Monitoring Biodegradation Capacity of Organic Pollutants in the Environment

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This research was conducted under the auspices of the Netherlands Research School for the Socio-Economic and Natural Sciences of the Environment (SENSE).

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Thesis
submittted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 19 October 2010
at 4:00 p.m. in the Aula

Author: Bastian Mattijs van der Zaan
Title: Monitoring Biodegradation Capacity of Organic Pollutants in the Environment
PhD Thesis Wageningen University, Wageningen, the Netherlands (2010)
158 pages
With references - with summaries in Dutch and English
ISBN: 978-90-8585-777-8

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Chapter 1

General introduction

1.1 Introduction

The environment has become contaminated with organic pollutants throughout the world by human activities. Chlorinated aliphatic hydrocarbons and aromatic hydrocarbons are important groups of these organic pollutants. Although they are formed naturally by biological or geochemical processes, since the start of the industrial revolution these compounds entered the environment in excess, due to leakage, improper disposal, or accidents (Häggblom and Bossert, 2004; Juttner and Henatsch, 1986; Smidt and de Vos, 2004). For example, more than one-third of the 1.4 million gasoline storage tanks in the United States are leaking aromatic hydrocarbons (Ulrich *et al.,* 2005), and the amount of chlorinated aliphatic hydrocarbons, which reached surface waters in the Netherlands was about 11000 ton in 2001 (Dijk, 2005). These compounds form a long-lasting danger in the environment to human health and ecosystem functioning, because of toxic and carcinogenic effects, persistence, and bioaccumulation (Vogel *et al.,* 1987). Therefore, effective strategies for removal or control pollution are essential for ecosystem health.

It has been estimated that more than 400,000 sites in the Netherlands are potentially contaminated, and that about 15,000 sites are indicated by the authorities as 'urgent cases with acute risks' (RIVM, 2008). Risk analysis must indicate as soon as possible how many of these locations should be decontaminated. In 2008, 370 million euros were spent in the Netherlands to clean-up (remediate) soil systems and for related research (RIVM, 2008). Often, polluted soils were excavated and stored in a depot. However, in built environments or large industrial locations, soil removal is undesirable, very expensive and often even not feasible.

Organic pollutants are also removed from the environment by various natural processes: dilution, sorption, volatilization, and chemical or biological transformation. Together, these natural processes of pollutant removal are summarized by the term 'natural attenuation' (Röling and van Verseveld, 2002). Although all of these processes may reduce the environmental risk of pollutants, only chemical or biological transformation result in alteration of chemical structures of pollutants, and can result in harmless products. However, under prevailing environmental conditions chemical transformation is often a slow process with half-life times of several decades (Jeffers *et al.,* 1989). Therefore, biological transformation and degradation of organic pollutants (biodegradation) is an important process in nature (Mulligan and Yong, 2004). A highly diverse group of microorganisms biodegrades a wide range of compounds, and uses specific catabolic pathways for pollutant biodegradation to safe products. As a result, bioremediation based on biological transformation and biodegradation is an effective way to decontaminate polluted locations (Lovley, 2003; Watanabe, 2001). It is a much less expensive alternative for excavation of polluted soil, without the need for disposal elsewhere, and independent on type of land-use.

The U.S. Environmental Protection Agency (EPA) indicated 129 compounds that form a potential hazard for the environment, and listed them as priority pollutants. This list mainly contains chlorinated aliphatic and aromatic compounds. In Europe, the European Union selected 33 priority pollutants based on the EPA-list, including benzene and 1,2-dichloroethane (European Union, 2000). Extensive research during last decades has resulted in better knowledge of biodegradation processes of these pollutants, for example for 1,2-dichloroethane (1,2-DCA), vinyl chloride (VC), tetrachloroethene (PCE), and benzene. This knowledge resulted in bioremediation strategies for these compounds to decontaminate polluted areas in the environment by using microorganisms (Watanabe, 2001). However, also geochemical conditions under which biodegradation processes occur in the environment, like redox potential, pH, temperature, availability of electron acceptors and donors, moisture, bioavailability, and presence of toxic components are crucial. Unfortunately, the local geochemical conditions at polluted locations are often heterogeneous and unknown. As a result, biodegradation is not observed, or biodegradation processes occur, which are not understood (LaPat-Polasko *et al.,* 2005; Volpe *et al.,* 2009). A better understanding of the interaction between biodegradation of organic pollutants and geochemical conditions, however, can be expected to make the application of biodegradation as remediation strategy more successful.

1.2 Factors affecting biodegradation in the environment

Biodegradation of organic pollutants in the environment is affected by geochemical and biotic factors (Fig. 1). Geochemical conditions are crucial for microbial degradation processes, because these are mediated by enzymatic activity and are restricted to a narrow range of optimal conditions (Pandey *et al.,* 2009). An important abiotic factor is availability of alternative electron acceptors. In the aerobic environment, oxygen is the preferred electron acceptor for biodegradation of hydrocarbons to harmless products (Dijk, 2005; Nyyssonen, 2009; Vaillancourt *et al.,* 2006). This is because oxygen results in a higher growth yield for microorganisms, compared to other terminal electron acceptors (Lovley, 1991). However, below the top layers of sediment and soil systems, (the thickness of which can range from some μm to several cm), and also in groundwater, oxygen is hardly present (Coates *et al.,* 1997; Saponaro and Bonomo, 2003; Schink, 2006). In the top layer oxygen is consumed by chemical and biological processes, and the influx from the atmosphere is low. In the absence of oxygen, microorganisms can oxidize organic compounds with alternative electron acceptors, like nitrate, iron(III), sulfate, and CO_2. The energy yield obtained from anaerobic oxidation of hydrocarbons is lower than from aerobic oxidation, and growth rates are lower under anaerobic conditions (Madigan *et al.,* 2003).

