chemical oxidation, as observed in [Chapters 6 and 7](#), was also mitigated by bioremediation but, at least in the case of peat in [Chapter 6](#), did reduce the remediation efficiency. Although these temporary changes were proven to naturally return to baseline conditions without interference, they do represent a disruption due to chemical oxidation.

Although the aforementioned subsurface changes were reversible, some impacts of chemical oxidation were found to be permanent (Figure 10.4). Such irreversible changes have long-term implications for soil characteristics, the microbial community, and thus subsurface processes. For example, extended periods of groundwater acidification and high oxidation-reduction potentials indicate a depletion of the soil's natural buffering capacity and oxidant demand ([Chapters 7 and 9](#)). While pH and oxidation-reduction potential did return towards pretreatment conditions, this came at the cost of the subsurface's capacity to buffer future fluctuations

in groundwater characteristics. Oxidation of SOM, the cause of increased DOC concentrations, permanently changes soil structure. As investigated in [Chapter 7](#), SOM characteristics were altered due to ISCO, indicating a permanent change in SOM quality. Additionally, release of carbon and nitrogen due to chemical oxidation ([Chapters 5 and 7](#)) irreversibly changed the available substrate and nutrient pools, thereby effecting soil function and microbial community composition.

In addition to changing SOM, chemical oxidation also impacted inorganic soil constituents (Figure 10.4). In [Chapter 7](#), changes to subsurface mineralogy due to persulfate treatment were investigated using a variety of techniques. Here, oxidation of metal sulfides to produce amorphous iron precipitates not only changed the soil structure, but also could potentially cause the mobilization of heavy metal contaminants. Also, the deposition of remediation products changes the subsurface. Gypsum, from persulfate-derived sulfate, was found as a precipitate, as noted in [Chapter 7](#), and manganese oxides, from permanganate, were reduced as an electron acceptor, as shown in [Chapter 8](#). The remaining concentrations of each of these remediation end-products can impact subsurface permeability, stability of mineral species, and oxidation-reduction potential.

In this dissertation, it was found that both contamination and remediation treatments exert selective pressure on the microbial community. When comparing contaminated and uncontaminated locations, dissimilar community diversity was observed in [Chapter 4](#). In TPH-contaminated locations, analysis indicated a statistically significant decrease in diversity and increase in the relative abundances of *Chloroflexi*, *Firmicutes*, and *Euryarchaeota*. Likewise, in [Chapter 3](#), higher *alkB* abundances were observed in TPH-contaminated soils as compared to uncontaminated soils of similar type. These findings indicate some of the implications of contamination on a microbial community and are a step towards better illuminating the results of such selective pressure.

In addition to contamination, remediation treatments also exert a selective pressure on the microbial community. As described in section 2.2, the relative enrichment of certain degraders following chemical oxidation is an example of the selective pressure that remediation treatments can have on microbial communities. On the other hand, chemical oxidation can fully disrupt portions of the population, as seen for *D. mccartyi* in [Chapters 8 and 9](#). Also, changes in the substrate and nutrient concentrations described above due to chemical oxidation surely influence microbial community composition. For example, in [Chapter 6](#) a higher abundance of *Betaproteobacteria* was observed following chemical oxidation. These copiotrophs

may have been more capable of rapidly utilizing mobilized aqueous carbon and nitrogen species released by chemical treatment [64]. Finally, the addition of electron acceptors derived from chemical oxidants impacts the microbial community. Electron acceptors such as oxygen (from hydrogen peroxide), iron(III) (used as catalyst in Fenton’s reagent), sulfate (from persulfate), and manganese oxides (from permanganate) change the oxidation-reduction potential and select for microbes capable of utilizing these species. This was observed in [Chapter 8](#), where a shift in the community from fermenters such as *Clostridia* to manganese reducers such as *Geobacter* was detected upon stronger chemical oxidation treatments. Together, these results give important insights into the interactions between environmental parameters and microbial community structure and ecology.

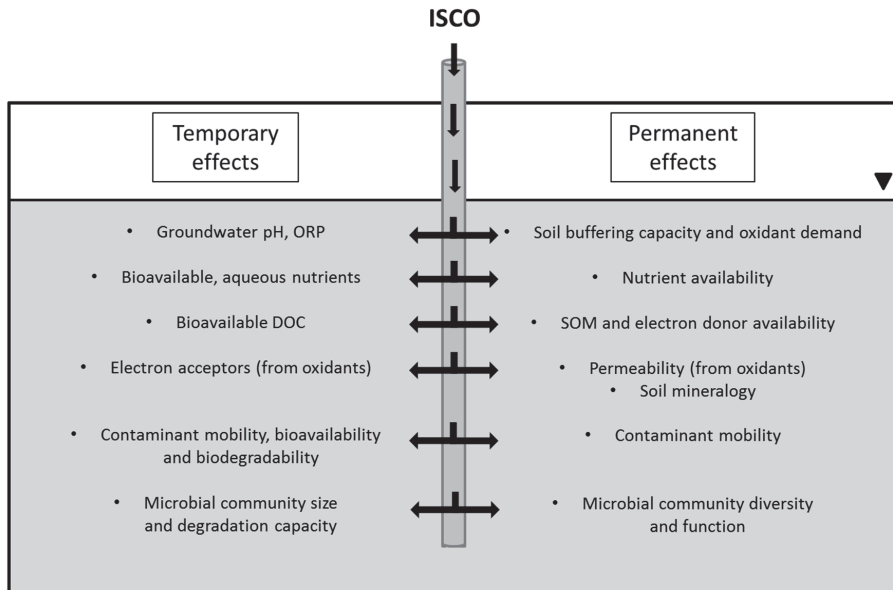


Figure 10.4 Schematic of temporary and permanent effects of ISCO on the subsurface. The parameters described are grouped to highlight the fact that some temporary effects are symptomatic of permanent changes to the subsurface.

