Living is no laughing matter:
you must live with great seriousness
like a squirrel, for example-
I mean without looking for something beyond and above living,
I mean living must be your whole occupation.

Nazım Hikmet RAN

to my family...

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Interaction between chemical and biological degradation of chlorinated aliphatic hydrocarbons

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) of Applied Biological Sciences

Titel van het doctoraat in het Nederlands:

Interactie tussen chemische en biologische afbraak van gechloreerde alifatische koolwaterstoffen

Cover illustration: *In situ* chemical oxidation

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Notation Index

ATP	adenosine tri-phosphate
BDL	below detection limit
CAH	chlorinated aliphatic hydrocarbon
cDCE	*cis*-1,2-dichloroethene
CSIA	compound-specific isotope analysis
DGGE	denaturing gradient gel electrophoresis
DM	dry matter
DNA	deoxyribonucleic acid
DNAPL	dense non-aqueous-phase liquid
DOC	dissolved organic carbon
EPA	Environment Protection Agency
ERH	electrical resistive heating
FCM	flow cytometry
GC	gas chromatograph
ISCO	*in situ* chemical oxidation
MCL	maximum contaminant levels
MS	mass spectrometer
MW	monitoring well
NM	not measured
NOD	natural oxidant demand
NOM	natural organic matter
ORP	oxidation-reduction potential
PAHs	polycyclic aromatic hydrocarbons
PCB	polychlorinated biphenyl
PCE	tetrachloroethene
PCR	polymerase chain reaction
PM	potassium/sodium permanganate
PS	sodium persulfate
qPCR	quantitative PCR
RDase	reductive dehalogenase
RNA	ribonucleic acid

SEE	steam enhanced extraction
SMOC	Standard Mean Ocean Chloride
TCA	1,1,2-trichloroethane
TCE	trichloroethene
TCH	thermal conductive heating
TEA	terminal electron acceptor
trans-DCE	*trans*-1,2-dichloroethene
VC	vinyl chloride
VPDB	Vienna-Pee Dee Belemnite
VITO	Vlaamse instelling voor technologisch onderzoek
ZVI	Zero valent iron

Table of Contents

Chapter 1. Introduction and research objectives

Protection of the integrity of groundwater supplies is crucial for the environment and the health of human beings. Chlorinated aliphatic hydrocarbons (CAH) are compounds that are used widely for degreasing activities, and are threatening the groundwater quality. For the remediation of contaminated groundwater bodies, the design of an efficient and cost-effective technology requires (a) a comprehensive understanding of the nature and extent of the contamination, and (b) an attentive evaluation of the available remediation technologies. Technologies that have been used for the *in situ* clean-up of CAH contaminated sites comprise bioremediation, chemical oxidation as well as chemical reduction. In the current work, the added value of combining bioremediation and chemical oxidation concurrently (chemical plus biological treatment) or successively (chemical followed by biological treatment) was studied. Chemical oxidation is a relatively fast process that is most efficient for moderate to high pollutant concentrations, while biodegradation is a slow and most efficient at low to moderate concentrations. Therefore, combined remediation approaches may provide a good solution to achieve regulatory limits.

A first crucial question is related to the impact of *in situ* chemical oxidation (ISCO) on the structure and activity of the subsurface microbial community, and specifically on the CAH degrading microbial population of the groundwater and aquifer compartment. The impacts of the different types of chemical oxidants on a variety of anaerobic or aerobic microorganisms are described in literature. More specifically, the application of permanganate, persulfate and Fenton's reagent on CAH biodegradation and changes in the microbial community structure were investigated (Gardner et al., 1996; Kastner et al., 2000; Klens et al., 2001; Hrapovic et al., 2005; Sercu et al., 2013). However, particular responses of *Dehalococcoides mccartyi* spp. to the most commonly used chemical oxidants, permanganate and persulfate, have not been studied at 16S rRNA and *reductive dehalogenase (RDase)* gene level both in DNA and mRNA samples. Data on recovery of the microorganisms after the application of ISCO are also rare.

In this dissertation, the interaction between chemical and biological remediation of CAHs and their impact on ecosystem and in turn, the indigenous microorganisms, have been investigated. The main research questions have been set as "What is the impact of chemical oxidants on the activity and structure of indigenous microorganisms?", "Will there be any

recovery in the microbial activity under post oxidation conditions?", "Will there be any difference in the microbial community structure under pre- and post oxidation conditions?". More specifically, the research goals were to study (i) the activity, (ii) the recovery potential, and (iii) the changes in the structure of the microbial community before, during and after the oxidation process in *Dehalococcoides* containing systems. To achieve these goals, the complexity of the studied systems was increased from liquid dechlorinating enrichment cultures over aquifer microcosms towards a field study (Figure 1.1, step 2 and step 3). In the field, an *in situ* mesocosm approach was used that allowed to get a clear picture of the impact of ISCO on the activity and diversity of the microbial community in these different ecosystems.

A second research question is linked to analytical methods and approaches to detect and discriminate effects of different pollutant degradation mechanisms on contaminant removal. Because of the dynamic nature and heterogeneity of groundwater systems, decreases in the contaminant concentrations might not be attributed to only one degradation mechanism (e.g., chemical oxidation, biodegradation, …). On the one hand, molecular tools were considered to examine the activity of CAH degrading species and related genes. For this purpose, the dynamics of mRNAs in a CAH degrading enrichment culture were investigated (Figure 1.1, step 1). On the other hand, the potential of compound specific isotope analyses (CSIA) was evaluated (Figure 1.1, step 4). Past research has indicated the applicability of CSIA (i) for the identification of biodegradation potential of CAHs contaminated sites and (ii) for discriminating different removal mechanisms. However, the applicability of the dual isotope approach (C, Cl) to distinguish degradation of cDCE by anaerobic biodegradation and oxidation via permanganate, which is one part of the current study. has never been investigated.

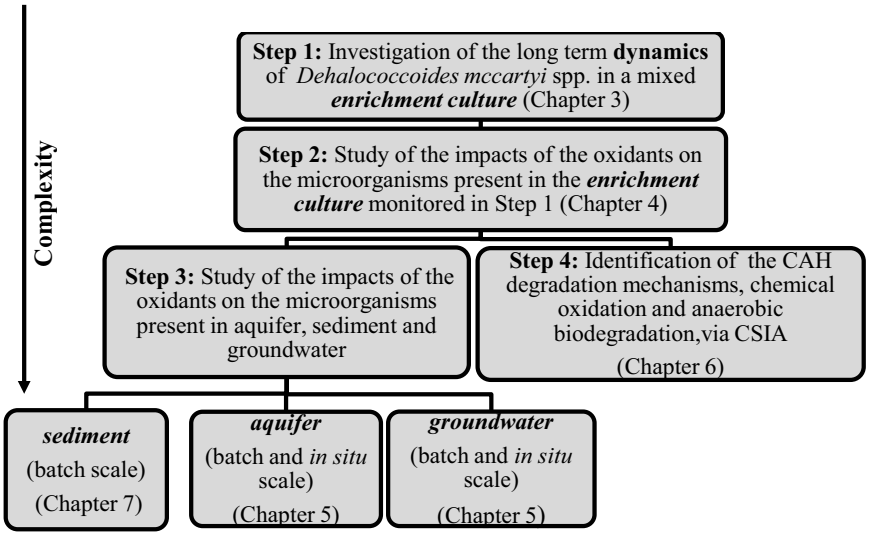

Figure 1.1. Schematic presentation of the road map followed in this dissertation (CAH: chlorinated aliphatic hydrocarbons; CSIA: compound specific isotope analysis).

The work is structured in eight chapters. A short summary of literature data is provided in Chapter 2. In Chapter 3, the dynamics of mRNAs in a CAH degrading culture were studied. More specifically, metabolic responses of *Dehalococcoides mccartyi* spp. in an enrichment culture under TCE exposure were monitored over a *long term* period via 16S rRNA, *tceA, vcrA* and *bvcA* gene copy and transcripts numbers (qPCR) together with ATP production and live/dead cell determination. The dynamics of the genes during TCE degradation formed *the basis* to study the effect of the chemical oxidants on the CAH degrading microorganisms of this enrichment culture in Chapter 4. More particularly, the dechlorinating activity and recovery potential of the organisms in the same enrichment culture were studied during and after exposure to different concentrations of the chemical oxidants, permanganate and persulfate. In Chapter 5, potential and different effects of the permanganate on microorganisms present in aquifer were discussed in microcosm and *in situ* scales. Moreover, in Chapter 5, the effect of the oxidants on the microbial community structure was studied by RNA based DGGE analysis. Chapter 6 focused on a dual isotope approach (carbon, chlorine) to differentiate between chemical oxidation and anaerobic degradation mechanisms in groundwater at batch and *in situ* level. Finally, in Chapter 7, the impact of the chemical oxidants on the indigenous microorganisms and on the release of heavy metals from three physico-chemically different sediment systems was investigated in a batch scale microcosm test. General perspectives, discussions and conclusions are presented in Chapter 8.

Chapter 2. Literature review

2.1. Groundwater Contamination

It is estimated that there are 300,000-1.5 million sites in the European Union which may have contaminated soil and groundwater bodies needing some form of remediation. The amount and variety of hazardous substances have drastically increased in the last 50 years. Because of the increase in industrial and agricultural activities and transfers of wastes, not only the traditional industrialized countries, but other countries have widespread soil and groundwater pollution (Gualandi, 2006). Subsequently, subsurface and groundwater contamination has created a big industry for remediation technologies. Conventional technologies include ex-situ or on-site removal of contaminants through excavation of soil or pump-and-treat remediation of groundwater, respectively. However, the high costs and health risks associated with removal of contaminated material lead to a shift towards *in situ* technologies for remediation, where chemical or biological pathways are remained within the subsurface environment (EPA, 1998).

The most common organic groundwater pollutants are chlorinated aliphatic hydrocarbons (CAHs) such as perchloroethene (PCE), trichloroethene (TCE) and 1,1,1-trichloroethane (TCA). They are widely used in various industrial processes, mainly as cleaning solvents in dry cleaning operations (TCE) and semiconductor manufacture. Careless storage, handling and disposal as well as their high chemical stability have contributed to the status of CAHs as most frequently encountered subsurface contaminants (Aulenta et al., 2006). Although these compounds are largely immiscible with the groundwater at ambient conditions, their concentrations greatly exceed maximum contaminant levels (MCLs) of drinking water standards. By the processes of dissolution and groundwater transport, the pollutants disperse to generate plumes of contaminated groundwater (Knauss et al., 1999).

According to the European Environmental Agency (EEA, 2010), it is estimated that in Europe, about 72, 000 sites are contaminated with chlorinated hydrocarbons. Moreover, projections indicate that the total number of contaminated sites may extend with more than 50% in 2025 while remediation is progressing relatively slow (www.eea.europa.eu).

2.1.1. Chlorinated Aliphatic Hydrocarbons (CAHs)

Since the beginning of the twentieth century, chlorinated aliphatic hydrocarbons have been manufactured in large amounts and extensively used in industrial, military, agricultural and household applications. The widespread use of CAHs is based on their desirable properties including low cost, easy availability, excellence as solvents, chemical stability and fire safety. The widespread use, careless handling and storage, ignorance of health effects and environmental dangers, and the lack of regulations over decades of extensive use, led to wide-ranging groundwater contamination. They form dense non-aqueous phase liquids (DNAPL), having a specific gravity heavier than water (Huling and Weaver, 1991). Exposure to CAHs is of public concern because they are toxic, several are classified as potential human carcinogens and some, such as vinyl chloride (VC), are proven human carcinogens (Löffler et al., 2013). The main characteristics of some selected CAHs are provided in Table 2.1.

Table 2.1. Physico-chemical properties and clean-up threshold limits of PCE, TCE, *cis*-dichloroethene (cDCE), vinyl chloride (VC) and 1,1,1-TCA.

	PCE	**TCE**	***cis*-DCE**	**VC**	**1,1,1-TCA**
Formula	C_2Cl_4	C_2HCl_3	$C_2H_2Cl_2$	C_2H_3Cl	$C_2H_3Cl_3$
Water solubility at 25°C (g/L)	0.15	1.0	3.5	2.7	4.4
Boiling point (°C)	121	87	60	-14	74
Melting point (°C)	-19	-73	-80	-153	-30
Molecular weight (g/mol)	165.8	131.4	96.9	62.5	133.4
Density at 20°C (g/mL)	1.62	1.47	1.26	0.91	1.34
Henry's law constant (atm·m^3/mol)	1.3×10^{-2}	1.0×10^{-2}	3.4×10^{-2}	5.6×10^{-2}	8.0×10^{-2}
log K_{OC}	2.32-2.38	2	1.56-1.69	1.75	1.91-1.95
log K_{OW}	3.40	2.29	1.86	0.60	2.49
Groundwater clean-up threshold (μg/L)[a]	40	70	50	5	500

[a]For Flanders (Vlaamse regering, 2008) (after Hamonts et al., 2009)

There are several remediation technologies for the treatment of CAHs both in the source and in the plume as summarized in Table 2.2.

Table 2.2. Commonly used *in situ* and on-site remediation technologies for source and plume remediation of CAH pollution

Remediation technology		Type of remediation			
			Mass transfer	**Mass destruction**	**Contaminant phase**
Physical	Excavation	on-site/off-site	X		All
	Pump-and-treat	*in situ*/on-site	X		Primarily dissolved
	Soil-vapor extraction	*in situ*/on-site	X		All
	Multiphase extraction	*in situ*/on-site	X		All
	Air sparging	*in situ*/on-site	X		Dissolved and vapor
	Thermal treatments	*in situ*/on-site	X	X	Primarily DNAPLs
	In situ flushing	*in situ*/on-site	X		All but vapor
Chemical	ISCO	*in situ*		X	DNAPL and dissolved
	Chemical reduction, permeable reactive barriers or direct injection of reactant	*in situ*		X	DNAPL and dissolved
Bioremediation	Enhanced aerobic or anaerobic degradation	*in situ*		X	Dissolved
	Enhanced reductive dechlorination	*in situ*		X	All, but primarily dissolved
	Monitored natural attenuation	*in situ*		X	Dissolved

Adapted from Bjerg et al., 2013 (DNAPL: Dense Non Aqueous Phase Liquid, ISCO: *In situ* chemical oxidation).

2.2. Physical Remediation of CAH Contaminated Sites

2.2.1. Pump-and-Treat

Pump-and-treat systems for the remediation of groundwater came into wide use in the early to mid-1980s. The traditional pump-and-treat system includes a series of recovery (extraction) wells to pump the contaminated groundwater up to the subsurface for treatment (Figure 2.1), where it is treated chemically, physically or biologically. The treated groundwater is re-injected through injection wells into the aquifer. Continuous pumping of contaminated groundwater provides hydraulic to prevent their migration. Pump-and-treat based remediation of CAH contaminated groundwater is often inefficient due to slow diffusion and continuous

dissolution from NAPLs and desorption of residual contaminants trapped in soil (Langwaldt and Puhakka, 2000).

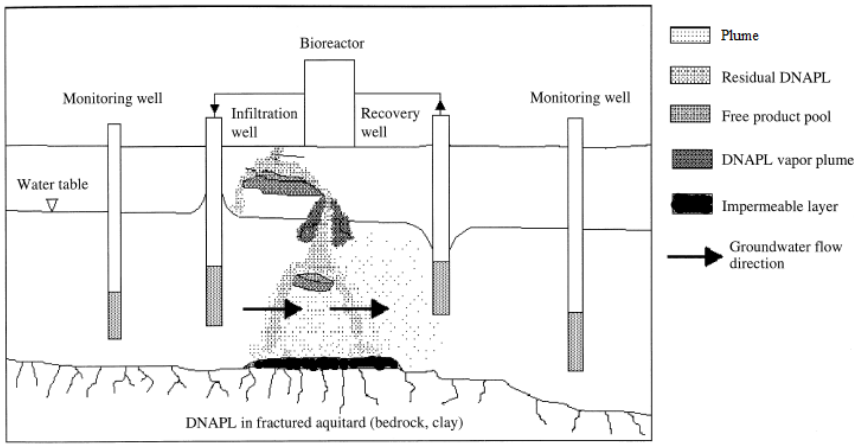

Figure 2.1. Scheme of a pump-and-treat system (DNAPL: dense non-aqueous phase liquid) (after Langwaldt and Puhakka, 2000).

2.2.2. Air Sparging

Air sparging is an remediation technology for the treatment of volatile organic contaminants. During the implementation a gas, usually air, is injected into the saturated soil zone below the lowest level of the contamination plume. Due to the effect of buoyancy, the injected air rises towards the surface. As the air comes into contact with the contamination, it strips the contaminant and/or stimulates *in situ* biodegradation. The transport mechanisms include advection, dispersion, and diffusion. The mass transfer mechanisms include volatilization, dissolution, and adsorption/desorption (Reddy, 2008). However, air sparging cannot be used for the treatment of confined aquifers and cannot be used if free products exist since these free products must be removed prior to air sparging (EPA, 1994).

2.2.3. Thermal Treatment

In situ thermal heating technologies are applied to enhance contaminant transport in order to volatilize, mobilize, or degrade contaminants. Currently, three thermal treatments are applied: steam enhanced extraction (SEE), electrical resistive heating (ERH) and thermal conductive heating (TCH). SEE is best suited for moderate to high permeability zones, in which steam is injected into the source zone (Heron et al., 2005). ERH, in which electrical current is passed

through the contaminated zone to increase the subsurface temperature, is particularly suited for the treatment of low permeable media. TCH, in which surface or subsurface conductive heating elements are used to create a high-temperature zone, differs from the other heating methods (SEE and ERH) in that it does not rely solely on steam as a heat source or water as a conductive path (Friis et al., 2006).

2.3. Chemical Remediation of CAH Contaminated Sites

2.3.1. Chemical Reduction

Remediation of CAH contaminated sites by chemical reduction is mainly achieved by using zero-valent iron (Fe^0, ZVI) in nanoscale or granular form in mainly permeable reactive barriers or *in situ* ZVI zones (Cundy et al., 2008; Dolfing et al., 2008). There has also been growing interest in micro-scale ZVI particles because of their higher reactivity and potential to be injected in the subsurface as a slurry. However, the application of such fine particles (micro- and nano) is challenging because of the aggregation of nano-sized ZVI particles making them less mobile and less reactive and the sedimentation of microscale ZVI in the slurry reservoir, tubing and injection wells (Velimirovic et al., 2013). These obstacles can be solved by increasing the stability and dispersion using different polymers such as guar gum, xanthan gum, starch and carboxymethyl cellulose.

The ZVI barriers may consist of a funnel-and-gate system designed to direct water through a treatment zone, continuous treatment walls, or wells spaced to maximize contact with the barrier material. Contaminant removal is affected by the presence of carbonate precipitates or by the composition of the groundwater (Hønning, 2007). *In situ* ZVI permeable barriers are successfully implemented for the treatment of groundwater contaminated with chlorinated solvent (Dries et al., 2005; Cundy et al., 2008) or heavy metals (Gadhi et al., 2002). The ZVI technology is very effective for the removal of contaminants with a variety of chemical characteristics due to the occurrence of multiple interactions such as reduction, (co) precipitation, and sorption. Chlorinated aliphatics are reductively dechlorinated by a direct electron-transfer mechanism from the corroding Fe^0 surface to the CAH, following the reaction (Eqn.1) (Dries et al., 2005).

$$Fe^0 + RCl + H^+ \rightarrow Fe^{2+} + RH + Cl^- \quad \text{(Eqn.1)}$$

2.3.2. Chemical Oxidation

Chemical oxidation is a process in which the oxidation state of a substance is increased. In general, the oxidant is reduced by accepting electrons released from the transformation (oxidation) of target and non-target reactive species. Oxidation of organic compounds may include oxygen addition, hydrogen abstraction (removal), and/or withdrawal of electrons with or without the withdrawal of protons (Huling and Pivetz, 2005). Groundwater contaminants are ultimately converted into carbon dioxide and water. Table 2.3 lists the relative strengths of commonly used oxidants. The reactions, considered as thermodynamically favorable based on E^0 values, may be impractical under field conditions. The rates of the oxidation reactions are dependent on several variables including temperature, pH, concentration of the reactants, catalysts, reaction by-products, natural organic matter (NOM), humus and minerals like iron, etc. NOM, humus and minerals are important parameters since they contribute to the natural oxidant demand (NOD) of the treated matrix so their concentrations correspond to a direct estimate of the oxidant consumption by organic and inorganic components in the matrix (soil or water). Such an estimation is valuable in determining oxidant dosage and is typically conducted on uncontaminated samples (background). Therefore, in any ISCO application, the final oxidant dose should be designed by considering the amount needed for NOD next to the amount needed to remediate the contaminant of concern.

Table 2.3. Strength of the oxidants

Chemical species	Standard oxidation potential (V)	Relative strength (chlorine = 1)
Hydroxyl radical (•OH)*	2.8	2.0
Sulfate radical ($\bullet SO_4^-$)	2.5	1.8
Ozone	2.1	1.5
Sodium persulfate	2.0	1.5
Hydrogen peroxide	1.8	1.3
Permanganate (Na/K)	1.7	1.2
Chlorine	1.4	1.0
Oxygen	1.2	0.9
Superoxide ion ($\bullet O^-$)*	-2.4	-1.8

*These radicals can be formed when ozone and H_2O_2 decompose (ITRC, 2001).

Stable contaminants can be oxidized only with the stronger oxidants, but these oxidants are also consumed quickly in the subsurface, limiting the distance the oxidant can travel. Less reactive oxidants are more stable and can be transported over greater distances in the subsurface. Therefore, the volume of aquifer to be treated is an important variable to consider

when choosing an oxidant. The solubility of the oxidant in water is also important because it limits the mass of oxidant that can be injected per volume of injection fluid (ITRC, 2001).

Currently, chemical oxidation is a relatively mature technology for the remediation of contaminated groundwater, including source zones and plumes (Siegrist et al., 2011). The downgradient contamination plume is often a secondary priority and may not involve ISCO because of the large area of contamination relative to the source area, lower oxidation efficiency, and greater cost. Commonly, application of ISCO is not preferred in the groundwater plume extending downgradient from the source zone because of the small size of source zones compared to the downgradient plume. In the source zone the oxidant can be applied at high concentration by focusing in specific source area locations, and as a result, greater oxidation efficiencies are achieved as compared to downgradient zones. Under such conditions, larger quantities of contaminant can be transformed using lower quantities of oxidant and at lower cost. (Huling SG and Pivetz, 2005).

An overview of the most used chemical oxidants and their main characteristics is provided below.

2.3.2.1. Permanganate

Permanganate (MnO_4^-) is reported to be effective in the remediation of CAH contaminated groundwater bodies, and has been widely applied for *in situ* and ex situ remediation. It can degrade chlorinated ethenes and is more persistent than most other oxidants. Permanganate can move further from the point of injection since it is less reactive and slower than more reactive oxidants. In addition, permanganate is relatively safe to handle in the field and does not produce large quantities of heat and gases. Potassium permanganate (Mn^{7+}) is reduced to manganese dioxide (MnO_2) (Mn^{4+}) which precipitates as indicated in the following reactions (Tsail et al., 2008).

Under acidic conditions the oxidation half-reactions are:

$$MnO_4^- + 4H^+ + 3e^- \rightarrow MnO_2 + 2H_2O \qquad E^o = 1.68\ V$$

$$MnO_4^- + 8H^+ + 5e^- \rightarrow Mn^{2+} + 4H_2O \qquad E^o = 1.51\ V$$

Under alkaline conditions, the half-reaction is:

$$MnO_4^- + 2H_2O + 3e^- \rightarrow MnO_2 + 4OH^- \qquad E^o = 0.60\ V$$

Complete oxidation of chlorinated ethenes has been observed to generate chloride ion Cl^- and carbon dioxide as in the following reactions (Kao et al., 2008):

$$4KMnO_4 + 3C_2Cl_4 + 4H_2O \rightarrow 6CO_2 + 4MnO_2(s) + 4K^+ + 12Cl^- + 8H^+$$

$$2KMnO_4 + C_2HCl_3 \rightarrow 2CO_2 + 2MnO_2(s) + 3Cl^- + H^+ + 2K^+$$

$$8KMnO_4 + 3C_2H_2Cl_2 \rightarrow 6CO_2 + 8MnO_2(s) + 8K^+ + 2OH^- + 6Cl^- + 2H_2O$$

$$10KMnO_4 + 3C_2H_3Cl \rightarrow 6CO_2 + 10MnO_2(s) + 10K^+ + 3Cl^- + 7OH^- + H_2O$$

Permanganate has been applied for the removal of chlorinated ethenes e.g., PCE, TCE, DCEs, and VC both in laboratory and field studies. For example, in the study of Huang et al. (2002), 1060 mg/L of TCE was completely dechlorinated by 2 g/L permanganate in the aquifer matrix of a column test. In another test, a two-dimensional physical model was designed to simulate a sand aquifer overlying a clay aquitard to assess the capability of 10 g/L permanganate for removal of 50 mg/L of PCE (MacKinnon and Thomson, 2002). Moreover, 500 µg/L, 32 mg/L; and 87 µg/L of TCE were treated with permanganate (0.2-2 g/L) in the batch studies of Kao et al. (2008) and Lee et al. (2009), respectively. Batch scale experiments showed a rapid and complete mineralization of contaminants by permanganate and resulting in the environmentally safe end products MnO_2, K^+ etc.(Yan and Schwartz, 1999).

Schnarr et al. (1998) were the first to explore the use of permanganate for remediation purposes at field scale. They reported complete oxidation of PCE and TCE (10-100 mg/L) to carbon dioxide and chlorine under the exposure of 10 g/L permanganate in a lab scale test. Two field experiments were also conducted. In the first test, 1 L of PCE that was added to a confined area (1640 mg of PCE) was completely removed within 120 days by flushing through 100 L per day of a 10 g/L $KMnO_4$ solution. In the second test, 8 L of a mixed PCE/TCE DNAPL was added to a test cell and after 290 days of flushing with 10 g/L permanganate (MnO_4^-), 62% of the contaminants was oxidized. Siegrist et al. (1999) successfully employed solid $KMnO_4$ (400-600 kg) emplacement in fractured silty clay soils to prevent vertical leaching of TCE at low and moderate aqueous TCE concentrations (53-480 mg/L) and >99% degradation was recorded.

2.3.2.2. Persulfate

Persulfate is one of the strongest oxidants for destroying organic contaminants such as chlorinated solvents. The mechanism to oxidize organic contaminants is radical reaction.

Sodium persulfate ($Na_2S_2O_8$) is commonly used to form persulfate while the low solubility of potassium persulfate ($K_2S_2O_8$) limits its application as a remediation agent (Tsail et al., 2008). Persulfate salts dissociate in water to persulfate anions that are strong but relatively stable oxidants according to activated persulfate :

$$Na_2S_2O_8 \rightarrow 2Na^+ + S_2O_8^{2-}$$
$$S_2O_8^{2-} + 2e^- \rightarrow 2SO_4^{2-} \qquad \text{(Hønning, 2007)}$$

The ability of persulfate to generate free radicals is crucial for remediation applications. If activated by heat (35-130 ^oC) or a transition metal (M^n) persulfate can initiate a free radical pathway through the formation of the sulfate radical according to the following reactions.

$$S_2O_8^{2-}(\text{heat/UV}) \rightarrow 2{\bullet}SO_4^-$$
$$S_2O_8^{2-} + M^n \rightarrow {\bullet}SO_4^- + SO_4^{2-} + M^{n+1}$$

Persulfate may also be activated by alkaline pH activation involving the addition of a concentrated base, such as sodium hydroxide (NaOH) or potassium hydroxide (KOH). The raise of the pH into a strongly alkaline range (pH 11–12) initiates decomposition of the oxidant to form radicals, although the exact nature of the mechanism is unknown. After activation, due to the high concentration of hydroxide anions (OH^-) at alkaline pH, propagation reactions promote conversion of sulfate radicals to hydroxyl radicals via the reaction (Petri et al., 2011):

$${\bullet}SO_4^- + OH^- \rightarrow SO_4^{2-} + {\bullet}OH$$

The stoichiometry of the direct sodium persulfate oxidation of TCE requires three moles of sodium persulfate per mole of TCE as illustrated below:

$$3Na_2S_2O_8 + C_2HCl_3 + 4H_2O \rightarrow 2CO_2 + 9H^+ + 3Cl^- + 6Na^+ + 6SO_4^{2-}$$

The performances of $Na_2S_2O_8$ and Fe(II)-EDTA-catalyzed $Na_2S_2O_8$ (1 g/L) for the treatment of chlorinated solvents in groundwater and soil-groundwater matrices were evaluated in the bench-scale test of Dahmani et al. (2006). The extent of destruction was 74% for PCE, 86% for TCE and 84% for cDCE by $Na_2S_2O_8$ alone and 68% for PCE, 76% for TCE, and 69% for cDCE by Fe (II)-EDTA catalyzed $Na_2S_2O_8$ in a reaction period of 120 h. Laboratory column and batch experiments were conducted to test an oxidation barrier system to remediate gasoline-contaminated groundwater (0.1-0.8 mg/L) with persulfate (20 g/L) (Liang et al., 2011). Moreover, although persulfate is a recent agent used in ISCO, it has already been

applied during remediation projects at several contaminated sites (Cronk, 2006; Smith et al., 2006). For instance, Smith et al. (2006) investigated the removal of TCE, TCA and DCE by alkaline activated persulfate. The sequential use of $Na_2S_2O_8$ and MnO_4^- at pilot-scale was conducted to assess the efficacy of each oxidant for the destruction of TCE, DCE, and VC (Sperry et al., 2002). Forty g/L of persulfate solution (645 kg $Na_2S_2O_8$) was injected into a 340 m^3 test zone into three wells (2.5 to 3.0 L/min per well) for 4 days at 8 h/day. Moreover, the treatment train initiated with the catalyzation of hydrogen peroxide (Fenton's reaction) followed by the activation sodium persulfate resulted in 77-99% removal of VOC (5 mg/L) in the groundwater (Cronk et al., 2006).

2.3.2.3. Ozone

The use of ozone in remediation is unique since it involves the application of a gas. There are two distinct forms of *in situ* ozone application including vadose zone injection of ozone gas and ozone sparging below the water table (Tsail et al., 2008). The use of ozone in remediation is performed by either direct or indirect oxidation. The direct oxidation is typically used in water treatment under acidic pH and the indirect approach in remediation with neutral to basic pH, with both methods yielding a strong oxidant as following (Hønning, 2007).

$$O_3 + 2H^+ + 2e^- \rightarrow O_2 + H_2O \text{ (direct)}$$
$$O_3 + OH^- \rightarrow O_2 + \bullet OH \text{ (indirect)}$$

On the other hand, hydroxyl radicals are very unstable and have a very short half-life. Due to its high reactivity and instability, ozone is produced on site. To ensure radical formation, hydrogen peroxide is often added to the ozone/water system resulting in enhanced •OH generation (ITRC, 2005).

$$2O_3 + H_2O_2 \rightarrow 3O_2 + 2\bullet OH$$

Due to the high, direct reactivity with alkene bonds, all chloroethenes have high rates of reaction with ozone. For example, the study of Sunder and Hempel (1997) showed the complete removal of PCE and TCE (100-1000 μM) by ozone oxidation in a lab scale test.

2.3.2.4. Hydrogen Peroxide/Fenton's Reagent

Hydrogen peroxide can be applied through either direct or indirect oxidation, typically at low hydrogen peroxide concentrations. Hydrogen peroxide can work on its own through direct

oxidation, but at low concentrations (<0.1%) it is not kinetically fast enough to degrade the contaminants before it decomposes (Chamarro et al., 2001).

Fenton's Reagent is a mixture of hydrogen peroxide (H_2O_2) and ferrous iron salts that reacts to form hydroxyl radicals (•OH) ferric iron (Fe^{3+}), hydroperoxyl radicals ($•HO_2$) and/or superoxide radicals ($•O_2^-$) (Waddell and Mayer, 2003; Hønning, 2007) according to the following reactions:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + •OH$$
$$•OH + H_2O_2 \rightarrow H_2O + •HO_2$$
$$•HO_2 \rightarrow •O_2^- + H^+ \qquad pKa = 4.8$$

Superoxide, dominant at neutral pH, and hydrogen peroxide also may contribute to hydroxyl radical formation by the overall equation:

$$H_2O_2 + •O_2^- \rightarrow O_2 + OH^- + •OH$$

The study of Yeh et al. (2003) explored the Fenton-like oxidation of TCE existing as DNAPL form in natural sands of various iron content. After passing 7 pore volumes of 1.5 or 3% H_2O_2 solution through the aquifer sand columns, the residual TCE concentration was 12.0 and 2.6% of the initial concentration, respectively.

2.4. Biological Remediation (Bioremediation) of CAH Contaminated Sites

Bioremediation of CAHs can occur through natural processes (intrinsic bioremediation) or by enhancing the natural mechanisms (enhanced bioremediation) (Vogel et al., 1987). Chloroethenes can be biodegraded under anaerobic and aerobic conditions. All related degradation mechanisms are presented and explained below.

2.4.1. Aerobic Biodegradation of CAHs

Aerobic oxidation is the microbial breakdown in which the compound serves as an electron donor and as a primary growth substrate for the microbial community. Electrons that are generated by the oxidation of the compound are transferred to an electron acceptor such as oxygen (EPA, 2000). It was shown in several studies that chlorinated ethenes can be degraded by aerobic oxidation (Hartmans and De Bont, 1992; Ryoo et al., 2000; Coleman and others, 2002; Davis et al., 2009) and by co-metabolic processes (Murray and Richardson, 1993; Pooley et al., 2009; Conrad et al., 2010). In the cometabolic oxidation, the contaminant is

oxidized incidentally by an enzyme or cofactor produced during microbial metabolism of another compound (Figure 2.2). In such a case, the oxidation of the contaminant does not yield any energy or growth benefit for the microorganism involved in the reaction (EPA, 2000).

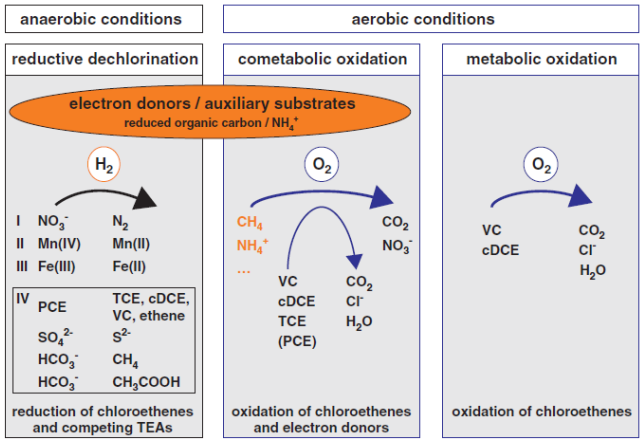

Figure 2.2. Comparison of reductive dechlorination with cometabolic and metabolic oxidation in terms of need for auxiliary substrates as electron donors and competing reactions (after Tiehm and Schmidt, 2011) (TEA: Terminal Electron Acceptor).

As the least chlorinated of the chloroethenes, VC has the greatest tendency to undergo oxidation and was the first CAH shown to serve as primary substrate for growth and metabolism under aerobic conditions. Rapid microbial degradation of VC including mineralization, has been observed in aquifer microcosms under aerobic conditions (Bradley, 2003). VC is also mineralized to carbon dioxide by pure cultures of aerobic bacteria such as *Mycobacterium* sp., *Rhodococcus* sp., *Actinomycetales* sp., or *Nitrosomonas* sp. (Lee et al., 1998). Moreover, Gossett et al. (2010) showed aerobic oxidation of VC (16-42 µmol/bottle) in VC oxidizing transfer cultures derived from two chloroethene contaminated sites, as well as in microcosms constructed from sediment and groundwater from one of these sites. In the test, VC oxidation was sustained at dissolved oxygen concentrations below 0.02 and 0.1 mg/L, respectively. This supports the possibility that, at least, at some sites, apparent loss of VC from what are thought to be anaerobic zones might, in fact, be due to aerobic pathways occurring under conditions of low oxygen flux. *In situ* aerobic cometabolism of TCE and cDCE by toluene-utilizing microorganisms was evidenced in the study of Azizian et al.

(2007) which was conducted in a shallow alluvial aquifer contaminated with 60-281 μg/L of cDCE and 112-514 μg/L of TCE. The sulfate, nitrate, oxygen and pH values were measured as 9.7-11 mg/L, 1.85-3.1 mg/L, 4.9-6.6 mg/L and 6.98-7.15, respectively.

2.4.2. Anaerobic Biodegradation of CAHs

Under anaerobic conditions, CAHs can be transformed by four processes (El Fantroussi et al., 1998), i.e. anaerobic oxidation, fermentation, co-metabolic degradation, and metabolic organohalide respiration .which will be explained in detail in the following sections.

2.4.2.1 . Anaerobic oxidation

The lesser chlorinated ethenes, VC and cDCE, can be anaerobically oxidized to CO_2 and chloride under iron- and manganese-reducing conditions, respectively. In these anaerobic reactions, the chlorinated ethene serves as the electron donor and oxidized to CO_2; iron or manganese serves as the electron acceptor (McCarty and Ellis, 2002). Since the presence of DCE and VC in groundwater is generally associated with anaerobic conditions, the potential importance of oxidative microbial degradation of chloroethenes under environmental conditions was not realized until microbial oxidation of VC under Fe(III)-reducing conditions was demonstrated in 1996 (Bradley and Chapelle, 1996). In their study, addition of Fe(III) as Fe-EDTA to anaerobic aquifer microcosms resulted in rapid mineralization (15-34%) of [1,2-^{14}C] vinyl chloride to $^{14}CO_2$ within a period of 84 h. However, possible degradation pathways and enzymes or microorganisms involved in the degradation mechanism were not reported in this study.

The possibility of anaerobic oxidation of DCE and VC to non-toxic products under anaerobic conditions might have important implications for natural attenuation of CAHs in groundwater systems. For instance, the combination of reductive dechlorination of PCE and TCE under anaerobic conditions followed by anaerobic microbial oxidation of DCE and VC might provide another microbial pathway for complete degradation.

2.4.2.2. Fermentation

In fermentative metabolism a dehalogenated intermediate serves as electron acceptor (Janssen et al., 2001). While *Dehalobacterium formicoaceticum* ferments dichloromethane to acetate and formate, *Acetobacterium dehalogenans* uses chloromethane and produces acetate as a fermentation end product (Smidt H and M de Vos, 2004). In addition, evidence has been

obtained for the occurrence of fermentative dehalogenation of halogenated ethenes and ethanes, including VC and 1,2-DCA (Bradley and Chapelle, 1999).

2.4.2.3. Co-metabolic reductive dechlorination

In cometabolic reductive dechlorination, the contaminant is reduced incidentally by an enzyme or cofactor produced during microbial metabolism of another compound. In such a case, the reduction of the contaminant does not yield any energy or growth benefit for the microorganism involved in the reaction. Cometabolic reductive dechlorination of PCE and TCE was first observed in methanogenic cultures. Under conditions of anaerobic growth, methanogens and other microbial groups harbor abundant reduced transition-metal cofactors that fortuitously dechlorinate PCE and TCE (Löffler et al., 2013).

Enzymes containing tetrapyrrole cofactors like factor F_{430}, iron porphyrins, and cobalamins have been described to dehalogenate chloroethenes. In addition, reductive dechlorination of chloroethenes can be catalyzed by corrinoids and factor F_{430} without the involvement of proteins. Because many enzymes of methanogenic archaea and other anaerobic bacteria contain these tetrapyrrole cofactors in high amounts, cometabolic dechlorination reactions are most likely catalyzed by these cofactors (Tiedje and Löffler, 1999). For example, TCE is co-metabolically transformed into cDCE, *trans*-DCE, VC and ethene by the carbon monoxide dehydrogenase enzyme of the acetotrophic methanogen *Methanosarcina thermophila* (Jablonski and Ferry, 1992). Methyl-coenzyme M reductase in *Methanobacterium thermoautotrophicum* catalyzes the reductive dechlorination of 1,2-DCA to chloroethane and ethene (Holliger et al., 1992), while reduced *c*-type cytochromes co-metabolically transform carbon tetrachloride to chloroform in *Shewanella putrefaciens* 200, a facultative anaerobic iron-reducing bacterium (Hamonts, 2009). Especially cometabolic dechlorination of PCE and TCE has been observed, in contrast significant cometabolic conversion of DCE's and VC to ethene was never reported. A reason might be that the dechlorination rates drop by an order of magnitude with each chlorine removed (Tiedje and Löffler, 1999).

2.4.2.4. Metabolic organohalide respiration

Metabolic reductive dechlorination includes the replacement of chlorine with hydrogen which requires the activity of a dehalogenase enzyme, an electron donor (usually molecular hydrogen), an electron acceptor (the chlorinated ethene), and a carbon source (usually acetate). The reaction is thermodynamically favorable under anaerobic conditions. PCE is

sequentially degraded by organohalide respiration to ethene or ethane, through intermediates such as TCE, cDCE, *trans*-DCE, 1,1-DCE and VC (Chambon et al., 2013). The rate of reductive dechlorination decreases with decreasing numbers of chloroatoms.

Several groups of bacteria such as *Desulfomonile*, *Dehalobacter*, *Desulfitobacterium* and *Desulfuromonas* are recognized to be able to degrade PCE via TCE to the end product cDCE. However, only bacteria of the *Dehalococcoides* group are capable of performing the complete reductive dechlorination from PCE to ethene (Tiehm and Schmidt, 2011). Several isolates of the genus *Dehalococcoides* can grow with halogenated alkanes and alkenes, and chlorinated benzenes, biphenyls, and dioxins. While PCE dehalogenating bacteria generally dechlorinate this carcinogenic compound to cDCE, *Dehalococcoides mccartyi* (*D. mccartyi*) strain 195 has been the first bacterial pure culture that completely dehalogenates PCE to ethene. Yet, the dechlorination steps from PCE to VC are metabolic while the dechlorination of VC is a slow, cometabolic reaction. *D. mccartyi* strain FL2 shows a physiology similar to that of *D. mccartyi* strain 195, but cannot convert PCE to TCE at high rates (Taş et al., 2009). Other two strains of *D. mccartyi*, VS and BAV1, use cDCE and VC, respectively, as electron acceptor for growth. Strain BAV1 also dechlorinates *trans*-DCE, 1,1-DCE, and 1,2-dichloroethane, whereas PCE and TCE are converted only co-metabolically. Another group of *Dehalococcoides* spp., strain CBDB1, has the ability to grow with halogenated aromatic compounds as terminal electron acceptors (Smidt and De Vos, 2004). Recently, a novel *Dehalococcoides* containing culture that dechlorinates PCE to ethene was enriched from a contaminated anaerobic aquifer in Bitterfeld (Eastern Germany). From this culture the PCE degrading strain *D. mccartyi* BTF08 was isolated (Cichocka et al., 2010).