10.2.4 Importance of molecular tools

Rapid developments in the area of culture-independent biomolecular tools have revolutionized our capacity to understand microbial diversity and function in response to specific ecosystems. Throughout this dissertation, DNA-based analyses were utilized to understand microbial community diversity and determine the capacity for biodegradation (Figure 10.5). This represents a new age of design and application of soil remediation techniques. While some biomolecular techniques have been previously used to confirm chlorinated ethene degradation capacity, soil remediation projects typically do not incorporate molecular tools into monitoring plans [232]. The results presented in this dissertation indicate that, when properly used, molecular techniques can be used to better understand subsurface processes and steer remediation projects.

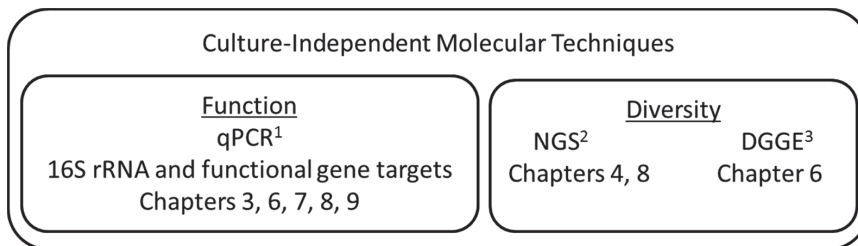


Figure 10.5 Summary of molecular tools utilized in this dissertation.

¹ quantitative Polymerase Chain Reaction

² Next Generation Sequencing

³ Denaturing Gradient Gel Electrophoresis

Function-based molecular techniques were used to confirm the presence of degraders and their degradation genes in experiments with TPH and chlorinated ethene contamination. Quantitative PCR targeted at either the 16S rRNA gene of specific degraders or at functional genes encoding for degradation enzymes gave insight into the capacity for bioremediation. In some instances, qPCR could confirm that factors other than biodegradation capacity were limiting bioremediation. For example, by showing that both the population size and *alkB* abundance were sufficient for biodegradation in [Chapter 3](#), it could be more definitively concluded that bioavailability restricted further TPH bioremediation. Similarly, the absence of bioremediation following permanganate treatment in [Chapter 6](#) could also not be attributed to microbial biodegradation capacity, as *alkB* was also detected in these

microcosms. In other cases, qPCR was used to identify chemical treatments which could be coupled with bioremediation and quantitatively define enrichment of degradation capacity. In Chapters 6, 7, 8, and 9, an increase in the relative abundance of some degraders was observed following certain chemical oxidation treatments (Figures 10.2 and 10.3). The ability to couple the results of biomolecular assays targeting specific degraders with results on degradation rates and geochemical conditions is a step forward in our understanding of both subsurface processes and execution of soil remediation technologies. The results of Chapters 7 and 9, in which regular monitoring of microbial biodegradation capacity was used to understand and direct the treatment, offer a glimpse into the future of soil remediation. With their improved reliability and decreasing costs, biomolecular tools will increasingly be applied in the future to steer soil remediation projects.

Whereas function-based assays can only be used to target known degraders and their functional genes, diversity-focused biomolecular tools can be used to illuminate the composition of the entire microbial community. Currently, only a small portion of the microbial community has been identified, and even less is known about the environmental pressures that select for certain segments of the community and specific functions. Expanding our understanding of microbial community diversity will improve our fundamental understanding of microbial ecology, while simultaneously identifying environmental conditions, community compositions, and individual species associated with improved biodegradation. In this dissertation, DGGE and NGS of the 16S rRNA gene were used to investigate microbial community diversity and its association to environmental conditions. For example, in Chapter 6, DGGE and sequencing analysis identified shifts in the microbial community following specific chemical treatments and during eight weeks of incubation. Similarly, NGS of microbial communities *in situ* at a TPH-contaminated location gave insights into the selective pressure of contamination on the microbial community. Finally, in Chapter 8 NGS was utilized to identify portions of the population that support the regeneration of OHR following chemical oxidation. Each of these studies was designed to yield both insights into community structure and function within certain environmental conditions while simultaneously linking community diversity to the presence and degradation of contamination. These types of studies will prove critical as we continue to explore microbial ecology and capacity for biodegradation.

10.3 Future perspectives

10.3.1 Improving prediction capacity for coupled ISCO and ISB systems

While the advantages of coupling ISCO with ISB have been confirmed, further research is required to improve the application of this treatment train. A major challenge in any *in situ* remediation project is the predictability of the treatment and outcomes. In the field, treatments are often robustly executed and thus include excesses of all remediation products in order to ensure high remediation efficiencies. In contrast, coupling ISCO with ISB is most advantageous when a mild chemical treatment is applied [103, 255, 274]. While this can reduce costs, the uncertainty of the outcome of less-engineered systems is a major drawback which can preclude implementation in the field. Extending our understanding of the effects of ISCO on the subsurface system and on a subsequent ISB phase is integral to advancing our capacity to predict the outcomes of biphasic treatment. This predictive power is essential to further developing the combination of chemical oxidation and bioremediation into a full-fledged treatment. For this, strong collaboration should be cultivated between scientific institutions and practitioners in the soil and water sector in order to promote innovation and understanding.

The overall outcomes of coupling ISCO with ISB are highly dependent on the system size, soil type, contaminant characteristics, and geochemistry. Similar variability in the results of treatments was observed in this dissertation. For example, persulfate completely inhibited microbiological activity in batch experiments in [Chapter 6](#); however, it was compatible with bioremediation in field applications in [Chapters 7 and 9](#). Similarly, the two soils tested in [Chapter 6](#) reacted differently to identical chemical oxidation treatments. Whereas a pretreatment with Fenton's reagent or modified Fenton's reagent was advantageous to the bioremediation phase in fill, mobilization of TPH in the peat soil upon chemical oxidation reduced the overall remediation efficiency. These differences in the outcomes of biphasic treatment highlight the necessity for complete testing of soils and treatments on a variety of scales (microcosm, column, and pilot) prior to application in the field.