To summarize, while many bacteria have been found to dechlorinate PCE and/or TCE to cDCE, only some bacteria of the genus *Dehalococcoides* have been documented to be capable to dechlorinate PCE to ethene, as summarized in Figure 2.3.

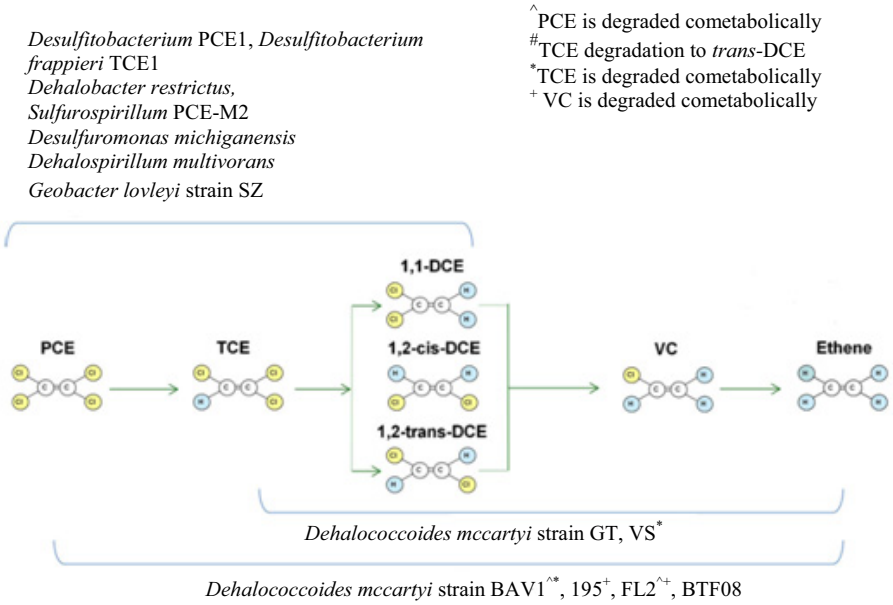

Figure 2.3. Bacterial strains performing sequential reductive dechlorination steps by dehalorespiration (after Chambon et al., 2013).

Reduction of chloroethenes by *D. mccartyi* strains is mediated by membrane-bound reductive dehalogenase enzymes (RDases), while hydrogenase enzymes (H_2ases) are likely involved in the stripping of electrons from H_2 (Nijenhuis and Zinder, 2005). Reductive dehalogenases are characterized in halorespiring organisms and are the key catalysts in the halorespiration of PCE, TCE, VC and 1,2-DCA (Maillard et al., 2003; Smidt and de Vos, 2004; Marzorati et al., 2007). In *D. mccartyi* strain 195, RDases PceA and TceA have been shown to catalyze the reduction of PCE to TCE and TCE to ethene, respectively. However, recent studies have shown that transcripts of other RDases, including DET1559 and DET1545, can be detected at relatively high numbers in actively PCE respiring cultures (Rahm and Richards, 2008). *D. mccartyi* 195 also contains a second enzyme, TCE-RDase, which is the only known RDase that is able to catalyze the complete dechlorination of TCE, DCEs, and VC to ethene (Magnuson et al., 2000). In addition, the RDases responsible for catalyzing VC transformation to ethene have been identified in strain VS and BAV1, and are encoded by the *vcrAB* gene in strain VS and the *bvcAB* gene in strain BAV1 (McMurdie et al., 2009; Taş et al., 2009).

A number of *in situ* bioremediation studies have been reported for the removal of chlorinated compounds through metabolic organohalide respiration. For instance, the potential use of intrinsic and/or enhanced bioremediation to remediate PCE and TCE to innocuous ethene and ethane with a complete conversion in 7 months was documented in the field study of Arthur and Claycomb (2005). The indicator parameters and corresponding ideal ranges were recorded as sulfate (< 20 mg/L), TOC (> 10 mg/L), ethane, ethene, and methane, total ferrous iron (Fe^{2+}), pH (6.0-8.5), dissolved oxygen (<0.5 mg/L), and oxidation-reduction potential (ORP, from −300 to +750 mV). Moreover, the natural attenuation over a 4 month period of an alluvial aquifer contaminated with PCE (2-11 mg/L), TCE (12-56 mg/L), and cDCE (2-14 mg/L) was documented (Courbet et al., 2011). In another study, enhanced bioremediation was successfully performed at a low permeability clay till site contaminated with TCE (102,000 $\mu g/m^3$ in pore air, 740 mg/L in groundwater, and 62 mg/kg in sediment) by direct push injection of molasses and dechlorinating bacteria. The performance was investigated by long-term groundwater monitoring and after 4 years of remediation an average mass reduction of 24% was estimated (Damgaard et al., 2013).

2.5. Interaction between abiotic and biotic remediation of CAH contaminated sites

There are several technologies available to remediate groundwater and aquifer systems contaminated with CAHs. Usually, individual treatment options (e.g., only chemical oxidation or only bioremediation) are preferred at *in situ* applications. Indeed, chemical and physical processes are often used to treat huge masses of pollutants in shorter time. Yet, it is difficult to achieve regulatory limits due to the heterogeneous or low-permeability formations, and large plumes in the subsurface. Similarly, bioremediation technologies require bioavailability and insights in microbiology. However, concurrent or successive application of ISCO and biodegradation might be the right option in some cases considering the amount of contaminants, site conditions, biological potential/capacity of the site, etc. Although most of the studies in literature focused only on the effects of the oxidants on the microorganisms, only a few documented the effects on the contaminant removal after concurrent or successive applications. For instance, in the batch test of Goi et al. (2006), 80% of removal of shale (5.5 g/kg) and transformer oils (17.4 g/kg) was achieved by biotreatment after the application of H_2O_2 to Fe-containing soil. However, in the same study, the removals after biodegradation alone were recorded as 52% and 50% of shale and transformer oils, respectively. In another example, catalyzed hydrogen peroxide (CHP) was applied (1,574–8,862 mM H_2O_2 and 47–

180 mM Fe(II)) to soil microcosms contaminated with PAH (mainly 258 g/kg of phenanthrene and 228 g/kg of fluoranthene) (Valderrama et al., 2009) and removal efficiencies were documented. In this study, overall PAH removal was recorded as 75% under chemical oxidation followed by biodegradation compared to values of 45 and 71%, estimated after biodegradation and oxidation, respectively. Only one study investigated the combined effect of ISCO and biodegradation on the removal of TCE (1-10 mg/L) from a field site (Sercu et al., 2013). The oxidizing conditions (from 9-90 mV to 95-691 mV), the absence of VC, and undetectable *Dehalococcoides* spp. suggested the absence of ongoing reductive dechlorination in most of the wells after permanganate injection (> 10 mg/L). However, the low concentrations of cDCE suggested partial reductive dechlorination in the wells with the highest TCE concentrations, but at low rates.

Therefore, the interaction between chemical and biological treatment is really an important issue especially during combined/sequential treatment applications. There are several ways in which ISCO could be beneficial to natural attenuation by generating reaction byproduct that can increase the bioavailability of some nutrients, resulting in increased microbial growth. Moreover, the injection of oxidants might result in the addition of various terminal electron acceptors including dissolved oxygen from H_2O_2 and O_3; SO_4^{2-} from $S_2O_8^{2-}$; and Mn^{4+} from MnO_4^- which might induce activity of oxygen, sulfate and manganese reducing microorganisms, respectively (Heimann et al., 2005; Huling and Pivetz, 2005).

Some of the studies presented in Table 2.4 support positive effects of ISCO on the subsequent contaminant biodegradation processes. For instance, Miller et al. (1996) observed, after the application of 150-300 g/kg of Fenton's reagent on pendimethalin contaminated soil, that the organic matter released into the solution was biodegradable and served as substrate for microbial growth. Although an overall decrease in heterotrophic diversity was observed after oxidant exposure, an increase in the *Pseudomonas* spp. number was recorded suggesting that Fenton's treatment created favorable conditions for microorganisms of interest for bioremediation. Ozonation (1.5 mg/L ozone) followed by biodegradation was also efficient in the transformation of PAHs (366-1205 μg/g) since ozonation generated water-soluble and oxygenated PAH metabolites, which were more biodegradable compared to the parent compound (Nam et al., 2000). In another study, biological treatment was combined with chemical oxidation with ozone to enhance the degradation of PAHs in contaminated soil (Derudi et al., 2007). In that study, 10.2 mg/kg of PAH contaminated soil was first treated

biologically after which ozonation (2 g ozone/h, for a period of 16 h) was applied as a polishing step. In another study ozonation resulted into decreased numbers of heterotrophic, alkane-degrading, and phenanthrene-degrading bacteria (from 10^8 to 10^4, 10^7 to 10^3, and 10^6 CFU /g soil to below detection limit, respectively) after 900 min exposure (Jung et al., 2005). However, after 9 weeks, a recovery was observed.

Despite the potential benefits of chemical oxidation processes towards stimulation of microbial degradation processes, there are also some possible negative effects. For example, residual oxidant due to unproductive consumption caused by matrix demand, might alter the pH and redox potential affecting the indigenous microbial community (Hrapovic et al., 2005). For many environmental contaminants, biodegradation is the result of microbiologically catalyzed redox reactions in which the contaminant compound is transformed while serving either as an electron donor or as an electron acceptor in a biochemical reaction. Thus, *in situ* redox conditions are a primary determinant of the efficiency of biodegradation for many environmental contaminants (Bradley and Chapelle, 2010). For example, in the field study of Klens et al. (2001), the redox potential increased from 100 mV to 800 mV after injection of a 0.7% solution of $KMnO_4$ followed by decreased numbers of aerobic and anaerobic heterotrophs, nitrate reducers, sulfate reducers, and methanogens. Aerobic heterotroph counts in groundwater increased substantially within 6 months. It is a fact that under anoxic conditions, chlorinated ethenes are subject to reductive dechlorination. However, the efficiency of dechlorination may change under methanogenic, sulfate, iron(III), and nitrate-reducing conditions which are a result of the oxidants (Friedman and Gossett, 1989). Dechlorination of PCE and TCE to cDCE is favored under mildly reducing conditions such as nitrate or iron (III) reduction, whereas the transformation of cDCE to VC or VC to ethene requires more strongly reducing conditions such as methanogenic conditions (Distefano et al., 1991). Therefore, not only shifts in the number/activity of the microorganisms but also the geochemical parameters (pH, redox value, TOC, type and concentration of terminal electron acceptors) should be monitored during post-oxidation phase since they might cause possible shifts in the microbial diversity, and in turn, a competition between different species might be observed. In this sense, the results of the study of Hrapovic et al. (2005) might be a good example to observe the negative effect of permanganate on reductive dechlorination. Following permanganate (2.5 g/L) application which resulted in rapid but incomplete removal of 920 mg/L of TCE, biological activity was not recorded following the addition of distilled water amended with ethanol and acetate. The reductive dechlorination was only observed

when bioaugmentation was applied and the reducing conditions were achieved in the system. The effects of heat-activated 0.1, 1 and 10 g/L of persulfate on indigenous microorganisms and microcosms augmented with *Pseudomonas putida* KT2440 were studied in laboratory batch reactors with aquifer material (Tsitonaki et al., 2008). No significant toxicity effect was noted for indigenous microorganisms after a 2-day exposure but the number of augmented cells was decreased from 2.96×10^6 cells/g soil to 3.35×10^5 cells/g soil under 10 g/L of chemical oxidant.

Shifts in the microbial community structure are also an important parameter since the community might change completely after oxidant application. In the study of Sutton et al. (2013) differences in community structure were observed following chemical oxidation in lab microcosms. Ninety two g/L $KMnO_4$, 139 g/L $Na_2S_2O_8$, 150 mg/L Fe^{2+} and 5% H_2O_2 Fenton's reagent and modified Fenton's reagent were used to treat total petroleum hydrocarbons (TPH, 11.1-25.4 g/kg) contaminated soil. Total bacterial 16S rRNA gene abundance was reduced one order of magnitude and/or below detection limit. Moreover, a reduction of bacterial biodiversity was also recorded right after Fenton's application in a TPH contaminated pilot plant (Silva-Castro et al., 2013). Macbeth et al. (2005) observed a decreased microbial diversity in groundwater (PCE,17-190 μg/L) samples one year after permanganate application. Moreover, after permanganate exposure, *Dehalococcoides* spp. were not detected in any of the wells. However, as oxidant concentrations decreased below a certain threshold, a few microbial populations were stimulated; as oxidant concentrations decreased further, additional populations were stimulated resulting in an increased biomass and diversity. Diversity even recovered to pre-ISCO levels.

Generally, in most of the literature studies, a reduction in the number/activity of the microorganisms was observed after treatment with a chemical oxidant but such a reduction was mostly followed by a recovery. However, the time for reactivation differed from 60 h to one year in these studies (Table 2.4) which may change due to test conditions, type of microorganisms, concentration and type of oxidant.

Table 2.4. Studies regarding the effects of ISCO on biological processes

Oxidant	Concentration of oxidant	Scale	Target contaminant	Type of microorganisms	Reactivation of micro.	Time to recover	Observed Changes	Reference
CHP	150-360 g/kg	Lab, soil	Pendimethalin	Aerobic	yes	~60 h	Increased biomass, decreased diversity	Miller et al., 1996
$KMnO_4$	40 g/L	Field, soil and groundwater	TCE (10-1,112 mg/L), cDCE (600-22,000 mg/L)	Anaerobic, aerobic	NOE	NA	No observed negative effects to aerobic degradation	Gardner et al., 1996
CHP	0.77–0.2 g/L	Lab, culture	-	*X. flavus*	NOE	NA	Mineralization decreased when $[H_2O_2]$ >77 mg/L	Büyüksönmez et al., 1999
CHP	10-20 g/L	Lab	PAHs, PCP	Aerobic	yes	6 days	Decrease of cell concentrations first, then increase	Allen & Reardon, 2000
Fenton's reagent	50 g/L	Field, aquifer	TCE, PCE	Methanotrophs	yes	10 months	Rebound in microbial biomass/activity	Kastner et al., 2000
$KMnO_4$	7 g/L	Field, groundwater and soil	TCE	Anaerobic, aerobic	yes	6 months	Rebound of aerobic and anaerobic biomass	Klens et al., 2001
Ozone	300 g/L	Lab, soil	PAHs (0.6 mg/g)	Aerobic	NOE	2 weeks	Successful coupling observed (chemical and biological)	Nam et al., 2001
$KMnO_4$	NR	Field, aquifer	TCE	Anaerobic, aerobic	yes	1 year	Short-term increased biomass	Azadpour-Keeley et al., 2004
Fenton's reagent	NR	Field, groundwater	PCE (4.5 mg/L), VC(0.8mg/L)	Anaerobic	yes	< 6 months	Decreased efficiency of reductive dechlorination	Chapelle et al., 2005
$KMnO_4$	2.5 g/L	Lab, groundwater and aquifer	TCE (920 mg/L)	Anaerobic	yes	6–7 months	Slight rebound of TCE dechlorination	Hrapovic et al., 2005
Ozone	NR	Lab, soil	Petroleum hydrocarbons (2500 mg/kg)	Heterotrophic, phenanthrene and alkane degrading	yes	9 weeks	One or two orders of magnitude increase in cell number	Jung et al., 2005

$KMnO_4$	NR	Field, groundwater	PCE	Anaerobic	yes	1 year	Increased biomass, decreased diversity	Macbeth et al., 2005
Fenton's reagent	10-200 nM	Lab, soil	PCE (120 mg/kg)	Aerobic	yes	NA	No decrease in activity	Ndjou'ou et al., 2006
CHP	NR	Lab	PAHs	NR	yes	1 week	Slight decrease in bacterial biomass following oxidation	Sahl and Munakata-Marr, 2006
Heat activated persulfate	0.1, 1, 10 g/L	Lab	NA	*P. putida*	NOE		Detrimental effect to *P. putida* survival	Tsitonaki et al., 2008
$Na_2S_2O_8$	20 g/L	Lab	NA	Phenanthrene degrading bacteria	yes	30 days	Rapid recovery of both abundance and activity	Richardson et al., 2011
$Na_2S_2O_8$ $KMnO_4$	50, 150, 300 g/kg sediment	Lab	mineral oil, PAHs and heavy metals	Indigenous microor.	NOE	NA	Decrease (2 log units) in the number of 16S rRNA gene of total bacteria	Doğan et al., 2013
Fenton's reagent		Lab, culture	Hexachloroethane and PCE (0.12 mM)	Anaerobic microorganisms	yes	3-7 days	Decrease in diversity first, then observed increase in diversity	Jho et al., 2013
$NaMnO_4$	> 10 mg/L	Field, groundwater	TCE(1-10 mg/L)	Indigenous microor.	yes	1 year	Significant reduction in activity, recovery in several week	Sercu et al., 2013
Fenton's reagent	NR	Pilot, soil	Hydrocarbons (20g/kg)	Heterotrophic	yes	28 days	Reduction in bacterial diversity, stimulation in activity	Silva-Castro et al., 2013
Fenton's, $Na_2S_2O_8$	8.3-14 g/kg	Lab, soil	Petroleum hydrocarbons (11.1-25.4 g/kg)	Indigenous microor.	yes	8 weeks	Reduction in activity, recovery in several weeks	Sutton et al., 2013

NR =Not reported; NOE = No observed effect; NA = Not applicable; PCE = tetrachloroethene; TCE = trichloroethene; cDCE = *cis*-dichloroethene PAHs = Polycyclic aromatic hydrocarbons; PCP = Pentachlorophenol; CHP = catalyzed hydrogen peroxide.

2.6. Methods for Quantification of CAH Biodegradation

Since the bioremediation of CAH contaminated sites is mediated by the indigenous microbial community or introduced organisms, monitoring of contaminant degradation requires the integration of quantitative geochemical measurements with measurements of the abundance and activity of the key microorganisms involved in the degradation process. In addition, understanding the mechanisms responsible for biodegradation allows the enhancement of these processes through an engineering approach. Although geochemical data can help researchers to understand environmental conditions and the fate and transformation rates of contaminants, collecting microbial data is essential since the structure and function of microbial communities exhibit significant spatial and temporal variability (Weis and Cozzarelli, 2008). Therefore, several analytical, microbial and molecular methods have been used to quantify CAH biodegradation such as compound specific isotope analysis (CSIA), flow cytometry (FCM), fluorescence *in situ* hybridization (FISH), quantitative polymerase chain reaction (qPCR), RNA analysis, etc. Some of these techniques are explained in the following sections.

2.6.1. Quantitative PCR (qPCR)

Quantitative real-time PCR (qPCR) is a powerful nucleic acid quantification technique enabling researchers to answer questions concerning the growth and dehalogenating abilities of microbial populations in environmental systems (Cupples, 2008). The applications of qPCR in dehalogenation processes provide opportunities to make a connection between number of species and dehalogenating activities, to investigate interactions between dehalogenating bacteria and other species and to analyze the effects of growth factors and physico-chemical (field) conditions on dehalogenating bacteria (Ding and He, 2012).

However, the quantification of the 16S rRNA gene does not necessarily refer to dehalogenation ability because of the sequence similarity between *D. mccartyi* spp. Therefore, 16S rRNA-targeted qPCR is frequently inadequate for characterizing dehalogenating ability and, as a result, quantification of the reductive dehalogenase genes via qPCR is gaining importance. Specific primers have been designed for targeting the *pceA* encoding PCE reductases from *D. mccartyi* strain 195 and CBDB1 (Behren et al, 2008); the TCE to VC reductive dehalogenase gene *tceA* of *D. mccartyi* strains 195 and FL2, and the VC reductive dehalogenase genes, *vcrA*, from *D. mccartyi* strains VS and GT and *bvcA* from *D. mccartyi*

strain BAV1 (McMahone et al. 2009). In addition, enumeration of the reductive dehalogenase gene expression via reverse transcriptase qPCR indicates the dehalogenating activity.

2.6.2. ATP Analysis

The quantification of the concentration of Adenosine tri-phosphate (ATP) can be used as a complementary method for viability assessment (Hammes et al., 2008). Measurements of biomass are important parameters in environmental microbiology and can be estimated from the measurement of (i) cell biovolume converted into carbon (based on direct measurements of bacterial cell carbon content, cell number, and biovolume and estimation of a conversion factor; Bratbak, 1985) or (ii) from the analysis of individual cell components which are highly correlated to biomass (e.g. protein or ATP concentration). A typical method for ATP quantification involves the chemical and/or enzymatic extraction of ATP from bacterial cells, followed by the measurement of light emission derived when the dissolved ATP reacts with the Luciferine Luciferase complex (Hammes et al., 2010) (Figure 2.4).

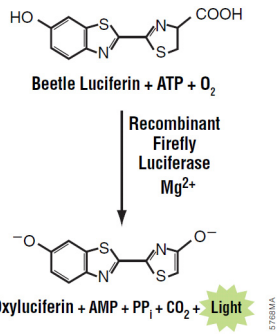

Figure 2.4. The luciferase reaction. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of the Mg^{2+}, ATP and molecular oxygen.

2.6.3. Compound Specific Isotope Analysis (CSIA)

The determination of compound-specific stable isotope ratios is a well-established tool to assess biodegradation of organic compounds in groundwater (Hunkeler et al., 1999). Biological reactions in subsurface aquifer are often accompanied by dissolution, volatilization, and sorption which make it difficult to link changes in chloroethene concentration to biological conversion. One solution to overcome this problem might be the quantification of changes in the stable carbon, chlorine and hydrogen isotope ratios of the chloroethenes. When organic contaminants are degraded in the environment, the ratio of

stable isotopes often changes; the stronger and less reactive isotopes are enriched as lighter isotopes are degraded through biodegradation. The extent of degradation can be recognized and predicted from these changes (Lee et al., 2007). CSIA applications on chlorinated ethenes are numerous including studies to discriminate between biotic and abiotic reductive dechlorination and aerobic biodegradation (Nijenhuis et al., 2005; Liang et al., 2007(a); Abe et al., 2009; Elsner et al., 2008).

Contaminant-specific isotope measurements do not only offer the possibility to qualitatively detect, but also to quantitatively estimate the extent of contaminant degradation. Since transformation causes isotope fractionation, greater changes in isotope values are expected for a greater extent of degradation. For example, the Rayleigh equation links degradation-induced shifts in isotope ratios to the extent of degradation.

$$\delta^{13}C_{compound} = [\,(^{13}C/\,^{12}C)_{compound} - (^{13}C/\,^{12}C)_{ref}] \,/(^{13}C/\,^{12}C)_{ref}$$

$$= (^{13}C/\,^{12}C)_{compound}/\,(^{13}C/\,^{12}C)_{ref}\text{-}1$$

$$(^{13}C/\,^{12}C) \,/\, (^{13}C/\,^{12}C)_0 = [(\,\delta^{13}C+1)/\,(\delta^{13}C_0+1)] \qquad \text{(Elsner, 2010)}$$

In which $(^{13}C/^{12}C)_0$ is the isotope ratio of a given organic compound when it has not yet been degraded. $(^{13}C/^{12}C)$ is the isotope ratio of the same compound after a certain extent of degradation has occurred, and f is the fraction of the compound remaining at this stage of degradation. $\delta^{13}C_0$ and $\delta^{13}C$ are the isotope ratios expressed in the Delta notation (Elsner, 2010).

Even though different mechanisms are generally associated with a different ε, and estimated numbers of ε may distinguish degradation pathways in lab experiments (Hirschorn et al., 2004), it is usually not possible to identify the reaction mechanism at *in situ* applications based on isotope data of a single element only. Conversely, multiple isotope analysis (e.g., $^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$) may provide unique insight into contaminant degradation pathways (Elsner, 2010; Cretnik et al., 2013).

Chapter 3. Long term dynamics of *Dehalococcoides* spp. and *tceA* and *vcrA* gene copies and transcripts under TCE exposure

Abstract

This study aimed at monitoring the dynamics of phylogenetic and catabolic genes of a dechlorinating enrichment culture before, during, and after complete dechlorination of chlorinated compounds. More specifically, the effect of 23 µmol trichloroethene/bottle (TCE) and 5.6 mM lactate on 16S rRNA and RDase gene abundances and activity of an enrichment culture was investigated for 40 days. The results revealed that total ATP concentrations increased from 2 to 5 ng/mL after the addition of TCE and lactate. Although *tceA* and *vcrA* gene copy numbers were relatively stable in DNA extracts over time, *tceA* and *vcrA* mRNA abundances were up-regulated from undetectable levels to 2.96×10^4 transcripts/mL and 6.33×10^4 transcripts/mL, respectively, upon exposure to TCE and lactate. While *tceA* gene transcripts decreased over time with TCE dechlorination, the *vcrA* gene was expressed steadily even when the concentration of vinyl chloride was at undetectable levels. In addition, ratios between catabolic and phylogenetic genes indicated that *tceA* and *vcrA* gene carrying organisms dechlorinated TCE and produced cDCE and VC, while *vcrA* gene was mainly responsible for the dechlorination of VC in the later stage of TCE degradation.

Chapter redrafted after: Doğan-Subaşı E, Bastiaens L, Leys N, Boon N, Dejonghe W (2013). Quantitative and functional dynamics of Dehalococcoides spp. and its tceA and vcrA genes under TCE exposure. Biodegradation, DOI 10.1007/s10532-013-9676-8

1. Introduction

Chlorinated solvents like tetrachloroethene (PCE) and trichloroethene (TCE) are among the most abundant groundwater contaminants. Moreover, vinyl chloride (VC), which is a biodegradation product of PCE and TCE, is even more toxic and highly carcinogenic (Kielhorn et al., 2000) and its accumulation may cause serious problems in ecosystems. Bioremediation, where reductive dechlorination of the chlorinated ethenes is coupled to bacterial growth, is a well established technique to remove these pollutants from the environment (He et al., 2003; Lee et al., 2006). Although numerous bacterial isolates with reductive dechlorination capacity have been described (Smidt and M. de Vos, 2004), only *Dehalococcoides mccartyi* spp. (*Dhc* spp.) can reductively dechlorinate PCE and TCE beyond dichloroethene (DCE) to VC and finally harmless ethene (Maymo-Gatell et al., 1997; Holmes et al., 2006). *Dhc* spp. has reductive dehalogenase (RDase) genes that encode the proteins responsible for the respiration of chlorinated ethenes. The *tceA* gene encodes a reductive reductase (*Dhc mccartyi* 195 and FL2) catalyzing the transformation of TCE to cDCE and VC (Magnuson et al., 2000; He et al., 2005). Other well-studied RDase encoding genes detected in *D. mccartyi* strains VS and GT, and *D. mccartyi* BAV1, respectively, are *vcrA* and *bvcA* which are involved in the conversion of cDCE to VC (*vcrA* gene, Behrens et al., 2008) and VC to ethene and ethane (*vcrA* and *bvcA* genes, Magnuson et al. 2000; He et al., 2003; Müller et al., 2004; Van der Zaan et al., 2010).

Several studies have been performed regarding the dynamics of 16S rRNA and RDase gene copy (Holmes et al. 2006; Sung et al., 2006) and transcript numbers (Johnson et al., 2005b; Lee et al., 2006; Rahm et al., 2006; Behrens et al., 2008; Lee et al., 2008) in response to TCE exposure. For instance, in a TCE degrading anaerobic enrichment culture, the number of 16S rRNA of *Dhc*, *tceA* and *vcrA* gene copies were measured as 3.70×10^7, 2.80×10^7 and 1.10×10^7 copies/mL, respectively, during degradation of 880 µM of TCE (Holmes et al., 2006). Johnson et al. (2005b) indicated that *tceA* transcripts increased approximately from 1.0×10^9 to 4×10^{10} transcripts/mL when a *Dehalococcoides* containing enrichment culture was exposed to 400 µM of TCE. Maximum levels of *tceA* transcripts were obtained after 3 h and stayed constant for at least 24 h. Rahm et al. (2006) demonstrated that in an enrichment culture containing *D. mccartyi* strain 195, *tceA* gene transcripts increased during a 3 h dechlorination period with 60 µM of PCE. However, great temporal variability was noted since the expression levels of RDases depended on time of sampling and individual target expression

did not appear to correlate with the instantaneous dechlorination rate of specific chlorinated ethenes. In another study performed with a *Dehalococcoides*-containing enrichment culture, 240 μM of TCE was fully converted to ethene in 4 days. One day after TCE addition, the number of *tceA* and *vcrA* transcripts increased from 0.001 to 10 transcripts/gene. These levels of transcripts were constant over the 6 days monitoring period (Lee et al., 2006). The study concluded that while gene expression correlated generally to the presence of chlorinated ethenes, there was no apparent direct relationship between RDase-encoding transcript numbers and respective rates of TCE dechlorination. On the other hand, long term degradation of chlorinated solvents and related change in RDase gene expressions were only studied in a field study where the groundwater was contaminated with TCE (50-80 μM) and cDCE (60-100 μM) (Lee et al., 2008). Through a 1-year monitoring period, the *tceA, vcrA* and *bvcA* genes were expressed in the range of 0.1-10, 1-10 and 0.1-1 transcripts/gene, respectively. In general, these studies indicated that it is essential to quantify the abundances but most importantly transcripts of the phylogenetic and mainly functional genes to monitor the dechlorination activity of a site (Müller et al., 2004; Lee et al., 2006; Ritalahti et al., 2010; Van der Zaan et al., 2010). The methods for their detection and quantification at DNA level are also commercially available but not at RNA level (SERDP, 2005; Scheutz et al., 2008; URL-1).

This study addresses the key metabolic responses of an anaerobic dechlorinating enrichment culture, and more specifically its chlorinated ethene degrading microbial population, during long term exposure to TCE. To the best of our knowledge this is the first time that the dynamics of the related genes were investigated over a long term period of 40 days and more in particular before, during and after complete dechlorination of TCE. For this purpose, general (flow cytometry (FCM), total ATP, and 16S rRNA gene of total bacteria) as well as specific (16S rRNA and RDase encoding genes of *Dhc* spp.) biomarkers were used, both at presence and activity level.

2. Materials and Methods

2.1. Bacterial culture

The used microbial culture was enriched from sediment taken from the Zenne River, Vilvoorde, Belgium (Hamonts et al., 2009) via transfers in an anaerobic mineral medium (Haest et al., 2011) during 5 years. Briefly, 180 g of sediment were shaken in 1.6 L of anaerobic mineral medium overnight in a 2-L glass bottle. After 6 h of sedimentation,

supernatant was taken and transferred into fresh anaerobic mineral medium with an inoculum/medium ratio of 1:10 with addition of 30 µM PCE, 52 µM cDCE and 5.6 mM lactate as electron acceptors and donor, respectively.

2.2. Experimental set-up

Active enrichment culture (900 mL) was transferred into two 1-L glass bottles capped with pre-autoclaved Teflon lined septa. After a lag phase of three weeks, the bottles were spiked with 23 µmol TCE (99%, Acros Organics, New Jersey, USA) and 5.6 mM lactate (50%, Merck, Germany). All bottles were kept at 28 °C throughout the experiment. Subsamples were taken in function of time to monitor the evolution of the chlorinated aliphatic hydrocarbon (CAH) concentrations and the biomarkers.

To investigate whether the increase in ATP observed in the mixed culture (see Results section) was due to the activity of *Dhc* spp. (growing on TCE and lactate) or due to the activity of the total microbial community (growing on lactate/TCE or lactate), an additional test was performed in triplicate. In 160 mL pre-autoclaved serum bottles with Teflon lined septa, 100 mL of mixed culture was brought together with only 23 µmol TCE, only 5.6 mM lactate, or both 5.6 mM lactate and 23 µmol TCE. The ATP production and remaining CAH-concentrations were measured each day for a period of 11 days.

2.3. Chemical analyses

Aqueous samples (5 mL) taken each day from the 1 L bottles were transferred into 10 mL glass vials, capped with pre-autoclaved Teflon lined septa and amended with 100 µL of phosphoric acid (85%, Merck, Germany) to stop the microbial activity. The concentrations of TCE, cDCE, VC, methane, ethene and ethane were measured by GC-FID (Varian GC-FID (CP-3800)), as described by Hamonts et al. (2009), reflecting the CAH concentrations in the liquid phase of the 1-L bottles. The corresponding concentrations in the gas phase were calculated via the Henry's law (Gossett, 1989) using the dimensionless H_C values for 28 °C for TCE (0.475), cDCE (0.155), VC (1.189) and ethene (1.80). The CAH concentrations are expressed as the sum of the measured amounts in the liquid phase and calculated amounts in the gas phase.

2.4. DNA and RNA extractions

DNA was extracted as described by Hendrickx et al. (2005) by using 2 mL aqueous sample without concentrating the cells. The RNA was isolated from 2 mL of sample by MO-BIO RNA PowerSoil® Total RNA Isolation Kit according to the manufacturer's recommendations. The RNA was treated with DNase in two successive steps (2µL of DNase in each step) with the DNA-*free* kit (Ambion, Austin, TX, USA) to remove contaminating DNA. The DNase treated RNA samples were subjected to PCR amplification with primers 63F (CAGGCCTAACACATGCAAGTC) and 518R (ATTACCGCGGCTGCTGG), and amplification products were analyzed on a 1.5% (wt/vol) agarose gel to assess DNA contamination. Reverse transcription was performed with GeneAmp® RNA PCR Core Kit (Applied Biosystems). Master mix consisted of 2 µL of random hexamer primers (50 µM, Applied Biosystems), 4 µL 10×PCR buffer, 4 µL dNTP mix (2.5 mM), 1.6 µL $MgCl_2$ (25 mM), 2 µL RNase inhibitor (20 U/µL) and 2 µL MuLV reverse transcriptase (50 U/µL). Finally, 15 µL of master mix was mixed with 12 µL of RNA sample (DNase treated) and 12 µL nuclease free water. The reverse transcription was proceeded in a T3 Thermocycler (Biometra, Germany) with an incubation time of 45 min at 48 °C followed by 55 min at 99 °C. cDNA was obtained in a final volume of 39 µL and stored at -80 °C until qPCR analyses.

2.5. Quantitative PCR analyses

The qPCR reaction was performed in volumes of 12.5 µL, containing 6.25 µL 2xABgene SYBR Green mix (AB gene, Epsom, Suirrey, UK), 2.5 µL of each primer and 1.25 µL DNA template. In case a TaqMan probe was used, 6.25 µL ABGene qPCR mix (ABgene, Epsom, Surrey, UK), 0.375 µL of each primer, 0.375 µL probe, 3.875 µL nuclease free water (MO-BIO, Carlsbad, California, USA) and 1.25 µL DNA template were used. Primers and probes are provided in Table 3.1 with indication of the applied concentrations of each primer and probe that were synthesized by Operon Biotechnologies GmbH (Cologne, Germany). qPCR was performed in a Westburg Rotor-Gene 3000 (Corbett Research, Sydney, Australia). A calibration curve, ranging from 10^2 to 10^8 gene copies per µL of template DNA, was achieved using 10-fold serial dilutions of pure plasmid DNA carrying either a cloned bacterial and *Dhc* 16S rRNA gene or *tceA*, *vcrA* and *bvcA* genes of *Dehalococcoides mccartyi* strain 195, strain VS and BAV1, respectively. Experiments were performed in triplicate along with a template-free control. The algorithm calculated the efficiency, threshold cycle (C_T), slope and intercept as well as R^2 (>0.99) values.

2.6. ATP analyses

ATP was measured using the BacTiter-Glo Microbial Cell Viability Assay (G8231, Promega Corporation, Dübendorf, CH) and a Luminoskan Ascent Luminometer (Thermo Labsystems, USA). The BacTiter-Glo reagent was prepared according to the manufacturer's guidelines. One hundred µL of culture was transferred to white wall 96-multiwell plates (Nunclon Delta, Denmark) and 100 µL of reagent at 38 °C was added. After 1 min of incubation at room temperature, the measurement was performed with an integration time of 10 sec. The standard curve was prepared in the same way, by adding 100 µL of 38 °C reagent to 100 µL of ATP standard solutions ranging between 10^{-6} M to 10^{-12} M ATP including blank. Active cell determination was based on converted ATP amounts using the assumptions that one cell contains 20 fg of carbon (Griebler et al., 2002) and 1 ng/mL ATP = 250 ng/mL cell carbon (Karl, 1993).

Table 3.1. Overview of primers and TaqMan probes used in this study.

Primers/probes	Sequence (5'-3')	Target gene	Amplicon length (bp)	Primer/probe concentration (nM)	References
Eub341F	5'-CCTACGGGAGGCAGCAG-3'	16S rRNA gene of *Bacteria*	194	400	Muyzer et al., 1993; Smits et al. (2004)
Eub534R	5-'ATTACCGCGGCTGCTGGC-3'			400	
Dhc1200F	5'-CTGGAGCTAATCCCCAAAGCT-3'	16S rRNA gene of *Dehalococcoides* spp.	72	300	He et al. (2003), Ritalahti et al. (2006)
Dhc1271R	5'-CAACTTCATGCAGGCGGG-3'			300	
Dhc1240Probe[b]	5'-FAM-TCCTCAGTTCGGATTGCAGGCTGAA-TAMRA-3'			300	
TceA1270F	5'-ATCCAGATTATGACCCTGGTGAA-3'	*tceA* gene of *D. mccartyi* strain 195 and FL2	67	100	Johnson et al. (2005), Ritalahti et al. (2006)
TceA1336R	5'-GCGGCATATATTAGGGCATCTT-3'			100	
TceA1294Probe[b]	5'-FAM-TGGGCTATGGCGACCGCAGG-TAMRA-3'			100	
Vcr1022F	5'-CGGGCGGATGCACTATTTT-3'	*vcrAB* gene of *D. mccartyi* strain VS and GT	72	100	Ritalahti et al. (2006)
Vcr1093R	5'-GAATAGTCCGTGCCCTTCCTC-3'			300	
Vcr1042Probe[b]	5'-FAM-CGCAGTAACTCAACCATTTCCTGGTAGTGG-TAMRA-3'			100	
Bvc925F	5'-AAAAGCACTTGGCTATCAAGGAC-3'	*bvcA* gene of *D. mccartyi* strain BAV1	93	100	Ritalahti et al. (2006)
Bvc1017R	5'-CCAAAAGCACCACCAGGTC-3'			200	
Bvc977Probe[b]	5'-FAM-TGGTGGCGACGTGGCTATGTGG-TAMRA-3'			100	

[b] All TaqMan probes have 6-carboxyfluorescein (FAM) as a reporter fluorophore on the 5' end, and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) as quencher on the 3' end.

2.7. Flow cytometry analyses

Using live-dead staining with SYBR Green and Propidium iodide (PI), cells were enumerated through FCM. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. The SYBR Green stain generally labels all bacteria in a population both with intact membranes and damaged membranes when used alone. In contrast, PI penetrates only bacteria with damaged membranes, causing a reduction in the SYBR Green stain fluorescence when both dyes are present (Invitrogen, 2004). A stock stain solution was prepared by adding 10 µL SYBR Green (1:100 dilution in DMSO, Invitrogen AG, Basel, Switzerland) and 20 µL PI (20 mM, Invitrogen AG, Basel, Switzerland) to 970 µL filtered (0.2 µm) DMSO. Ten µL of sample, 10 µL $Na_2EDTA.H_2O$ (500 mM, pH8) solution and 10 µL of stock stain solution were added to 970 µL of sterile water, leading to a one hundred-fold dilution of the sample. The mixture was incubated at room temperature in the dark for 15 min and then introduced to a Coulter Epics XL flow cytometer with an air-cooled argon ion laser of 15 mW output and a fixed wavelength excitation of 488 nm. The instrument was equipped with two light scatter detectors that measured forward (FSC) and side scatter (SSC) and four fluorescence detectors that detected appropriately filtered light at green (FL1, 525 nm) and red (FL3, 620 nm; for PI) wavelengths.

2.8. Statistical analyses

Data were analyzed using the statistical analysis package of Microsoft Excel 2007. *t*-tests were performed based on the null hypothesis that the two sets (two replicates) were statistically the same and significance levels were expressed as $p > 0.05$. The p values higher than 0.05 revealed that the hypothesis was statistically true and the replicates were the same.

3. Results

3.1. Dechlorination of trichloroethene

After a starvation period of three weeks without any electron donor and acceptor, 23 µmol (40 µM) TCE and 5.6 mM lactate were added to each duplicate enrichment culture. The CAH concentrations determined in function of time are summarized in Figure 3.1. TCE was gradually dechlorinated within 10 days. Accordingly, the production and subsequent dechlorination of cDCE and VC was observed in the reactors. VC was still present until day 28 but at amounts less than 0.6 µmol/bottle. No ethane was detected, but ethene production indicated the occurrence of complete dehalogenation in the system.

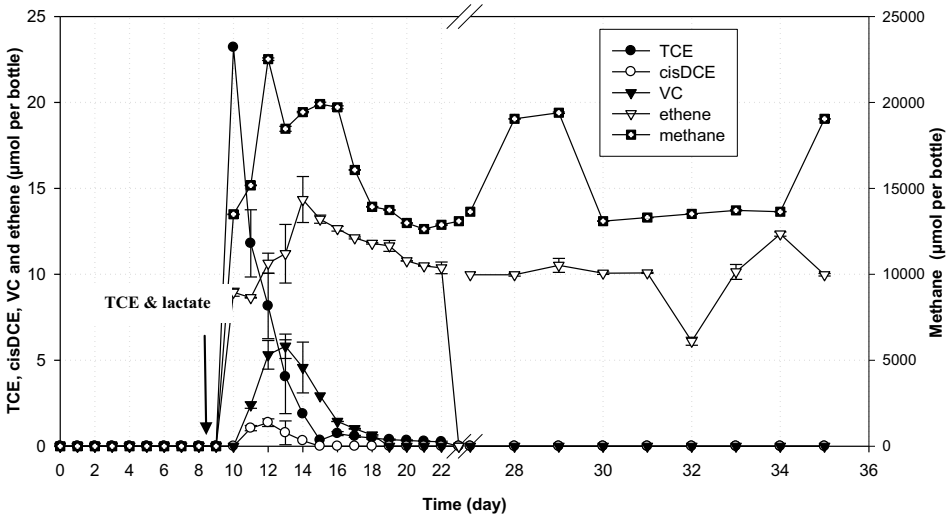

Figure 3.1. Amount of chlorinated ethenes, ethene and methane (in µmol/bottle) measured before, during and after TCE biodegradation. Concentrations are reported as averages of duplicate reactors, error bars represent analytical error (*p>>0.05*) (Detection limit: 5 µg/L for TCE, cDCE and VC; 0.5 µg/L for methane and ethene).

Next to dehalogenation activity, also a high methanogenic activity was recorded shown by an increase in methane concentration from 0 to 22.5 mmol/bottle, 4 days after the addition of TCE and lactate. In the following 26 days, the methane concentration gradually decreased and reached a stable concentration of about 13 mmol/bottle on day 30 (Figure 3.1).

3.2. Determination of active and living cells and total ATP

Measurements by flow cytometry indicated that the levels of living and dead cells were constant throughout the experiment, with an average value of 7.35×10^7 living cells/mL (Figure 3.2) and 3.79×10^6 dead cells/mL (not shown).

Total ATP concentration of total bacteria was around 2 ng/mL before addition of TCE. The addition of TCE and lactate on day 8 induced an activity and ATP was produced to values of 4-5 ng/mL ATP as shown in Figure 3.2. Once TCE and lactate were degraded on day 18, the concentration of ATP decreased and stayed almost constant at an average value of 2.5 ng/mL until the end of the experiment. Active cells were estimated from ATP copies per cell based on assumptions reported in the studies of Karl (1993) and Griebler et al. (2002), and were

around 10^6 cells/mL in the lag phase. An increase to 1.13×10^7 cells/mL was recorded right after TCE and lactate addition and the numbers were almost constant until day 26. However, a decrease to 1.95×10^6 cells/mL was observed on day 28 after which the numbers stayed constant.

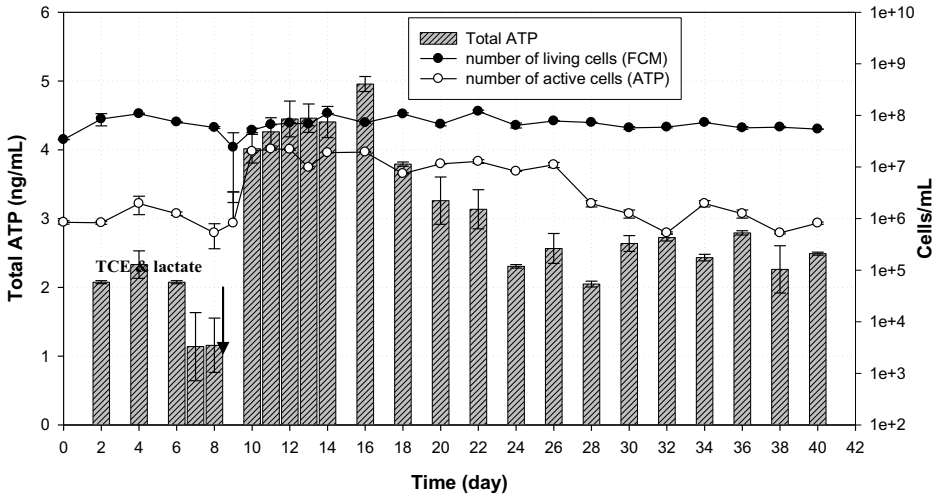

Figure 3.2. Concentration of total ATP, number of living and active cells based on ATP concentration and live/dead flow cytometry (FCM) measurements throughout the test. Concentrations are reported as averages of duplicate reactors, error bars represent the analytical error (Detection limit: 0.01 ng/mL ATP).

However, since we worked with a mixed culture, the increase in total ATP amount could be due to an increase in the specific activity of the CAH dechlorinating population and/or an increase in the activity of the total population. Therefore, a similar but more detailed test was performed with the enrichment culture where the response of microorganisms to the addition of TCE and lactate were tested jointly, but also separately in parallel (data not shown). While total ATP did not increase in the test conditions without any amendments or where only TCE was added; the total ATP concentration increased from 0.5 ng/mL to 2.5-3.0 ng/mL in the test conditions amended with lactate (with or without TCE). This ATP level stayed constant for 11 days in the reactors including only lactate while it decreased to a stable concentration of 1.5 ng/mL already on day 2 in the reactors amended with lactate and TCE. Next to an increase in the ATP amount, an increase in methane concentration was also recorded which was higher in

the lactate (9868 µM on day 11) than in the lactate and TCE amended (2693 µM on day 11) culture.

3.3. Determination of gene copies and transcripts

qPCR measurements in DNA extracts revealed that during the starvation period, bacterial and *Dhc* 16S rRNA genes. and; *tceA* and *vcrA* gene copies were present in the reactors (Figure 3.3A) with average values of 5.38×10^8, 3.07×10^7, 2.10×10^7, and 1.12×10^8 copies/mL, respectively. The *bvcA* gene was not detected. When TCE and lactate were added on day 8, the number of 16S rRNA gene copies of bacteria and *Dhc* spp. and the catabolic genes *tceA* and *vcrA* in DNA extracts were quite constant. However, from day 16 onwards, the numbers of all these genes showed fluctuations.

On the other hand, at mRNA level, 16S rRNA gene transcripts of total bacteria increased gradually from 9.11×10^6 to 8.99×10^7 transcripts/mL directly after TCE and lactate addition on day 8. These numbers then decreased to 1.53×10^6 transcripts/mL on day 14 and finally stabilized at a level of 10^4-10^5 transcripts/mL when all TCE was dechlorinated on day 20 (Figure 3.3B). The 16S rRNA gene of the *Dhc* spp. was induced somewhat slower as the transcripts increased only six days after TCE and lactate exposure. This maximum number of 1.78×10^6 transcripts/ mL was only kept for 2 days since it decreased between day 18 and 26 and finally reached a steady state on day 26 with an average value of 5.33×10^4 transcripts/mL. Although the 16S rRNA gene of the *Dhc* spp. was expressed during the whole experiment (between values 1.34×10^3-1.36×10^6 transcripts/mL), *tceA* and *vcrA* gene transcripts were not detected during the starvation period. However, the *tceA* gene was up-regulated to 2.96×10^4 transcripts/mL only right after TCE and lactate addition (Figure 3.3B). The values were below detection limit (10^3 transcripts/mL) eight days after TCE exposure when TCE was almost not detectable anymore in the culture (0.6 µmol on day 16). Similarly, a direct increase in *vcrA* gene transcript (to 4.73×10^4 transcripts/mL) was recorded on day 10. In contrast to the *tceA* gene transcripts, the *vcrA* gene was continuously expressed until day 32. More specifically, the number of *vcrA* transcripts increased from 4.73×10^4 transcripts/mL to 2.42×10^5 transcripts/mL from day 8 to day 12, decreased to 4.41×10^4-10^5 transcripts/mL on day 14 but stabilized at a level of 10^4 transcripts/mL until day 32 after which the *vcrA* transcripts were below the detection limit (10^3 copies/mL).

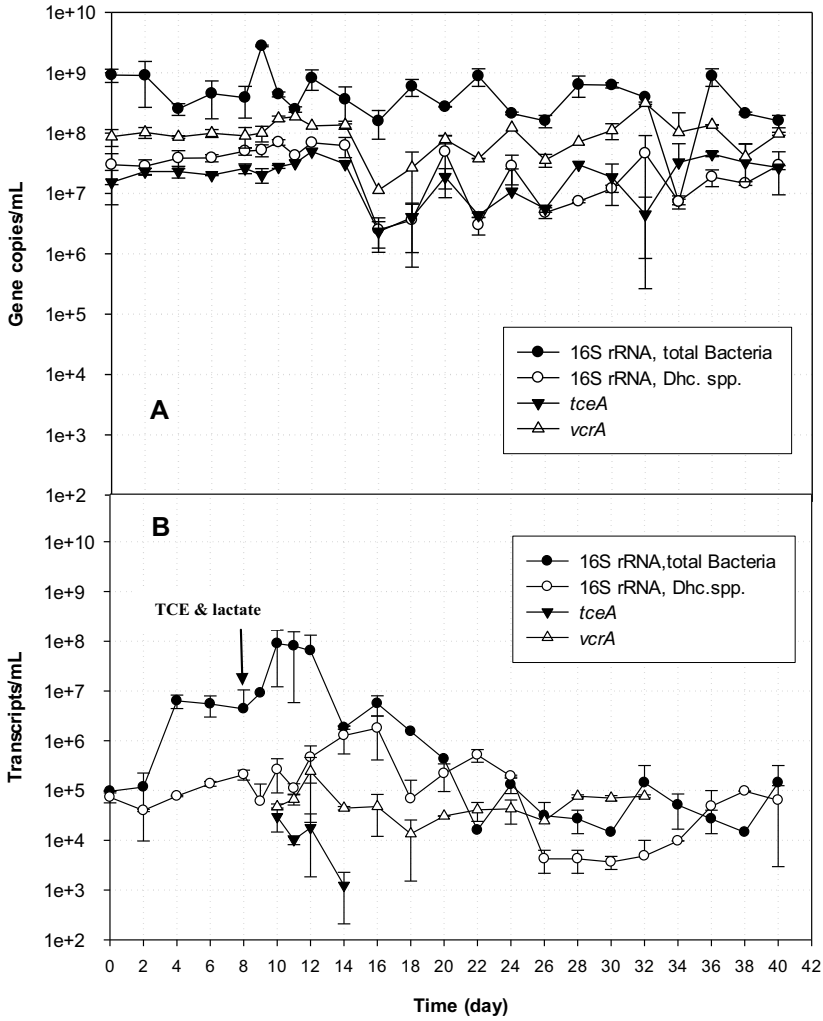

Figure 3.3. 16S rRNA of total bacteria and *Dehalococcoides mccartyi* spp.; *tceA* and *vcrA* gene copies and transcripts over time in DNA (A) and RNA (B) extracts. Each value represents the average of the results of triplicate real-time PCRs performed on two independent DNA/RNA extractions from biological replicates (n = 6) (Detection limits: 10^2, 10^3 and 10^3 copies or transcripts/mL for 16S rRNA, *tceA* and *vcrA,* respectively).

The changes in the ratios of 16S rRNA gene of *Dhc* spp./16S rRNA gene of total bacteria, *tceA*/16S rRNA gene of *Dhc* spp., and *vcrA*/16S rRNA gene of *Dhc* spp. both in DNA and RNA extracts over time are given in Figure 3.4A and B. The 16S rRNA gene copy numbers of *Dhc* spp. were about 20% of those detected for the total bacterial community (Figure 3.4A1), while on gene transcript level this percentage was generally higher (40% to 600%, Figure 3.4B1). When looking at the RDase genes relative to the number of 16S rRNA genes of *Dhc* spp., it was clear that the *vcrA*/Dhc ratios were higher than the *tceA*/Dhc ratios both in DNA (Figure 3.4A2 and A3) and RNA (Figure 3.4B2 and B3) samples. At DNA level, *tceA*/Dhc and *vcrA*/Dhc ratios were quite constant till day 14; however, between day 16 and 40, higher values for both ratios were detected. In this period, lower numbers of 16S rRNA genes of *Dehalococcoides* spp. were often recorded (Figure 3.3A). Ratios at RNA level indicated that *tceA* and *vcrA* gene transcripts were abundant in the system in the first period of the experiment (day 10 to 12) while *vcrA* gene transcripts were majorly present in the culture in a later period (day 26 to 32) (Figure 3.4B2 and B3).

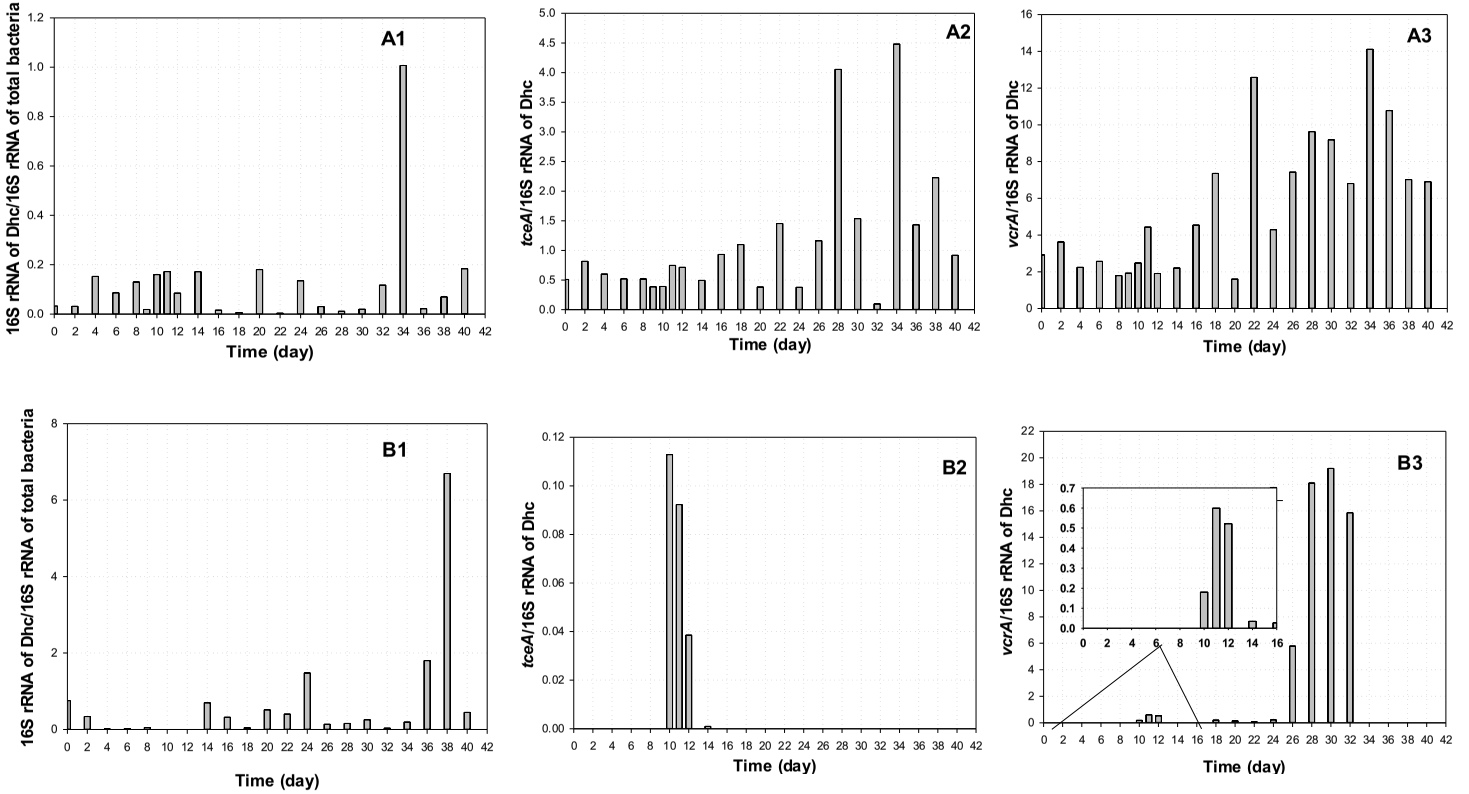

Figure 3.4. Ratio of 16S rRNA of *Dehalococcoides* spp./ 16S rRNA of total bacteria, *tceA*/16S rRNA of *Dehalococcoides* spp., and *vcrA*/16S rRNA of *Dehalococcoides* spp. in DNA (A) and RNA (B) extracts. Note that for Y axes different concentration scales are used.

4. Discussion

In this study, the functional responses of a *Dehalococcoides mccartyi* spp. containing enrichment culture under TCE and lactate exposure were investigated. The rapid consumption of TCE after the 3-week starvation period (lack of TCE and lactate and the immediate production of cDCE, VC, and non-toxic ethene confirmed the dechlorination capacity/activity of the culture (Figure 3.1). The detection of methane after lactate addition could be linked to the activity of methanogens in the system next to the *Dhc*. spp. This was an expected outcome since the culture itself was enriched from sediment where many bacterial and archaeal populations are present (Schippers and Neretin, 2006).

In contrast to the pure culture study of Dolfing (1990), we could not link ATP production to the dechlorination potential in our mixed culture. In their study, ATP production was linked to the degradation of 3-chlorobenzoate and the concentration of ATP increased from 3 to 10 nmol/mg protein as the pure strain *Desulfomoline tiedjei* (strain DCB-1) degraded 3.2 mM of 3-chlorobenzoate in the presence of 6 mM of formate. Our results indicated that the increase in ATP concentrations under TCE and lactate exposure was due to the induction of activity of the total microbial community and cannot be linked to a specific increase in the activity of the *Dhc* spp. In addition, based on the methane production, a part of the observed ATP was produced by the methanogenic population. ATP based measurements of the activity are therefore too general and cannot be used to monitor the dechlorination potential in a mixed culture. Nevertheless, they can be used as an indicator of the overall activity in a mixed culture and as a monitoring tool for inhibitory effects by different factors (e.g., pH changes, chemical oxidant, …) on the total activity (Doğan-Subaşı et al., 2013).

A comparison between the number of living cells (via FCM) and biologically active cells (via ATP) showed that the number of living cells was constant during the whole experiment (7.35×10^7 cells/mL) and the values were approximately the same as for the active cells (1.15×10^7 cells/mL) (Figure 3.2). However, it was noticed that a decrease in metabolic activity led to a reduction in the number of active cells starting from day 28 even though the number of living cells stayed constant. As living cells do not necessarily have an active metabolism and the estimation of biologically active cells (via ATP) was based on some assumptions (the amount of cell carbon), ATP was found to be a more sensitive tool than FCM to monitor the general microbial activity in the system.

Gene expression profiles via analyses of RNA extracts, correlated with the physiological activity of the cells. The 16S rRNA gene transcripts of total bacteria and *Dhc* spp. increased (right) after the addition of TCE and lactate indicating the induction in microbial metabolism (Figure 3.3B). Although the number of 16S rRNA transcripts of *Dhc* spp. can be related to increased metabolic activity, they could not be directly linked to TCE dechorination potential since they were also expressed in the lag phase (in absence of TCE and lactate). On the other hand, the transcript numbers of the *tceA* gene increased immediately after TCE addition, and subsequently, decreased to the numbers below the qPCR detection limit when TCE was removed. The study of Johnson et al. (2005b) revealed that *tceA* gene expression was independent of the concentration of chlorinated ethenes although the culture was exposed to a sum of TCE and cDCE concentrations ranging from 2.2 µM to 333 µM. A similar trend was also observed for *vcrA* gene transcripts since the gene was induced right after cDCE production on day 10. However, although the *vcrA* gene was only expected to be expressed during the period of cDCE and VC degradation (Holmes et al., 2006), it was also detected after day 28 when VC was not detectable anymore (Figure 2.3B). Between day 28 and 32, trace amounts of VC may have been present in the system at non-detectable levels but sufficiently high to induce *vcrA* gene expression. In literature, half-rate coefficients for the dechlorination of TCE and cDCE by a *Dhc* containing microbial culture were reported as 1.4 and 3.3 µM, respectively (Haston and McCarty, 1999), suggesting that concentrations in the lower µM range may still induce the expression of the *vcrA* gene. Another possible explanation for the long term presence of the *vcrA* gene transcript might be related with the stability and associated longevity of the *vcrA* gene transcript. In this sense, the findings of Amos et al. (2008) might have indicated that the detection of mRNA can be linked to its longevity (i.e., slow mRNA turnover) in the system.

The measured abundances of genes were in line with values provided in previous studies. Holmes et al. (2006), for example, reported constant values of 10^7-10^8 copies/mL for the 16S rRNA gene of *Dhc* spp., *tceA* and *vcrA* genes in a TCE degrading enrichment culture. In different systems (e.g., aquifer, groundwater etc.), gene copy numbers ranged from 10^4 to 10^8 copies/mL (or g aquifer) (Behrens et al., 2008; Lee et al., 2008; Bælum et al., 2013). Previous studies performed with TCE exposed enrichment cultures observed gene expression levels of 10-100 transcripts per gene (Johnson et al., 2005a,b) or 10^{10}-10^{11} transcripts/mL (Rahm and Richardson, 2008). In these studies, an internal standard with known mRNA copy number was used to estimate the total RNA loss during extraction and to correct the final measured

numbers by taking this RNA loss into account. Therefore, the much lower ratios detected in our study (0.01-0.4 transcripts/gene, 10^3-10^5 transcripts/mL) might be attributed to the mRNA losses in the extraction procedure performed in the absence of an internal standard. On the other hand, our results are still in agreement with studies of Lee et al. (2006), Amos et al. (2008) and Bælum et al. (2013) as lower transcripts per genes (0.001-10) or per mL (10^3-10^6 transcripts /mL) were also detected in their studies with dechlorinating cultures.

The pronounced shifts in the qPCR *tceA*/Dhc and *vcrA*/Dhc ratios over time in the DNA and RNA extracts provides information on the dynamics of *tceA* and *vcrA* genes during dechlorination of TCE and its daughter products in our culture. Based on analyses performed on DNA and RNA extracts, the majority of the dechlorinators present in the culture were *vcrA* carrying organisms since the *vcrA*/Dhc ratios were higher than the *tceA*/Dhc ratios throughout the test (Figure 2.4A/B2 and Figure 3.4A/B3). The decrease in the number of 16S rRNA genes of *Dehalococcoides* (Figure 3.3A) and the increases in the *tceA*/Dhc and especially in the *vcrA*/Dhc ratios between days 16 and 40 in DNA samples (Figure 3.4A2 and A3), might indicate that the relative abundance of the *tceA* and *vcrA* carrying organisms increased in our culture between day 16 and 40. DNA (Figure 3.4A) but mainly RNA (Figure 3.4B) profiles clearly indicated that organisms having *tceA* and *vcrA* genes were active from day 10 to 12 which could indicate that these organisms worked together to dechlorinate TCE and its daughter products completely. However, at a later stage in the dechlorination process (day 26 to 32), the increase and dominance of the *vcrA*/Dhc ratio indicated that the low concentrations of VC mainly induced the *vcrA* gene. To investigate if the *tceA* and *vcrA* genes were expressed simultaneously, the dynamics of these genes should have been investigated during the growth of our culture on these individual compounds (i.e., growth on TCE to cDCE or on VC to ethene) as was also performed in the study of Holmes et al. (2006).

5. Acknowledgment

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Chapter 4. Dechlorination activity of a dechlorinating enrichment culture during and after chemical oxidant treatment

Abstract

Chemical oxidants, like potassium permanganate (PM) and sodium persulfate (PS), are regularly used in soil remediation, however, their compatibility with a coinciding or subsequent biotreatment is poorly understood. In this study, different concentrations of the oxidants PM (0.005 to 2 g/L) and PS (0.01 to 4.52 g/L) were applied and their effects on the abundance, activity, and reactivation of a trichloroethene (TCE) dechlorinating enrichment culture were investigated. Expression of the *tceA*, *vcrA* and 16S rRNA genes of *Dehalococcoides mccartyi* spp. were detected in the test systems treated with 0.005 to 0.01 g/L PM and 0.01 to 0.02 g/L PS. However, at higher concentrations of 0.5 to 2.0 g/L PM and 1.13 to 4.52 g/L PS no gene expression was recorded, neither were indicator molecules for total cell activity (ATP) detected. Dilution did not promote the reactivation of dechlorination in any of the test systems when the redox potential was still above -100 mV. Similarly, inoculated cells (10^{10} copies/mL) did not dechlorinate TCE above -100 mV. When the redox potential was decreased by addition of $Na_2S.9H_2O$ to -300 mV and the reactors were bioaugmented for a second time, dechlorination activity recovered, but only in the reactors treated with PS concentrations of 1.13 and 2.26 g/L. In conclusion, our results show that chemical oxidants can be combined with a reductive dechlorination biotreatment at concentrations below 0.5 g/L PM and 1.0 g/L PS.

Chapter redrafted after: Doğan-Subaşı E, Bastiaens L, Boon N, Dejonghe W (2013). Microbial dechlorination activity during and after chemical oxidant treatment. Journal of Hazardous Materials, 262, 598-605.

1. Introduction

Chlorinated ethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), are persistent groundwater pollutants which are toxic and mobile in groundwater systems and therefore form a serious risk for human health and the environment (Van der Zaan et al., 2010). *In situ* chemical oxidation (ISCO) is a promising technology to degrade pollutants like PCE and TCE in the subsurface (Tsitonaki et al., 2008). Potassium permanganate ($KMnO_4$, PM) and sodium persulfate ($Na_2S_2O_8$, PS), are effective oxidants in treating chloroethene contaminated soil and groundwater systems (Azadpour-Keeyley et al., 2004; Sahl et al., 2007). Complete oxidation has been observed to generate chloride ion (Cl^-) and carbon dioxide, leaving no chlorinated ethenes, according to the following reactions:

$$2KMnO_4 + C_2HCl_3 \rightarrow 2CO_2 + 2MnO_2(s) + 3Cl^- + H^+ + 2K \quad \text{(Kao et al., 2008)}$$

$$3Na_2S_2O_8 + C_2HCl_3 + 4H_2O \rightarrow 2CO_2 + 9H^+ + 3Cl^- + 6Na^+ + 6SO_4^{2-} \quad \text{(Tsitonaki et al., 2010}$$

Despite the potential benefits of chemical oxidation processes, there are also some possible drawbacks. For example, reaction by-products might induce an altered pH and redox potential which in turn may affect the indigenous microbial population in the subsurface (Hrapovic et al., 2005). The oxidants can also be directly harmful to microorganisms by attacking a variety of cellular components (Imlay and Linn, 1988; Büyüksönmez et al., 1998).

Implementation of ISCO as a single treatment strategy may not be the solution for chlorinated aliphatic hydrocarbon (CAH) contamination in the groundwater and aquifer systems since i) soil heterogeneity, ii) flow and mass transfer reductions due to precipitation of by-products (like MnO_2), and iii) carbon dioxide generation might prevent the efficient delivery of the oxidants in the subsurface, which in turn might decrease the contaminant removal efficiency (Hrapovic et al., 2005). Therefore, a concept of "treatment train" can be considered in which ISCO and biodegradation processes are coupled to achieve contaminant concentrations below regulatory limits (Richardson et al., 2011). Generally, in this approach, biodegradation follows the ISCO treatment as a polishing step. As the application of excessive amounts of oxidants might inhibit or kill the indigenous microorganisms, a good understanding of the impact of chemical oxidants on CAH biodegradation activity is needed to combine chemical and biological treatment.

Although several studies have investigated the effect of oxidants on microbial communities (e.g., *Xanthobacter flavus* FB71 (Büyüksönmez et al., 1998), *Pseudomonas putida* KT2440 (Chapelle et al., 2005), heterotrophic (Jung et al., 2005; Tsitonaki et al., 2008), alkane-degrading (Studer et al., 2009), phenanthrene-degrading bacteria (Richardson et al., 2011)) in different systems (such as aquifer and culture), only a limited number of studies has investigated the impact of PM or PS on dechlorination activity of microorganisms (Hrapovic et al., 2005; Sahl et al., 2007; Sercu et al., 2013). These studies revealed that simultaneous or successive application of abiotic and biotic processes for the treatment of different compounds may be possible using Fenton (Büyüksönmez et al., 1998), ozone (Jung et al., 2005), PM (Sahl et al., 2007; Doğan et al., 2013) and PS (Richardson et al., 2011; Doğan et al., 2013) reactions up to certain oxidant concentrations, but subsequent bioaugmentation with active culture was a necessity in some cases (Hrapovic et al., 2005). In these lab-scale and *in situ* studies performed with aquifer, sediment or culture samples, basic analyses such as acetate consumption, plate counts, phospholipid fatty acids, and mineralization of ^{14}C phenanthrene were conducted to investigate the effect of the oxidants. However, to the best of our knowledge, the effect on the RDase genes at mRNA level has been investigated for the first time in our study.

In the present study, we investigated the effects of different doses of the oxidants PM and PS on the dechlorination activity of an enrichment culture containing *Dehalococcoides mccartyi (D. mccartyi)* spp. The impact of the chemical oxidants was evaluated based on CAH-removal, general activity of the cells (ATP) and transcription of specific dehalogenating genes (mRNA). Moreover, the possible recovery of the dehalogenation activity and impact of bioaugmentation after the oxidant exposure were studied.

2. Materials and Methods

2.1. Bacterial Culture

The microbial culture used in this study was enriched from sediment taken from the Zenne River, Vilvoorde, Belgium (Hamonts et al., 2009) via transfers in an anaerobic mineral medium (Haest et al., 2011) during 5 years at 28 °C (Chapter 3). Shortly, 180 g of sediment and 1.6 L of anaerobic mineral medium were shaken overnight in a 2-L bottle, followed by 6 h of sedimentation. Supernatant was taken and transferred into fresh anaerobic mineral medium with an inoculum/medium ratio of 1:10 with addition of 30 µM PCE (99%, Acros Organics, New Jersey, USA), 52 µM *cis*-dichloroethene (cDCE) (99%, Acros Organics,) and

5.6 mM lactate (50%, Merck, Germany) as electron acceptors and donor, respectively, during a period of 5 years.

2.2. Experimental Conditions

The test was set up in 160 mL pre-autoclaved duplicate serum bottles with pre-autoclaved Teflon lined septa. Each test bottle was filled with 120 mL of microbial culture with a headspace of 40 mL and stirred horizontally on an Edmund Bühler SM30-control shaker (125 rpm/min) at room temperature in the dark. The test was divided into the following phases:

Phase I (Day 0-44): Different amounts of oxidants were added to the bottles to study their impact on the dechlorination activity of the microbial culture. Final concentrations of 0.005, 0.01, 0.5, 1.0 and 2.0 g/L PM (stock solution from 98%, Sigma-Aldrich) and 0.01, 0.02, 1.13, 2.26 and 4.52 g/L PS (stock solution from 97%, Sigma-Aldrich) were investigated. Control reactors including culture but no oxidants were also set up. Each reactor contained 50 µM of TCE and 5.6 mM of lactate on day 0.

Phase II (Day 44-67): Once the chemical oxidants were depleted during Phase I, the reactors were re-spiked with 100 µM of TCE and 5.6 mM lactate to investigate the dechlorination activity of the microorganisms after contact with PM or PS.

Phase III (Day 67-89): In this phase, the effect of dilution or bioaugmentation as a recovery strategy for the chemical oxidant affected microbial culture was studied. For this purpose, one set of the duplicate reactors which were exposed to 0.5, 1.0 or 2.0 g/L of PM and 1.13, 2.26 or 4.52 g/L of PS in Phase I, was diluted (1/10) with fresh culture medium (A series), while the B series were bioaugmented with active culture (concentrated cells of Zenne culture, Chapter 3, 10^{10} copies/mL of 16S rRNA of *D. mccartyi*), resuspended in 1 mL of medium ensuring final concentration of 10^8 copies/mL in each reactor.

Phase IV (Day 89-124): The redox potential was decreased in all reactors to -300 mV using $Na_2S.9H_2O$, and both series A and B were monitored for dechlorination activity.

Phase V (Day 124-160): In this phase, only the reactors in B series were bioaugmented for a second time and dechlorination was monitored in all reactors until day 160.

Phase VI (Day 160-200): As a final step, the test systems with lack of dechlorination activity (i.e., PM 0.5 to 2.0 g/L (A&B) and PS 1.13 to 4.52 g/L (A) and 4.52 g/L (B)) were centrifuged (25,000 x g, 20 min), supernatants (containing all dissolved by-products) were removed and the pellets were re-suspended with 26 mL fresh medium containing 5.6 mM of

lactate in pre-autoclaved 37 mL serum bottles. 100 µM of TCE was added and the activity of the bacteria was monitored for another 40 days.

2.3. Analyses of chlorinated ethenes

Headspace analyses of the test systems were performed on a Varian GC-FID (CP-3800) equipped with a Rt-U plot column (J&W Scientific) for the detection of methane, ethene and ethane, or a split–splitless injector followed by a Rt-X column (30 m length, 0.53 mm ID and 3 µm film thickness, Restek, Bellefonte, Pennsylvania, USA) and a DB-1 column (J&W Scientific, USA) for analysis of CAHs. Standards for CAHs, ethene, ethane, and methane were prepared by adding a known amount of each compound to a serum bottle with the same headspace to liquid ratio as the test bottles.

2.4. Extraction of nucleic acids

DNA and RNA were extracted as described in Chapter 3.

2.5. Quantification of nucleic acids

The quantitative PCR (qPCR) reaction was performed as described in Chapter 3. Primers and probes are provided in Table 3.1 with indication of the applied concentrations of each primer and probe synthesized by Operon Biotechnologies GmbH (Cologne, Germany). The algorithm calculated the efficiency, threshold cycle (C_T), slope and intercept as well as R^2 (>0.99) values.

2.6. ATP analyses

ATP is an indicator molecule for general cell activity and its concentration was measured using the BacTiter-Glo Microbial Cell Viability Assay (G8231, Promega Corporation, Dübendorf, CH) and a Luminoskan Ascent Luminometer (Thermo Labsystems, USA) as reported in Chapter 3.

3. Results

3.1. Dechlorination of TCE

After a starvation period of three weeks without any electron donor and acceptor, 50 µM TCE and 5.6 mM lactate were added to each reactor and PM and PS in different final concentrations were amended to obtain the appropriate concentration range stated in the experimental part. In Phase-I, TCE was gradually dechlorinated to VC and stoichiometrically converted to ethene between day 2 and day 44 in the test systems with 0 (control, without

oxidant), 0.005, and 0.01 g/L of PM and 0.01, and 0.02 g/L of PS (Figure 4.1-B1, B2&C1,C2). TCE was undetectable in the reactors having higher PM concentrations (0.5, 1.0 and 2.0 g/L) on day 2 (Figure 4.1-A1, A2). Yet, the production of VC and ethene was not observed under these concentrations. The decrease in TCE concentration took place over time in the reactors exposed to the highest PS doses (1.13, 2.26 and 4.52 g/L) and was followed by the production of ethene but in trace amounts (1.1 μM) compared to the stoichiometric amounts in the control (38 μM) (Figure 4.1-C2). In Phase-II, 100 μM of TCE and 5.6 mM lactate were again added to the test systems on day 44. While TCE and lactate were stoichiometrically consumed between day 44 and day 60 in the control, and in the reactors supplemented with 0.005 and 0.01 g/L of PM and 0.01 and 0.02 g/L of PS, the TCE level remained constant till day 60 in the reactors exposed to 0.5, 1, 2 g/L of PM and 1.13, 2.26, 4.52 g/L of PS (Figure 4.1-A1, A2). Since any decrease in TCE concentration did not occur in the reactors with these higher PM and PS concentrations, it was concluded that the oxidants were not reactive anymore in Phase II.

In Phases III to VI (day 67-160), we tried to reactivate the microbial population by diluting the chemical oxidants or their by-products, by bioaugmentation or by decreasing the redox potential. In these phases, only the changes in TCE concentration were monitored as a marker for the dechlorination activity. Dechlorination was still inhibited in the set-up that had received the highest doses PM and PS (0.5, 1.0 and 2.0 g/L PM and 1.13, 2.26 and 4.52 g/L PS) in the test systems that were bioaugmented with fresh, active, dechlorinating bacteria (Zenne culture, 10^{10} copies/mL of 16S rRNA) in Phase III, series B (Figure A1). Lowering of the oxidation reduction potential (ORP) till -300 mV did not result in a decrease in the TCE concentration in any of these reactors (Phase IV, B series). However, after a second bioaugmentation was implemented in Phase-V, TCE dechlorination was recorded in the reactors that had received 1.13 and 2.26 g/L of PS but not in any of the other reactors amended with PM (Figure A1). In the A series reactors, the reaction medium was diluted to simulate groundwater infiltration that might cause dilution of the oxidants in *in situ* systems. The results revealed that the dechlorination was not induced by this process as TCE concentrations remained constant at 80 μM between day 67 and 152 under both high (Phase III) and low redox (Phase IV) conditions (data not shown).

In the final Phase VI of the test, all potentially soluble inhibiting by-products were removed by centrifugation and re-suspension of the bacterial pellet in fresh medium. Within 40 days,

the re-spiked TCE was dechlorinated in the bioaugmented reactors with 0.5 and 1 g/L of PM (Figure A2), however, recovery of bacterial cells was not observed in the reactors that had been exposed to 2 g/L of PM and 4.52 g/L of PS when either dilution or bioaugmentation was applied (Table 4.1).

Table 4.1. Overview of the bacterial responses recorded in the reactors that were amended with ≥0.5 g/L of permanganate and ≥1.13 g/L of persulfate in terms of reductive dechlorination activity

Experimental Steps	**Permanganate**						**Persulfate**					
Concentration (g/L)	0.5		1		2		1.13		2.26		4.52	
Phase I (oxidants+TCE+lactate)	(-)		(-)		(-)		(-)		(-)		(-)	
Phase II (respike of TCE and lactate)	(-)		(-)		(-)		(-)		(-)		(-)	
	Dilution			Bioaug.			Dilution			Bioaug.		
Concentration (g/L)	0.5	1	2	0.5	1	2	1.13	2.26	4.52	1.13	2.26	4.52
Phase III (dilution/bioaugmentation + high ORP)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Phase IV(decreased ORP)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Phase V (2nd bioaugmentation, decreased ORP)*	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(-)
Phase VI (centrifugation+resuspension+TCE +lactate)	(-)	(-)	(-)	(+)	(+)	(-)	(+)	(-)	(-)	NA	NA	(-)

* 2nd bioaugmentation was applied only in the previously biaugmented reactors. (-) = no reductive dechlorination, (+) = reductive dechlorination, NA = not applicable, Bioaug. = bioaugmented

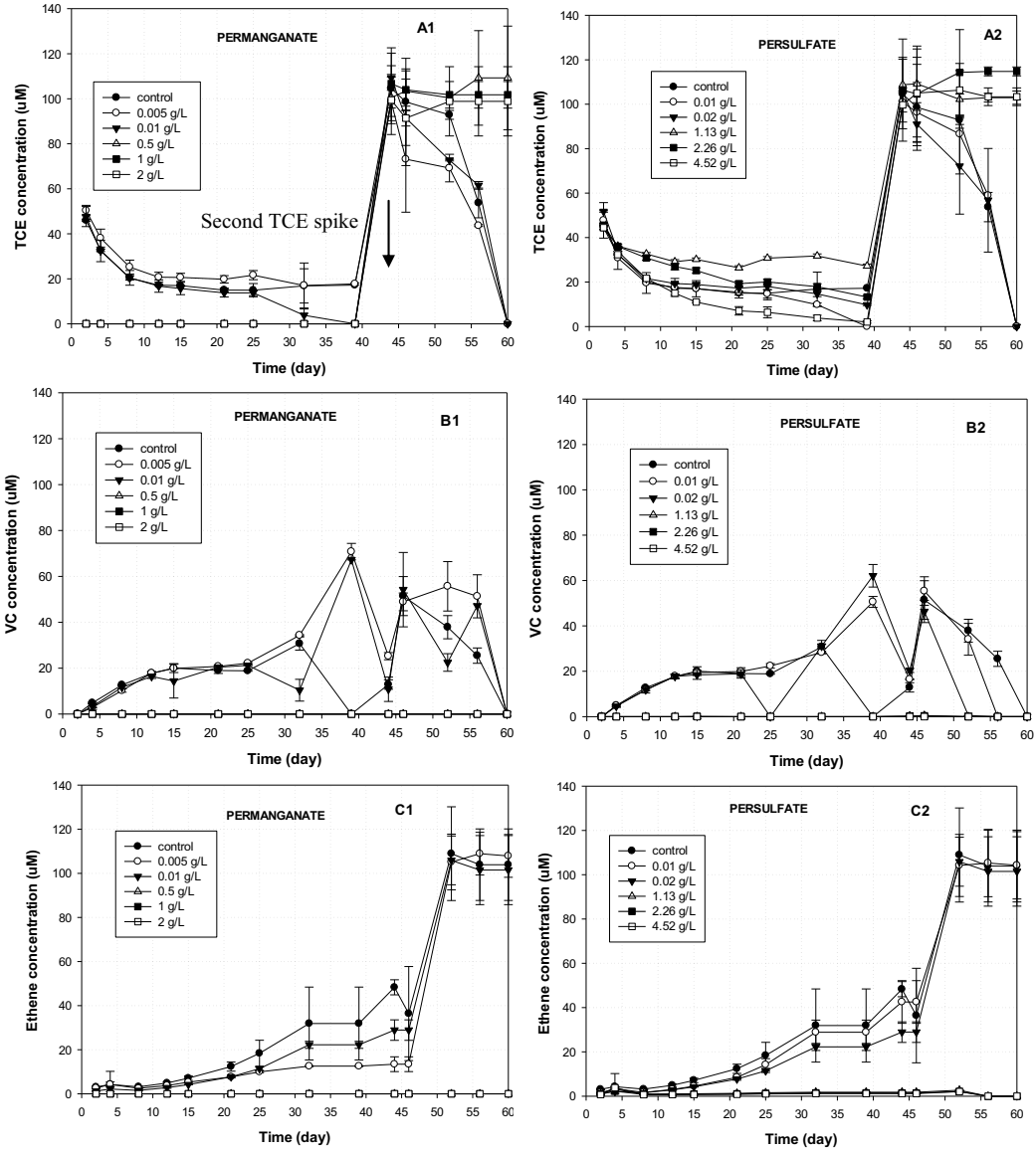

Figure 4.1. Molar concentrations of TCE,VC and ethene before, during and after oxidant treatment. Values are reported as averages of duplicate reactors, error values represent analytical error (Please note that 100 μM TCE and 5.6 mM lactate were re-added on day 44).

3.2. ATP concentrations

In Phase I and II, the measured concentrations of ATP before (culture without any electron acceptor and donor) and after the addition of TCE and lactate were 0.8 ng/mL and 3.1 ng/mL, respectively. Once TCE and lactate were consumed in the control, the values decreased to 1.7 ng/L and stayed constant at 1.3 ng/mL till day 44 (Figure 4.2). The same trend as in the control was observed in the reactors amended with 0.005 and 0.01 g/L of PM and 0.01 and 0.02 g/L of PS. Nevertheless, significant lower ATP concentrations (< 0.40 ng/mL) were measured when the culture was exposed to higher concentrations of oxidants (> 0.5 g/L of PM and >1.13 g/L of PS). The ATP concentration decreased with increased use of oxidant concentration (Figure 4.2).