A better understanding of the influence of chemical oxidants on soil and contaminant characteristics is required to improve knowledge to a point that allows proper prediction of the outcomes of treatments. Whereas previous focus has been placed on the oxidative and pH stress placed on the microbial community, this dissertation indicated that the impacts of chemical oxidation go beyond temporary disruption of microbial function. Changes in soil, groundwater, and contaminant

characteristics were shown to impact the bioremediation step and thus influenced the overall treatment efficiency. For example, it was found that the mobilization of DOC and nutrients observed in [Chapters 5, 6 and 7](#) due to chemical oxidation can serve as substrates and nutrient reserves for the microbial community. While these aqueous components contributed to biological activity, DOC was utilized as an alternative electron donor to biodegradation of TPH, which would increase the amount of additional electron acceptor and nutrient amendments required. The uncertainty of the necessity of, and additional costs associated with, these amendments is an example of an area in which further work is required to improve our ability to predict the outcomes of treatment.

Similarly, future work should focus on improving our capacity to predict the extent of contaminant mobilization after chemical oxidation. Mobilization of TPH ([Chapters 6 and 7](#)) and low concentrations of toxic heavy metals ([Chapter 7](#)) can pose an environmental risk. While mobilization of organic contaminants can increase bioavailability and biodegradability, thereby improving the overall remediation efficiency, mobilization does come with additional costs. Bioremediation can to some extent reduce the concentration of mobilized organic contaminants; however, extending the length of treatment and implementing additional mitigation strategies does increase the cost of the treatment. Being able to predict the extent of mobilization and thus to design proper mitigation is essential to improving the robustness of coupled ISCO and ISB.

Finally, additional research is required to better establish the timing of chemical oxidant additions and nutrient amendments. Whereas regeneration has been observed without amendment ([Chapters 7 and 9](#)), full scale field applications of coupled ISCO and ISB will most likely require amendments to support bioremediation, and treatment may even require multiple chemical oxidation steps. Proper monitoring of the subsurface must go beyond determining the current conditions and pursue the goal of predicting future amendment types and requirements. For example, in [Chapter 7](#), it was shown that an increase in pH and decrease in oxidation-reduction potential indicated a return towards pretreatment conditions; these data were used to determine the timing of a second persulfate injection and subsequent nutrient amendment. Similarly, in [Chapter 9](#), rebound of aqueous PCE concentrations indicated that a bio-polishing step could be used to ensure remediation at the site. This information, combined with biomolecular analyses of the microbial community showing that full OHR capacity had been disrupted, could be used to design electron donor or bioaugmentation events to ensure full PCE degradation to ethene.

The capacity to determine which parameters must be monitored in order to utilize the optimal type and timing of additions is required to further improve the application of remediation. Whereas clearly the key parameters and types of amendments will be contaminant and to some extent site specific, increased experience and a good understanding of subsurface processes will contribute to our capacity to predict the course of remediation projects. For this, it is essential to perform experiments in which equal focus is placed on both the remediation efficiency as well as a fundamental understanding of the processes occurring. Research of this nature, especially on field scale, is essential to improving the capacity to make decisions based on subsurface processes rather than above ground boundary conditions.

10.3.2 Opportunities for investigating subsurface systems

While the focus of this dissertation was *in situ* soil remediation, the research scope went beyond a strict examination of contaminant degradation and remediation efficiencies. Rather, explicit attention was placed on a fundamental investigation of the subsurface ecosystem as an interesting and scientifically relevant area for research. As described in the introduction, investigations considered the subsurface as an integrated system where the influence of a variety of parameters were considered (Table 10.1). Within this framework, microbial community diversity was considered especially relevant, from both a soil remediation and a microbial ecology perspective. Additionally, focus was placed on investigating systems contaminated with chlorinated solvents and performing field investigations. While the results of this dissertation do contribute to improved understanding of subsurface systems, this work reflects only a small portion of potential areas of research. Considering the increasingly diverse toolbox of research techniques and equipment available to investigators, and the need for a thorough understanding of complex and valuable subsurface systems, the opportunities for scientifically- and socially-relevant research are numerous.

Contaminated locations and remediation projects are attractive systems for more fundamental scientific studies on the subsurface. Contamination and remediation products influence soil and groundwater geochemistry. They exert selective pressure on the microbial community in a manner which is otherwise seldom encountered, thereby making these systems especially interesting from a scientific perspective. Whereas previously it was difficult to properly measure and describe changes in subsurface parameters, new analytical techniques are increasingly making high resolution and highly accurate investigations into the subsurface possible. For example,

advances in the available types and sizes of electrodes allow for high resolution determination of aqueous phase components [180]. Similarly, high-resolution soil characterization with advanced spectroscopy and microscopy methods allows for illumination of the biogeochemically relevant surfaces of soil minerals and organic matter [205]. Such measurements together give insight into the characteristics of niche environments in heterogeneous subsurface systems beyond what can be achieved with bulk groundwater and soil measurements. Finally, recent advances in the availability and application of high-throughput, culture-independent techniques allow for the illumination of microbial community composition and function [48]. While further progress is required in the resolution of these techniques [287], such “-omics” fields as metagenomics and transcriptomics give important insight into the influence of soil and groundwater geochemistry on microbial diversity and activity. These techniques will prove essential to furthering our understanding of soil biogeochemistry and sustainable use of the subsurface.