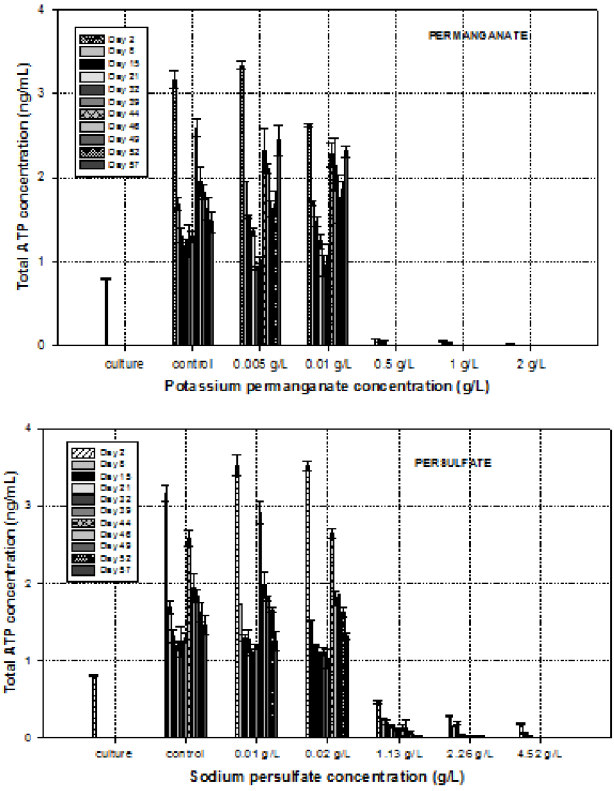

Figure 4.2. ATP concentrations measured in function of time. Concentrations are reported as averages of duplicate reactors, error bars represent analytical error (Detection limit: 0.01 ng/mL ATP). (Please note that 100 µM TCE and 5.6 mM lactate were re-added on day 44).

3.3. Quantification of Gene Copies and Transcripts

The abundances of the copies and transcripts of 16S rRNA genes of bacteria and *D. mccartyi* spp., and of the catabolic genes *tceA* and *vcrA* in DNA and RNA samples extracted from the different reactors on day 8, in Phase I, are shown in Figure 4.3. In DNA samples, 1 and 2 log unit decreases in 16S rRNA gene of total bacteria, compared to non-treated control, were recorded after the cells were exposed to 0.005 g/L and ≥ 0.01 g/L of PM, respectively (Figure 4.3, DNA). Three log unit decrease in bacterial 16S rRNA gene copy number was observed starting from lowest concentration of PS. The same trend was also observed for 16S rRNA gene of *D. mccartyi* spp. in DNA extracts; the number of genes decreased from 4.07×10^6 to 10^4-10^5 copies/mL under the application of both oxidants. However, the amount of reductive dehalogenase genes *tceA* and *vcrA* did not change significantly when the concentrations were ≤ 0.5 g/L of PM and ≤ 1.13 g/L of PS. Yet, these genes were not detected at the concentrations of 1 and 2 g/L of PM and 2.26 and 4.52 g/L of PS. The effect of the oxidants was more pronounced in the RNA than DNA extracts. The decrease in 16S rRNA transcripts of total bacteria and the catabolic genes *tceA* and *vcrA* were limited for the conditions ≤ 0.01 g/L PM and ≤ 0.02 g/L PS**.** Although the concentration of 16S rRNA transcripts of *D. mccartyi* spp. were 2.07×10^8, 1.31×10^8 and 9.21×10^7 transcripts/mL for the conditions control, 0.005 and 0.01 g/L PM, respectively, we observed a 3 log unit decrease under the exposure of 0.01 and 0.02 g/L PS compared to the control. Both 16S rRNA and catabolic gene transcripts were at undetectable levels as the concentrations increased above 0.5 g/L PM and 1.13 g/L PS (Figure 4.3, RNA).

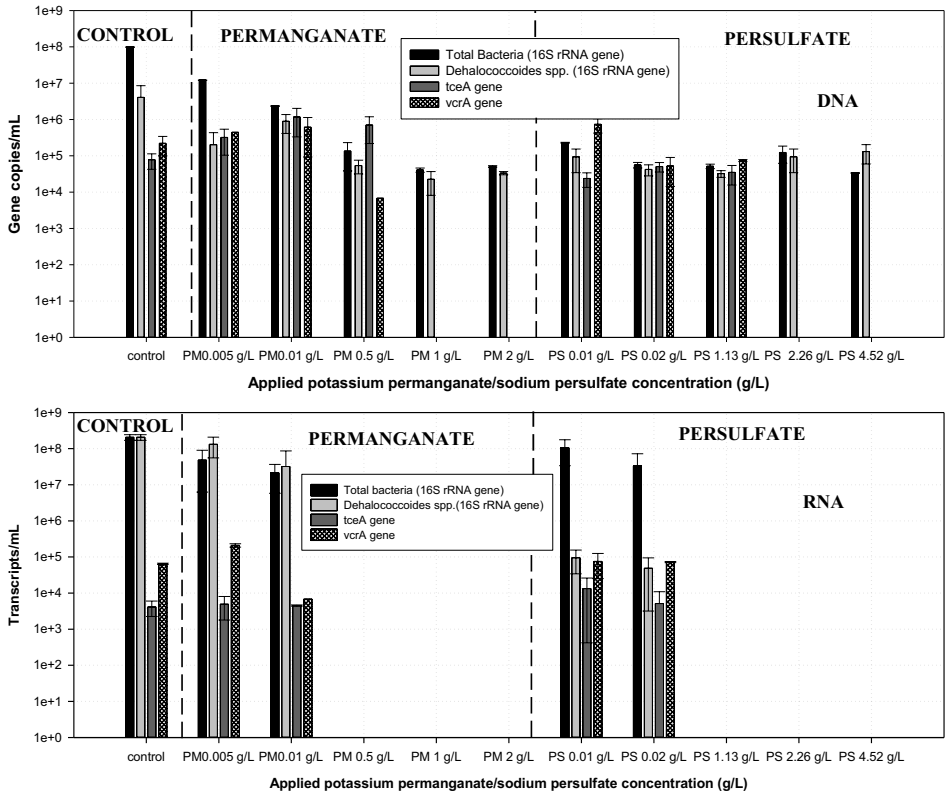

Figure 4.3. Bacterial and *Dehalococcoides mccartyi* spp 16S rRNA, *tceA,* and *vcrA* gene copies and transcripts on day 8 after oxidant addition in DNA (A) and RNA (B) extracts. Values represent the average of triplicate real-time PCRs performed on DNA/RNA extractions from two independent biological replicates (n = 6) (Detection limits: 10^2, 10^2 and 10^3 copies/mL for 16S rRNA, *tceA* and *vcrA,* respectively).

3.4. Change in redox and pH values

The change in redox and pH values in function of time was monitored in Phase I and II of the experiment. While the control reactor kept reducing conditions (-297 to - 243 mV) throughout the test, changes were recorded only in the reactors under oxidant exposure. Although the values increased from -300 mV to approximately -200 mV under the application of 0.005 and 0.01 g/L of PM, much less reducing conditions (-87 mV, -55 mV and -30 mV) were observed in the reactors amended with 0.5, 1 and 2 g/L PM, respectively. In the reactors amended with 1.13, 2.26 and 4.52 g/L PS, highly oxidative conditions (196 mV, 250 mV and 290 mV, respectively) were obtained. However, the change was not significant under lower amounts of

added PS (i.e., 0.01 and 0.02 g/L; -247 mV and -236 mV, respectively) (Figure 4.4). The pH values were almost constant throughout the test and ranged from 7.8 to 8.1 for PM and from 7.7 to 7.9 for PS amended reactors, while the pH was 7.7 in the control reactor (data not shown).

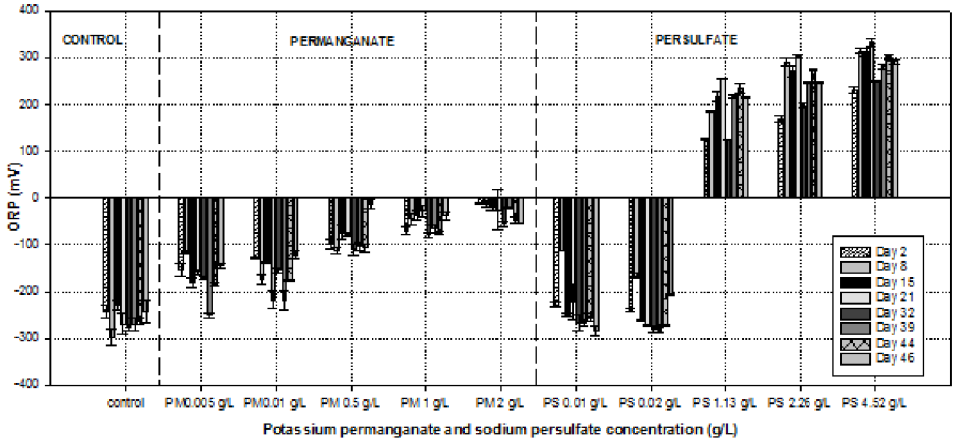

Figure 4.4. Redox values measured in function of time in the reactors.

4. Discussion

The aim of this study was to assess the effects of low and high PM and PS concentrations on the survival and activity of *D. mccartyi* spp. in a dechlorinating enrichment culture. Approaches that were used comprised the monitoring of the (i) concentrations of remaining CAHs and CAH products formed, (ii) ATP, reflecting general microbial activity, and (iii) abundances and transcripts of specific RDase genes. As no aquifer material was present in the batch reactors, the findings are considered as worst case since microbial protection of niches were lacking. Moreover, as un-activated PS was used, $\bullet SO_4^-$ radicals, which are more oxidative and aggressive to bacteria than SO_4^{2-} ion, were not expected to be formed (Tsitonaki et al., 2010). The rather slow degradation of TCE in PS amended reactors indicates that PS indeed did not react through free radicals but through direct electron transfer which is relatively slow (Siegrist et al., 2011). Although MnO_4^- and $S_2O_8^{2-}$ are known to decrease the pH (Tsitonaki et al., 2010), the buffering capacity of the culture medium prevented pH drops below pH 7.5 and it remained within the optimal pH range (6.8 – 7.5) for dechlorinating activity of *D. mccartyi* spp. (Siegrist et al., 2011).

Addition of **low** concentrations of PM (≤ 0.01 g/L) and PS (≤0.02 g/L) had a rather limited impact. TCE removal followed by VC and ethene production points towards at least partial biotic TCE degradation in these systems. The clear biodegradation of second TCE and lactate spike in Phase-II showed that post-chemical biodegradation is possible after amendment of these low PM and PS concentrations. Redox values increased with PM concentration, but remained in all cases below -200 mV. Measured ATP concentrations also showed that microorganisms survived under these moderate oxidant treatments. In literature, improved reductive dechlorination in case of addition of low amounts of oxidants was reported and explained by oxidation of large organics to more bioavailable simple molecules (Doğan et al., 2013). This effect was not observed in the current study as the experiment was set up in liquid medium without aquifer material.

Furthermore, despite the 1-3 log unit decreases of the bacterial and *D. mccartyi* spp 16S rRNA genes abundances (Figure 4.3, DNA), the numbers of the RDase genes, *tceA* and *vcrA,* were constant at low oxidant concentrations. One possible explanation is the oxidant susceptibility of a different strain of *D. mccartyi* spp., having 16S rRNA gene but not *tceA* and/or *vcrA* genes as explained in a previous study (Lee et al., 2006). DGGE analyses indeed indicated the presence of at least two strains in our culture (Figure 4.5). These strains might have reacted differently to the exposure to the chemical oxidants. The expression of all targeted genes over a period of 60 days indicated that oxidant concentrations of 0.01 g/L PM and 0.02 g/L PS did not have an adverse impact on the cells as time proceeded and followed almost the same trend as the control (Figure A3). Another culture-based study reported 40.3% survival for peroxide-acclimated bacteria after the application of 0.3 g/L H_2O_2 and 5 nM Fe(II) after a contact time of 60 seconds (Büyüksönmez et al., 1998).

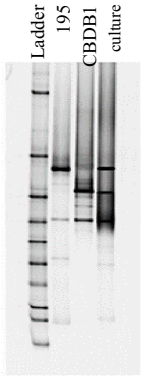

Figure 4.5. DNA-based DGGE profile of strain *Dehalococcoides mccartyi* 195, *Dehalococcoides* CBDB1 and the dechlorinating enrichment culture.

In contrast, **high** concentrations of PM (0.5-2.0 g/L) and PS (2.26-4.52 g/L) did show detrimental effects. The lack of VC and/or ethene implied that reductive dechlorination was stopped and TCE was rather oxidized to CO_2 in Phase-I. The rise of ORP values up to -30 mV and +290 mV after PM and PS exposure, respectively, might have had substantial contribution to the observed inhibition of the reductive dechlorination process (Chen, 2004), next to the formation of the by-products MnO_2(s) and SO_4^{2-} (Heimann et al., 2005). Unreacted PM and PS can also react with water and produce MnO_2(s) and O_2, respectively, at neutral pH conditions which in turn may affect reductive dechlorination (ITRC, 2001). In our study, high doses of PM and PS did have an irreversible negative effect on the CAH degraders as dilution nor decreases in ORP values could re-establish CAH biodegradation. ATP was at undetectable levels, or only trace amounts of ATP were recorded which can be attributed rather to the activity of methanogens (trace amounts of methane were detected at these high oxidant concentrations - data not shown) than to activity of CAH reducers. However, under excessive oxidant stress, 16S rRNA genes were still detected in DNA samples. This is in agreement with previous studies stating the persistence of intact DNA during harsh conditions in sediments (Leloup et al., 2009; Doğan et al., 2013). Our study indicated that detection of residual DNA fragments was still possible even in culture-based systems without protection of sediment or soil particles. The non-detectable amounts of *tceA* and *vcrA* copies and transcripts indicated the sensitivity of these organisms to the oxidant stress.

In our study, 0.5 g/L PM and 1.13 g/L PS were identified as **intermediate** concentrations at which gene transcripts were undetectable levels, but still allowed detection of *tceA* and *vcrA*

gene copies in DNA samples. Furthermore, revival of the dehalogenating activity was observed in the reactors amended with 1.13 g/L PS after decrease of the ORP values and dilution, but not for PM amended reactors. The reason for this inhibition can be the MnO_4^- oxidation at carbon-carbon double bonds which may result in the formation of α-hydroxyketones, diols and/or epoxides that may induce losses in membrane function and subsequently cause cell death (Waddell and Mayer, 2003). Moreover, toxic effect of PM which is known for its disinfectant properties, has been reported (Sahl et al., 2007). The microbial activity up to certain oxidant doses (e.g.,0.77–0.2 g/L PS, 0.1-10 g/L PS, 20 g/L heat activated PS) was recorded in other studies although some decreases in activity were observed (Büyüksönmez et al., 1998; Jung et al., 2005; Tsitonaki et al., 2008; Richardson et al., 2011; Doğan et al., 2013). For instance, following PM oxidation (0.1, 1 and 10 g/L) an inhibition of reductive dechlorination was recorded in the column study of Sahl et al. (2007); yet, after passing sterile growth medium, a rebound in dechlorination activity was observed without the need for bioaugmentation.

In our study, the necessity for bioaugmentation was also investigated. For the systems exposed to 0.5-2.0 g/L PM and 2.26-4.52 g/L PS, the necessity for bioaugmentation to achieve post-chemical bioremediation was indicated, but it only led to CAH biodegradation after reinstalling reducing redox conditions (Figure A1). More specifically, the second bioaugmentation (Phase-V) was successful in the reactors exposed to 1.13 and 2.26 g/L PS, but not for the reactors exposed to the highest PS (4.52 g/L) dose and to PM (0.5-2.0 g/L). A possible reason can be inhibiting by-products that were still present in the reactor systems. Removal of by-products present in the liquid phase (sulphate, O_2,…) by centrifugation and re-suspension of the pellets in fresh medium (Phase VI) did result in recovery of the augmented bacteria in the reactors exposed to 0.5 and 1 g/L PM, indicating that (i) inhibiting soluble compounds were formed, and (ii) precipitated $MnO_{2(s)}$ was not toxic in the associated doses (Figure A2). However, the dehalogenating microorganisms in the reactors amended with the maximum PM and PS concentrations could not be re-activated despite bioaugmentation. They may have already been irreversibly impacted by the soluble inhibiting substances (SO_4^{2-}) or non-soluble toxic compounds (like $MnO_{2(s)}$). The results show that oxidant doses should be carefully selected in order not to lose the activity of microorganisms.

The different approaches used to evaluate the activity and survival of CAH-degrading bacteria were complementary. Although ATP is linked with the activity of the total microbial

community present in the system, and not specifically of *D. mccartyi* spp. (see Chapter 3), it was an important biomarker since its detection is related to the integrity of the cytoplasmic membrane in the viable cells (Tsitonaki et al., 2008). Quantification of catabolic DNA and especially RNA targets were used to investigate the effect of the oxidants on the dechlorination activity, and to support the CAH removal data.

5. Conclusions

Our study showed that the maximum oxidant doses for combined or successive abiotic and biotic dechlorination should be less than 0.5 g/L PM and 1.13 g/L PS. Higher doses inhibited the reductive dechlorination activity and even killed the cells. The ORP was identified as a crucial parameter in this process, as well as soluble (SO_4^{2-}) and non-soluble by-products ($MnO_{2(s)}$). The negative impact of PM was more pronounced than PS because PS was not activated and radical formation was not achieved. While the removal of SO_4^{2-} did stop the inhibition, precipitates of $MnO_{2(s)}$ still kept their toxic effect on *D. mccartyi* spp. When oxidants were added first time into the system, the cells were destructed which might have been considered as direct effect. However, change in redox potential and the produced by-products had indirect effects which blocked possible survival of the microorganisms. The impact of these oxidants might be less in heterogeneous aquifer systems since the protection of the bacteria can be ensured by the soil particles. However, it should be noted that bacteria in groundwater are generally under direct exposure of the oxidants which might result in negative influences on bacteria as observed in our study. Therefore, the oxidant dose should be carefully chosen to assure the occurrence of bioremediation after chemical oxidation especially at sites under stagnant flow conditions. A balance between different parameters in the subsurface should be set to minimize the aggressive impacts of the chemical oxidants on the microbiology. When it is necessary to use excessive amounts of chemical oxidants, the aggressive impacts may be decreased by controlling the boundaries of the plume so that oxidants can react only inside the plume without disturbing the microorganisms present outside of the plume. In the center of the plume, the change in the activity and structure of the microorganisms should be monitored at post-oxidation stage.

6. Acknowledgment

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Chapter 5. The impact of chemical oxidants on indigenous microorganisms in aquifer and groundwater containing batch and *in situ* systems

1. Introduction

The contamination of chlorinated aliphatic hydrocarbons (CAHs) in the soil and groundwater systems and their associated risks were presented in previous chapters of this thesis. Chemical oxidation and bioremediation, as well as their coupled use, were identified as possible remediation approaches (Chapter 4). However, chemical oxidation and bioremediation processes need to be compatible to some degree to exploit their coupled use. More specifically, the CAH degrading microorganisms need to survive after the chemical treatment to start a subsequent polishing bioremediation phase without bioaugmentation. In Chapter 4, we reported the impact of chemical oxidant on the removal of TCE, and on various microorganisms in suspensions in the absence of aquifer material (Chapter 4). In the absence of aquifer material, the maximum oxidant doses for combined or successive chemical and biological degradation should be less than 0.5 g/L permanganate and 1.13 g/L persulfate since higher doses inhibited the microbial activity and even killed the cells.

With emphasis on dechlorinating organisms, the aim of the current study was to evaluate the impact of oxidants on soil microbial populations in environmental matrices closer to reality. Kastner et al. (2000) investigated the effect of Fenton's reagent on methanotrophic growth in a trichloroethene (TCE)/perchloroethene (PCE)-contaminated aquifer. The results revealed that Fenton's reagent process can be designed to operate at a higher pH (e.g., ~ 4.5) and/or lower hydrogen peroxide concentration to minimize detrimental effects on the microbial community. This approach could provide an optimal environment to couple advanced oxidation processes with bioremediation technologies. Other reports on *in situ* applications with permanganate in CAH contaminated aquifer (Klens et al., 2001; Azadpour-Keeley et al., 2004; Sahl and Munakata-Marr, 2006) resulted in a decreased microbial activity right after the injection but followed by recovery at the long term (see table 2.4).

Within the scope of the FP7 UPSOIL project, a large lab-scale batch experiment was performed where the impact of three oxidizing agents (sodium permanganate (PM), sodium persulfate (PS) and/or alkaline activated persulfate (PS-A), each in three different

concentrations 5, 10, 20 g oxidant/kg aquifer) on CAH degradation by the indigenous microbial community in an aquifer material was examined (Simons et al., 2012). The evolution of the concentration of contaminants and breakdown products (PCE, TCE, *cis*-dichloroethene (cDCE), vinyl chloride (VC), dichloromethane, 1,2-dichloroethane, toluene, ethylbenzene, ethene, ethane, acetylene) were monitored over time and the results revealed that (1) chemical oxidation did have a negative impact on anaerobic biodegradation, (2) redox potential, which significantly increased after oxidant application, was a crucial parameter, (3) aerobic biodegradation might be more practical directly after chemical oxidation due to the oxidized aquifer conditions, and that (4) reduction of the redox potential by lactate addition was needed to obtain anaerobic biodegradation after oxidant exposure (Simons et al., 2012). In the current study, the impacts of the chemical oxidants, PM, PS and/or PS-A on the activity of the microbial community in the presence of aquifer material were investigated. Therefore, groundwater and aquifer samples from the UPSOIL batch test were taken over time and subjected to molecular analyses.

Furthermore, an *in situ* PM injection was also performed at a field site in the framework of the UPSOIL project to evaluate the PM injection with a newly developed MIP-IN detection-injection device (Uyttebroek et al., 2012, Bastiaens et al., 2013). Meanwhile, groundwater and aquifer samples were taken before and at different time points after the PM-injection to evaluate the impact of PM on the activity and structure of microbial community. In the current study, the effect of the oxidants on the microbiology in the samples collected from the batch test and field site were examined using qPCR and RNA-based DGGE analyses.

In summary, specific aims of this study were (i) to evaluate the short term impact of different doses of chemical oxidants under realistic environmental conditions, (ii) to evaluate the recovery of the CAH degradation for a post-chemical bioremediation, and (iii) to investigate the responses of introduced cells under post-chemical conditions only at batch level.

2. Material and Methods

2.1. Set-up of microcosms

The large scale laboratory UPSOIL microcosm test was prepared in 160-mL glass serum bottles containing homogenized 37 g (wet) aquifer and 70 mL of CAH contaminated anaerobic groundwater (Groundwater 1) from the field (Table 5.1) as described by Simons et

al. (2012). The bottles were sealed with Teflon-lined butyl rubber stoppers and stirred and incubated at 12°C.

Table 5.1. Composition of the groundwater and aquifer

	Groundwater 1	**Groundwater 2**	**Aquifer**
	(µg/L)	**(µg/L)**	**(µg/kg DS)**
Dichloromethane (DCM)	600000	< DL	67
Tetrachloroethene (PCE)	850	< DL	< DL
Trichloroethene (TCE)	< DL	< DL	< DL
Cis-Dichloroethene (cDCE)	18000	2227	49
Vinylchloride (VC)	< DL	329	134
Chlorethane (C)	< DL	< DL	858
Toluene (T)	832	131	10
Ethylbenzene (EB)	1800	1744	404

DL: detection limit

An overview of the experimental conditions relevant for this study is given in Table 5.2. Activation of PS was achieved via addition of 0.3-1.1 ml of a 25% of NaOH solution to the test systems. A 40% sodium permanganate (used in *in situ* application) and 20% sodium persulfate stock solution was used.

Table 5.2. Description of the test systems (Simons et al., 2012)

Reactor name	**Condition**
poisoned	Contained formaldehyde 37% (0.9 mL) and $HgCl_2$ (0.8 mL)
control	No addition
stimulated	Lactate addition (50%)
PM5	Sodium permanganate, final concentration of 5 g oxidant/kg aquifer
PM10	Sodium permanganate, final concentration of 10 g oxidant/kg aquifer
PM20	Sodium permanganate, final concentration of 20 g oxidant/kg aquifer
PS5	Sodium persulfate, final concentration of 5 g oxidant/kg aquifer
PS10	Sodium persulfate, final concentration of 10 g oxidant/kg aquifer
PS20	Sodium persulfate, final concentration of 20 g oxidant/kg aquifer
PS-A5	Activated persulfate, final concentration of 5 g oxidant/kg aquifer
PS-A10	Activated persulfate, final concentration of 10 g oxidant/kg aquifer
PS-A20	Activated persulfate, final concentration of 20 g oxidant/kg aquifer

The test was separated into two phases (Figure 5.1):

Phase 1 (Day 1-100): In this phase of the test, the removal of the main pollutant (cDCE) by chemical oxidation was targeted. The reactors were spiked with the oxidants on day 0 and day 50. Samples for quantifying gene copies and transcripts were taken on day 15 and 72.

Phase 2 (Day 100-450): In this phase, the groundwater in the reactors was replaced with less contaminated fresh groundwater (Groundwater 2, Table 5.1) on day 100 to investigate the

biodegradation of newly added pollutants. Lactate was added as electron donor for the CAH-degrading bacteria, but also to reduce the redox potential which was too high (+120-300 mV) after the chemical oxidation phase. Once the redox potential was lowered, a bacterial culture (same culture used in Chapter 3) capable of degrading chlorinated ethenes was added to selected test bottles on day 226, in order to study the effect of bioaugmentation on the degradation of CAHs. Slurry samples were taken for DNA and RNA analyses on day 129 (after groundwater addition) and day 289 (from bioaugmented and not bioaugmented reactors).

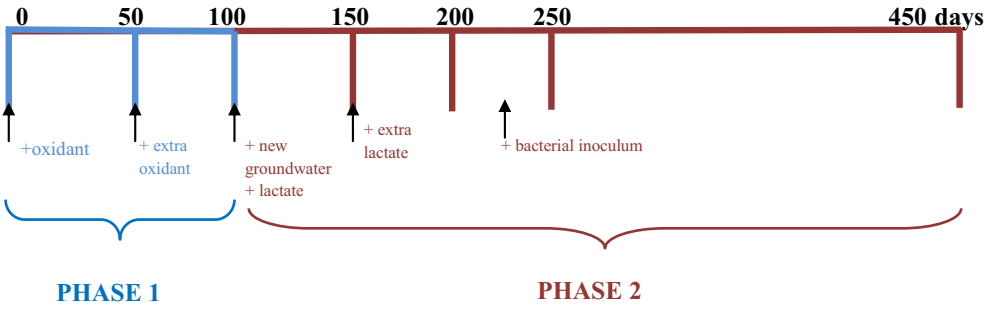

Figure 5.1. Overall of the different phases in the batch test

The evolution of the PCE, TCE, cDCE and VC concentrations and ORP values during Phase 1 and Phase 2 for the test conditions with permanganate and persulfate are summarized in the appendix, respectively (Bastiaens et al., in preparation).

2.2. Field applications

2.2.1. Site description and permanganate injection

The UPSOIL site is located in an industrial area near Antwerp, Belgium. At this site, a heterogeneous geology has been documented with dredged sand from 0 to 3 m below ground level (bgl), alluvial deposits of loamy sand and sandy loam with shells from 3 to 8 m bgl, sand with glauconite from 8 to 25 m bgl and a non-permeable clay layer at 25 m bgl (Uyttebroek et al., 2012). The groundwater flow direction is irregular but it flows mainly southwards (Figure 5.2) and a mixture of pollutants, mainly cDCE, was detected in the groundwater (Table A1). The cDCE concentrations detected in the groundwater ranged from 41 to 18,000 µg/L in all the wells. Via direct push technology, 1896 L and 900 L of a 83 kg PM/m^3 solution were injected at two locations (IN1 and IN2, 2 m apart), between 2.1-5.1 m bgl and 5.1-6.0 m bgl,

respectively. As high injection pressures were used, preferential flow paths were created (Uyttebroek et al., 2013). The injection spots were surrounded by monitoring wells as summarized in Figure 5.2. Some of them (MW5a, MW6b and MW8b) were directly impacted by PM based on visual observations of the groundwater which turned purple due to PM injection (Figure 5.2), while others (MW5b and MW7b) were impacted only indirectly.

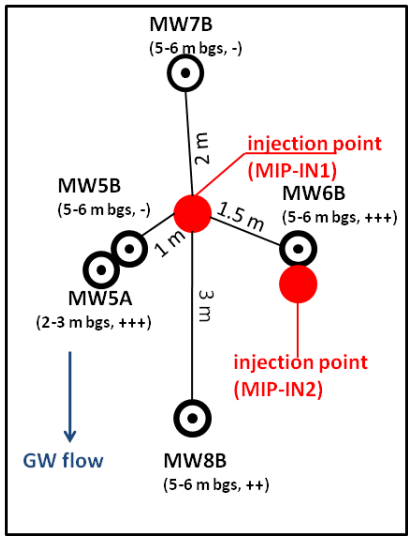

Figure 5.2. Location of the monitoring wells, depth of the filters and distances from the two injection points (+ /- indicates the degree of PM impact) (adapted from Uyttebroek et al., 2012).

Groundwater samples were collected one day before PM injection and 7, 15, 82, 305 and 443 days after PM injection using a peristaltic pump and polyethylene sample tubes (Eijkelkamp, Giesbeek, The Netherlands), following purging until field parameters stabilized. Groundwater samples for DNA and RNA extractions were collected with a peristaltic pump in 500 mL dark glass bottles, filled without leaving a headspace from bottom to top and allowing overflow equal to two times the bottle volume.

2.2.2. Mesocosms

In situ mesocosm systems were prepared to monitor the dynamics of the microbial community in the polluted aquifers as described previously bu Hendrickx et al. (2005). A mesocosm system consisted of a permeable membrane pocket filled with aquifer material (from field) (Table 5.1) placed within a polypropylene holder. A train of different of mesocosms was

inserted below groundwater level in the monitoring wells; the depths were chosen according to the depths of the filter screens (Figure 5.2). The details about the mesocosm system can be found in Hendrickx et al. (2005). Two months after placing the mesocosms into the monitoring wells MW5a, MW5b, MW6b, MW7b and MW8b, the first mesocosm in each well was recovered (just before the PM injection). Further, mesocosms were harvested 15, 82, 305 and 443 days after PM injection.

2.2.3. Undisturbed soil core samples (Liners)

Five months after the injection, undisturbed core samples were taken from the aquifer nearby monitoring well MW6b from 0 to 8 m bgl using a geoprobe device. Based on the MnO_2 concentrations measured in the liner (Uyttebroek et al., 2012), subsamples for the molecular analyses were taken at 197, 275, 525 and 662 cm depth in the core.

2.3. Analyses of chlorinated ethenes and manganese

The analyses of chlorinated ethenes in microcosms were performed as explained in Chapter 4. Manganese concentrations were determined by total destruction (HNO_3/HCl/HBF_4) and fixation according to CMA/2/II/A.3 and measurements were performed by ICP-AES according to CMA2/I/B.1.

2.4. Extraction and quantification of DNA and RNA

DNA and RNA were extracted and qPCR was performed according to the method described in Chapter 3. Instead of using 2 mL of liquid sample, 2 g of slurry and aquifer samples were used during extractions for the batch and mesocosm tests, respectively. The DNA or RNA extracted from aquifer samples was dissolved in 50 µL of buffer solution and the results were converted to copies/ g slurry unit at the end. The groundwater samples were filtered over 0.45 µm filters (Millipore, Molsheim, France) using a membrane filtration unit (Pall Life Sciences, New York, USA). The aquifer and membranes containing filtrated groundwater were stored at -80 ^{o}C until the extraction. The primer sets are given in Table 3.1 of Chapter 3.

3. Results and Discussion

3.1. Lab-scale test

Phase 1.

In Phase 1, it was targeted to investigate the short term effects of the oxidants on the dehalogenating activities by RNA-based qPCR analyses. A significant part of the CAH was degraded via oxidation by PM, yet the application of PS and PS-A was less successful in the removal of the contaminants (Simons et al., 2012, Figure A4). Although inhibition on the organisms was expected right after the aggressive oxidant injection, such an effect was not observed in the number of 16S rRNA *Dehalococcoides mccartyi* spp. (*Dhc* spp.) and *tceA* gene transcripts on day 15, which were at levels of 10^3-10^4 transcripts/g slurry as shown in Figure 5.3, except for the condition with 20 g/kg PM. Yet, it is important to note that for the first 50 days, the amount of injected oxidants was below or equal to the natural oxidant demand (NOD) of the aquifer. This meant that the oxidants were also consumed for the matrix demand resulting in less stress on microorganisms so that an inhibition was not observed. However, the number of gene transcripts remained almost at the same level (10^3-10^4 transcripts/g slurry) even also after the second injection of chemical oxidants on day 50. On day 72, before groundwater replacement, the detection of *vcrA* and *bvcA* gene transcripts at levels of 10^3-10^4 transcripts/g slurry was recorded except for the condition with 20 g/kg of PM.

At this point, a new index, transcript/gene ratio (mRNA/DNA), is helpful to normalize the expression data, subsequently to have a better insight into activity since the nucleic acid extraction efficiency may vary between different species and the matrix effect is different for extracellular anthropogenic mRNA and intracellular target mRNA (Bælum et al., 20013). In our study, the relatively low mRNA/DNA ratios of 16S rRNA gene of *Dhc* spp. and *tceA* on day 15; *vcrA* and *bvcA* genes on day 72 ranging between 0.012 and 0.352 transcripts/gene indicated limited dechlorination activity of the cells at that time (Figure 5.4) as compared to literature values. The reason might be attributed to the increase in the redox values from -200 mV to +200-300 mV after oxidant exposure (Simons et al., 2012). Moreover, the activation of persulfate with NaOH forms sulfate, hydroxyl, and superoxide radicals that are more aggressive to microorganisms (Furman et al., 2010) which explained the low transcripts per gene number under PS-A exposure on day 15 in Phase 1 for 16S rRNA of *Dhc* spp., *vcrA* and

bvcA genes. Lee et al. (2008) reported mRNA/DNA values between 0.8 and 10 transcripts/gene in a bioaugmented and biostimulated contaminated sandy site. Moreover, higher values have been reported under natural biodegradation conditions in groundwater samples with values between 10 and 17 transcripts/gene (Damgaard et al., 2013) and up to the value of 115 transcripts/gene (Courbet et al., 2011). However, the ratios in our study remained less than 0.5 transcripts/gene for oxidant exposed test systems until day 100 (until fresh groundwater was introduced to the system). Therefore, detected gene transcripts at levels of 10^3-10^4 transcripts/g slurry can be considered as possible background signals as also observed in the study of Bælum et al. (2013) in which biostimulated and bioaugmented microcosms were set up with clay till sediment. The same low mRNA/DNA values were also observed in the control systems (without oxidant) indicating a possible lag phase of the indigenous microorganisms. Yet, the ratios were higher than 0.5 transcripts/gene for the control systems at the end of Phase 1 except for the *tceA* and *vcrA* genes suggesting the start of dechlorination together with CAH degradation data (Figure A6).

Phase 2:

We also monitored the dechlorination activity of the microorganisms after the addition of less contaminated groundwater (Phase 2) with a target to investigate the biodegradation potential after chemical oxidation. At the beginning of Phase 2 (day 100), dechlorination of CAHs was not observed in the conditions where oxidant was added, even not under the exposure of lowest oxidant amount (5 g/kg) and also not when lactate was added as electron donor. The CAH biodegradation recovered only after several extra additions of lactate resulting in an ORP value lower than –150 mV, pointing out the importance of redox potential (Figure A5). At the first sampling event after groundwater replacement (day 129), increases in the mRNA/DNA ratios of 16S rRNA of *Dhc* spp. and *bvcA* genes from 1.0 to 2.0 were recorded in the control (without chemical oxidant) and in the test systems spiked with 5g/kg of PM and PS (Figure 5.4). Such an increase in mRNA/DNA ratios might be attributed to the activity of the organisms present (10^4 transcripts/mL for 16S rRNA genes of *Dhc* spp.) in the added groundwater in the oxidant exposed systems. In the control systems, however, the reason for increased mRNA/DNA values may be both the activity of indigenous microorganisms and microorganisms that were present in the added groundwater. On the other hand, in the test systems with higher oxidant concentrations (15-20 g/kg), the mRNA/DNA values did not

increase after groundwater addition indicating the inhibitory effect of the higher amount of by-products of the oxidants, high redox and pH conditions.

Bioaugmentation did not improve the dechlorination under the oxidant concentrations of 10 and 20 g/kg (Simons et al., 2012) (Figure A6). The adverse impact of the oxidants was noticeable at these higher concentrations (10 g/kg and 20 g/kg) since the genes were transcribed at background levels (≤0.5 transcript/gene) suggesting that dechlorination activity of the augmented microorganisms was inhibited by high ORP values caused by the oxidants (Figure 5.4). However, increases in the ratios up to 1.50-3.50 transcripts per genes on day 289, in both non-bioaugmented and bioaugmented test systems (control, stimulated, 5 g/kg for each oxidant), were in agreement with the CAHs depletion and ethene production at that time suggesting induced activity of the cells under reducing redox conditions (Figure A5 and A6).

One other important point to discuss is that higher mRNA/DNA values for 16S rRNA genes of *Dhc* spp., *tceA* and *vcrA* genes were detected in the test systems with 5 g/kg of PM and PS than the values detected in the control. This difference might be attributed to the induction of microbial activity in the presence of more bioavailable, simpler organic carbon which was produced from the complex organics by the chemical oxidants. In general, the mRNA/DNA ratios estimated in our study were indeed lower than the values measured in the previous field studies but they were in line with the values (1.7 and 2.1 transcripts per gene) determined by Bælum et al. (2013) in which biodegradation of TCE was monitored over time. These differences could be due to different stress responses of bacteria to their environment.

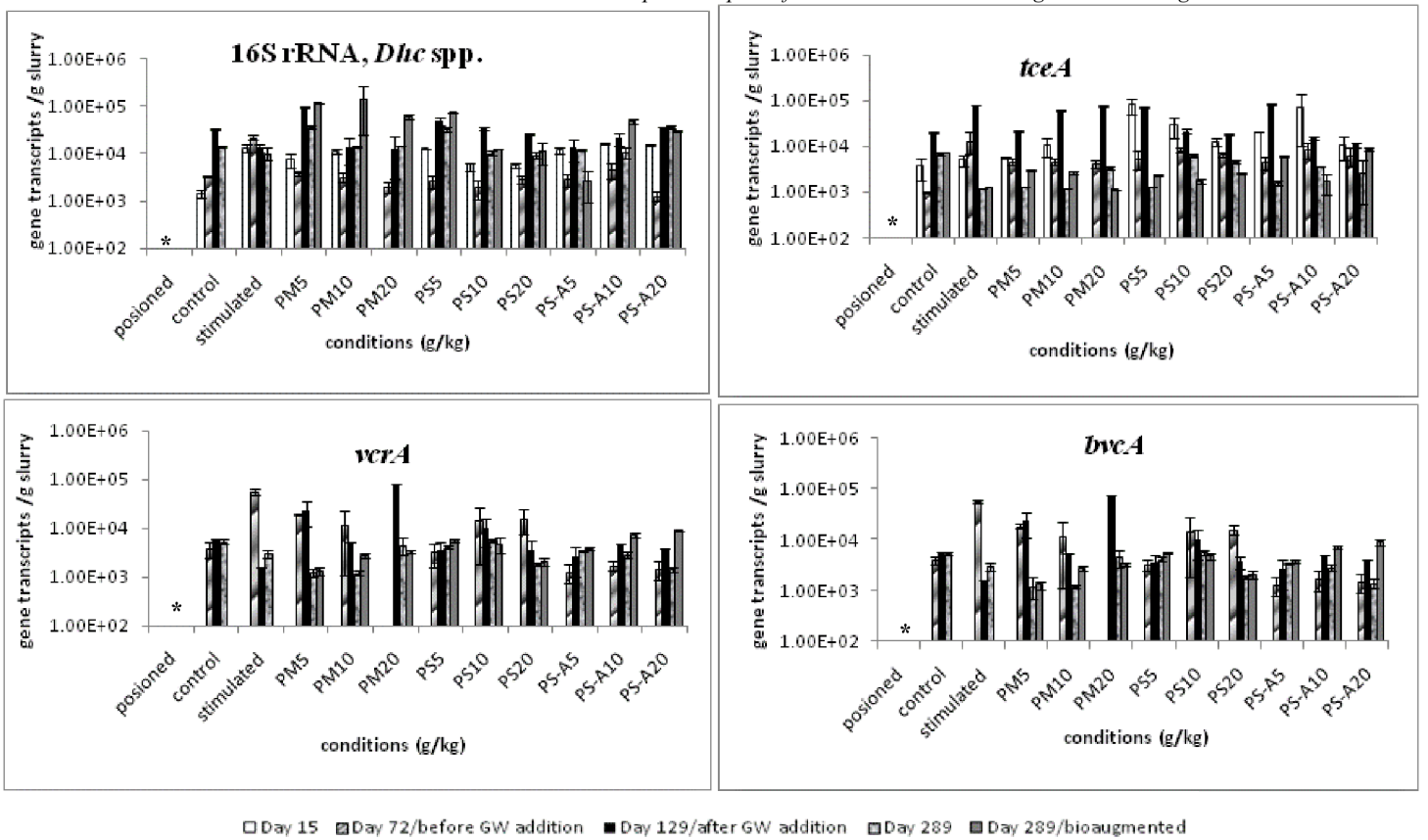

Figure 5.3. The number of gene transcripts under the application of sodium permanganate (PM), sodium persulfate (PS) and alkaline activated sodium persulfate (PS-A) in batch test (Each bar represents the average of the results of triplicate real-time PCRs performed on two independent RNA extractions (n = 6) (Detection limits: 10^2 ,10^2 and 10^3 copies/mL (4.99×10^1 , 4.99×10^1 and 4.99×10^2 copies/ g slurry) for 16S rRNA, *tceA* and *vcrA,* respectively; * below detection limit).