In addition to a growing tool box of techniques available for research into subsurface systems, the increasing need for such research is evident. Whereas industrial contamination was previously considered to be a scientifically separate system from more traditional agriculturally-focused soil science investigations, these fields are increasingly overlapping. On the one hand, growing population size and thus dwindling space availability necessitate the reuse of contaminated locations for urban or agricultural purposes. On the other hand, acknowledgment of the diffuse presence of such materials as pesticides, s in agricultural areas proves that soil contamination and remediation are not limited to urban activities. The importance of the subsurface for agriculture and urban uses, for filtration of contaminants in the water cycle, and as a resource for drinking water necessitates thorough investigations into soil and groundwater. Thus, multi-disciplinary research illuminating subsurface processes must be executed that integrates the fields of soil remediation and soil science.

When properly executed, such integrated studies have the capacity to answer scientifically and socially relevant research questions. For example, the increasing awareness of the threat of micropollutants in the environment will necessitate a thorough understanding of the subsurface processes occurring in contaminated locations. The knowledge gained from decades of research on single-contaminant industrial locations will have to be translated into recommendations on dealing with diverse and diffuse micropollutant contamination. An integrated understanding of subsurface geochemistry, biochemistry, and microbiology will be required to determine the factors essential to sequestration and remediation of micropollutants.

The field of micropollutants is one example of the growing focus being placed on utilizing engineered natural systems for the treatment of waste streams and the sustainable production of resources. For such systems, a thorough understanding of the processes occurring in the subsurface is necessary to properly utilize and protect soil systems as well as for the mimicking of natural processes in engineered *ex situ* reactors. While microbial capacity to catalyze a variety of reactions has been acknowledged, significant research is required to determine the community composition and environmental conditions required for such transformations. Considering the extensive microbial diversity present in soil, these systems represent a pool of environmentally relevant enzymes which may prove essential to sustainable treatment processes and closing resources cycles.

The opportunities to utilize the subsurface are numerous. The more practical use of subsurface processes for contaminant degradation is one highly relevant example. However, fundamental research into subsurface processes will prove essential to furthering our capacity to utilize natural systems to solve environmental problems. Investigations into soil and groundwater contamination and remediation offer the opportunity to research essential microbiological and geochemical processes occurring within well-characterized subsurface systems and their relationship to surface and aquatic processes as an integral part of the global environment.

Chapter 11

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Appendices

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Appendix 2 Summary of diesel contaminated soils used in this dissertation. Differences in contaminant concentrations, bioavailability, and chemical characteristics reported in each chapter can be explained by differences in subsamples taken for each experiment and by changes in the soil sample during storing.

Depth cm bgl	Groundwater level cm bgl	Soil Matrix	Chapter 3		Chapter 4	Chapter 5	Chapter 6	
			Location	Name	Name			
0-100	230	Fill	I	FL	A1	soil		
130-230		Fill	I	FH	A2			Fill
0-70		Fill					B1	
80-150	170	Clay	II	CL	B2			
150-170		Peat			B3			
150-250		Peat	II	PH	B4		Peat	
20-90	200	Fill	V	FU	C1			
90-180		Peat	V	FU	C2			
200-220		Peat			C3			
0-130	200	Fill			D1			
130-160		Clay	III	CU	D2			
170-200		Clay	III	CH	D3			
200-230		Peat	III	PL	D4			
230-250		Sand	III	SH	D5			
40-120	120	Fill			E1			
120-130		Fill			E2			
80-150	190	Sand	IV	SU	F1			
190-220		Sand	IV	SU	F2			
50-120	unknown >180	Fill			G1			
120-160		Fill			G2			
160-180		Fill			G3			
10-40	40	Peat			H1			
40-70		Peat			H2			
70-100		Sand			H3			
10-40	240	Sand			I1			
70-240		Sand			I2			

Appendix 3 Overview of primers used for qPCR assays for OHRB and *rdh* genes.

Primer	Sequence	Target 16S rRNA / gene	Reference
341F 534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGC	All Bacteria	[174]
Dco728F Dco944R	AAGGCGGTTTTCTAGGTTGTCAC CTTCATGCATGTCAAAT	<i>Dehalococcoides mccartyi</i>	[244]
Dre441F Dre645R	GTTAGGGAAGAACGGCATCTGT CCTCTCCTGTCTCAAGCCATA	<i>Dehalobacter</i>	[244]
Geo557F Geo822R	GCGTGTAGGCGGTTTTSTTAA TACCCGCRACACCTAGTACT	<i>Geobacter</i>	[11]
Dsb406F Dsb619R	GTACGACGAAGGCCTTCGGT CCCAGGGTTGAGCCCTAGGT	<i>Desulfitobacterium</i>	[244]
TceA1270F TceA1336R	ATCCAGATTATGACCCTGGTGAA GCGGCATATATTAGGGCATCTT	<i>tceA</i>	[99]
Bvc925F Bvc1017R	AAAAGCACTTGCTATCAAGGAC CCAAAAGCACCACCAGGTC	<i>bvcA</i>	[209]
Vcr1022F Vcr1093R	CGGGCGGATGCACTATTTT GAATAGTCCGTGCCCTTCCTC	<i>vcrA</i>	[209]

Nederlandse Samenvatting

De ondergrond is een systeem dat bestaat uit organische, anorganische en microbiologische componenten, die samen verschillende processen uitvoeren zoals nutriënten cycli, koolstofvastlegging en waterfiltratie. Bestaand onderzoek heeft zich vooral gericht op het begrijpen van het samenspel van deze dynamische microbiologische, geochemische en fysische processen. Verontreiniging van de ondergrond als gevolg van stedelijke en industriële activiteiten vormt een ernstig milieurisico. Om deze locaties te zuiveren en in hun oorspronkelijke staat te herstellen is het noodzakelijk dat efficiënte saneringstechnologieën ontwikkeld worden. Echter, de aanwezigheid en de behandeling van deze verontreinigingen biedt ook mogelijkheden voor wetenschappelijk onderzoek naar fundamentele onderwerpen op het gebied van de microbiële ecologie en bodemgeochemie. Dit proefschrift beschrijft *in situ* chemische oxidatie (ISCO) en *in situ* bioremediatie (ISB) van organische verontreinigingen, met een primaire focus op het verklaren van de dynamische microbiologische en geochemische processen die optreden tijdens de sanering (Hoofdstuk 1).