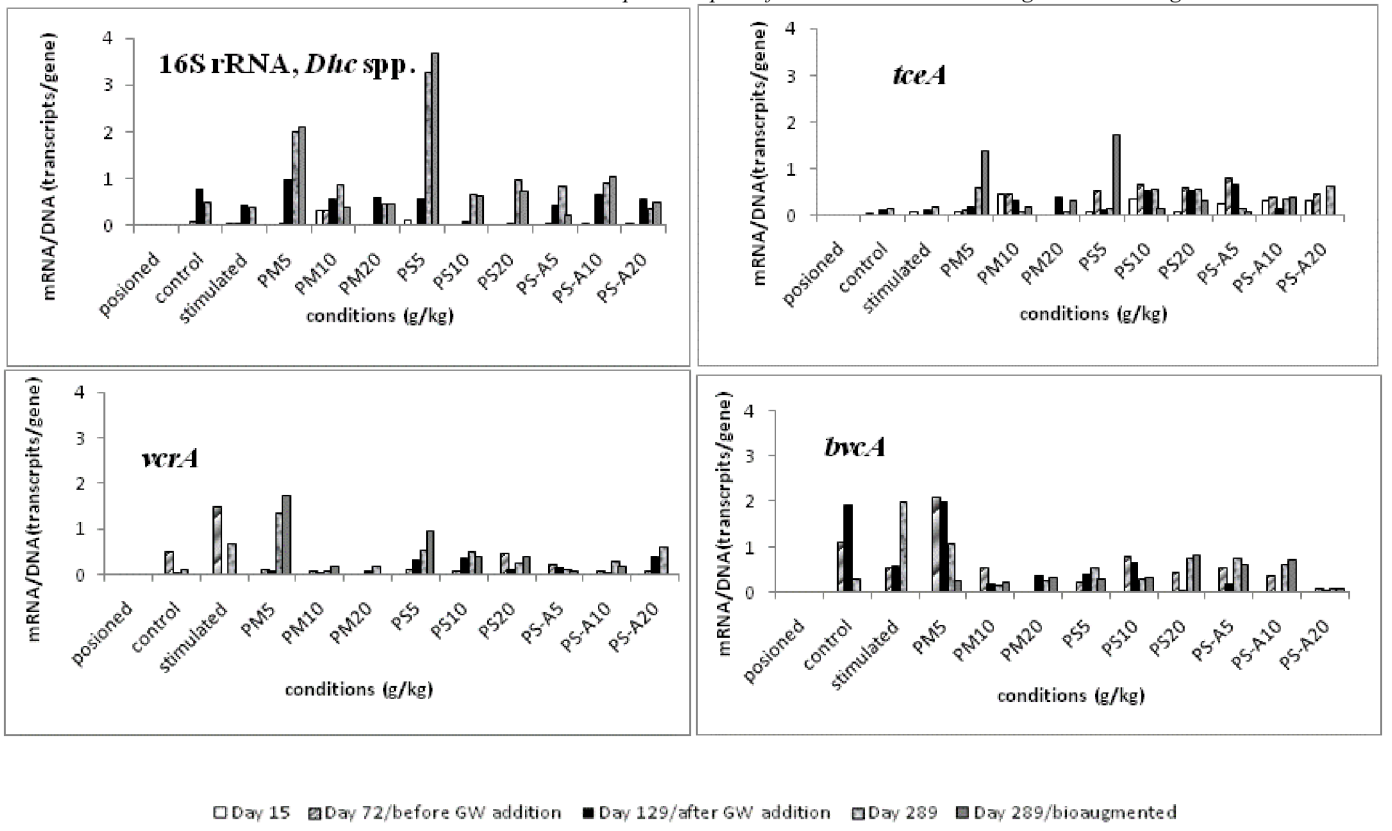

Figure 5.4. mRNA/DNA ratios under the application of sodium permanganate (PM), sodium persulfate (PS) and alkaline activated sodium persulfate (PS-A) in batch test.

3.2. Field test

3.2.1. Groundwater and mesocosm samples

In this study, mRNA quantification, demonstrating the presence but most importantly the activity of *Dhc* spp., was used to track the recovery of organohalide respiring bacteria (OHRB) in aquifer and groundwater samples after PM application at a field site. The expression of the 16S rRNA gene of *Dhc* spp. detected in groundwater samples at levels of 10^3 transcripts/mL suggested their activity (Figure 5.5-A1). The number of gene transcripts were below the detection limit (10^2-10^3 transcripts/mL) 15 days after the injection in the directly impacted wells (Figure 5.5, MW5a, MW6b and MW8b) indicating the limited activity of *Dhc* spp. under oxidant exposure (Figure 5.5A1, 2 and 3). The lack of gene transcripts in the wells MW5b and MW6b before injection may be linked to limited activity of *Dhc* spp. However, from day 15 onwards, the microorganisms recovered and the transcripts of 16S rRNA of *Dhc* spp., increased again to 10^4-10^7 transcripts/mL 82 days after the injection. It is notable that the number of transcripts at well MW6b on day 82 were lower than the values measured in the other wells since MW6b was more impacted by PM and recovery of the bacteria was slower. The number of gene transcripts was below the detection limit on day 15 also in the wells MW5b and MW7b which were not directly impacted by PM. The reason might be the increase in the redox value from -58 mV to 159 mV which in turn may inhibit *Dhc* spp. indirectly. Yet, the transcripts of 16S rRNA of *Dhc* spp. did increase again between day 15 and 82 at ORP values of - 130 mV. On the other hand, the RDase genes were stimulated from day 82 onwards, as the number of transcripts were detected at levels of 10^2-10^4 transcripts/mL for both *tceA* and *bvcA* genes.

The trends for the gene transcript numbers in the aquifer samples, in the mesocosm systems, were similar as in the groundwater samples (Figure 5.5-B1, 2 and 3). The 16S rRNA gene of *Dhc* spp. was expressed at levels of 10^3 transcripts/g aquifer in all examined wells before PM injection. After PM injection, on day 15, the number of gene transcripts was below the detection limit indicating again the detrimental effect of the oxidant on the indigenous microorganisms. The recovery of the microbial activity was observed again from day 82 onwards, with gene transcripts levels of 10^3-10^8 transcripts/g aquifer.

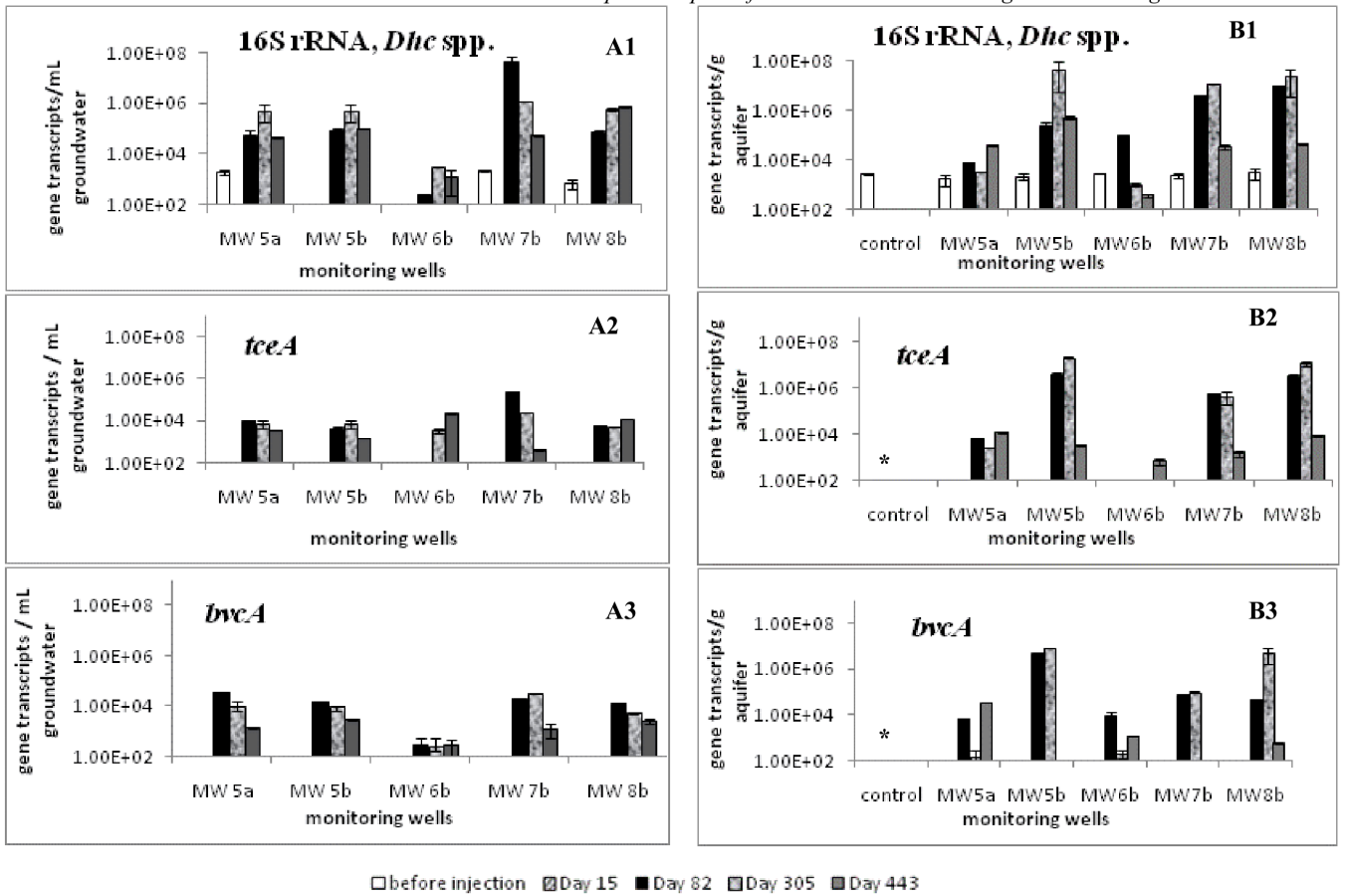

Figure 5.5. Number of gene transcripts of the 16S rRNA gene of *Dehalococcoides mccartyi* spp. (1), *tceA* (2) and *bvcA* (3) at *in situ* test: comparison of groundwater (A) and aquifer (B) samples taken from different wells. Control is the aquifer sample taken from the field before injection and used for filling the microcosms and used in the batch test. The aquifer sample in control was only analyzed once. Each value represents the average of the results of triplicate real-time PCRs performed on two independent RNA extractions ($n = 6$) (Detection limits: 10^2,10^2 and 10^3 copies/mL for 16S rRNA, *tceA* and *vcrA,* respectively; * below detection limit).

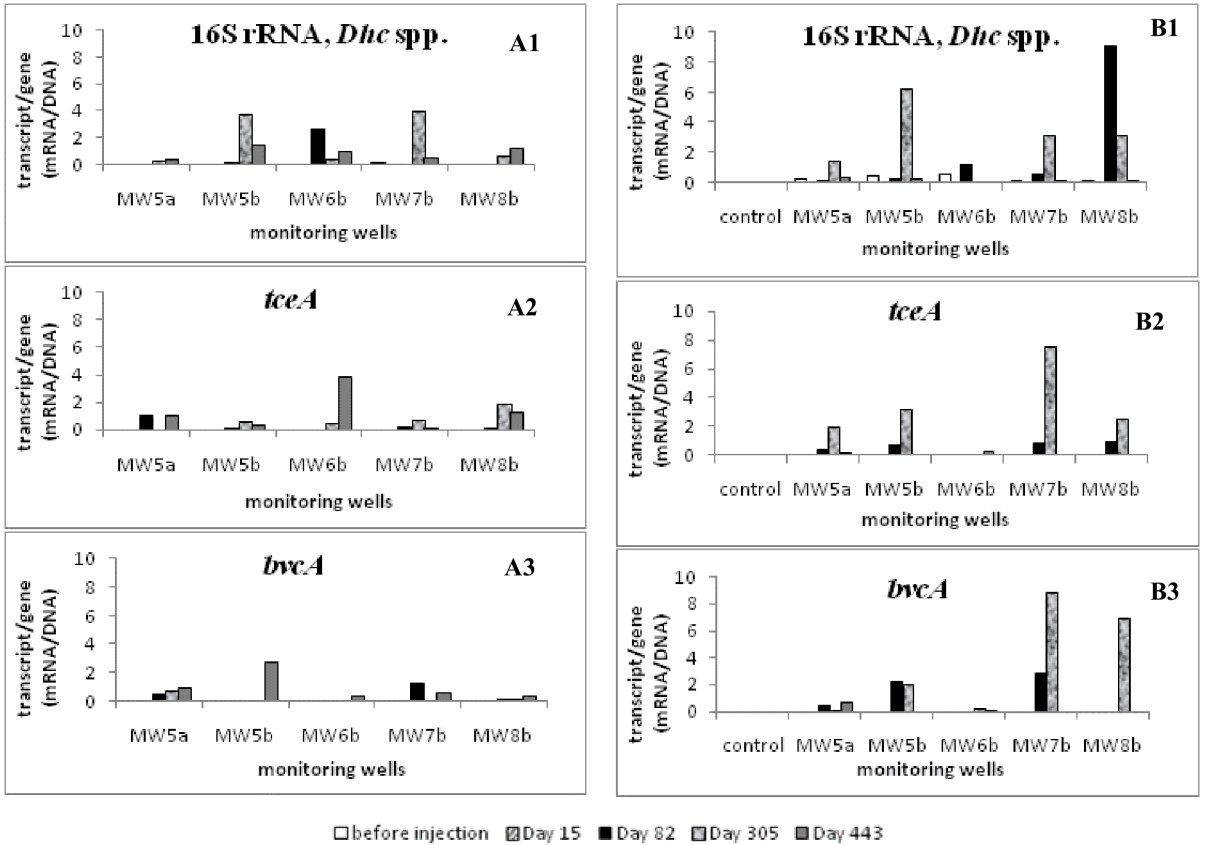

Figure 5.6. mRNA/DNA ratios after *in situ* test: comparison of groundwater (A) and mesocosm aquifer (B) samples taken from different wells.

mRNA/DNA ratios ranged between 0.04-3.98 transcript/gene for groundwater and 0.06-8.84 transcript/gene for aquifer samples (Figure 5.6). The stress on the microorganisms caused by PM was more obvious in the impacted wells MW5a, MW6b and MW8b since the values of the mRNA/DNA for the genes 16S rRNA of *Dhc* spp., *tceA* and *bvcA* were lower (0.02-1.20) than the values estimated for the wells MW5b and MW7b (0.05-9.05) on day 82 onwards for both aquifer and groundwater samples. One important point to address is the difference between the number of transcripts detected in the groundwater and aquifer samples. In general, the mRNA/DNA values in aquifer samples were higher than the values estimated in groundwater samples indicating the protective ability of soil particles for microorganisms against chemical oxidant stress.

In general, the absence of *vcrA* gene in the wells indicated that throughout the test cDCE dechlorination was performed by organisms carrying *bvcA* gene. Interestingly, the detection of *vcrA* in DNA extracts before the injection suggested its presence, however, undetectable copy numbers from day 15 to 443 (data not shown) in all monitoring wells indicated high sensitivity of the organisms carrying *vcrA* gene to PM. The fact that *tceA* and *bvcA* gene transcripts were steadily detected after day 82 and not before PM injection shows that the initial ISCO by PM injection could be considered as an important stimulatory step for organisms carrying *tceA* and *bvcA* genes and subsequent effective biological polishing. This might be due to high DOC availability (Chapter 6, Figure 6.3B) provided after PM injection which is known to stimulate not only OHRB but also fermenting bacterial groups that are considered as the drivers of organohalide respiration by providing electron donors (Smidt and de Vos, 2004) and corrinoids (Men et al., 2013) for *Dhc* spp.

3.2.2. Undisturbed soil core samples

The correlation between Mn concentrations and gene transcripts is important since $MnO_{2(s)}$ might have inhibitory effect on the bacteria as proven earlier in our study (Chapter 4). The manganese concentrations in the aquifer samples taken from the different depths of the liner (197, 275, 525 and 662 cm), that was sampled near the permanganate impacted well MW6a, were measured and the results are shown in Figure 5.7. Only 16S rRNA genes of total bacteria were expressed at a level of 3.18×10^2 transcripts/g aquifer when manganese concentration was 1510 mg/kg DM at a depth of 197 cm. The RDase genes were at undetectable levels suggesting the inhibitory effect of the by-product MnO_2(s) on *Dhc* spp. as

observed in Chapter 4. When Mn concentration levels were below 270 mg/kg DM, the RDase genes were expressed in the system (Figure 5.7). Therefore, we can conclude that the inhibitory effect of $MnO_{2(s)}$ on the microorganisms is highly depend on its concentration as indicated in this test and also in Chapter 4.

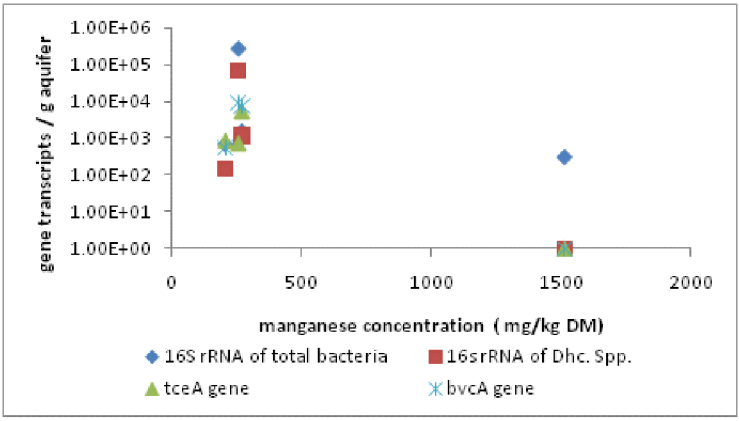

Figure 5.7. The concentration of manganese vs gene transcripts of 16S rRNA genes of *Dehaloccoides mccartyi* spp. and bacteria, *tceA* and *bvcA* genes in the liner near well MW6B.

3.2.3. Bacterial community structure

RNA based PCR-DGGE analysis targeting the total bacterial community was applied on the groundwater and mesocosm aquifer samples before and after the exposure to PM. In aquifer samples, there were no dominant bands before the injection and therefore the DGGE profiles on day 15 cannot be attributed to the oxidant impact in the wells MW5a, MW6b and MW8b (Figure 5.8A). Shifts occurred in the monitoring wells MW5a, MW5b and MW7b, from day 82 onwards however, might be caused by the change in the microbial dynamics due to the groundwater flow over time.

In groundwater samples, on the one hand, the effect of PM on the active microbial community was noticed in the oxidant impacted wells MW5a, MW6b and MW8b on day 15 (Figure 5.8B). Some bands became dominant after the exposure. Near MW6b the diversity of the active population decreased clearly on day 15 and even day 82.

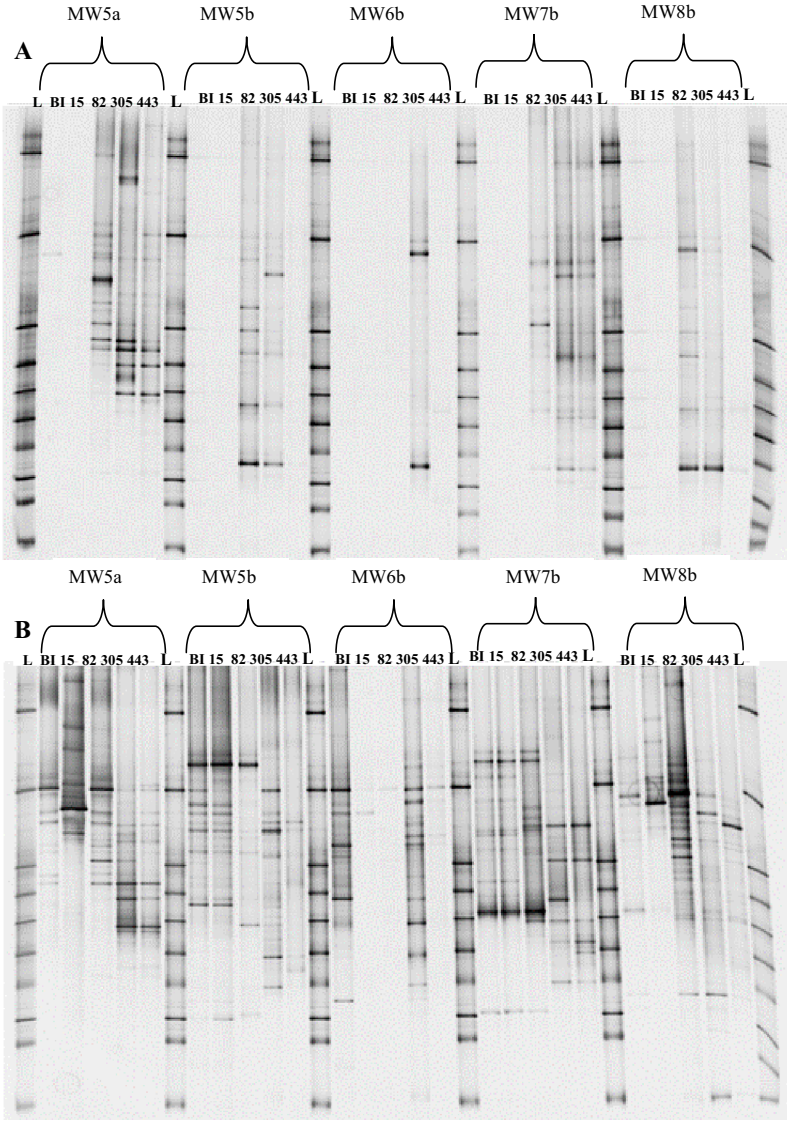

Figure 5.8. RNA-based 16S rRNA gene DGGE profile of the bacterial community in the monitoring wells over time in aquifer (mesocosm) (A) and groundwater (B) samples (L: Ladder; BI: Before injection; 15, 82, 305, 443 are days after injection).

On the other hand, the active microbial community was not affected in the un-impacted wells, MW5b and MW7b, on day 15 as expected. During the post-chemical phase, from day 82 on, shifts in the population were recorded in all five wells which might be attributed to the change

in the microbial dynamics again due to the groundwater flow over time and due to the heterogeneity of the site. An important observation is that the soil microorganisms seemed to recover rapidly after the PM injection, but the microbial population structure was not exactly the same as the one at the pre-oxidation conditions for all the monitoring wells.

4. Conclusions

The following conclusions can be drawn based on the results of this test:

- Chemical oxidation of CAHs was possible and compatible with biodegradation at low doses of oxidant (5 g /kg aquifer). However, in closed batch systems, biodegradation was negatively impacted after oxidant exposure at high doses of 15 and 20 g/kg oxidants and recovery of the bioactivity was not possible in some cases.
- Under more dynamic field system, recovery of CAH biodegradation without bioaugmentation was possible. The application of ***excessive*** amount of oxidants caused stress on the indigenous microorganisms in the field after the injection, but recovery was recorded after 82 days.
- The expression of the genes under aggressive oxidation conditions may be attributed to the background signals but the mRNA/DNA ratio indicated the detrimental effect of the higher oxidant amounts on gene expression, i.e., microbial activity. In this sense, 16S rRNA gene of *Dhc* spp. and *tceA* genes may be considered as the best indicator genes for evolution of CAH degradation.
- The inhibitory effect of by-product MnO_2(s) on *Dehalococcoides mccartyi* spp. in aquifer systems was suggested.

5. Acknowledgment

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Chapter 6. Dual element (C, Cl) isotope fractionation to distinguish between different removal processes of cDCE after in situ permanganate injection

Abstract

Permanganate (PM) injection is applied to treat groundwater contaminated with chlorinated ethenes. In this study compound specific isotope analyses (CSIA) were performed to distinguish between dilution, chemical oxidation and biodegradation of *cis*-1,2-dichloroethene (cDCE). Firstly, cDCE was degraded in lab scale batch tests focusing on separate removal process. The carbon and chlorine isotope enrichment factors measured during PM oxidation in liquid medium were ε_C = -26.2‰ ± 0.9‰ and ε_{Cl} = +0.20‰ ± 0.02‰, respectively. During anaerobic biodegradation with aquifer and groundwater from a cDCE contaminated site, enrichment factors of ε_C = -17.9‰ ± 0.6‰ and ε_{Cl} = -3.3‰ ± 0.1‰ were calculated. Dual isotope slopes and in particular the inverse chlorine isotope effect of the oxidation by PM was the key to distinguish between biodegradation and chemical oxidation. In a next step, the dual isotope approach was tested in the field before and up to 443 days after a pilot scale PM injection. The results showed that chemical oxidation and anaerobic biodegradation could be distinguished via chlorine measurements, while carbon isotope values remain important to detect dilution. Our study indicated, for the first time, the potential of dual isotope approach for distinguishing cDCE oxidation by PM and anaerobic biodegradation.

Chapter redrafted after: Doğan-Subaşı E, Elsner M, Qiu S, Cretnik S, Atashgahi S, Shouakar-Stash O, Boon N, Dejonghe W, Bastiaens L (2014). Dual element (C, Cl) isotope fractionation to distinguish between different removal processes of cDCE after in situ permanganate injection, submitted.

1. Introduction

Contamination of groundwater by chlorinated aliphatic hydrocarbons (CAHs) like trichloroethene (TCE) and *cis*-1,2-dichloroethene (cDCE) is a common environmental problem due to their toxicity and potential carcinogenicity. Although *in situ* bioremediation has recently become a well-established technique to remove these compounds at contaminated sites, there is no guaranty for successful application under heterogeneous field conditions with mixed pollution and/or residual free phase (Lee at al., 2007). Alternatively, a combination of *in situ* chemical oxidation (ISCO) followed by bioremediation might be a cost-effective approach to remove CAH contamination in the subsurface and reduce their concentrations below regulatory limits (Richardson et al., 2011; Doğan et al., 2013). Sodium permanganate (PM) has been applied as an effective oxidant for CAHs remediation in contaminated soil and groundwater systems (Schnarr et al., 1998).

When applying ISCO in the field, a decrease in the concentrations of CAHs could be due to degradation or transformation processes, but also for instance to dilution by displacement of contaminated groundwater by the injected solution (Hunkeler et al., 2003). Compound specific stable isotope analysis (CSIA) is a promising tool to distinguish between these processes (Hunkeler et al., 2003; Meckenstock et al., 2004) since physicochemical processes such as dispersion, sorption, dilution or volatilization of CAHs have minor impact on pollutant's isotope ratios (Schmidt et al., 2004), whereas biodegradation (Hunkeler et al., 1999; Abe et al., 2009; Fletcher et al., 2011) and chemical oxidation (Poulson and Naraoka, 2002; Hunkeler et al., 2003) result in pronounced changes in isotope ratios. Generally, due to stronger and less reactive molecular bonds formed by heavy isotopes in comparison with light isotopes, the heavy isotopes are degraded more slowly and become enriched in the remaining and not yet reacted fraction of the original contaminant (Lee et al., 2007; Elsner, 2010). This enrichment can be used as evidence for the occurrence of a degradation process and can be quantitatively expressed by the isotope enrichment factor (ε) that can be determined for a specific compound and a specific degradation process in laboratory experiments based on the Rayleigh equation (Elsner et al., 2005). In addition, if isotope ratios are determined for more than one element, they may even allow to distinguish between different transformation mechanisms that may occur during remediation, which is of paramount importance for field data interpretation (Zwank et al., 2005). Therefore, besides geochemical data and molecular-

based bioassay methods, isotopic analysis can provide complementary insight on underlying CAH removal processes at contaminated sites.

Although different mechanisms are generally associated with a different ε, and even though such values may distinguish degradation pathways in lab experiments (Hirschorn et al., 2004), it is usually not possible to identify the reaction mechanism in the field based on isotope data for a single element only. In this case, application of multiple isotope analysis (e.g., $^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$) can provide better insight into contaminant degradation pathways (Elsner, 2010; Cretnik et al., 2013). Indeed, several studies indicated that dual isotope plots have the potential to distinguish different transformation pathways (Elsner et al., 2005; Abe et al., 2009; Hunkeler et al., 2011). For example, Abe et al. (2009) applied the dual isotope approach (C, Cl) to distinguish between aerobic oxidation and anaerobic reductive dechlorination reactions for cDCE and vinyl chloride (VC). In another study, dual isotope (C/Cl) was used to differentiate abiotic transformation of cDCE by zero-valent iron (ZVI) and biotic degradation of the cDCE by KB-1, a commercially available *Dehalococcoides mccartyi* containing culture (Audí-Miró et al., 2013). A dual isotope approach was also documented for field scale biodegradation of cDCE (Hunkeler et al., 2011) and TCE (Lojkasek-Lima et al., 2012). However, to the best of our knowledge, there are no studies on the application of the dual element isotope approach (C, Cl) to evaluate cDCE biodegradation and/or chemical oxidation by PM.

In this study, we evaluated the possibility of using stable isotope analyses of two elements ($^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$) to distinguish between different scenarios of cDCE mass reduction i.e.(1) dilution, (2) degradation by chemical oxidation using PM and (3) biodegradation. The study site was an industrial area near Antwerp, Belgium that underwent *in situ* PM treatment. Due to heterogeneous distribution of the contaminants (CAHs, toluene, ethylbenzene) in time and space already before the pilot PM injection, monitoring of pollutant concentrations was not a viable option to document the impact of PM. cDCE was the main pollutant at the site and used as indicator compound. To lay the basis for interpretations of field data, cDCE isotope enrichment factors (ε_C, ε_{Cl}) during chemical oxidation by PM and anaerobic biodegradation were first determined in lab scale microcosm tests. Next, groundwater samples from the experimental field site were collected before and after PM injection and subjected to C and Cl isotope analyses. The dual element isotope slopes estimated from the lab scale test were used

as reference to identify the concurrent modes of degradation (chemical oxidation vs. reductive biodegradation) in the field.

2. Material and Methods

2.1. Laboratory experiments

2.1.1. Oxidation experiments of cDCE by permanganate

To determine the carbon and chlorine isotope enrichment factors for cDCE during oxidation by PM, batch experiments were set up in duplicate in closed 250 mL bottles equipped with Mininert valves (Supelco, Bellefonte, Pennsylvania, USA). Each bottle contained 150 mL distilled water with a final cDCE concentration of 60 mg/L (99%, Acros Organics). Forty percent(w/v) of sodium permanganate solution (same solution as used for *in situ* injection) was added to each bottle with a final concentration of 240 mg/L. Control bottles were filled with 150 mL of aqueous cDCE solutions (60 mg/L) without PM addition. For the analyses of CAHs and carbon and chlorine isotope ratios of cDCE, 10 mL subsamples were taken from each bottle every two minutes for a period of 20 minutes and subdivided in 2 mL samples in 2 mL GC vials containing 0.1 mL of a quenching solution (0.5 M sodium thiosulfate and 0.06 M calcium nitrate, used to stop the reactivity of the PM).

2.1.2. Anaerobic biodegradation experiments of cDCE

Microcosms for anaerobic biodegradation were prepared in 160 mL glass serum bottles containing 40 g (wet) homogenized aquifer material and 70 mL anaerobic groundwater from the field site located in an industrial area near Antwerp, Belgium. They were sealed with Teflon-lined butyl rubber stoppers. The field material was known to comprise a cDCE biodegradation potential (Simons et al., 2012). All the preparations were performed under nitrogen in a glove box. The bottles were purged with a N_2/CO_2 (80/20%) mixture to remove any preexisting volatile contaminants from the sediment/groundwater. Subsequently 15 mg/L cDCE was injected into each bottle. cDCE concentration in microcosms was monitored via direct measurement of the headspace, and sub-samples were taken for carbon and chlorine isotope measurements when the concentration of cDCE dropped to 10, 7, 5, 2 and 1 mg/L. These samples were kept at -20 ^{o}C until isotope measurements after increasing pH with NaOH.

2.2.Field Test

2.2.1. Site description and permanganate injection

The details about the field site, the concentrations of pollutants, PM injection process are provided in Chapter 5. Groundwater samples were collected one day before PM injection and 7, 15, 33, 55, 82, 305 after the injection. Samples for stable carbon/chlorine isotope analysis were collected in 250 mL amber bottles without leaving headspace, and immediately treated with NaOH pellets to reach pH 10 in order to stop microbial activity. Ten mL of groundwater samples were taken and filtrated through Millipore Millex-HA Filter (0.45 μm, 25 mm) and stored at -20 °C for dissolved organic carbon (DOC) analyses.

2.2.2. Compound specific isotope analysis

Carbon isotope analysis of cDCE was conducted by the method described by Milosevic et al. (2013). Chlorine isotopes of cDCE were determined according to the method presented in Audí-Miró et al. (2012). The carbon isotopic signature ($\delta^{13}C$) of the cDCE internal standard was 25.5‰ ± 0.2‰. The Cl isotopic signature ($\delta^{37}Cl$) of the cDCE external standards, cisF and IS-63, were -1.52‰ and +0.07‰, respectively.

$$\delta^{13}C = [\ (^{13}C/^{12}C)_{compound} - (\ ^{13}C/^{12}C)_{VPDB}]/\ (\ ^{13}C/^{12}C)_{VPDB} \quad (1)$$

$$\delta^{37}Cl = [\ (^{37}Cl/^{35}Cl)_{compound} - (\ ^{37}Cl/^{35}Cl)_{SMOC}]/\ (\ ^{13}Cl/^{12}Cl)_{SMOC} \quad (2)$$

where VPDB and SMOC are the international reference standards of Vienna Pee Dee Belemnite and Standard Mean Ocean Chloride, respectively (Audí-Miró et al., 2012).

2.2.3. Calculations of isotope enrichment factors

Carbon and chlorine isotope enrichment factors (ε) were calculated according to the Rayleigh equation:

$$\delta^{x}C = \delta^{x}C_0 + [\ \varepsilon_x \cdot \ln f\ ] \quad (3)$$

Where $\delta^{x}C_0$ is the carbon or chlorine isotope composition of the compound at time zero and $\delta^{x}C$ is the shift in the carbon or chlorine isotope composition from time zero to time t and f is the fraction of substrate remaining at time t (Cretnik et al., 2013).

2.3. Chemical analyses

CAHs were analyzed for the lab test via direct headspace analyses on a Varian GC-FID (CP-3800) as described in Chaper 4. The concentrations of PCE, TCE, cDCE, VC, methane, ethene and ethane in the field samples were measured by Thermo Finnigan Trace GC-MS equipped with a DB5-MS column (60 m length, 0.25 mm inner diameter and 0.25 μmfilm thickness, J&W Scientific, Folson, California, USA) as described by Hamonts et al. (2009). DOC was determined from samples as the difference between total dissolved carbon and dissolved inorganic carbon, measured with a Shimadzu TOC-5050A analyzer equipped with an ASI-5000 auto-sampler.

3.Results and Discussion

3.1. Laboratory reference experiments

3.1.1. Carbon and chlorine isotope enrichment during cDCE oxidation by permanganate and anaerobic biodegradation

Batch experiments were performed to determine the carbon and chlorine isotope enrichment factor of cDCE during oxidation by PM (distilled water) and anaerobic biodegradation (aquifer microcosms). The obtained carbon and chlorine isotope values of cDCE were plotted according to the Rayleigh equation (3) and the calculated isotope enrichment factors are presented in Figure 6.1. Negative controls (without PM) did not show any change in cDCE concentration or its carbon and chlorine isotope signatures (data not shown).

The carbon isotope enrichment factor of cDCE during anaerobic biodegradation was ε_C = -17.9‰ ± 0.6‰ (Figure 6.1A) which was in agreement with previous studies where ε_C ranged from -16.9‰ to -31.9‰ (Lee et al., 2007; Carreon-Diazconti et al., 2009; Abe et al., 2009; Fletcher et al., 2011). On the other hand, during permanganate oxidation of cDCE, a value of ε_C = -26.2‰ ± 0.9 ‰ was determined in our test (Figure 6.1A), which also falls within the range of values (ε_C = -17.0‰ to -31.9‰) reported before for the oxidation of different chlorinated ethenes by PM (Poulson and Naraoka, 2002; Hunkeler et al., 2003). The large and similar carbon isotope enrichment factors for anaerobic biodegradation and oxidation by PM indicate that changes in carbon isotope values in field samples may be caused by either process. Hence, carbon isotope analysis alone may not be useful to make a distinction between these degradation mechanisms in the field.

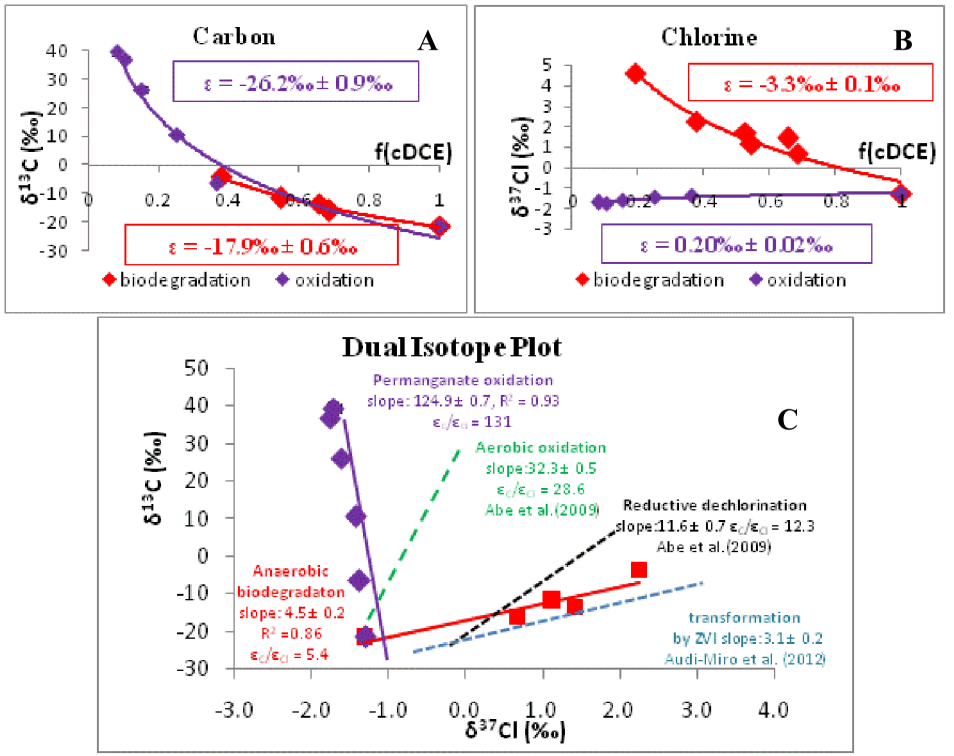

Figure 6.1. Isotope fractionation patterns in cDCE of $\delta^{13}C$ (A) and $\delta^{37}Cl$ (B) measured during degradation by biodegradation and chemical oxidation. Data points are from duplicate batches per experiment. **(C)** Dual isotope plot for batch permanganate oxidation and anaerobic biodegradation. The slopes are given and compared to the results of Abe et al. (2009) and Audí-Miró et al. (2012).

The chlorine isotopic enrichment factor observed during anaerobic biodegradation (ε_{Cl} = -3.3‰ ± 0.1‰, Figure 6.1B) was significantly larger than the one reported by Abe et al. (2009) (ε_{Cl} = -1.6‰ to -1.4‰). A lower $\varepsilon_C/\varepsilon_{Cl}$ value observed in our study compared to Abe et al. (2009) can therefore be attributed to these larger chlorine enrichment factors since the carbon isotope enrichment factors were practically the same (ε_C = -17.9‰ ± 0.6‰ (our study) vs. ε_C = -18.5‰ (Abe et al., 2009)) (Figure 6.1C). While this indicates a notable underlying difference in the cDCE reductive dechlorination mechanism between microorganisms, biodegradation still shows significant chlorine isotope enrichment which may serve as an indicator to identify the C-Cl bond cleavage associated with reductive dehalogenation. In contrast, our study provides the first data on chlorine isotope enrichment of cDCE during oxidation by PM. Here the observed chlorine isotopic enrichment factor of ε_{Cl} = 0.20‰ ± 0.02‰ was (i) very small and (ii) even inverse (i.e., ^{37}Cl reacted faster) (Figure 6.1B). This is consistent with secondary isotope effects, since a C-Cl bond is not directly broken in the initial step of permanganate oxidation of ethenes (Houk and Strassner, 1999) (Figure 6.2). Also the inverse chlorine isotope effect is consistent with the mechanism, since the number of substituents increase around the C-Cl bond in the transition state. The C-Cl bending vibrations are therefore more constrained which may lead to inverse Cl isotope effects. Such effects are well established for C-H bending vibrations, for example, in amide hydrolysis (Marlier, 2001). Therefore, the large carbon isotope fractionation reflected a primary carbon isotope effect in the formation of C-O bonds with PM, while the chlorine did not participate in this first step of the reaction and caused only small, secondary isotope effects. Therefore, our data show that secondary chlorine isotope effects are rather insignificant in this reaction (Figure 6.2). As a result, when compared to carbon isotope enrichment, the additional evidence from chlorine isotope effects can, hence, distinguish the two mechanisms (reductive dechlorination vs. oxidation by PM). An additional test was also performed to determine carbon and chlorine isotope enrichment factor of TCE during oxidation by PM (data not shown). The same pattern, i.e. large carbon and small, negligible chlorine isotope fractionation, was observed at the end. As carbon isotope enrichment factor was calculated as ε_C = -24.9‰ ± 0.9 ‰, the enrichment value for chlorine was ε_{Cl} = 0.06‰ ± 0.02‰.

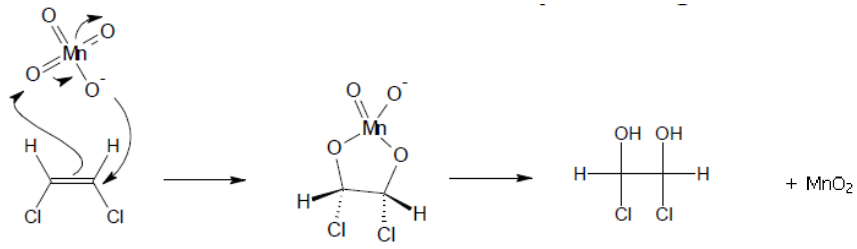

Figure 6.2. Proposed mechanism for cDCE oxidation via permanganate (after Morasch and Hunkeler, 2009).

In particular, the dual (C, Cl) isotope plot was found suitable for differentiating between the considered degradation pathways, because the relative shift in isotope ratios of two elements varied significantly depending on the reaction mechanism (Figure 6.1C). The observed slope of 124.9 ± 0.7 for chemical oxidation in our experiment is much higher than the slope observed for anaerobic biodegradation (4.5 ± 0.2) (Figure 6.1C), the slopes observed by Abe et al. (2009) for reductive dechlorination (11.4 ± 0.6) and aerobic oxidation (32.3 ± 0.5), and by Audí-Miró at al. (2013) for cDCE transformation by ZVI (3.1 ± 0.2). Pronounced differences in dual element (C, Cl) isotope slopes of different mechanisms therefore delineate a promising new way to discriminate biotic and abiotic degradation of chlorinated ethenes. Here, for the first time we applied this approach to distinguish between cDCE transformation by permanganate oxidation vs. anaerobic biodegradation.