Het gebruik van sterke oxidanten tijdens ISCO veroorzaakt grote veranderingen in de eigenschappen van zowel de verontreiniging als de bodem en heeft invloed op de grootte, structuur en functie van de microbiële populatie. Toch kan ISCO, mits correct gebruikt, een nuttige voorbereiding op ISB zijn. Literatuurstudie toont aan dat bestaand onderzoek vooral gericht is op (1) het optimaliseren van de chemische oxidatie behandeling om een volgende stap in het proces van biologische afbraak te ondersteunen, en (2) het vaststellen van de wijzigingen die nodig zijn om bioremediatie op gang te brengen na een ISCO behandeling (Hoofdstuk 2). In sommige gevallen kan volledige bioremediatie niet worden bewerkstelligd doordat de biobeschikbaarheid van de verontreiniging of de redox potentiaal van de ondergrond ontoereikend is. Dit zijn allebei obstakels die kunnen worden overwonnen door de inzet van ISCO behandelingen. Een overzicht van bioremediatie efficiëntie is uitgevoerd met behulp van een aantal door diesel verontreinigde bodems, bestaande uit vier bodemmatrix types (Hoofdstuk 3). Door de bodems onder optimale omstandigheden voor biodegradatie te incuberen, moleculaire analyses van microbiële biologische afbraakcapaciteit op basis van een alkaan mono-oxygenase gen (*Alkb*) uit te voeren en de biobeschikbaarheid van de overgebleven diesel te meten, kon worden aangetoond dat volledige biologische afbraak waarschijnlijk wordt geremd door biobeschikbaarheid. Daarnaast is een analyse uitgevoerd van de samenstelling van

de microbiële populatie door middel van pyrosequencing van het 16S rRNA-gen fragment. Hierdoor wordt een beter begrip mogelijk van het samenspel tussen de geochemische parameters van de bodem, diesel eigenschappen en microbiële diversiteit op een verontreinigde locatie ([Hoofdstuk 4](#)). Uit de resultaten blijkt dat er een significante vermindering van de microbiële diversiteit in verontreinigde monsters is ontstaan en dat er een sterke associatie is tussen de aanwezigheid van diesel en strikte anaerobe micro-organismen, wat aangeeft dat de redox potentiaal een aanmerkelijke hindernis vormt voor natuurlijke afbraak. Samen laten deze secties zien dat ISCO voorafgaand aan ISB bodem sanering kan bevorderen.

De koppeling van chemische oxidatie met de aerobe biologische afbraak van door diesel verontreinigde grond is onderzocht in laboratorium- en veldexperimenten. Experimenten met Fenton's reagens en Modified Fenton's reagens richten zich op het begrijpen van de geochemische veranderingen die optreden als gevolg van chemische oxidatie en hun invloed op de volgende bioremediatie stap ([Hoofdstuk 5](#)). Chemische oxidatie breekt organische stof in de bodem af, hetgeen leidt tot het vrijkomen van opgeloste voedingsstoffen en organisch koolstof. Deze organische koolstof concurreert als substraat met diesel tijdens het biodegradatie proces. Veranderingen in de microbiële populatiestructuur en diesel afbraakcapaciteit zijn onderzocht tijdens bioremediatie na een chemisch behandeling met Fenton's reagens, Modified Fenton's reagens, permanganaat en persulfaat in twee verschillende door diesel verontreinigde bodems ([Hoofdstuk 6](#)). De totale saneringsefficiëntie is afhankelijk van het bodemtype, zoals blijkt uit de lagere saneringsefficiëntie in veengrond door de mobilisatie van diesel door chemische oxidatie in vergelijking met de ophooglaag, waarin weinig mobilisatie van de verontreiniging is waargenomen. DGGE fingerprinting laat zien dat in de diverse chemische behandelingen verschillende microbiële populaties ontwikkelen. Kwantificering van de aanwezigheid van de 16S rRNA en *Alkb*-genen tonen een verrijking van de biodegradatiecapaciteit na chemische behandeling, vergeleken met de biotische controle. Tot slot is de microbiologische en geochemische dynamiek van ISCO met persulfate gevolgd door een nutriënt- gestimuleerde ISB onderzocht in een door diesel verontreinigde locatie ([Hoofdstuk 7](#)). Grondwater parameters zoals pH en redox potentiaal geven aan dat zich na ISCO ongunstige omstandigheden voor microbiële groei ontwikkelen. Dit wordt bevestigd door een waargenomen afname van 1-2 ordes van grootte in zowel microbiële populatie (16S rRNA-gen) als biologische afbraak capaciteit (*Alkb*). Hoewel de zuurtegraad van het grondwater op den duur afneemt en de microbiële populatie zich herstelt, leiden de wijzigingen in de organische stof structuur en de oxidatie van metaalsulfiden tot een permanente verandering van de bodem.