3.2.Field Results

3.2.1. Changes in redox potential and dissolved organic carbon at the field

After injecting 2,800L of 83 kg PM/m³ solution in the pilot test area (Chapter 5, Figure 5.1), shifts in redox potential and DOC concentrations were monitored in the monitoring wells (Figure 6.3). Monitoring wells MW5a, MW6b and to a lesser extent MW8b were significantly impacted by the PM, as visually observed by the dark purple color of the groundwater just after the injection (Chapter 5, Figure 5.1, indicated with +/-). This was also supported by redox measurements, since 7 days after the injection the redox values increased from -58 mV to 159 mV, -94 mV to 89 mV and -112 mV to 49 mV in MW5a, MW6b and MW8b, respectively. This effect was observed till day 33, when PM was no longer visually observed. Analogous trends in redox potentials were reported in previous studies as a result of PM treatment (Sahl et al., 2007; Siegrist et al., 2011; Doğan et al., 2013). Reducing redox

conditions resumed after day 33 in these monitoring wells (Figure 6.3A). Monitoring wells MW5b and MW7b were not directly affected as no PM was visually observed. Explanations could be the differences in the filter depth and IN1 injection depth as well as the heterogeneous distribution of the oxidant in the subsurface. Nevertheless, a temporary increase in ORP values on day 7 was also observed suggesting an indirect effect of injected PM solution at these spots possibly due to influx of mobile oxidized groundwater when PM was already consumed. Yet, the measured values on day 15 and onwards indicated recovery of the reducing conditions (Figure 6.3A).

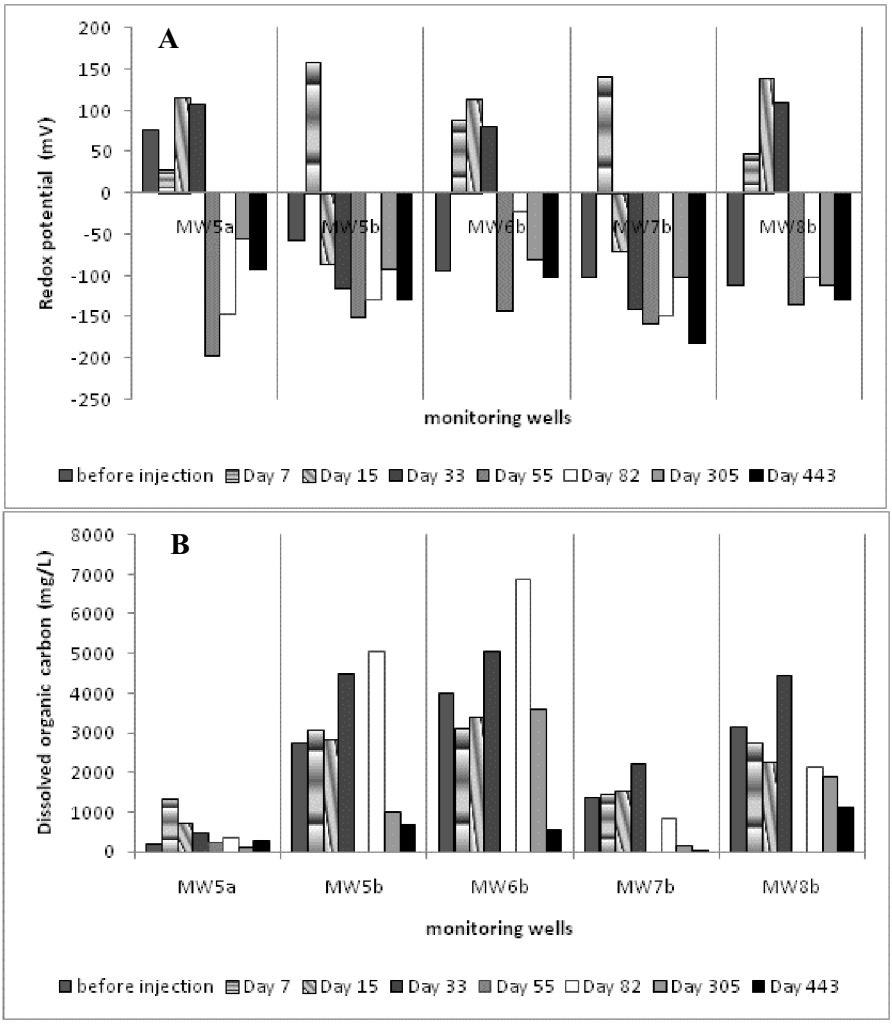

Figure 6.3. Redox values (A) and dissolved organic carbon (B) concentration in the monitoring wells in function of time (before injection means before injection of the permanganate, day 0).

The change in DOC concentrations was more dynamic in the total system with no clear distinction between directly impacted wells compared to the other wells (Figure 6.3B). Increases in DOC concentrations from 194 mg/L to 738 mg/L in the impacted well MW5a right after the PM injection might be attributed to the oxidation of complex organic matter into smaller soluble molecules.

3.2.2. *In situ* carbon and chlorine isotopic shift

Carbon and chlorine stable isotope values were determined in groundwater samples from the different wells to evaluate the added value of the dual isotope approach (1) for revealing whether cDCE degradation processes occurred in the test area, and (2) for differentiating between chemical oxidation by PM and anaerobic biodegradation. The average stable isotope values of cDCE obtained for monitoring well MW5b (not PM impacted) during the first 82 days ($\delta^{13}C$ = -29.6‰ ± 0.2 and $\delta^{13}Cl$ = 5.0‰ ± 0.1) were considered as the reference isotopic signature of the field throughout the test (Figure 6.4B)

After PM injection, CAH-concentrations and isotopic shifts in the groundwater samples collected from the directly PM impacted wells MW5a, MW6b and MW8b were studied (Figure 6.4, Table A1). In MW5a, there was a significant decrease in cDCE concentration from 30 µM to 6 µM after 15 days of PM injection (Figure 6.4A, Table A1). The relatively constant values of $\delta^{37}Cl$ (from 5.0‰ ± 0.1‰ to 4.8‰ ± 0.2‰) and $\delta^{13}C$ (from -29.9‰ ± 0.2‰ to -28.3‰ ± 0.2‰), however, point rather in the direction of dilution by the injected permanganate solution or other processes than degradation of cDCE (Table A3). Indeed, the disappearance of certain contaminants along the groundwater flow path might be caused by several processes such as adsorption on the aquifer matrix and dilution (Schmidt et al., 2004; Meckenstock et al., 2004), but these processes do not result in isotopic shifts as observed in our study. On the other hand, in MW5a, the carbon and chlorine isotope ratios changed from -26.7‰ to -24.1‰ and from 4.1‰ to 6.1‰, respectively, between day 33 and 55 suggesting that (i) cDCE was degraded and (ii) the underlying degradation pathway was anaerobic biodegradation, as evidenced by the changes in chlorine isotope values (no changes, or the opposite trend would have been expected in the case of permanganate degradation) (Figure 6.5).

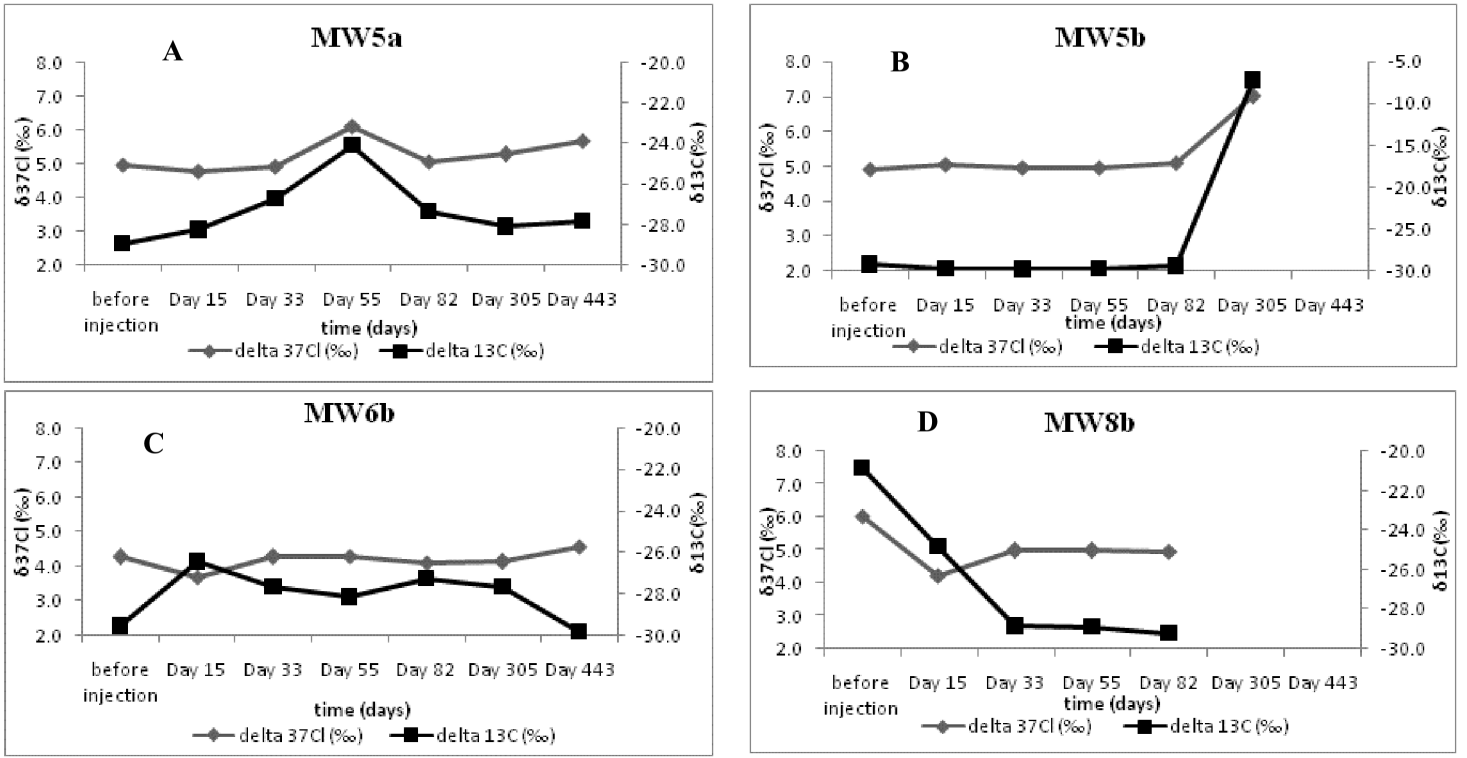

Figure 6.4. Carbon and chlorine isotope ratios in the monitoring wells (Note that for the Y axes, different concentration scales are used for carbon).

These δ^{37}Cl and δ^{13}C values were in agreement with the field study of Hunkeler et al. (2011) in which anaerobic reductive dechlorination was concluded as the removal mechanism. Reductive dechlorination is further supported by the presence of sufficient organic carbon at our site to stimulate anaerobic biodegradation, as well as by reducing redox conditions (-197 mV) on day 55.

Similar to MW5a, a 3.1‰ increase in δ^{13}C ratios for well MW6b on day 15 indicates degradation of cDCE (Figure 6.4C). In contrast to MW5a, however, a 0.6‰ decrease in δ^{37}Cl for well MW6b demonstrates that the cDCE concentration was diminished from 186 µM to 160 µM due to chemical oxidation by PM near that well at that time (Figure 6.4). From day 33 onwards, when PM was no longer reactive, isotopic signatures that were closer to the pre-chemical condition were again observed, although the slightly increased average δ^{13}C value (-28.2‰ ± 0.5‰) in this well may indicate some slowly ongoing biodegradation as compared to the pre-injection values (-29.5‰ ± 0.2‰)(Figure 6.4C).

Finally, in well MW8b, in which strong indication for cDCE biodegradation was found based on isotope ratios (-20.8‰ ± 1.1‰ for δ^{13}C and 6.0‰ ± 0.1‰ for δ^{37}Cl) before the injection, a decrease in δ^{37}Cl values by 1.8‰ was recorded 15 days after PM injection (Figure 6.4D). This, again, clearly indicated that chemical oxidation was the dominant mechanism that reduced cDCE concentrations from 213 mM to 18 µM at that time (Table A1). As time proceeded, the isotopic values in MW8b bounced back to the values of -28.8‰ ± 0.8‰ and 5.0‰ ± 0.1‰ for δ^{13}C and δ^{37}Cl, respectively (Figure 6.4D).

In well MW5b, which was not directly impacted by PM, δ^{13}C values were constant until day 82 (used as reference values), although cDCE concentration decreased from 302 to 250 µM between day 15 and day 82. Yet, a significant increase in δ^{13}C (from -29.4‰ to -7.3‰) and δ^{13}Cl values (from 5.1‰ to 7.0‰) were measured at day 305, giving strong evidence of the occurrence of anaerobic degradation of cDCE. A change in cDCE concentrations from 250 µM to 53 µM followed by production of VC and ethene supported this fact (Table A1). Unfortunately, no isotope data is available for MW7b because of the low cDCE concentrations. Based on the assumed isotopic signatures of the field (δ^{13}C = -29.0‰ ± 0.2; δ^{13}Cl = 5.0‰ ± 0.1), it can be concluded that a certain extent of anaerobic biodegradation took place throughout the course of the experiment in all the wells with the exception of day 15.

All the recorded $\delta^{13}C$ and $\delta^{13}Cl$ values measured in groundwater samples from the field were plotted in a dual isotope graph (Figure 6.5), where also the slopes determined in lab scale degradation tests are indicated. The results clearly indicate that chemical oxidation of cDCE occurred near monitoring wells MW6b and MW8b. Further, clear evidence for anaerobic biodegradation of cDCE was found for the wells MW5a, MW5b and MW8b. Identifying the dominant pathway from a dual element isotope plot also allowed us to choose the appropriate enrichment factor for deriving estimates of the degradation..

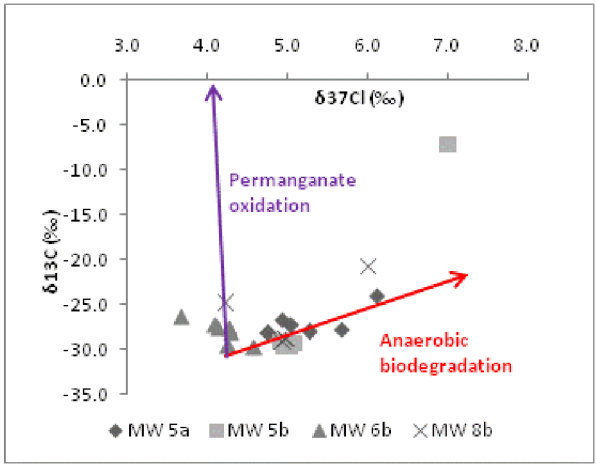

Figure 6.5. Dual isotope graph for *in situ* chemical oxidation test. The arrows were drawn as references according to the slopes of the batch scale tests.

Moreover, the extent of (bio)degradation occurred in each well was estimated for each sampling time (Table A3), where only values that showed a 2.0‰ shift in carbon and 0.8‰ shift in chlorine isotope values were considered (Hunkeler et al., 2008). Based on this approach, the degree of chemical oxidation was estimated as 20% in well MW5a on day 15 while the extent of biodegradation was estimated as 24% (via carbon) and 29% (via chlorine) on day 55 supporting the mechanisms already identified. In the unimpacted well MW5b, 71% (via carbon) and 47% (via chlorine) of biodegradation were estimated on day 305 (Table A3). In contrast, the degradation mechanism in wells MW6b and MW8b was clearly chemical oxidation giving estimates of degradation of 95% and 97% for MW6b and MW8b, respectively (Table A3). However, in the current study, these different observations may be explained by the heterogeneity of the site, rebound of the CAH contamination in the post-chemical phase.

4. Conclusions

This study presents the first results on dual isotope approach (C, Cl) to evaluate the occurrence of biodegradation and chemical oxidation of cDCE. The dual isotope approach presented in our study and in the previous studies indicate the possibility of distinguishing between different mechanisms during cDCE degradation as presented in Figure 2C. In other words, the mechanisms of chemical oxidation by PM, anaerobic degradation, aerobic oxidation, reductive dechlorination were clearly had different slopes ($\varepsilon_C/\varepsilon_{Cl}$ values) in dual isotope plot suggesting its applicability. The results of our study indicate that dual isotope approach might be a promising tool to identify/differentiate the mechanisms involved in the degradation of chlorinated ethenes in the field. This approach could be particularly interesting for evaluating the fate of contaminants in the sites under combined treatment; such as chemical oxidation followed by biodegradation.

5.Acknowledgment

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Chapter 7. Impact of chemical oxidants on the heavy metals and the microbial population in sediments

Abstract

Chemical oxidation was applied to treat three contaminated sediments contaminated with mineral oil, polycyclic aromatic hydrocarbons (PAHs) and heavy metals and containing an organic matter content ranging from 2.4 to 7.6%. The natural oxidant demand of the sediments was determined during treatment with two different types of oxidants (potassium permanganate and sodium persulfate), and the effect of these oxidants on the release of heavy metal and on the microbial community was examined. The natural oxidant demands of the sediments under persulfate treatment were lower (30-100 g/kg) than those under permanganate treatment (50-450 g/kg). Cr was released during the application of permanganate whereas Zn and Pb were released under persulfate treatment. qPCR results showed that permanganate and persulfate, both at a concentration of 150 g/kg, caused a decrease (2 log units) in the number of bacterial 16S rRNA genes in the sediment with the lowest organic matter content. However, ATP concentration, considered as a biomarker for total overall microbial activity, was below detection limit in all sediments when treated with at least 150 g/kg oxidant. Only permanganate induced a shift in the microbial community structure.

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1. Introduction

Mixtures of organic and heavy metal contaminants present in the sediments pose a risk to aquatic life, human health, and wildlife throughout the world due to their toxicological and adverse effects (Ho et al., 2002). Therefore, remediation of these contaminated sediments is often necessary in order to prevent environmental risks to the ecosystem.

For most pollutions, numerous remediation techniques are available such as *in situ* chemical oxidation (ISCO), which is especially useful for chemical destruction of organic pollutants after injection of a strong chemical oxidant into the subsurface (Tsitonaki et al., 2008). Depending on the type of pollution and sediment, different oxidants like ozone, hydrogen peroxide, persulfate and permanganate or catalyzed oxidants like Fenton's reagent are injected into the subsurface. Each of these oxidants has its beneficial characteristics as well as some limitations (e.g., persistence, reactivity, etc.). While hydrogen peroxide and Fenton's reagent have the potential to complete the remediation in a relative time (high reactivity), permanganate oxidizes the pollutants over an extended period (low reactivity). Permanganate is also stable, easy to handle, and applicable over a wide pH range (Hønning, 2007). Persulfate is relatively persistent and can be transported over considerable distances (USEPA, 2004; Hønning, 2007; Liang et al., 2007(b); Waldemer et al., 2007; Tsitonaki et al., 2008). Both persulfate and permanganate are less damaging to microorganisms than Fenton's reagent and hydrogen peroxide (Tsitonaki et al., 2008) and are capable of oxidizing chlorinated solvents, petroleum hydrocarbons, PAHs, and BTEX.

Apart from the above mentioned organic pollutants, there are also a variety of sedimentary reductants, such as organic matter and/or ferrous, manganous and sulfidic species, present in the contaminated sediments. These sedimentary reductants often react with the oxidants (e.g. permanganate and persulfate) and hamper their performance. The consumption of the oxidants by the sedimentary reductants is defined as the natural oxidant demand (NOD) and is expressed as the mass of oxidant consumed per mass of dry solid (Mumford et al., 2005; Tsitonaki et al., 2008). The NOD affects the diffusion of the chemical oxidant in the sediment matrix resulting in the need of higher oxidant demands. Therefore, interaction between demand and supply of the oxidants is essential to select site specific dosing requirements and delivery system (Sra et al., 2007).

Despite its potential benefits in treating contaminated sediments, chemical oxidation processes can also lead to undesired effects in sediments. For example, residual oxidant due to unproductive consumption induces geochemical effects such as altered pH and redox potential which in turn can affect the metal release in the soil or sediments (Crimi and Siegrist, 2005). In fact, more than 90% of the metal load in aquatic systems is bound on particulates like suspended matter and sediments (Calmano et al., 1993) and the application of chemical oxidants may cause considerable metal release leading to problems in the river ecosystem. In addition, indigenous microbial populations can be affected via a direct (destruction of the cell) or indirect way (changes in redox conditions, organic matter, pH, …). Therefore, the undesired effects of the oxidants such as release of heavy metals and alteration in the microbial community should be examined and controlled in order to improve the efficiency of *in situ* treatment systems such as bioremediation.

To the best of our knowledge, only a limited number of studies has investigated the effect of chemical oxidants on the microbial community in soil and groundwater by using basic analyses such as acetate consumption (Chapelle et al., 2005; Tsitonaki et al., 2008), plate counts (Jung et al., 2005; Studer et al., 2009), phospholipid fatty acids (Brown et al., 2009) and mineralization of ^{14}C phenanthrene (Richardson et al., 2011). However, no research has used molecular methods to provide a better understanding of the effects of ISCO treatment on the diversity abundance, and activity of the microbial system in sediments. Furthermore, the comparison of the desired and undesired effects of permanganate and persulfate on the microbial community is also a novel aspect of this study.

The aim of this study was to investigate the effect of the oxidants potassium permanganate ($KMnO_4$) and sodium persulfate (NaS_2O_8) on the behavior of the heavy metals and the indigenous microbial community in three sediments with different organic matter, heavy metal and organic pollutant content. More specifically, the effects on the number of the 16S rRNA gene of total bacteria was quantified with qPCR, while the microbial metabolic activity and diversity were investigated by ATP and PCR-DGGE analysis, respectively.

2. Materials and Methods

2.1. Sediment samples

Sediment samples were taken from a depth of 0 to 50 cm from three different locations in Belgium i.e., Zandwinningsput (ZWP, a sludge pond in Antwerp), Berendrechtsluis (BDS, a part of the Port of Antwerp) and Ieper-Ijzer kanaal (IIK, a canal close to Ieper). Samples were stored under anaerobic conditions (nitrogen atmosphere) at 4°C until use.

2.2. Physico-chemical measurements

The pH and redox values were measured by a pH-meter (WTW Microprocessor, pH meter) and redox probe (WTW-SenTix MIC-D and SCHOTT instrument). Dry and organic matter were determined with methods EN 14346:2006 and EN 13137:2001. Physico-chemical characteristics of the sediments are given in Table 7.1.

Table 7.1. pH, organic, dry matter, clay, organic contaminant, and total heavy metal content of the sediments

Sediment Characteristics	IIK	BDS	ZWP
Organic matter content (%)	7.6	6.9	2.4
Dry matter content (%)	32.2	38.0	59.7
pH	8.3	8.5	8.4
Clay content (%)	7.6	9.0	5.4
Organic contaminant content (mg/kg DM)			
Mineral oil	2300	210	640
PAH 16	18.32	<8	5
PCBs	0.100	<D.L	0.043
Total heavy metal content (mg/kg DM)			
Al	45000	51000	29000
As	20	32	38
Cd	2.20	4.40	7.70
Cr	170	120	110
Fe	33000	44000	35000
Cu	91	66	67
Hg	2.70	0.71	1.20
Pb	140	91	190
Ni	53	28	26
Zn	720	410	530

D.L: Detection Limit**; DM:** Dry Matter**; IKK:** Ieper-Ijzer kanaal**; BDS:** Berendrechtsluis**; ZWP:** Zandwinningsput

2.3. Chemical oxidation experiment

The laboratory scale chemical oxidant batch experiments were conducted in 50 mL plastic Falcon tubes. Two oxidants i.e., $Na_2S_2O_8$ (7% anaerobic (N_2) solution) and $KMnO_4$ (25%

anaerobic (N_2) solution) were used to determine the natural oxidant demand. Sediment (10 g fresh weight) was added into each tube and then the required amount of oxidants was subsequently added to obtain the concentration ranges shown in Table 7.2. Various amounts of water were added in order to have the same total volume of oxidant, water and sediment sample in every tube. Preliminary tests were conducted to quantify the optimal oxidant concentration ranges for the different sediments. The test was set up in the anaerobic chamber (N_2) to eliminate the effect of oxygen on the system and the tubes were wrapped with Teflon tape to prevent gas exchange. The tubes were kept in the dark to prevent photo-induced degradation of oxidants and were shaken horizontally at 100 rpm for 14 d. After a contact time of 14 d, the tubes were centrifuged at 1566 × g for 10 min to separate the sediment and liquid part. The presence of unreacted permanganate or persulfate in the supernatant was detected spectrophotometrically.

Table 7.2. Chemical load ranges of the reactors [$Na_2S_2O_8$ (7% solution) and $KMnO_4$ (25% solution)]

Reactor number	**(IIK) g oxidant/kg DM**	**(BDS) g oxidant/kg DM**	**(ZWP) g oxidant/kg DM**
	$Na_2S_2O_8$		
1(control)	0	0	0
2	25	25	10
3	50	50	20
4	75	75	30
5	100	100	40
6	130	130	60
7	160	160	-
	$KMnO_4$		
1(control)	0	0	0
2	150	150	50
3	300	300	100
4	450	450	150
5	600	600	225
6	750	750	300
7	-	1000	-

DM: Dry Matter; IKK: Ieper-Ijzer kanaal ; BDS: Berendrechtsluis; ZWP: Zandwinningsput

2.4. Colorimetric determination of persulfate and permanganate concentration

For the determination of persulfate concentration, 10 mL of H_2SO_4 (5 M) and 100 µL $Fe(NH_4)_2(SO_4)_2.6H_2O$ (0.4 N) solutions were added into tubes containing 1 mL of supernatant. After a mixing period of 40 min at 200 rpm, 200 µL of KSCN (0.6 N) was added to the samples to get the final color. The color was measured spectrophotometrically at a wavelength of 261 nm (Genesys 6, Thermo Spectronic). The concentration of permanganate was directly measured in the supernatant at a wavelength of 525 nm (APHA, 2005). Standard concentration series of permanganate and persulfate were prepared in the range of 25-1000 mg/L.

2.5. DNA extraction, qPCR/PCR and DGGE analyses

DNA was extracted from 2 g of sediment slurry as described by Hendrickx et al. (2005) and the extraction was conducted in duplicate for each sample. The number of bacteria was quantified by real time qPCR. The qPCR reaction was performed in a volume of 12.5 µL, containing 6.25 µL 2xABgene SYBRGreen mix (AB gene, Epsom, Suirrey, UK), 2.5 µL of each primer (EUB341F [CCTACGGGAGGCAGCAG] and EUB534R [ATTACCGCGGCTGCTGGC]), resulting in a final primer concentration of 400 nM, and 1.25 µL DNA template. DNA templates were diluted ten-fold prior to analysis. qPCR runs were performed in a Westburg Rotor-Gene 3000 (Corbett Research, Sydney, Australia). A calibration curve, ranging from 10^2 to 10^8 gene copies per µL of template DNA, was achieved using 10-fold serial dilutions of pure plasmid DNA carrying the cloned bacterial 16S rRNA gene of *Hydrogenophaga*. The algorithm calculated the efficiency, threshold cycle (C_T), slope and intercept as well as R^2 (>0.99) values.

Amplicons of PCR reaction with bacterial primers (Boon et al., 2002) were separated by DGGE as previously described (Muyzer et al., 1993). Briefly, a 8% (wt/vol) polyacrylamide gel with a denaturing gradient of 35-65% (where 100% denaturant contains 7 M urea and 40% formamide) was loaded with 15 µL of the PCR products. Electrophoresis was performed at a constant voltage of 120 V for 15 h in 1× TAE buffer (40 mM Tris-acetate (pH 7.4), 20 mM sodium acetate, 1 mM EDTA) at 60°C on an INGENY phorU-2DGGE apparatus (INGENY International BV, Goes, The Netherlands). The gels were stained with GelRed (10000×) nucleic acid gel stain (Biotum, VWR, Belgium).

2.6. Metal concentrations and leachability determination

After centrifugation at 1566 × g for 10 min, 10 mL of supernatant from each tube was filtrated over a 0.45 µm filter paper, acidified with HNO_3 (2% (v/v)) and analyzed by ICP-AES (Jarrell-Ash Autocomp 750) (Vanbroekhoven et al., 2007). In the DIN-S4 analysis, sediment samples were put into distilled water and shaken for 24 h at 20±5 °C (CEN, 2002). Afterwards, the supernatant was filtrated over a 0.45 µm filter paper and leachable metal amounts were measured again by ICP-AES. Although the heavy metal concentrations were measured in the water phase, all results were expressed as mg metal/kg DM based on the amount of sediment used so that the results can be compared to the VLAREA standards (URL-2).

2.7. ATP analysis

ATP was measured using the BacTiter-Glo Microbial Cell Viability Assay (G8231; Promega Corporation, Dübendorf, CH) and a Luminoskan Ascent Luminometer (Thermo Labsystems, Waltham, MA). The BacTiter-Glo reagent was prepared according to the manufacturer's guidelines. ATP free water and reagent were heated to 38°C and were kept at this temperature. Sediment (25 mg) was added into eppendorf tubes after which 200 µL of ATP free water and 100 µL of reagent were added and mixed for 10 sec in a mixer. The tubes were transferred to the heat block and incubated at 38°C for 2.5 min. After this incubation period, the samples were centrifuged for 1 min at 440 × g and 200 µL of supernatant was transferred to white wall 96-multiwell plates (Nunclon Delta, Denmark) and measured with an integration time of 10 sec. The standard curve was prepared in the same way, by adding 100 µL reagent to 200 µL of ATP standard solutions ranging between 10^{-6} M to 10^{-12} M ATP. Active cell determination was based on converted ATP amounts using the assumptions that one cell contains 20 fg of carbon (Griebler et al., 2002) and 1 ng/mL ATP corresponds to 250 ng/mL cell carbon (Karl, 1993).

3. Results and Discussion

3.1. Natural oxidant demand of the sediments

The NOD values obtained, when the sediments were treated with persulfate, were lower than the values obtained, when the sediments were treated with permanganate (Table 7.3). Persulfate is indeed a stronger oxidant than permanganate (oxidation potential of 2.01 V for persulfate compared to 1.70 V for permanganate) (Liang et al., 2004; Tsitonaki et al., 2008)

and thus lower amounts are sufficient to oxidize the organic matter (Kao et al., 2008). For both oxidants, the lowest NOD was observed in sediment ZWP and the highest in sediment BDS. The NOD of the sediment IIK was in between (Table 7.3). The NOD values obtained in this study were much higher than those regularly observed for soil matrices. For example, other studies reported NOD values between 0.06-53 g/kg DM in case of permanganate treatment for sandy soils (Crimi and Siegrist, 2005; Hønning, 2007; Tsitonaki et al., 2008). Dahmani et al. (2006) reported values ranging between 0.1 g/kg DM and 0.3 g/kg DM for a soil having an organic matter content of 0.07 % and treated with $Na_2S_2O_8$. One reason for the higher NOD values in this study could be the amount of indigenous reductants like humus (Hønning, 2007) and especially Fe^{2+}, which are generally much higher in sediments (between 8 000 mg/kg DM and 458 000 mg/kg DM (Hous and Denison, 2002)) than in soils (between 1 800 mg/kg DM and 22 700 mg/kg DM (Dahmani et al., 2006; Hartog et al., 2005; Hønning, 2007; Sra et al., 2007)). In our study, the iron concentrations in the investigated sediment samples ranged between 33 000 mg/kg DM (IIK sediment) and 44 000 mg/kg DM (BDS sediment) (Table 7.1) and were thus also relatively high. Since permanganate and persulfate are not as selective as the other oxidants, they also oxidized iron which resulted in elevated NOD values.

Table 7.3. The natural oxidant demand (NOD) values of the sediments treated with permanganate and persulfate

		Permanganate	**Persulfate**
Sediment sample	**Organic matter content**	**NOD (min-max)**	**NOD (min-max)**
	(%)	(g oxidant/kg DM)	(g oxidant/kg DM)
Zandwinningsput ZWP	2.4	50-100	30-40
Berendrechtsluis BDS	6.9	300-450	50-100
Ieper-Ijzer kanaal IIK	7.6	150-300	50-100

NOD: Natural oxidant demand; DM**:** Dry Matter

Another reason of observing high NOD values could be the relatively higher amounts of organic matter that are present in the sediments compared to the soil. Other studies have indeed observed increasing NOD values with increasing organic matter contents in soils (Hønning, 2007; Mumford et al., 2005). However, it was not always the case in our study. The sediment BDS, having 6.9 % of organic matter, had higher NOD values than that of IIK

with an organic matter content of 7.6 %. One reason might be the age of the sediment BDS. Sediment age has an effect on the distribution and reactivity of sedimentary reductants, resulting in a preferred degradation of labile components over time (Hartog et al., 2005). Compared to the IIK sediment, the BDS sediment came from a part of a harbor that was continuously being dredged resulting in a fresh material and therefore organic matter was more prone to oxidation and leading to higher NOD values. In addition, the amount of iron in the BDS sediment was the highest of the three sediments (Table 7.1) which might also lead to an increased oxidant demand as mentioned here above.

3.2. Effect of chemical oxidants on heavy metals

One of the disadvantage at the end of chemical oxidation processes might be metal release from the sediments. Due to a potential risk of metals As, Cd, Cr, Cu, Pb, Ni and Zn to the environment, the metal concentration in the water phase of the control (no addition of chemical oxidant), and minimum and maximum oxidant demand conditions were measured after the contact time of 14 d (Table 7.4). The results were compared to Flemish threshold concentration standards (VLAREA standards) (URL-2) which indicate the amount of metals (in mg metal/kg of dry sediment) at toxicity level (Table 7.4). In addition, since especially the leachable fraction of the heavy metals might be a risk (Stephens et al., 2001; Tack et al., 1999), this fraction of non-treated sediments was determined by a DIN-S4 extraction (Table 7.4) and compared with the heavy metal amounts released under chemical oxidant treatment. After exposure of the sediments to the oxidants, the As, Cu and Ni concentrations were below the VLAREA limits (data not shown) and thus posed no risk.

After treatment with permanganate, the amount of total Cr in the water phase of all the three sediments was higher than the limit allowed by VLAREA (> 0.50 mg Cr/kg DM). Since the leachable amounts of Cr in the non-treated sediments were below the amounts released after the oxidant exposure, increases in heavy metal concentration in the water fraction were due to the application of permanganate. The highest release of Cr was observed in sediment IIK in which 28% of the total Cr was released after exposure to the maximum oxidant dose. The lowest heavy metal release was determined in sediment ZWP (6.6% of total Cr). Since the pH values increased only by 1 unit in the presence of permanganate, it probably did not play a role in the Cr oxidation and release. The increase in redox state, from negative values to > 495 mV, are considered as favorable for the oxidation of Cr (III) to Cr (VI). In three other studies

(Li et al., 2002; Crimi, and Siegrist, 2003 and Kozubek et al., 2012), the oxidation of Cr (III) to Cr (VI) was indeed established by measuring the concentration of these two valence states of Cr namely Cr (III) and Cr (VI) after ISCO applications. Moreover, MnO_2 has a high adsorption capacity for metal ions thus potentially providing a local surface environment in soils in which coupled processes of Cr (III) oxidation and manganese oxide reduction may take place (Edmond and Dhanpat, 1987). In addition, as the oxidation of organic matter promoted disintegration of sediment structure, it increased the access of the oxidized solution to Cr mineral phases and accordingly its oxidation and subsequent release from the sediments. Apart from $KMnO_4$, Rock et al. (2001) indicated that the addition of peroxide (H_2O_2) to different soils contaminated with Cr under different pH conditions also resulted in the oxidation and subsequent release of Cr from soil.

Contrary to the permanganate, different metals, Pb and Zn, were released from all the three sediments under persulfate treatment. The release of Zn from the sediment BDS was almost as high as the release from the IIK sediment. Approximately 2.4% of the total Zn was released from the sediment BDS upon exposure of the maximum oxidant concentration, whereas 2.7% of the total Zn was released from IIK sediment while only 0.35 % for ZWP (Table 7.4). On the other hand, the highest Pb release was noticed from the sediment BDS (6.7% of total Pb compared to 3.90 % in IIK and 1.46 % in ZWP). The higher Pb releases in sediment BDS can be attributed to the freshness of this sediment. It is clear that the presence of persulfate decreased the pH values (by at least 0.5 unit, Table 7.4). Accordingly, this decline in the pH and also the increase in redox potentials in BDS (-150 mV to 358 mV) and in IIK (-123 mV to 136 mV) caused elevated metal concentrations in the water phase (Table 7.4). In sediments, Zn and Pb are associated to the insoluble large molecular humic acids and sulfides under anaerobic conditions. Under aerobic conditions, with elevated redox values, the affinity between molecular humic acids, sulfides (i.e. ZnS, PbS) and heavy metals decreases which results in the release of the metals (Calmano et al., 1993; Guo et al., 1997). Similarly, the study of Miao et al. (2006) also suggested the release of Pb and Zn due to decreased pH (7.1 to 5.7) and increased redox potential (-200 mV to 500 mV).

Table 7.4. Results for the release of heavy metals after the addition of permanganate or persulfate to the ZWP, BDS and IIK sediments (25% $KMnO_4$ or 7% $Na_2S_2O_8$ added)

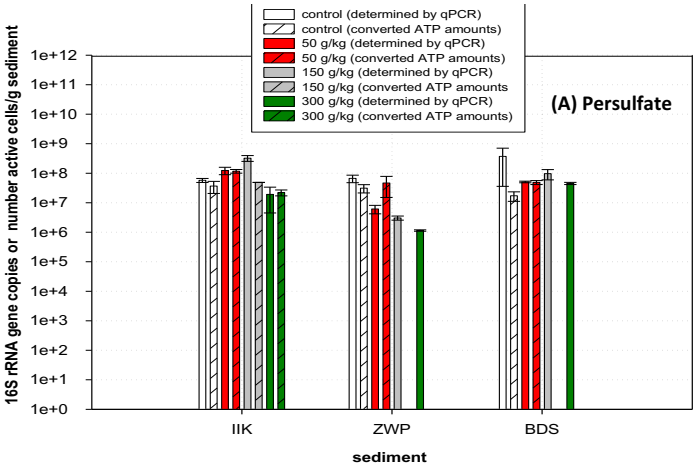
Permanganate				Persulfate				
Oxidant dosage	**pH**	**ORP**	**[Total Cr]**	**Oxidant dosage**	**pH**	**ORP**	**[Pb]**	**[Zn]**
(g/kg DM)		mV	(mg Cr/kg DM)	(g/kg DM)		mV	(mg Pb/kg DM)	(mg Zn/kg DM)
ZWP				**ZWP**				
0 (Control)	7.12	-104	<0.12	0 (Control)	7.12	-104	0.20	0.02
50	8.56	72	12.89	20	6.88	196	1.81	0.02
100	8.30	575	7.27	40	6.87	232	2.77	1.85
BDS				**BDS**				
0 (Control)	7.50	-150	0.30	0 (Control)	7.50	-150	0.37	0.04
300	8.20	40	27.30	50	6.73	295	3.70	0.04
450	8.10	560	28.30	100	6.59	358	6.11	9.87
IIK				**IIK**				
0 (Control)	7.57	-123	<0.26	0 (Control)	7.57	-123	0.43	0.04
150	8.45	-21	14.47	50	6.67	-54	3.02	0.04
300	8.30	495	48.51	100	6.66	136	5.47	19.32

Leachable metals (DIN-S4 extraction) (mg metal/kg DM)		Leachable metals (DIN-S4 extraction) (mg metal/kg DM)	
ZWP		**ZWP**	
0.08		<0.10	1.90
BDS		**BDS**	
0.06		<0.10	0.40
IIK		**IIK**	
<0.05		<0.10	0.46
VLAREA limits (mg metal/kg DM)		**VLAREA limits (mg metal/kg DM)**	
0.50		1.30	2.80

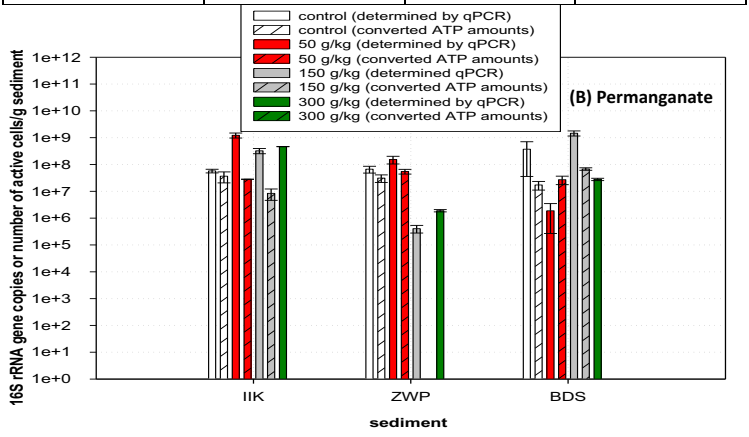
DM: Dry Matter; ORP: Oxidation Reduction Potential

3.3. Effect of chemical oxidants on the microbial community

qPCR analysis, targeting the 16S rRNA gene of bacteria, indicated that persulfate had the highest impact on the bacterial community present in the ZWP sediments. While with increasing amounts of persulfate almost no decrease in the gene numbers was observed in sediments BDS and IIK, a step-wise decrease was noted in sediment ZWP. The decline already started after the addition of 50 g/kg persulfate (1 log unit) and was the highest after the addition of 300 g/kg persulfate (2 log units) (Figure 7.1). In both sediments (ZWP and BDS) the microbial activity decreased below the ATP detection limit after the addition of 150 g/kg persulfate. As expected, the effect of persulfate is higher at ATP than at 16S rRNA gene level (Baumler et al., 2008). For the sediments, the effect of persulfate increased in the order ZWP > BDS > IIK. The reason might be the low organic content of ZWP sediment which led to insufficient protection of the microorganisms. Moreover, the applied persulfate concentrations of 150 g/kg and 300 g/kg were much higher than the NOD of this ZWP sediment (30-40 g/kg), which means that the leftover of the unreacted oxidant was much higher in the ZWP than in the sediments BDS and IIK. This could result in a higher oxidant stress on bacteria present in the ZWP and a higher decrease in 16S rRNA gene numbers compared to the other two sediments. However, it is clear that the results based on DNA analysis do not correspond to those based on ATP analysis. An explanation could be the overestimation of 16S rRNA genes in DNA samples since the extracellular DNA may adsorb to the sediment particles resulting in a protection towards the chemical oxidant (Trevors, 1996). Persistence of intact DNA during rough conditions has been cited in literature (Leloup et al., 2009).



dosage persulfate (g/kg DM)	IIK ATP (ng/g)	ZWP ATP (ng/g)	BDS ATP (ng/g)
0	7.34 ±3.22	6.23 ±1.95	3.45±0.61
50	23.42±3.41	9.36±6.34	9.77±1.54
150	9.77±0.06	<0.0041	0.06±0.00
300	4.41±0.99	<0.0041	<0.0041



dosage permanganate (g /kg DM)	IIK ATP (ng/g)	ZWP ATP (ng/g)	BDS ATP (ng/g)
0	7.34 ±3.22	6.23 ±1.95	3.45±0.61
50	5.63±0.06	10.96±2.21	5.44±0.94
150	1.69±0.76	<0.0041	13.61±0.07
300	<0.0041	<0.0041	<0.0041

Figure 7.1. Comparison of **16S rRNA gene copies/g of sediment** as determined by qPCR, **active cells/g of sediment** based on converted ATP amounts using the assumptions in Karl (1993) and Griebler et al. (2002) and **amount of ATP** (ng/g) in sediments treated with 4 different amounts of persulfate or permanganate (0, 50, 150 and 300 g/kg DM).