De koppeling van een chemische oxidatiestap met anaerobe bioremediatie voor de behandeling van gechloroerde oplosmiddelen is onderzocht in laboratorium- en veldexperimenten. Organohalide respiratie (OHR) snelheden zijn onderzocht in vloeibare microkosmos experimenten met een permanganaat behandeling van tetrachlooretheen (PCE; [Hoofdstuk 8](#)). Resultaten geven aan dat milde permanganaat behandelingen de biologische afbraak enigszins lijken te stimuleren, zoals weergegeven in OHR snelheden. Sterke permanganaat doses, daarentegen, verstoren de PCE afbraak tot vinylchloride en veroorzaken een 2-4 orden van grootte vermindering van de aanwezigheid van organohalide respirerende bacteriën (OHRB) en reductieve dehalogenase genen (RDH). De microbiële populatie samenstelling op basis van pyrosequencing van 16S rRNA gen fragmenten geeft aan dat chemische oxidatie lijkt te selecteren voor *Deltaproteobacteria*, vooral *Geobacter*, en *Epsilonproteobacteria*, vooral *Sulfurospirillum*. De aanwezigheid van OHRB en RDH genen wordt ook waargenomen bij ISCO behandeling met persulfaat op een PCE en trichlooretheen (TCE) verontreinigde locatie ([Hoofdstuk 9](#)). Na ISCO behandeling werd een significante daling in de aanwezigheid van OHRB en RDH genen gemeten, waarschijnlijk is dit teweeg gebracht door grondwater aanzuring ($\text{pH} < 3$) en de hoge redox potentiaal ($> 500 \text{ mV}$). Hoewel de regeneratie van de OHRB populatie zes maanden na ISCO wordt waargenomen, blijken *Dehalococcoides mccartyi* en RDH genen niet aanwezig te zijn, hetgeen wijst op een langdurige verstoring van de microbiële capaciteit voor volledige natuurlijke afbraak.

De uitkomsten van dit proefschrift geven inzicht in de dynamiek van ondergrondse microbiologische en geochemische processen ([Hoofdstuk 10](#)). De meerwaarde van het koppelen van ISCO met ISB wordt ondersteund door de verbeterde saneringsefficiëntie die bereikt wordt wanneer chemische oxidatie wordt toegepast in plaats van alleen bioremediatie. Onderzoek met chemische behandelingen geven inzicht in de microbiële veerkracht. Micro-organismen zijn niet alleen in staat om te regenereren na sterke oxidatie behandelingen; in veel gevallen is ook een geringe toename in de afbraakcapaciteit waargenomen. Chemische oxidatie veroorzaakt veranderingen in de ondergrondse geochemie, waaronder de structuur van organische stof, mineralogie en samenstelling van het grondwater. Deze veranderingen kunnen permanente gevolgen hebben voor de microbiële populatie structuur en diens functie. Daarom zijn moleculaire technieken vereist om het effect van verontreiniging en sanering op microbiële populaties duidelijk te maken. Dergelijke kennis over de veerkracht en de gevoeligheid van bodem- en grondwater-ecosystemen onder stresssituaties draagt bij aan de ontwikkelingen van het vakgebied microbiële ecologie. Goed uitgevoerd

onderzoek naar deze ondergrondse systemen biedt bovendien het voordeel dat het inzichten biedt in relevante microbiologische en geochemische processen in de ondergrond. De resultaten van dit proefschrift vormen een belangrijke nieuwe stap richting het analyseren van verontreinigde locaties en bodemsanering vanuit een fundamenteel wetenschappelijk perspectief.

Deutsche Zusammenfassung

Der Untergrund ist ein System aus organischen, anorganischen und mikrobiologischen Bestandteilen, die zusammen verschiedene Prozesse wie Nährstoffkreislauf, Kohlenstoffbindung und Wasserfiltration unterstützen. Die bisherige Forschung hat sich auf das Verständnis des dynamischen Zusammenspiels dieser mikrobiologischen, geochemischen und physikalischen Prozesse fokussiert. Die Kontaminierung des Untergrundes durch Städtische- und Industrieaktivitäten stellt ein ernsthaftes Umweltrisiko dar, welches die Entwicklung wirksamer Sanierungstechniken erforderlich macht, die die betroffenen Stellen wieder in einen funktionierenden Zustand versetzen. Die Anwesenheit und Behandlung dieser Schadstoffe bieten Möglichkeiten für die Erforschung grundlegender Fragen auf dem Gebiet der mikrobiellen Ökologie und Bodengeochemie. Diese Arbeit beschreibt *in situ* chemische Oxidation (ISCO) und *in situ* biologische Sanierung (ISB) von organischen Verunreinigungen, mit Schwerpunkt auf der Aufklärung der dynamischen mikrobiologischen und geochemischen Prozesse, die während der Sanierung auftreten (Kapitel 1).

Die Verwendung starker Oxidationsmittel während ISCO verändert die Eigenschaften sowohl der Schadstoffe als auch des Bodens erheblich, und beeinflusst die Größe, Struktur und Funktion der mikrobiellen Population. Doch ISCO kann, richtig eingesetzt, eine sinnvolle Vorbereitung für die ISB sein. Eine Überprüfung der bisherigen Forschung zeigt, dass sich die bestehende Literatur auf (1) Optimierung der chemischen Oxidationsbehandlung zur Unterstützung des nachfolgenden biologischen Abbauprozesses und (2) auf die Ermittlung der Änderungen, welche nach einer ISCO-Behandlung erforderlich sind, um den biologischen Sanierungsprozess zu unterstützen, konzentriert (Kapitel 2). In einigen Fällen wird die vollständige biologische Sanierung verhindert, entweder durch die Bioverfügbarkeit der Verunreinigung oder des Redoxpotentials. Beide sind Hindernisse, die durch das Einsetzen von ISCO-Behandlungen überwunden werden können. Eine Übersicht der Effizienz der biologische Sanierung wurde mit Hilfe einer Anzahl von mit Diesel kontaminierten Böden, bestehend aus vier Arten von Bodenmatrix, durchgeführt (Kapitel 3). Durch Inkubation von Böden unter optimalen biologischen Abbaubedingungen, Ausführen von molekularen Analysen der mikrobiellen Abbaufähigkeit auf der Grundlage eines Alkans Monooxygenase-Gen (*aklB*) und Messen der Bioverfügbarkeit des verbleibenden Diesels, konnte gezeigt werden, dass der vollständige biologische Abbau vermutlich eher durch