Similar trends were observed under different concentrations of permanganate (Figure 7.1). The highest effect at 16S rRNA gene level was again observed in the sediment ZWP with 2 log unit of decrease after exposure to 150 and 300 g/kg permanganate. Organic matter content could again play a role here. In addition, the effect of the permanganate was again more apparent at ATP than at 16S rRNA gene level. However, after the addition of permanganate, a decrease in ATP level was not only observed in ZWP but also in the BDS and even in the IIK sediments. This decrease was only observed after the addition of the highest permanganate concentration of 300 g/kg.

To compare the effect of persulfate and permanganate on the microbial community, the equivalences of these two chemical oxidants should be taken into account (2.8 eq/kg and 2.5 eq/kg for 150 g/kg permanganate and 300 g/kg persulfate, respectively). Based on the qPCR and ATP results, the effects of permanganate and persulfate are similar in the ZWP and IIK sediments. For the sediment BDS, the effect of persulfate is higher than for permanganate but this effect is only seen at ATP and not at 16S rRNA gene level.

It is known that the destruction of DNA, cell death, protein and lipid membrane damage, decrease in activity and cell number may be observed under oxidant stress caused by chemical treatment (Bui and Cotton, 2002; Jung et al., 2005; Sahl and Munakata-Marr, 2006; Tsitonaki et al., 2010). However, it is also stated in literature that many anaerobic microorganisms contain enzymes such as superoxide reductase, superoxide dismutase and NADH oxidase which permit them to tolerate oxidant exposure (Cabiscol et al., 2000; Imlay and Linn, 1988; Lin et al., 2004). As a result, microbes may develop mechanisms to defend against the toxic effects of some oxidants as it is observed in our study. Bacteria in the sediments tolerated the oxidant stress up to a concentration of 150 g/kg. Yet, they lost their activity above this concentration for both oxidants. Microorganisms were more likely protected in the sediments due to the existing organic matter and reductants that also contributed to the consumption of oxidants in the system. Therefore, significant decreases in gene copy numbers were only observed under the application of higher concentrations of both chemical oxidants in this study.

Although different pollutants were detected in the sediments in our study (chemical oxidants, heavy metals, PAH, PCB and mineral oil), the observed negative effect on the microbial community was, in our opinion, mainly due to the chemical oxidants. The concentrations of these organic and inorganic pollutants in the present study are much smaller than the concentrations reported in the literature where in fact no effect of these contaminants on the microbial population was observed. For instance, an active microbial metabolism was still detected in a soil with 9 to 128 g Zn/kg (Chardin et al., 2002), concentrations that are much higher than in our study (1.85-19.32 g Zn/kg sediment). In our study the PCB and PAH concentrations in all the sediment samples were below VLAREA standards (0.5 mg/kg DM for PCB; 10 mg/kg DM for PAH), however for the mineral oil significant concentrations were observed in the IIK and ZWP sediments (2300 and 640 mg/kg DM, respectively). These concentrations were however still lower than the non-toxic mineral oil concentrations in the studies of Jorgensen et al., 2000 (700-2400 mg/kg DM) and Chanieau et al., 2003 (18 000 mg/kg DM). Mineral oil content, serving as carbon and energy source, might even have supported the growth of microorganism as stated in literature (Jorgensen et al., 2000; Ramsey et al., 2000; Chanieau et al., 2003).

In addition to qPCR and ATP, PCR-DGGE analysis targeting the total bacterial community was applied on the sediment samples before and after the exposure to chemical oxidants. From the DGGE profiles (Figure 7.2) we could deduce that the addition of PS did almost not have an effect on the structure of the bacterial community. Although some small shifts occurred in the PCR-DGGE pattern after the addition of the PS, the patterns in the condition with and without (see control) PS were almost the same. In contrast, permanganate induced some clear shifts in the DGGE pattern. Some bands appeared and became really dominant while other bands totally disappeared (Figure 7.2). We see that PM oxidized less organic matter than PS since the applied concentrations (50,150 and 300 g/kg) were lower than the NOD values of PM. Therefore, the (partly oxidized) organic matter that is left after PM application, might be more accessible to the microbial community resulting in the growth of (certain parts of) the microbial community. On the other hand, it might be that PM adversely influenced a certain part of the microbial community again inducing a shift in the diversity pattern.

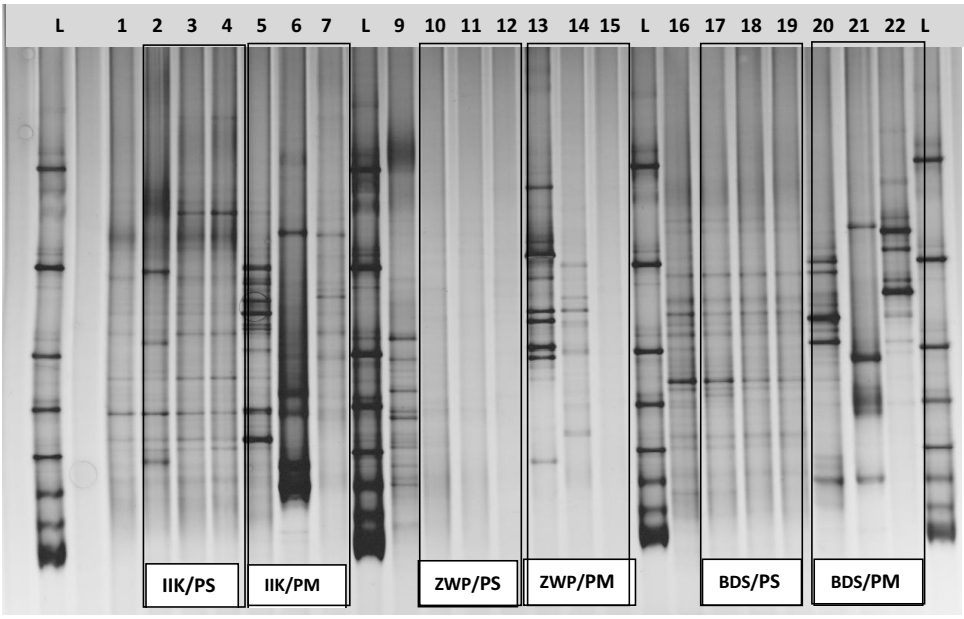


Figure 7.2. DGGE profile of bacterial community in IIK, ZWP and BDS sediments treated or not treated with persulfate PS and permanganate PM. Lanes 1,9,16 are controls (no addition of chemical oxidant) of IIK, ZWP, and BDS sediments respectively; following lanes represent increasing oxidant dosages namely 50 g /kg (lanes 2, 5, 10, 13, 17, 20), 150 g/kg (3, 6, 11, 14, 18, 21), and 300 g/kg (4, 7, 12, 15, 19, 22). PS:Persulfate, PM: Permanganate, L: Ladder.

4. Conclusions

The following conclusions can be drawn based on the results of this study:

The information on the amount, type and reactivity of the sedimentary reductants (like iron) as well as that of organic matter content and freshness of the sediments are crucial in the determination of the matrix demand and can eventually be quite helpful in planning the ISCO remediation strategies for contaminated sediments.

The application of persulfate led to the release of Pb and Zn due to a decrease in pH and an increase in redox potential. On the other hand, the oxidation of Cr (III) into the more water soluble Cr (VI) was recorded after exposure to permanganate. This indicated that the application of chemical oxidant in fact leads to the release of heavy metals. The high organic matter content tolerated the destructive effect of persulfate and permanganate on the microbial

community up to a concentration of 150 g/kg DM. On the other hand, low organic matter content did not ensure the protection of microorganisms. In all three sediments, the addition of both persulfate and permanganate resulted in a decrease in 16S rRNA gene numbers and activity, but only permanganate induced a shift in the structure of the microbial community.

When selecting a treatment for a polluted site, the interplay between the different pollutants but also the microbial community should be taken into account. The selected technique may indeed be optimal for one pollutant, but might result in the production of a side pollutant (which may even be more toxic than the original pollutant). Moreover, the effect of the oxidant on the microbial community should be taken into account, especially when bioremediation/biostimulation/bioaugmentation is still an option for the polishing of the residual contaminants.

5. Acknowledgments

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Chapter 8. General discussion, conclusions and perspectives

8.1. Introduction

Soil and groundwater systems offer a vast and complex habitat for diverse microbial populations. In these habitats, a variety of interactions may occur in different ways. For example, the organic material used by one species may be a metabolic by-product of a second species. In addition, the physico-chemical environment (pH, temperature, nutrient availability, etc.) can affect the total microbial community and competition/cooperation between different species may be observed (Griebler and Lueders, 2009; Guimarães et al., 2010). However, most of the soil and groundwater systems are not pristine as various pollutants are detected. The adaptation of the indigenous microorganisms to these contaminated conditions may be possible, but excessive amounts of the contaminants may have toxic effects and cause inhibition (Suthersan, 1999). Moreover, not only the contaminants or geochemical factors but also chemical oxidants that are injected for remediation purposes might be another disturbance factor for the environmental system (Figure 8.1). Contaminants persist in the subsurface because environmental conditions are not appropriate for microbial activity. The optimization of environmental conditions and identification of their effects on the responsible microorganisms are essential.

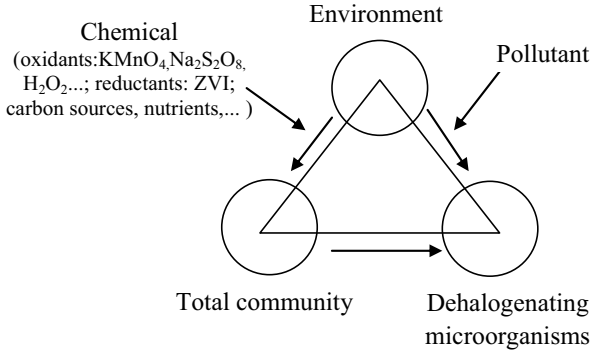

Figure 8.1. Interaction between environmental compartments (aquifer, groundwater) and microorganisms

Therefore, in this dissertation, the interaction between chemical and biological remediation of chlorinated aliphatic hydrocarbons (CAHs) and their impact on ecosystem and in turn, the

indigenous microorganisms has been investigated. The main research questions have been set as "What is the impact of chemical oxidants on the activity and structure of indigenous microorganisms?", "Will there be any recovery in the microbial activity under post oxidation conditions?", "Will there be any difference in the microbial community structure under pre- and post oxidation conditions?"

In this dissertation, the metabolic responses of microorganisms in an enrichment culture upon trichloroethene (TCE) exposure were investigated and the key genes involved and their long-term up- and down-regulation over time were identified (Chapter 3). To the best of our knowledge, we have presented *for the first time* the long-term dynamics (over a 40 days period) of catabolic reductive dehalogenase (RDase) genes before, during and after TCE exposure which enabled us to quantify the naturally occurring changes in the gene expression during TCE degradation process. Subsequently, after settings the results obtained in Chapter 3 as basis, the inhibitory effects of chemical oxidants, $KMnO_4$ and $Na_2S_2O_8$, on the activity of the CAH degrading microorganisms in the same enrichment culture under TCE exposure were studied in Chapter 4. Moreover, the possibility of microbial recovery, the potential of concurrent treatment, and role of bioaugmentation at post-oxidation stage were investigated in this part. The novel observations at m*RNA* level for both 16S rRNA and catabolic RDase genes led us to identify the negative impact of oxidant stress on the *microbial activity* but especially on the reductive dehalogenation activity (Chapter 4). On the other hand, the impacts of the oxidants, $NaMnO_4$ and $Na_2S_2O_8$ (cold and alkaline activated), on the indigenous microorganisms present in *aquifer* and *groundwater* were examined both in lab scale batch and *in situ* tests in Chapter 5. The inconsistency between the detection of mRNAs and the absence of anaerobic CAH degradation in the batch test led us to define the index "transcript per gene (mRNA/DNA)" to link the activity to the biological degradation in aquifer systems. The re-set of reducing redox conditions and prompt recovery of the microbial activity suggested the feasibility of the more dynamic *in situ* systems compared to the stagnant closed systems (Chapter 5). The promising results presented in Chapter 6 indicated the novel application of dual isotope approach (carbon and chlorine) that allowed us, *for the first time,* to differentiate between cDCE removal mechanisms of anaerobic biodegradation and chemical oxidation via lab scale batch microcosms and *in situ* tests. Finally, the chemical oxidant induced changes in the microbial activity and community structure were determined in *sediments* (Chapter 7). The results revealed the robustness of the microorganisms against the detrimental effects of the oxidant stress in the presence of sediment particles. The impacts

of $KMnO_4$ and $Na_2S_2O_8$ on the microbial diversity were compared *for the first time* indicating that only $KMnO_4$ induced shifts in the structure of the microbial community. This test also allowed us to assess the mobilization of heavy metals by the addition of these chemical oxidants.

8.2. Application of chemical and biological remediation technologies: combined approach

The goal of any remediation should be the removal of the pollutants so that they no longer pose unacceptable risks. In the long term, the sustainable use of groundwater resources depends on maintaining these sources at a level that enables continuous use (Evans and Furlong, 2003). *In situ* chemical oxidation (ISCO) and *in situ* bioremediation (ISB) are both well-established options to remediate CAHs contaminated groundwater and aquifer sites (Figure 8.2). It has already been documented in the literature that ISCO may *directly* affect the indigenous microorganisms by inhibiting or killing the microbial life but also affects biological processes *indirectly* by changing the environmental conditions such as pH, temperature, redox conditions and the concentration of electron acceptors and donors. Bioremediation is, on the other hand, a slow process and might not be effective to remediate sites contaminated with high pollutant concentrations. However, these two mechanisms can be applicable in a "coexisting/combined" manner so that a maximum removal of the contaminants can be achieved in the systems.

One indication that a combined strategy was feasible was given by Richardson and his colleagues (2011), who performed a test in which 20 g/L of persulfate solution was introduced to a continuous-flow column having 120 mg/kg PAH contaminated soil, followed with simulated unpolluted groundwater. This study illustrated that persulfate oxidation was compatible with subsequent bioremediation and the recovery of the overall microbial community and, more importantly, specific PAH-degrading bacterial groups in the soil matrix was possible within 500 days. The study of Sercu et al. (2013) focused on the immediate and short-term effects of $NaMnO_4$ on the indigenous subsurface microbial community composition in groundwater impacted by 1-10 mg/L of TCE. Their results suggested that ISCO might be combined with biodegradation assuming that the appropriate biogeochemical conditions (like oxygen and electron donor concentration, redox potential, etc …) can be established after a long time. As also observed in the field study presented in Chapter 5 of this dissertation, the recovery of the microbial activity was possible 82 days after permanganate

exposure. In all these studies, the chemical oxidation was always followed by bioremediation *(treatment trains)* and a rebound in the microbial activity was recorded although a long recovery time was needed. It should be noted that during such long recovery periods, concentration of the contaminants might increase again due to inefficient treatment of the pollutant source. Moreover, differences in the initial biomass concentrations, oxidative-stress resistance and availability of preferred substrate and/or electron acceptors can impact microbial recovery and diversity. Thus, an effective bioremediation may not be ensured during post oxidation in some cases (Hrapovic et al., 2005; Sahl and Munakata-Marr, 2006; Richardson et al., 2011).

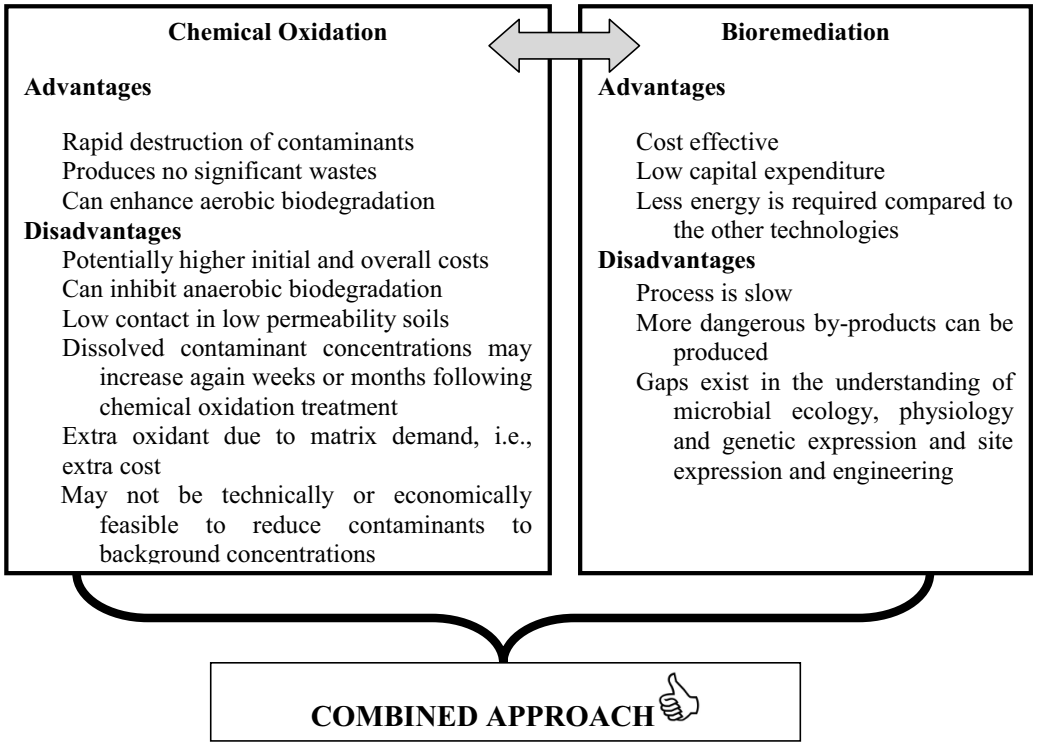

Figure 8.2. Advantages and disadvantages of chemical oxidation and bioremediation

Our proposed "combined approach" could be more promising by some new modifications. For example, the aggressive impact of oxidants may be used as a positive option in the remedial action to degrade a major part of the contamination in a short time. But in addition, in some cases, invasion of the inocula might be a problem in the ecosystems and the population which can degrade the target pollutant might be suppressed by the remaining community due to homeostasis (Sayara et al., 2011). This competition can be stopped by

removing/inhibiting the organisms from the system via aggressive oxidants. Subsequently, when the oxidants are not reactive anymore, introduction of a single strain or consortia of microorganisms with the desired catalytic capabilities might be more effective so that the improved bioremediation of the residual contaminant can be ensured. In this way, long microbial recovery periods can be eliminated from the total remediation process. Of course, the robustness of the augmented microorganisms may raise some questions since the adaptation of lab-grown cultures to the real *in situ* conditions may be an issue.

The concentration of target contaminant is also important in the treatment method selection. In general, monitored bioremediation is preferred in contaminated sites with low pollutant concentrations. On the other hand, aggressive chemical treatments are much more applicable, since excessive amount of pollutants might be inhibitory to the microorganisms and might block the bioremediation process. For the treatment of contaminated sites with intermediate pollutant concentrations, however, the combined approach might be a solution but a detailed site characterization including all geochemical (organic matter, pH, redox., etc.) and biological (abundance and activity of bacteria) properties should be performed for the best, cost effective remedial action (Figure 8.2).

The selection of the oxidant concentration is another important point to be considered for the combined/coexisting applications. The oxidant dose may be chosen in a way that the indigenous bacteria can still survive or recover after the oxidation process. As such, a "coexisting" (simultaneous ISCO and bioremediation) or a "combined" (ISCO followed by bioremediation) treatment might be applicable in an effective way. The negative effects of the oxidants are obvious, however, the key point is here to select the threshold oxidant concentrations at which both biodegradation and chemical oxidation may cope with the target contaminants. The threshold concentration may be determined by setting up very basic lab-scale microcosms treated with a range of oxidant concentrations. The lab scale approach indeed will not provide a perfect simulation of the field conditions but still can be beneficial. It has also been proven that ISCO applications might also have positive effects when the right dose is chosen next to the adverse effects. For instance, in a pilot test in which persulfate and permanganate were used to treat VOCs and TCE, an enhancement of biodegradation was observed after the oxidant exposure (Droste et al., 2002). The reasons of such a positive impact may be due to i) stimulation of sulfate-reducing bacterial growth by supplying sulfate (an electron acceptor which is a dissociation product of $Na_2S_2O_8$) and, ii) bioavailability of

simpler organic carbon for the bacteria by degrading the naturally occurring complex organic carbon present in the aquifer and in the VOCs via oxidants.

The effects of the chemical oxidants might be different depending on the environmental conditions in groundwater, aquifer or sediments. Natural recovery of microorganisms may be observed in some systems (aquifer, sediment) but only under *reducing* conditions. This dissertation also revealed that any planned bioaugmentation should be performed at these reduced conditions to stimulate the activity of anaerobic microorganisms and in turn to stimulate anaerobic bioremediation. Selection of the oxidant type is also crucial in the course of the remediation due to the different oxidant chemistries. For industries aiming large applications, permanganate and persulfate might be the best options. The former is stable, easy to handle, and applicable over a wide pH range (Hønning 2007) while the latter is relatively persistent and can be transported over considerable distances. In addition, both persulfate and permanganate are easy to handle and cost-effective compared to the Fenton's Reagent, hydrogen peroxide or ozone (Tsitonaki et al., 2010). Both are also less damaging to microorganisms than Fenton's reagent and hydrogen peroxide (Tsitonaki et al., 2008) but still have an adverse effects on the microbial community. Overall, the selection of proper ***oxidant type and dose***, assurance of ***reducing redox conditions*** and removal of ***inhibitory by-products*** are important aspects for a combined treatment approach.

8.3. Monitoring microbial responses under oxidant stress

Since intrinsic bioremediation is mediated by the indigenous microbial community, a proper evaluation of contaminant degradation in aquifers necessitates an approach that integrates qualitative and quantitative geochemical measurements of contaminant transformations with measurements of the *identity*, *abundance*, and *function* of the *key microorganisms* involved in the degradation process (Weiss and Cozzarelli, 2008; Azadpour-Keeley et al., 2009).

Under ISCO applications, responses of the microbiota may include stress or changes in diversity, community structure, biomass, and activity. Regardless of the basis for the remedial action, it is critical to recognize that subsurface microbial communities will respond to the presence of the contaminants or to the engineered manipulation of subsurface conditions. Therefore, molecular methods become important tools in the determination of the microbial community homeostasis caused by the environmental disturbances (pollution) or treatment applications (chemical oxidation) (Chikere, 2013). Several molecular (DNA and RNA based

qPCR and DGGE, next generation sequencing (454, illumina, SOLID)) and microbial (ATP, flow cytometry (FCM)) methods have been applied to identify and monitor the biodegradation potential of microorganisms under different environmental conditions. Indeed, the benefits of these methods are evident in the identification of the specific degradation pathways and they are also valuable to control bioremediation process occurring at the post-oxidation conditions (Bombach et al., 2010; Ding and He,2012; Maphosa et al., 2012). The combined treatment approach supported by molecular diagnostic monitoring tools has momentum in transforming an empirical approach into a precise, fine-tuned, and science-based technology for accelerated remediation of chlorinated ethene-contaminated sites (Ritalahti et al., 2005). The use of the molecular techniques may help in selecting the best remediation method. For example, the chemical oxidation might be an option when the abundances of the microorganisms are found to be too low for bioremediation. According to the report of ESTCP, for instance, efficient dechlorination and ethene production were unlikely when 16S rRNA gene of *Dehalococcoides* spp. were below 10^4 copies/L (ESTCP, 2011). In such a case, other remediation methods may be considered as an alternative option.

An important point to consider is that the results obtained from molecular analysis might be different for the samples taken from *different environmental compartments* (e.g., groundwater, aquifer, sediment). In groundwater samples, for example, ATP detection, DNA or RNA based qPCR and FCM might be dependable techniques to monitor the changes in microbial activity and abundances. Any of these techniques might be applicable according to the needs and budget. On the other hand, extra attention should be paid during the analysis of the samples originating from the aquifer and sediment compartments because the detection of intact DNA under aggressive oxidation conditions might lead to incorrect conclusions about the potential microbial activity in the system. Hence, such results should be supported by activity based analyses such as ATP or RNA measurements. Interestingly, some researchers revealed that the detection of mRNAs in the aquifer samples was also possible even though the microorganisms were not active and anaerobic dechlorination was not observed in the system (Bælum et al., 2013) as also observed in the test performed in Chapter 5. At this point, transcript per gene ratio (mRNA/DNA) might be a promising index to identify the current activity status of the cells. On the other hand, both DNA and RNA based DGGE analyses are still found to be useful tools to determine the shifts in the microbial community structure after ISCO in aquifer and sediment environments. In conclusion, the advantages and disadvantages of each method should be recognized considering the characteristics of the environment

(groundwater, sediment, etc.) and molecular/microbial data should be supported by geochemical and chemical data in order to have a complete contaminant removal in a combined treatment approach.

8.4. Engineering and practical aspects

A factor of primary importance in the success of ISCO is the effective delivery of the oxidant into the subsurface and achievement of oxidant-contaminant contact. Therefore, the injection process must be designed carefully, taking into account surface and subsurface site conditions and contaminant distribution. A number of oxidant delivery approaches may be used, such as injection wells, direct-push technology probes, sparge points, infiltration systems, recirculation systems, fracturing, mechanical mixing, and horizontal wells (Simpkin et al., 2011). Injections using direct-push technologies (DPTs) are conducted with equipment that drives small-diameter hollow rods into the ground. The use of this technology during field implementation provides greater flexibility in locating injection points compared to installation of permanent injection wells. It is also possible that the injection locations may be changed due to aquifer responses during injection and subsequent injections may also be optimized to directly address hot spots. Specific depth intervals that may be known to contain more contamination may also be more easily targeted with DPT probes which is a real advantage. However, when multiple injections are required, the cost advantage of DPT may be less compared to injection wells. Furthermore, DPT installations may not be effective in some formations, i.e., the presence of rocks or cemented layers may inhibit the application which again makes the well technology a viable option. However, new advances and larger rigs are being developed to overcome some of these limitations (Huling and Pivetz, 2005; Krembs, 2008). In this sense, an innovative detection-injection technology presented in Chapter 5 is a good example. This smart technology is based on the MIP-IN device, which combines detection of pollutants by a membrane interface probe (MIP) and a simultaneous correlated injection (IN) during direct push of the device using a Geoprobe®. The main advantage of this new technology is the nearly simultaneous coupling of detection of pollutants at a certain depth and injection of the reactive agent. In this way, the injected reagent is more targeted to the real location of the pollution with reduced remediation time and cost (Uyttebroek et al., 2012). Yet, again, each injection approach has characteristics that may make it most suitable for a particular oxidant and set of site conditions (Simpkin et al., 2011; injection tool used in Chapter 5).

A properly designed performance monitoring program is also essential to evaluate the achievement of operational and performance objectives and determines whether ISCO meets the treatment goals. A typical performance monitoring program may include three parts; baseline, delivery, and treatment monitoring.

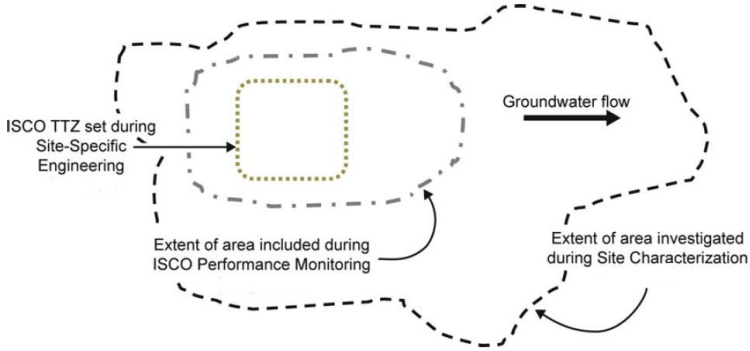

Figure 8.3. Illustration of how performance monitoring can relate spatially to site characterization. TTZ: Target Treatment Zone (after Palaia et al., 2011).

Baseline monitoring is a component of a performance monitoring that is carried out to determine pre-existing contaminant concentrations, provide a reference for comparison of data collected during and following the active oxidant delivery phases and to establish pre-ISCO geochemical and microbial conditions within and around the target treatment zone (TTZ) (Figure 8.3). On the other hand, monitoring of *oxidant delivery* is conducted to evaluate the nature and extent of oxidant distribution into the TTZ and any hydraulic effects related with fluid injection. Delivery monitoring includes physical measurements, sampling and analysis for parameters indicative of oxidant contact. Microbial activity monitoring can be a part of this process since then a complete picture about the responses of the microorganisms during oxidation may also be achieved as we already practiced in the field study presented in Chapter 5. Finally, treatment monitoring is typically initiated after oxidant delivery objectives are achieved and the goal is to determine achievements in the specific ISCO treatment goals set during the design phase. The degree of source mass removal, the occurrence of recontamination and/or rebound within a TTZ and changes in aquifer and water quality conditions due to ISCO are evaluated in this phase. Treatment phase is important also for the application of *treatment trains* since the possible shifts occurring in the field parameters may affect the biological processes. Therefore, biological data should be collected during treatment monitoring phase. Lack of adequate data on biological processes may lead to

ineffective treatments which may increase the cost for the long term applications. Moreover, given the fact that the cost of site closure is often an enormous burden on site owners and, by extension, to the overall economy, every reasonable tool needs to be considered to assist in new paradigms for site closure (Ritalahti et al., 2005). Industrial market is strongly linked to the methods for which the remediation success is more guaranteed by cost/benefit calculations of used money and time. However, although short term solutions are preferred by many firms, the remedial action should be considered in terms of short and long term effects, costs and benefits. In experienced hands, for example, molecular data can be produced more rapidly, with turn-around times of a few days for small numbers of samples. Rapid results provide practitioners with feedback on the performance of the system and allow operating parameters to be modified in a timely fashion which is a real advantage (Brockma, 1995). In this sense, the dialogue in between academic researchers and industrial CEOs must be intensified in a way that the long term benefits and profits of these applications are clearly explained (Sihna et al., 2013).

The cost is considered as one of the most important parameters by the practitioners in the remediation practices and should be estimated in detail. In the study of Simpkin et al. (2011), a cost estimation was performed for a *hypothetical* glacial till, heterogeneous and permeable site in which sodium persulfate was injected into 25 injection wells, over the source area. It was assumed that one full injection would be performed in the first year, and a second injection, with 75% of the full injection persulfate mass, in the second year. The costs include 3 years of operation and monitoring. The total present worth to treat 5663 m^3 of soil and 2265 m^3 of groundwater was calculated as €763,224 including €128,237 of capital, €360,823 of first year implementation and monitoring, €270,617 of implementation and monitoring cost (2nd and 3rd year) and €29,869 of post-closure costs (See Simpkin et al., 2011 for the details). Moreover, 2 scenarios were presented for the cost estimation of the hypothetical sites with different characteristics in the research of Harkness and Farnum (2010). They estimated overall total cost as €299,167 for enhanced *in situ* bioremediation (EISB), €294,074 for ISCO and €412,870 for air sparging (AS) applications in a residual source area contaminated with chlorinated ethenes (Case 1). On the other hand, the values were €426,353 for EISB, €1,112,163 for AS, €427,446 for phytoremediation, €423,802 for ZVI barrier and €368,413 for monitored natural attenuation (MNA) in Case 2 scenario. It is clear that the costs of different techniques may change according to the different factors (target contaminant, site conditions, operational conditions, etc.). For example, matrix demand is one factor affecting

the selection of oxidant dosing and delivery system and in turn, the cost. The key point is here to perform a detailed cost analysis and to select the best method for the remedy among the different alternatives.

In many of these studies, the costs were estimated by taking the design, capital, operations and maintenance, post-remediation and monitoring costs into account. Yet, some other factors should also be evaluated in a sustainability analysis of a project such as greenhouse gas emissions, energy consumption, reduction of risk to human health during implementation of the remedy. There is an increasing trend towards the use of sustainable remedial alternatives that are both cost-advantage and less intrusive/disruptive than many of the conventional approaches commonly used (Gordon, 2013). ISCO techniques are the preferred methods to convert the target contaminants (e.g., chlorinated solvents) into harmless and natural end products. However, the application of these techniques for the effective *in situ* treatment needs improvement in their environmental performance by identifying more sustainable options in process selection, design and optimization. In this sense, a holistic decision-making process and the use of life cycle assessment (LCA) to support the method selection are important. It should be noted that a remediation technology removes a local contamination, but also has environmental impacts on local, regional, and global scale, leading to the use of energy, chemicals, raw materials, and the generation of emissions and waste (Bjerg et al., 2013). Therefore, *primary* (local toxic impacts at site; e.g. TCE contamination) and *secondary* (impacts stemming from remediation; e.g. metal mobilization) impacts should clearly be defined in order to perform remedial action in a sustainable manner.

8.5. Future perspectives

Implementation of ISCO technology needs to be enhanced since it generally depends on injecting an oxidant into the site of contamination and just letting the natural movement of the water at the site to drive the spread of the oxidant. This method is not very effective because many of the oxidants, especially the stronger ones, are not very stable and react almost as soon as they are injected into the contaminated site, and not necessarily with the targeted substance (Stroo et al., 2010). Moreover, ISCO should be chosen and applied in a manner that minimizes the microbial interaction with concentrated chemical oxidants and directly reacts with the contaminant of concern. Such an application might be achieved by designing innovative injection apparatus which spread the oxidant only to the area that is contaminated.

One other key point is to determine *threshold* oxidant concentration which might cause insignificant disturbances but still allows microbial recovery/survival in the post-oxidation stage. For each oxidant, the predicted no effect concentration (PNEC) could be estimated for various microbial species and such a value (or index) might be achieved with a standard test that determines the concentration which brings about 50% mortality, for example. These tests may be conducted with pure or enrichment cultures to create base line data, however, more complex batch microcosm tests may be more practical to have comparable results with the *in situ* applications. By performing serial experiments, *allowable oxidant concentrations* can be estimated and such an index may help the practitioners during the application of combined treatment (ISCO + bioremediation).

Moreover, discovery of new organisms with new catabolic capabilities may advance the bioremediation and bioaugmentation processes. Designer microbes and directed lateral gene transfer might be useful genetic modification techniques for developing targeted, site-specific bioremediation strategies (Hug et al., 2013). Another option to increase the bioaugmentation capacity might be using carrier materials such as alginate, agarose, polyurethane, lentikats, or hollow fibers, each providing a temporary protective environment to the inoculum as presented in several literature studies (Stormo and Crawford, 1992; Durieux et al., 2000; Loh et al., 2000). Moreover, a novel slow-release inoculation approach using a catabolic strain encapsulated in open-ended tubes is also a promising bioaugmentation tool for contaminated sites, as it can enhance pollutant removal and can prolong the degrading activity in comparison with traditional inoculation strategies (Boon, 2010).

Summary

Chlorinated aliphatic hydrocarbons (CAHs) are one of the most prevalent groundwater contaminants in the industrialized world and therefore CAH polluted groundwater imposes environmental risks. Groundwater contamination by chlorinated solvents, particularly tetrachloroethene (PCE) and trichloroethene (TCE) is widespread. The large extent of groundwater contamination with these compounds has led to use of various innovative remediation technologies. Application of *In Situ* Chemical Oxidation (ISCO) using potassium permanganate ($KMnO_4$) and sodium persulfate ($Na_2S_2O_8$) for example, has been proven effective in remediating contaminated sites although this technique is not always effective enough to reach the regulatory objectives. When the objectives are not met, bioremediation can be considered as an alternative or secondary treatment tool. However, this combined approach of ISCO and bioremediation might have some disadvantages. The destructive effect of the oxidants on the indigenous microorganisms in the subsurface might be so severe that post-bioremediation cannot be achieved in the system. Moreover, a change in abiotic conditions, such as pH, redox potential and terminal electron acceptors (TEAs), might affect the dehalogenating bacteria and in turn, the efficiency of (reductive) dechlorination.

In this dissertation, we have studied three questions: i) what are the effects of the oxidants permanganate and persulfate on the microorganisms, especially on CAH degrading *Dehalococcoides mccartyi* spp., ii) is recovery of the microorganisms after oxidant exposure possible or not, and iii) what will be the profile of the microbial community after the exposure to the chemical oxidants, will it be the same as observed at pre-oxidation conditions or not?

Firstly, we investigated the long term responses of a *Dehalococcoides mccartyi* spp. containing enrichment culture under TCE (23 µmol/bottle) and lactate (5.6 mM) exposure but in absence of oxidants (Chapter 3). The results revealed that total ATP concentrations increased from 2 to 5 ng/mL after the addition of TCE and lactate to the culture. Although the number of 16S rRNA, *tceA* and *vcrA* genes were stable in DNA extracts over time, *tceA* and *vcrA* mRNA abundances were up-regulated from undetectable levels to 2.96×10^4 transcripts/mL and 6.33×10^4 transcripts/mL, respectively, only after exposure to TCE and lactate. While *tceA* gene transcripts decreased over time with TCE dechlorination, the *vcrA* gene was expressed steadily even when the concentration of vinyl chloride was at undetectable levels. In addition, ratios between catabolic and phylogenetic genes indicated

that *tceA* and *vcrA* gene carrying organisms dechlorinated TCE and its produced daughter products, while *vcrA* gene was mainly responsible for the dechlorination of the lower VC concentrations in a later stage of TCE degradation.

Subsequently, in Chapter 4, the impact of aggressive conditions, caused by $KMnO_4$ and $Na_2S_2O_8$, was investigated in the ***mixed enrichment culture*** of Chapter 3, still in well controlled liquid systems. Different concentrations of the oxidants $KMnO_4$ (0.005 to 2 g/L) and $Na_2S_2O_8$ (0.01 to 4.52 g/L) were applied to the culture and the reactors were monitored over time. Degradation of TCE and expression of the *tceA*, *vcrA* and 16S rRNA genes of *Dehalococcoides mccartyi* spp. were detected in the test systems having lower concentrations of 0.005 to 0.01 g/L $KMnO_4$ and 0.01 to 0.02 g/L $Na_2S_2O_8$. However, chemical degradation but no gene expression was recorded for the higher concentrations of 0.5 to 2 g/L $KMnO_4$ and 1.13 to 4.52 g/L $Na_2S_2O_8$. In addition, the destructive effects of the two chemical oxidants were recorded at mRNA level but not at gene level, indicating the persistence of intact DNA in the cultures under these higher chemical oxidant conditions. However, at lower concentrations of chemical oxidants, a ***combined*** chemical and biological degradation was possible. This test also showed the inhibitory impact of oxidizing redox conditions and by-products SO_4^{2-} and $MnO_{2(s)}$ on the activity of *Dehalococcoides mccartyi* spp. In addition, it was observed that bioaugmentation was necessary for the recovery of the dechlorination activity at $KMnO_4$ and $Na_2S_2O_8$ concentrations higher than 0.5 and 1.13 g/L, respectively.

In Chapter 5, the impact of permanganate on the soil microbial populations in environmental matrices closer to reality was examined. Firstly, the impact of the three oxidants $NaMnO_4$, $Na_2S_2O_8$ and alkaline activated $Na_2S_2O_8$ on the microorganisms was evaluated via a lab scale batch test (containing aquifer material and real groundwater) by following the activity of specific genes on RNA level via qPCR. At low doses of permanganate and persulfate (5 g/kg aquifer), it was shown that the activity of the *Dehalococcoides mccartyi* spp. was comparible with the non-exposed control. However, under exposure of 15 and 20 g/kg oxidants,. the relatively low mRNA/DNA ratios of 16S rRNA gene of *Dehalococcoides mccartyi* spp., *tceA*, *vcrA* and *bvcA* genes ranging between 0.012 and 0.352 transcripts/gene indicated limited dechlorination activity of the cells. The expression of the genes under aggressive oxidation conditions may be attributed to the background signals in this batch test. In a second part of Chapter 5, the effect of permanganate on the activity and structure of the microbial community was studied in the field. At a site in Belgium where a permanganate injection took

place, both aquifer (via mesocosms and liners) and groundwater samples were taken and subjected to molecular analyses. Right after the injection of an ***excessive*** amount of oxidants, the gene transcripts were below detection limit in both groundwater and aquifer samples indicating aggressive stress of the oxidants on the microbial community. However, after 82 days, microbial recovery was recorded in both environmental matrices. RNA-based DGGE profiles targeting total bacteria indicated shifts in the structure of the microbial community 15 days after the injection of permanganate in the oxidant impacted wells but recovery was recorded within approximately three months under stagnant flow conditions.

In Chapter 6, we investigated the possibility of distinguishing chemical oxidation and biological degradation of cDCE via compound specific stable isotope analyses (CSIA) after permanganate injection at a very heterogeneous field site (see Chapter 5). Based on lab scale batch experiments, carbon and chlorine isotope enrichment factors for cDCE oxidation by permanganate were determined as ε_C = -26.2‰ ± 0.9‰ and ε_{Cl} = +0.20‰ ± 0.02‰, respectively. Similarly, lab scale anaerobic biodegradation tests performed with aquifer and groundwater from the site revealed isotope enrichment factors for cDCE of ε_C = -17.9‰ ± 0.6‰ and ε_{Cl} = -3.3‰ ± 0.1‰. The observed inverse chlorine isotope effect of the oxidation by permanganate was an important tool to distinguish biodegradation and chemical oxidation of cDCE. The field data, collected before and after a pilot permanganate injection, showed that chemical oxidation and anaerobic biodegradation of cDCE could be distinguished via carbon and chlorine isotope measurements. Our study indicated that, ***for the first time***, dual isotope approach was successful in distinguishing oxidation by permanganate and anaerobic biodegradation in groundwater field samples.

In Chapter 7, $KMnO_4$ and $Na_2S_2O_8$ were applied to treat three contaminated **sediments**. All the sediments were contaminated with mineral oil, polycyclic aromatic hydrocarbons (PAHs) and heavy metals and had an organic matter content ranging from 2.4 to 7.6%. The natural oxidant demands of the sediments under persulfate treatment were lower (30-100 g/kg) than the ones treated with permanganate (50-450 g/kg). Cr was released during the application of permanganate whereas Zn and Pb were released under persulfate treatment. Quantitative PCR results showed that permanganate and persulfate, both at a concentration of 150 g/kg, caused a decrease (2 log units) in the number of 16S rRNA gene of total bacteria in the sediment having the lowest organic matter content. However, the total ATP, considered as a biomarker

for microbial activity, was below detection limit in all sediments in the presence of at least 150 g/kg oxidant. Only permanganate induced a shift in the structure of the microbial community.

In conclusion, interaction between biological and chemical degradation of CAHs in different environmental systems was studied in this dissertation. The oxidants $KMnO_4$ and $Na_2S_2O_8$ were found compatible with biodegradation at low doses, but had inhibitory/detrimental effects on the microorganisms at high concentrations. However, this adverse effect was more pronounced in the culture and groundwater-based systems than in the aquifer and sediment. Recovery of CAH degradation after oxidant exposure was not always possible, and bioaugmentation under ***reducing*** conditions was found necessary in a number of cases. In the field, recovery of CAH biodegradation was shown to occur at about 3 months after the injection. Finally, application of chemical oxidants may result in shifts in the microbial community structure.

Samenvatting

Gechloreerde alifatische koolwaterstoffen (CAHs) zijn één van de meest voorkomende grondwater verontreinigingen in de geïndustrialiseerde wereld. Hierdoor is CAH-verontreinigd grondwater vaak gekoppeld aan milieurisico's. Grondwater verontreiniging door gechloreerde solventen en met name perchlooretheen (PCE) en trichlooretheen (TCE) is wijdverspreid. De grote omvang van de grondwater verontreiniging met deze verbindingen heeft geleid tot het gebruik van diverse innovatieve saneringstechnologieën. Toepassing van *In Situ* Chemische Oxidatie (ISCO) met kaliumpermanganaat ($KMnO_4$) en natriumpersulfaat ($Na_2S_2O_8$) bijvoorbeeld, bleek effectief te zijn voor de sanering van verontreinigde terreinen. Wanneer echter onder bepaalde omstandigheden de saneringsnormen niet worden bereikt, kan bioremediatie worden beschouwd als een alternatieve of complementaire behandeling. Deze gecombineerde aanpak kan echter een aantal nadelen hebben. Het destructieve effect van de oxidanten op de autochtone bodem micro-organismen kan zeer groot zijn waardoor een biologische nabehandeling niet meer mogelijk is. Bovendien kunnen de veranderingen in abiotische omstandigheden, zoals pH, redox potentiaal en terminale elektronen acceptor (TEA), de dehalogeneringsactiviteit van de bacteriën beïnvloeden en dus de efficiëntie van de (reductieve) dechlorinering.

In dit proefschrift werden volgende drie vragen onderzocht: i) wat zijn de effecten van de oxidanten $KMnO_4$ en $Na_2S_2O_8$ op de micro-organismen, met name op CAH afbrekende *Dehalococcoides mccartyi* spp, ii) is het herstel van de micro-organismen na blootstelling aan de oxidanten mogelijk of niet en iii) wat is de structuur van de microbiële gemeenschap na de oxidant behandeling, is deze dezelfde als voor de toediening van de oxidantof niet?

Ten eerste onderzochten wedelange termijn respons van een *Dehalococcoides mccartyi* spp. bevattende aanrijkings cultuur na blootstelling aan TCE (23µmol TCE/fles) en lactaat (5.6 mM) maar niet aan oxidanten (Hoofdstuk 3). De totale ATP concentratie steeg van 2 naar 5 ng /mL na toevoeging van TCE en lactaat aan de cultuur. Hoewel het aantal 16S rRNA, *tceA* en *vcrA* genen in DNA extracten stabiel bleef in functie van de tijd, werden de *tceA* en *vcrA* mRNA hoeveel heden na blootstelling aan TCE en lactaat opgereguleerd van onder de detectieniveaus tot respectievelijk 2.96×10^4 en 6.33×10^4 transcripten/mL. Terwijl de *tceA* transcripten afnamen in functie van de tijd, kwam het *vcrA* gen continu tot expressie,

zelfswanneer de concentratie aan vinylchloride onder de detectie limiet lag. Bovendien gaven de verhoudingen tussen katabole en fylogenetische genen aan dat *tceA* en *vcrA* gen bevattende organismen TCE en zijn dochter producten afbraken, terwijl het *vcrA* gen voornamelijk verantwoordelijk was voor de dechlorinatievan de lagere VC concentraties in een later stadium van de TCE afbraak.

Vervolgens werd in hoofdstuk 4 de invloed van agressieve omstandigheden, veroorzaakt door $KMnO_4$ en $Na_2S_2O_8$, onderzocht in deze TCE afbrekende ***gemengde aanrijkings cultuur*** van hoofdstuk 3. Verschillende concentraties van de oxidanten $KMnO_4$ (0.005-2 g/L) en $Na_2S_2O_8$ (0.01-4.52 g/L) werden toegevoegd aan de cultuur en het effect op de microorganismen werd onderzocht in functie van de tijd. Afbraak van TCE en expressie van de *tceA*, *vcrA* en 16S rRNA genen van *Dehalococcoides mccartyi* spp. werden waargenomen in de culturen waaraan 0.005-0.01 g/L $KMnO_4$ en 0.01-0.02 g/L $Na_2S_2O_8$ werd toegevoegd. Chemische afbraak maar geen gen expressie werd gedetecteerd voor de hogere concentraties van 0.5 tot 2 g/L $KMnO_4$ en 1.13 tot 4.52 g/L $Na_2S_2O_8$. Deze effecten werden bovendien enkel waargenomen op mRNA en niet op DNA niveau. Dit toont de persistentie van het DNA onder deze agressieve omstandigheden aan. Bij lagere chemische oxidant concentraties was een ***gecombineerde*** biologische en chemische afbraak mogelijk. In deze test werd ook het inhiberende effect van oxiderende redoxcondities en de neven produkten SO_4^{2-} en $MnO_{2(s)}$ op de activiteit van *Dehalococcoides mccartyi* spp. aangetoond. Daarnaast werd opgemerkt dat bioaugmentatie noodzakelijk is voor het herstel van de dechlorinatie activiteit na toediening van de hogere $KMnO_4$ en $Na_2S_2O_8$ concentraties (hoger dan respectievelijk 0.5 en 1.13 g/L).

In hoofdstuk 5 werd het effect van chemische oxidantia op CAH biodegradatie bestudeerd onder meer realistische condities. Enerzijds werd de impact van $NaMnO_4$, $Na_2S_2O_8$ en alkalisch geactiveerd $Na_2S_2O_8$ op de micro-organismen bestudeerd via batchtesten met reëel aquifer materiaal en grondwater. Er werd aangetoond dat in deze systemen chemische oxidatie van CAHs mogelijk is en compatibel is met biodegradatie bij een lage dosis aan permanganaat of persulfaat (5 g/kg aquifer). Blootstelling aan 15 en 20 g/kg oxidanten in gesloten batchsystemen had echter een ernstig negatief effect op de biodegradatie van CAH. De relatief lage mRNA/DNA verhoudingen tussen 0.012 en 0.352 transcripten/gen voor het 16S rRNA gen van *Dehalococcoides mccartyi* spp., *tceA*, *vcrA* en *bvcA* genen gaven aan dat de dechlorinatie activiteit van de micro-organismen onder deze omstandigheden gelimiteerd

is. De expressie van de genen onder agressieve oxidatie omstandigheden kunnen worden toegeschreven aan de achtergrondsignalen in deze batch test. In een tweede deel van hoofdstuk 5 werd het effect van permanganaat op de activiteit en de structuur van de microbiële gemeenschap bestudeerd tijdens een test in het veld waar een permanganaat injectie plaats vond. Zo wel aquifer materiaal (via mesokosmossen en liners) als grondwater stalen werden bemonsterd en onderworpen aan moleculaire analyses. Net na de injectie van een grote hoeveelheid aan chemische oxidant, daalde de transcriptie onder de detectie limiet en dit zo wel in de grondwater als aquifer stalen. Ook al stemt dit overeen met een enorme stress van de oxidant op de microbiële gemeenschap, trad herstel op na 82 dagen. RNA gebaseerde DGGE profielen van de bacteriële gemeenschap vertoonden 15 dagen na depermanganaat injectie shifts in de structuur van de microbiële populatie. De populatie was ongeveer 3 maanden na deze injectie hersteld.

In hoofdstuk 6 zijn we nagegaan of chemische oxidatie en biologische afbraak van cDCE na permanganaat injectie in een zeer heterogene veld site van elkaar kunnen onderscheiden worden door component specifieke stabiele isotoop analyse (CSIA). Via batch experimenten op laboschaal werden de koolstof en chloorisotoop aanrijkings factoren voor de oxidatie van cDCE door permanganaat respectievelijk bepaald als ε_C = -26.2‰ ± 0.9‰ en ε_{Cl} = +0.20‰ ± 0.02‰. Bovendien bleek uit anaërobe biologische afbraak testen uitgevoerd met aquifer en grondwater van de site dat de isotoop aanrijkingsfactoren voor cDCE overeenstemmen met ε_C = -17.9‰ ± 0.6‰ en ε_{Cl} = -3.3‰ ± 0.1‰. Het waargenomen inverse chloor isotoop effect van de oxidatie door permanganaat was een belangrijk instrument om de biologische afbraak en chemische oxidatie van cDCE van elkaar te onderscheiden. Velddata bekomen voor en na een piloot injectie van permanganaat, toonden aan dat chemische oxidatie en anaërobe afbraak van cDCE van elkaar kunnen onderscheiden worden via koolstof en chloor isotoop metingen. Onze studie toonde voor het eerst aan dat deze duale isotoop aanpak kan worden gebruikt om oxidatie met permanganaat en anaërobe afbraak van elkaar te onderscheiden in grondwater stalen bekomen in het veld.

In hoofdstuk 7 werden $KMnO_4$ en $Na_2S_2O_8$ toegepast om drie verontreinigde sedimenten te behandelen. Alle sedimenten waren verontreinigd met minerale olie, polycyclische aromatische koolwaterstoffen (PAK's) en zware metalen en hadden een organisch stofgehalte

variërend tussen 2.4 en 7.6%. De matrix behoefte van de sedimenten onder persulfaat behandeling waren lager (30-100 g/kg) dan die behandeld met permanganaat (50-450 g/kg). Cr werd vrijgegeven tijdens de toepassing van permanganaat terwijl Zn en Pb werden vrijgesteld onder persulfaat behandeling. Kwantitatieve PCR resultaten toonden aan dat permanganaat en persulfaat, beide in een concentratie van 150g/kg, een afname (2 log eenheden) in het aantal 16S rRNA genen van de totale bacteriën in het sediment met het laagste gehalte aan organische stof veroorzaakten. Het totale ATP, beschouwd als een biomerker voor microbiële activiteit, was beneden de detectiegrens in alle sedimenten in aanwezigheid van ten minste 150 g/kg oxidant. Enkel permanganaat induceerde een verschuiving in de structuur van de microbiële gemeenschap.

In conclusie, in dit proefschrift werden de interacties tussen biologische en chemische afbraak van CAHs in verschillende ecologische systemen bestudeerd. De oxidanten $KMnO_4$ en $Na_2S_2O_8$ bleken bij lage concentraties compatibel te zijn met biodegradatie van CAHs. Bij hoge concentraties ontstonden remmende/nadelige effecten op de micro-organismen. Herstel na oxidant blootstelling was niet altijd mogelijk en bioaugmentatie onder reducerende omstandigheden kan noodzakelijk zijn. Dit nadelig effect was meer uitgesproken in de cultuur en grondwater gebaseerde systemen dan onder meer natuurlijke omstandigheden waar bufferend en beschermend aquifer materiaal of sedimenten aanwezig zijn. Tot slot werd aangetoond dat de toepassing van chemische oxidanten kan resulteren in verschuivingen in de structuur van de microbiële gemeenschap.

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EDUCATION

2005-2007	**M.Sc.,** Middle East Technical University, Department of Environmental Engineering, Ankara, Turkey. Thesis Title: Two-Phase Digestion of Semi-Solid Organic Wastes
2000-2005	**B.S.,** Middle East Technical University, Department of Environmental Engineering, Ankara,Turkey.

WORK EXPERIENCES

August 2009-August 2012	VITO (Flemish Institue for Technological Research), Researcher, Mol, Belgium,
October 2005- August 2009	Middle East Technical University, Department of Environmental Engineering, Teaching Assistant, Ankara, Turkey
October 2005- August 2009	Teaching Assistant for the courses: Unit Operations and Processes Laboratory, Pollution Prevention, Environmental Chemistry Laboratory, Water Supply Engineering

2004-2008	Researcher at the project "Organic Acid Production from Municipal and Agro-Industrial Wastes", The Scientific and Technical Research Council of Turkey, ÇAYDAG-104I127

PUBLICATIONS WITH PEER REVIEW

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- Doğan E., Accoe F., Boon N., Bastiaens L., Dejonghe W., 2011. " Chemical oxidation affects the quantity, diversity and activity of the microbial community in the sediments", 8th International Symposium of Subsurface Microbiology, 11-16 September, Garmisch-Partenkirchen, Germany, (poster presentation).
- Doğan E., Accoe F., Boon N., Bastiaens L., Dejonghe W., 2011. " Chemical oxidation affects the quantity, diversity and activity of the microbial community in the sediments", First international symposium on Microbial resource management in biotechnology: Concepts & applications, 30 June-1 July, Gent, Belgium, (poster presentation).
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SHORT COURSES AND WORKSHOPS

- Final Workshop of the Marie Curie Initial Training Network GOODWATER, German Research Center for Environmental Health GmbH (Helmholtz), Institute of Groundwater Ecology, 12 – 14 March 2012, Nueherberg, Germany.
- Training and Analyses on Compound Specific Isotope Analysis, German Research Center for Environmental Health GmbH (Helmholtz), Institute of Groundwater Ecology, 15-23 March 2012, Munich, Germany.
- GOODWATER Winter School, How to bring knowledge from the lab into the real world. Geological Survey of Denmark, 16-19 January 2012, Copenhagen, Denmark.
- EU environmental legislation & Patents and related intellectual property rights (IPR), Catholic University of Leuven, 10-13 October 2011, Leuven, Belgium.

- Training on Compound Specific Isotope Analysis, German Research Center for Environmental Health GmbH (Helmholtz), Institute of Groundwater Ecology, 6-16 September 2011, Munich, Germany.
- Second GOODWATER ITN Winter School "Monitoring of microbial markers in groundwater systems and different aspects of groundwater bioremediation" Gent University, Laboratory of Microbial Ecology and Technology (LabMET) and VITO, 10-14 January 2011, Gent and Mol, Belgium.
- Workshop on scientific writing-Understanding peer-reviewed publishing, Tress &Tress, 6-7 September 2010, Schliersbergalm, Germany
- Introductory Winter School of the Marie Curie Initial Training Network-GOODWATER ITN, Helmholtz Zentrum München (German Research Center for Environmental Health), Institut für Grundwasserökologie, 7 - 11 December 2009, Nueherberg, Germany.
- WISE- Waste in Social Environment, TC 6"Human- producer of waste and decision-maker for recycling systems" Marie Curie Actions I.A.R. RWTH Aachen University, 8-13 November, 2009, Madrid, Spain.
- Training Course on "Essential, expectations, and strategies for a successful PhD project" Marie Curie Initial Training Network GOODWATER ITN. Technical University of Denmark, Department of Environmental Engineering, 16 - 21 August 2009, Gilleleje, Denmark.
- Training Course on "Wastewater Treatment Plant Operation and Management" and "Water Reclamation and Reuse in the Mediterranean" UNEP/MAP-WHO; Middle East Technical University, Department of Environmental Engineering; Ministry of Environment and Forestry of Turkey- General Directorate of Environmental Management - Marine and Coastal Management Department, 30 June- 2 July 2009, Middle East Technical University, Ankara, Turkey (in organizing commitee)
- Cleaner Production: A tool for Green Competitiveness in the Turkish Industry, Workshop and Short Course Middle East Technical University, Department of Environmental Engineering, Workshop Series; Middle East Technical University, Ministry of Environment and Forestry, Regional Activity Center for Cleaner Production, 31 March-2 April 2008, Middle East Technical University, Ankara, Turkey (in organizing commitee)

- FISH Applications on Biotechnology, İstanbul Technical University and Bogaziçi University, Department of Environmental Engineering, 20-22 June 2007, İstanbul, Turkey.

Appendix

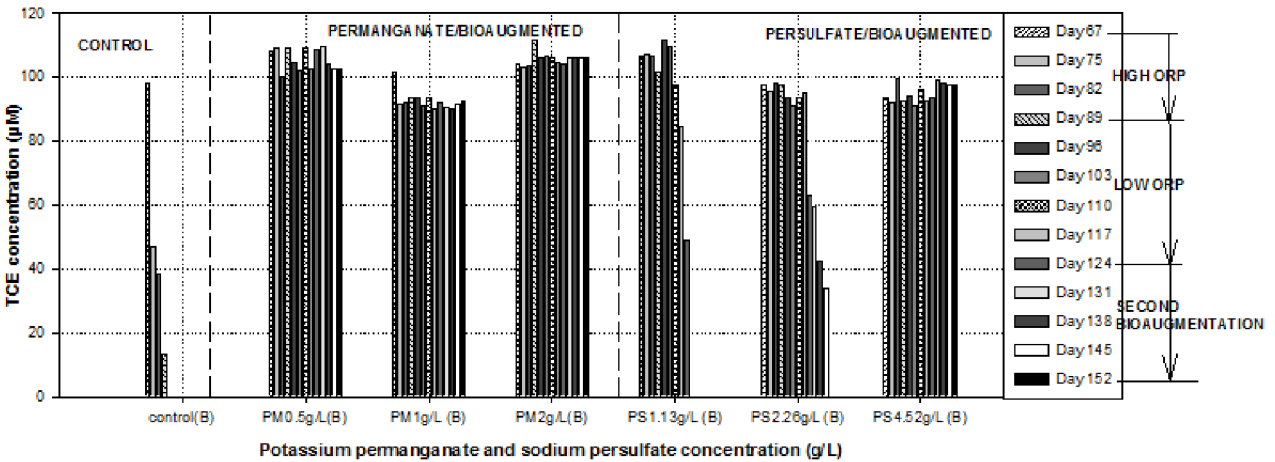

Figure A1. Change in TCE concentration in the test systems under the application of bioaugmentation over time at oxidizing and reducing redox conditions.

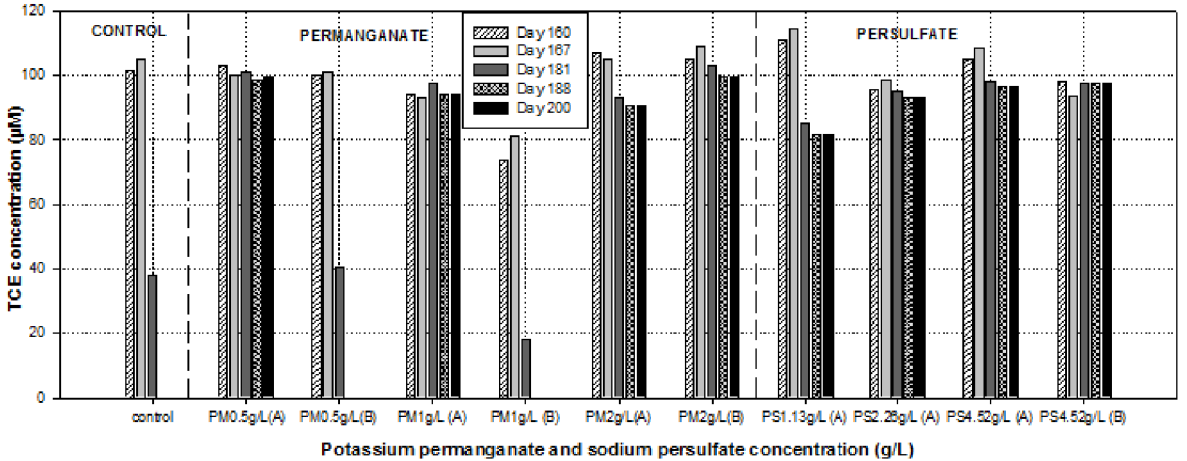

Figure A2. Change in TCE concentration in the test systems over time after centrifugation and resuspension process.

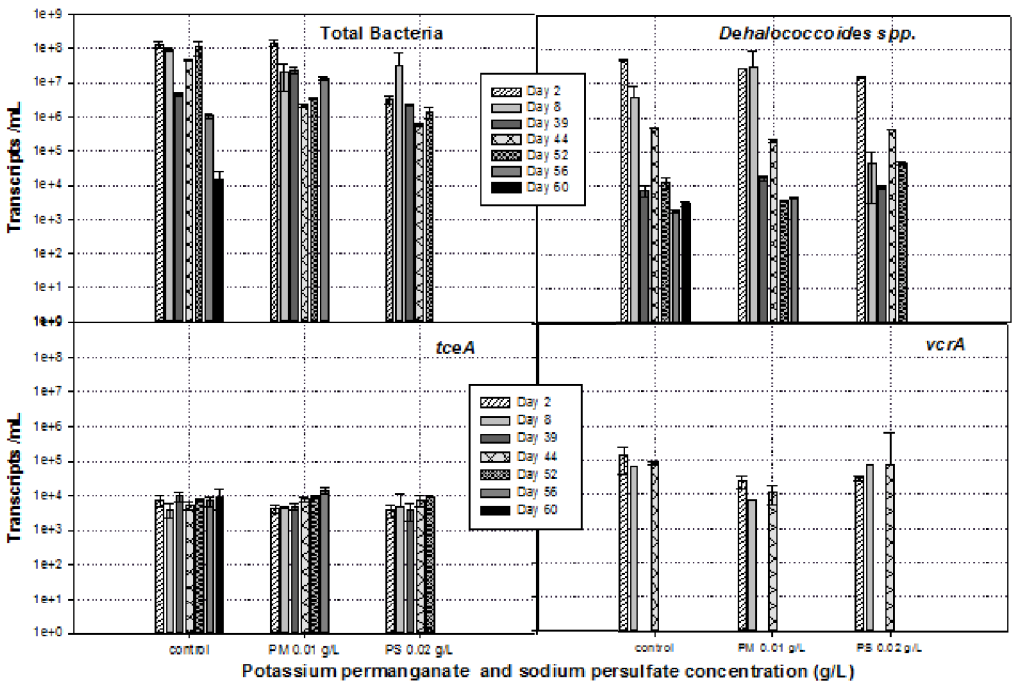

Figure A3. 16S rRNA of total bacteria and *Dehalococcoides* spp., *tceA,* and *vcrA* transcripts over time in RNA extracts. Each bar represents the average of the results of triplicate real-time PCRs performed on two independent RNA extractions (n = 6) (Detection limits: 10^2, 10^2 and 10^3 copies/mL for 16S rRNA, *tceA* and *vcrA,* respectively).

Table A1. PCE, TCE, cDCE, VC and ethene concentrations in each monitoring well over time

MW 5a							MW 5b					
Days	**PCE(µM)**	**TCE(µM)**	**cDCE(µM)**	**VC(µM)**	**Ethene(µM)**	**DOC(mg/L)**	**PCE(µM)**	**TCE(µM)**	**cDCE(µM)**	**VC(µM)**	**Ethene (µM)**	**DOC(mg/L)**
before injection	bdl	0	30	1	1	197	BDL	BDL	197	0	4	2728
15	0	0	6	0	0	738	0	0	302	4	4	2860
33	BDL	BDL	10	BDL	BDL	504	BDL	BDL	341	BDL	BDL	4467
55	BDL	BDL	9	BDL	BDL	247	BDL	BDL	288	BDL	BDL	3.45
82	0	0	10	1	1	350	0	0	251	4	3	5050
305	0	BDL	36	4	3	132	0	BDL	53	51	15	1030
443	bdl	0	27	5	5	254	BDL	BDL	2	3	13	673
MW 6b							**MW 7b**					
Days	**PCE(µM)**	**TCE(µM)**	**cDCE(µM)**	**VC(µM)**	**Ethene(µM)**	**DOC(mg/L)**	**PCE(µM)**	**TCE(µM)**	**cDCE(µM)**	**VC(µM)**	**Ethene (µM)**	**DOC(mg/L)**
before injection	51	5	309	BDL	1	3987	BDL	BDL	7	BDL	1	1367
15	178	3	186	1	1	3421	0	0	10	0	3	1566
33	NM	NM	160	NM	NM	5040	BDL	BDL	BDL	BDL	BDL	2233
55	NM	NM	186	NM	NM	4.44	BDL	BDL	BDL	BDL	BDL	1.64
82	16	3	154	0	1	6860	BDL	0	1	9	2	858
305	16	4	161	0	1	3607	BDL	BDL	0	0	0	149
443	7	2	241	1	1	547	BDL	0	0	0	0	15
MW 8b												
Days	**PCE(µM)**	**TCE(µM)**	**cDCE(µM)**	**VC(µM)**	**Ethene(µM)**	**DOC(mg/L)**						
before injection	2	1	213	82	12	3171						
15	0	0	18	12	2	2277						
33	BDL	BDL	170	BDL	BDL	4450						
55	BDL	BDL	135	BDL	BDL	3.59						
82	1	1	151	73	12	2130						
305	BDL	0	11	84	10	1904						
443	BDL	0	18	25	18	1107						

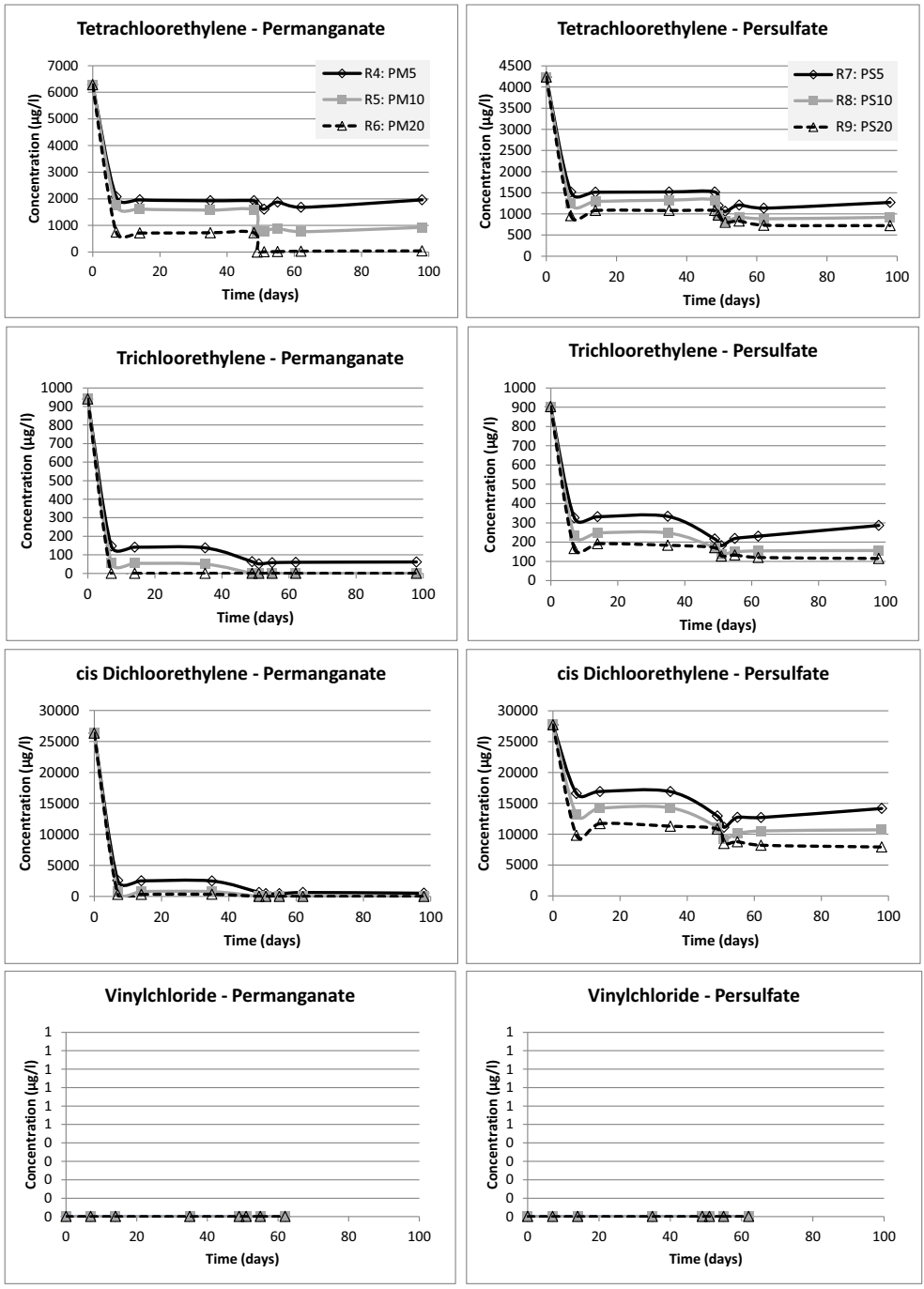

Figure A4. The evolution of the PCE, TCE, cDCE and VC concentrations and ORP values during Phase 1 for the test conditions with permanganate and persulfate (Bastiaens et al., 2013, in preparation).

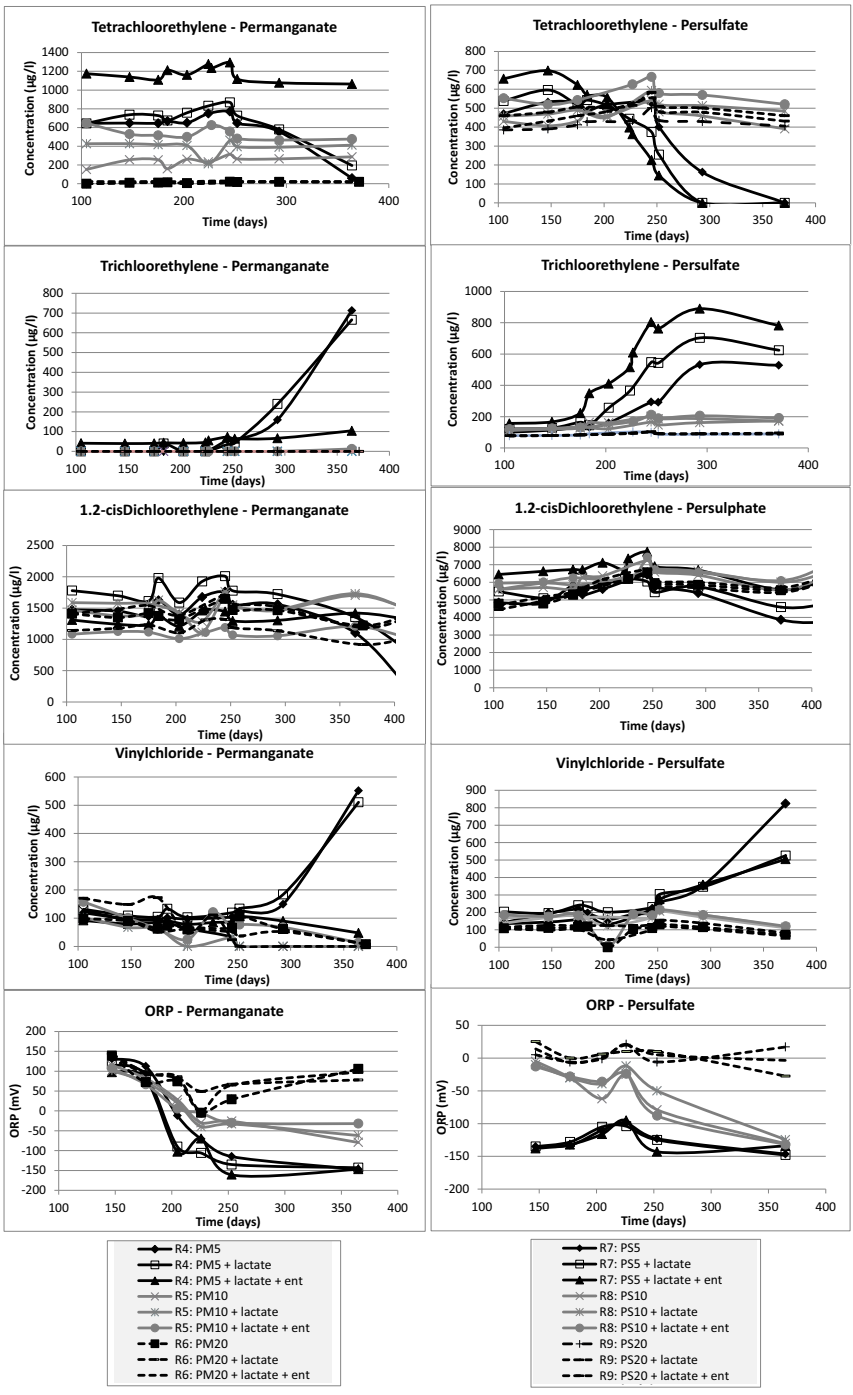

Figure A5. The evolution of the PCE, TCE, cDCE and VC concentrations and ORP values during Phase 2 for the test conditions with permanganate and persulfate (Bastiaens et al., 2013, in preparation).

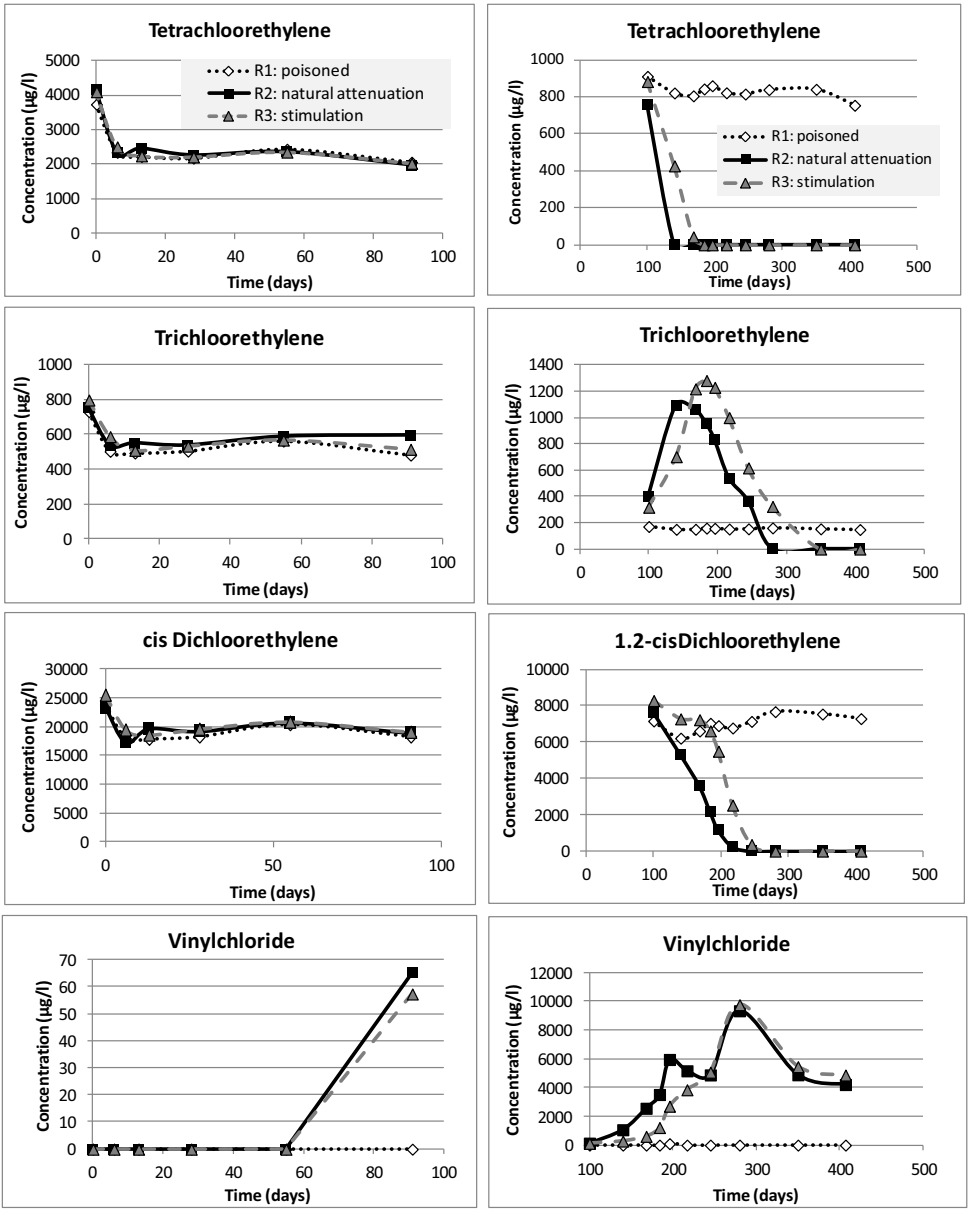

Figure A6. Evolution of CAH-concentrations in the controls in Phase 1 (left) and Phase 2 (right) (Bastiaens et al., 2013, in preparation).

Table A2. Carbon and chlorine isotope values for each monitoring well over time

MW 5a							
time (day)	$\delta^{37}Cl$ (‰)	stdv	$\delta^{13}C$ (‰)	stdv	cDCE(µM)	VC(µM)	Ethene(µM)
BI	5.0	0.0	-28.9	0.2	30	1	1
15	4.8	0.1	-28.3	0.2	6	1	0
33	4.9	0.1	-26.7	0.0	10	BDL	BDL
55	6.1	0.1	-24.1	0.7	9	BDL	BDL
82	5.0	0.0	-27.3	0.4	10	0	1
305	5.3	0.0	-28.1	0.1	36	4	3
443	5.7	0.1	-27.8	0.4	27	5	5

MW 5b							
time (day)	$\delta^{37}Cl$ (‰)	stdv	$\delta^{13}C$ (‰)	stdv	cDCE(µM)	VC(µM)	Ethene(µM)
BI	4.9	0.0	-29.2	0.2	197	0	4
15	5.0	0.0	-29.7	0.0	302	4	4
33	5.0	0.0	-29.8	0.0	342	BDL	BDL
55	4.9	0.0	-29.7	0.1	288	BDL	BDL
82	5.1	0.0	-29.4	0.2	251	4	3
305	7.0	0.0	-7.3	0.3	53	50	15
443	NM	NM	NM	NM	2	3	13

MW 6b							
time (day)	$\delta^{37}Cl$ (‰)	stdv	$\delta^{13}C$ (‰)	stdv	cDCE(µM)	VC(µM)	Ethene(µM)
BI	4.3	0.0	-29.5	0.0	309	bdl	1
15	3.7	0.0	-26.4	0.3	186	1	1
33	4.3	0.0	-27.7	0.9	160	NM	NM
55	4.3	0.0	-28.2	0.5	186	NM	NM
82	4.1	0.1	-27.2	1.2	154	0	1
305	4.1	0.1	-27.6	0.7	161	0	1
443	4.6	0.1	-29.8	0.1	241	1	1

MW 8b							
time (day)	$\delta^{37}Cl$ (‰)	stdv	$\delta^{13}C$ (‰)	stdv	cDCE(µM)	VC(µM)	Ethene(µM)
BI	6.0	0.0	-20.8	1.1	213	82	12
15	4.2	0.1	-24.8	0.0	18	12	2
33	5.0	0.0	-28.8	0.8	170	BDL	BDL
55	5.0	0.1	-28.9	0.1	135	BDL	BDL
82	4.9	0.1	-29.2	0.1	151	73	12
305	NM	NM	NM	NM	11	84	10
443	NM	NM	NM	NM	28	25	18

BI: before injection, NM: not measureable, BDL: below detection limit.

Table A3. Extent of biodegradation and chemical oxidation in the monitoring wells over time*

	MW5a				**MW5b**			
	Carbon isotope		**Chlorine isotope**		**Carbon isotope**		**Chlorine isotope**	
	Biodegradation(%)	Oxidation (%)	Biodegradation(%)	Oxidation (%)	Biodegradation(%)	Oxidation (%)	Biodegradation(%)	Oxidation (%)
Before injection	0	0	0	0	0	0	0	0
Day 15	4	3	-7	65	-3	-2	4	-81
Day 33	12	**8**	-1	**20**	-3	-2	1	-17
Day 55	**24**	17	**29**	-29256	-3	-2	1	-16
Day 82	**9**	7	**2**	-43	-1	-1	5	-141
Day 305	**5**	3	**9**	-335	**71**	57	**47**	-3252746
Day 443	**6**	4	**19**	-3261	-	-	-	-
	MW6b				**MW8b****			
	Biodegradation(%)	Oxidation (%)	Biodegradation(%)	Oxidation (%)	Biodegradation(%)	Oxidation (%)	Biodegradation(%)	Oxidation (%)
Before injection	0	0	0	0.00	**38**	28	**28**	-23413
Day 15	16	**11**	-20	**95**	22	**16**	-23	**97**
Day 33	**10**	**7**	0	-1	2	2	2	-37
Day 55	**8**	**5**	1	-19	2	1	2	-35
Day 82	**12**	**9**	-5	55	0	0	1	-14
Day 305	**10**	**7**	-4	47	-	-	-	-
Day 443	-2	-1	9	-388	-	-	-	-

*The extent of (bio)degradation (B) was calculated by equation $B = (1-f) * 100 = (1-(R_t / R_0)^{(1/\alpha-1)})*100$ (Meckenstock et al.,2004)

**The values were estimated by taking the $\delta^x C_0$ values measured in MW5b as reference.

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