die Bioverfügbarkeit als durch Nährstoff Abundanz, Elektronakzeptor Anwesenheit oder biologische Abbaukapazität, verhindert wird. Zusätzlich wurde eine Analyse der Zusammensetzung der mikrobiellen Population mittels Pyrosequenzierung des 16S-rRNA-Gen-Fragment durchgeführt. Dies ermöglicht ein besseres Verständnis des Zusammenspiels zwischen den geochemischen Parametern des Bodens, der Deseleigenschaften und der mikrobiellen Diversität an einem kontaminierten Standort (Kapitel 4). Die Ergebnisse zeigen eine signifikante Reduktion der mikrobiellen Diversität in kontaminierten Proben und einen starken Zusammenhang zwischen dem Vorhandensein von Diesel und strikt anaeroben Mikroorganismen, der anzeigt, dass das Redoxpotential ein signifikantes Hindernis für den natürlichen Abbau darstellt. Zusammen liefern diese Teilergebnisse einen überzeugenden Beweis für den Nutzen der Anwendung von ISCO vor ISB.

Die Koppelung chemischer Oxidation mit aerobem biologischen Abbau von dieselkontaminiertem Boden wurde sowohl in Labor- als auch in Feldexperimenten untersucht. Experimente mit Fentons-Reagenz und modifiziertem Fentons-Reagenz konzentrieren sich auf die Gewinnung des Verständnisses der geochemischen Veränderungen, die als Ergebnis der chemischen Oxidation auftreten und ihren Einfluss auf die biologische Sanierung (Kapitel 5). Chemische Oxidation baut organische Stoffe im Boden ab, wodurch Nährstoffe und organischer Kohlenstoff freigesetzt werden. Dieser konkurriert mit Diesel als Substrat während des biologischen Abbauprozesses. Veränderungen der mikrobiellen Populationsstruktur und Dieselabbaukapazität wurden während der biologischen Sanierung, gefolgt von Behandlungen mit Fentons-Reagenz, Permanganat, und Persulfat in zwei verschiedenen Dieselkontaminierten Böden untersucht (Kapitel 6). Die Effizienz der Gesamtsanierung ist abhängig von der Bodenart, da die Mobilisierung des Diesels, bedingt durch chemische Oxidation in Torf, eine verringerte Sanierungseffizienz im Vergleich zum Füllmaterial, bei dem eingeschränkte Mobilisierung beobachtet wurde, aufweist. Ein DGGE- Fingerabdruck zeigt die Entwicklung der unterschiedlichen mikrobiellen Populationen in den verschiedenen chemischen Behandlungen. Quantifizierung der Anwesenheit der 16S-rRNA- und *Alkb*- Gene zeigen eine Steigerung der biologischen Abbaukapazität nach der chemischen Behandlung, verglichen mit der biotischen Kontrolle. Schließlich werden die mikrobiologische und geochemische Dynamik der ISCO mit Persulfat, gefolgt von einer Nährstoff- stimulierten ISB in einer Diesel kontaminierten Standort, untersucht (Kapitel 7). Grundwasserparameter wie pH-Wert und Redoxpotential zeigen, dass sich nach der ISCO ungünstige Bedingungen für mikrobielles Wachstum entwickeln. Dies wird durch eine beobachtete Abnahme

von 1-2 Größenordnungen in beiden mikrobiellen Populationen (16S rRNA- Gen) und Abbaukapazität (*Alkb*) bestätigt. Obwohl der Säuregehalt des Grundwassers abnimmt und sich die mikrobielle Population erholt, sind die Änderungen der organischen Stoffe und die Oxidation von Metallsulfiden nicht wiederherstellbare Änderungen der Bodenbestandteile.

Die Koppelung einer chemischen Oxidation mit anaerober biologischer Sanierung für die Behandlung von chlorierten Lösungsmitteln wird in Labor- und Feldversuchen untersucht. Organohalide Atmungsgeschwindigkeiten (OHR) werden in flüssigen Mikrokosmos-Experimenten mit Permanganatbehandlung von Tetrachlorethen (PCE) untersucht (Kapitel 8). Die Ergebnisse zeigen, dass eine milde Permanganat-Behandlung den biologischen Abbau fördern kann, wie in den OHR-Geschwindigkeiten gezeigt, im Gegensatz zu starker Dosierungen von Permanganat, die den Abbau von PCE zu Vinylchlorid stört und eine 2-4 fache Reduktion in der Abundanz von Organohalogenid atmenden Bakterien (OHRB) und des reduktiven Dehalogenase-Genes (RDH) verursacht. Die Zusammensetzung der mikrobiellen Bevölkerung auf Basis von Pyrosequenzierung der 16S-rRNA-Genfragmente zeigt, dass die chemische Oxidation für *Deltaproteobacteria*, besonders *Geobacter* und *Epsilonproteobacteria*, insbesondere *Sulfurospirillum* selektieren scheint. Die Menge an OHRB und RDH- Genen wird auch bei einer ISCO-Behandlung mit Persulfat auf einem mit PCE und Trichlorethen (TCE) kontaminierten Standort beobachtet (Kapitel 9). Nach ISCO-Behandlung wurde eine signifikante Abnahme der Abundanz von OHRB und RDH-Genen gemessen, höchstwahrscheinlich verursacht durch Versauerung des Grundwassers ($\text{pH} < 3$) und das hohe Redoxpotential ($> 500 \text{ mV}$). Obwohl eine Regeneration der OHRB- Bevölkerung sechs Monate nach ISCO beobachtet wird, fehlen *Dehalococcoides mccartyi* und RDH- Gene, was auf eine langfristige Störung der vollständigen natürlichen Abbaukapazität hinweist.

Die Ergebnisse dieser Arbeit bieten einen Einblick in die Dynamik der unterirdischen mikrobiologischen und geochemischen Prozesse (Kapitel 10). Der Wert der Verknüpfung von ISCO mit ISB liegt in der verbesserten Effizienz der Sanierung, die durch die Anwendung der chemischen Oxidation statt nur der biologischen Sanierung erreicht wird. Forschung mit chemischen Behandlungen gibt Einblick in die mikrobielle Widerstandsfähigkeit. Mikroorganismen sind nicht nur in der Lage, nach starken Oxidationsbehandlungen zu regenerieren; in vielen Fällen ist auch eine leichte Erhöhung der Abbaukapazität zu beobachten. Chemische Oxidation verursacht Veränderungen der unterirdischen Geochemie, einschließlich der Struktur der organischen Materie, Mineralogie und der Zusammensetzung des Grundwassers.

Diese Veränderungen können dauerhafte Auswirkungen auf die mikrobielle Populationsstruktur und ihre Funktion haben. Daher sind molekulare Techniken erforderlich, um die Wirkung von Kontamination und Sanierung auf mikrobielle Populationen besser zu verstehen. Dieses Wissen über die Widerstandsfähigkeit und Empfindlichkeit der Ökosysteme Boden und Grundwasser unter Stresssituationen trägt zur Entwicklung der mikrobiellen Ökologie bei. Richtig ausgeführte Forschung über diese unterirdischen Systeme liefert nicht nur einen weitergehenden Beitrag zu dem Gebiet der Bodensanierung sondern auch darüber hinaus bedeutende Erkenntnis über grundlegende mikrobiologische und geochemische Prozesse in der Umwelt. Die Ergebnisse dieser Arbeit sind ein wichtiger, neuer Schritt in Richtung Untersuchung von kontaminierten Standorten und deren Sanierung aus einer grundlegend wissenschaftlichen Perspektive.

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About the Author

Nora Barbour Sutton was born January 9th, 1982 in San Francisco, CA, USA. In 2005, she graduated *cum laude* with Bachelor's Degrees in Biology-Chemistry and German Language from Claremont McKenna College, CA, USA. During this period, she worked on projects at the University of California at Berkeley and at the United



States Department of Agriculture's Research Services Unit. Her thesis research entitled 'Improving competent cell performance through preparation with cobalt complexes' integrated organic chemistry and microbiology research techniques to improve genetic transformation efficiencies in competent cells. During and after her undergraduate degree she studied and worked at Albert-Ludwigs-Universität Freiburg, Germany. After moving to The Netherlands in 2006, she completed a Master's degree *cum laude* in Environmental Geochemistry at Utrecht University in 2008. Her thesis, entitled "Characterization of geochemical constituents and bacterial populations associated with As mobilization in deep and shallow tube wells in Bangladesh", was performed in cooperation with Delft University of Technology and University of Dhaka, Bangladesh. The project sent

her to Bangladesh to research the influence of microbial community structure and geochemistry on the mobilization of arsenic in drinking water wells in the Ganges-Brahmaputra Delta. After graduation, she worked at Deltares in Utrecht, where she gained analytical experience and polished her Dutch skills. In 2009, she started a PhD at the department of Environmental Technology at Wageningen University, investigating chemical and biological *in situ* soil remediation. Her research interests focus on understanding the delicate interplay of microbial community structure and geochemistry and their association with bioremediation of organic contaminants in subsurface systems. She will continue with this area of research as a postdoctoral researcher in Environmental Technology, assessing the natural attenuation capacity of microorganisms to degrade micropollutants in the subsurface.



Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Nora Barbour Sutton

born on 9 January 1982 in San Francisco, United States

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 4 July 2014

the Chairman of the SENSE board

Prof. dr. Rik Leemans

the SENSE Director of Education

Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)



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A K A D E M I E V A N W E T E N S C H A P P E N



The SENSE Research School declares that **Ms. Nora Barbour Sutton** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 41.5 ECTS, including the following activities:

SENSE PhD Courses

- o Environmental Research in Context
- o Research Context Activity: Organizing Workshop on: Integrating in situ remediation technologies into land use cycles (Utrecht, 19 October 2011)

Other PhD Courses

- o Biological Processes for Resource Recovery in Environmental Technology
- o Environmental Biotechnology

Management and Didactic Skills Training

- o Supervision of MSc thesis student Daniel Hidalgo Lasso on "Understanding the effect of chemical oxidation on microbial activity and diversity to couple in situ chemical oxidation with bioremediation"

Oral Presentations

- o *Optimization of coupled ISCO and bioremediation at a Diesel Contaminated Railway Site.* Battelle bioremediation and sustainable environmental technologies conference, 27-30 June, 2011, Reno, USA
- o *Biodegradation of aged diesel in diverse soil matrixes: impact of environmental conditions and bioavailability on microbial remediation capacity.* 4th International Congress Eurosoil, 2-6 June, 2012, Bari, Italy
- o *Mobilization of organic carbon and nutrients during chemical oxidation impacts subsequent bioremediation of a diesel contaminated soil.* 12th international UFZ-Deltares conference on Groundwater-Soil-Systems and Water Resource Management (AquaConSoil), 16-19 April, 2013, Barcelona, Spain
- o *Coupling chemical and biological treatment: the advantages and risks.* National workshop: rehabilitation of contaminated soil and sites, 2 July 2012, Budapest, Romania
- o *Reliability of long-term bioremediation.* Monitoring, Control, and Evaluation of Remedial Efficiency, 21 March, 2012, Prague, Czech Republic

SENSE Coordinator PhD Education

Dr. ing. Monique Gulickx

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