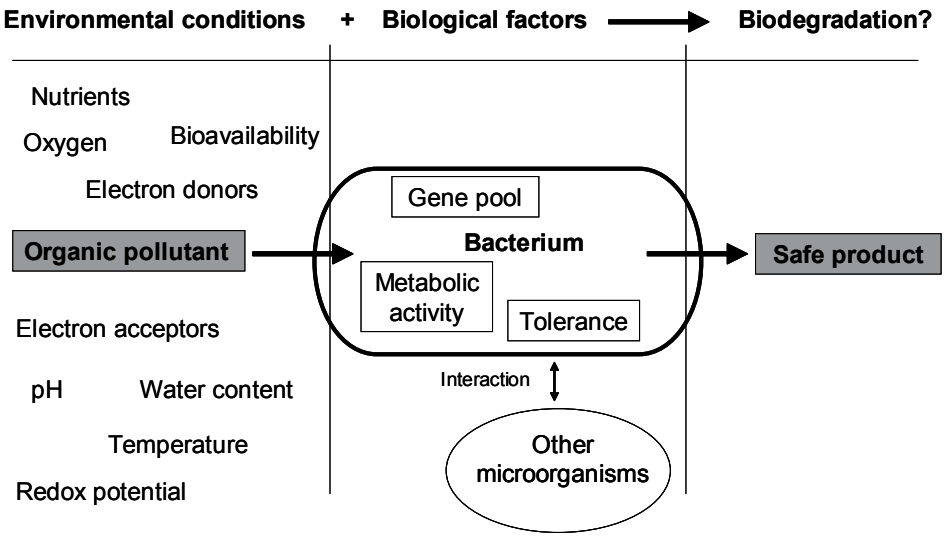


Figure 1: *Schematic overview of environmental and biotic factors that influence biodegradation of organic pollutants to products with reduced toxicity.*

Other important abiotic factors which influence biodegradation are pH, temperature, availability of nutrients, soil characteristics, moisture, pollutant concentration, presence of toxic components and bioavailability (Leahy and Colwell, 1990). The optimal conditions for biodegradation vary, depending on both type of pollutant and microorganism. For example, the optimal pH and temperature for aerobic benzene degradation by *Planococcus* strain ZD22 are 9.5 and 20˚C, respectively, but are 7 and 32˚C for *Rhodococcus pyrinovorans* (Jung and Park, 2004; Li *et al.,* 2006). The influence of environmental factors on biodegradation can be indirect, and is therefore often difficult to recognize. An example is the positive influence of toluene as co-substrate on biodegradation of petroleum-associated hydrocarbons (Ortiz *et al.,* 2006).

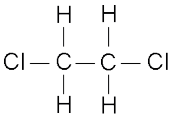
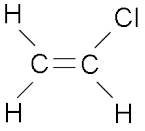
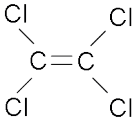
Presence and activity of microorganisms able to biodegrade specific organic pollutants is the most imported biotic factor for biodegradation of these compounds. Related to presence and activity, survival and migration of the right microorganisms is also important for successful biodegradation (Pandey *et al.,* 2009). The capacity of microorganisms to adapt to environmental changes is essential for sustained biodegradation in the environment (Fiorenza and Ward, 1997; Rittmann *et al.,* 2001). For most microorganisms, organic pollutants are an environmental stress factor. The ability of microorganisms to degrade pollutants is often related to resistance to the pollutant. Solvents, like toluene or PCE, accumulate easily in bacterial cell

membranes to toxic levels (Jaworska and Wayne Schultz, 1994). Microorganisms which are resistant to those pollutants developed strategies to deal with this accumulation and these microorganisms can be involved in the biodegradation of the organic solvent (Isken and de Bont, 1998; Ramos *et al.,* 2002).

1.3 Biodegradation processes

For the research described in this thesis, 1,2-dichloroethane (1.2-DCA), vinyl chloride (VC), tetrachloroethene (PCE), and benzene, were selected as model compounds for chlorinated as well as aromatic hydrocarbons, respectively, as they are among the most prevalent environmental pollutants, and because different biodegradation pathways for these compounds are known, and corresponding microorganisms are described. Table 1 summarizes physiochemical properties, production and source data of these compounds.

Table 1: *Properties of the environmental chemicals selected for research described in this thesis. Data obtained from Material Safety Data Sheet (MSDS) and (Field and Sierra-Alvarez, 2004; Weissermel and Arpe, 2008)*

	1,2-DCA	VC	PCE	Benzene
Chemical structure				
Molecular Weight	98.9	62.5	165.8	78.1
Water solubility (mg/L)	8700	2700	150	1780
Vapor pressure (mmHg)	64	2280	15	75
Production (in million tons per year, in the year 2000)	20*	31	0.3	30
Natural source	Not known	Oxidation of organic soils	Vulcanic activity,	Vulvanic activity, forest fire
Anthropegenic source	Solvent, intermediate PVC production	Industrial PVC production	Solvent (textile and metals cleaning), chemical intermediate	Industrial intermediate, solvent
Toxic effects	Liver damage, probably carcinogenic	Carcinogenic	Liver and kidney damage, probably carcinogenic	Carcinogenic, causes narcosis, reduction in blood pressure

**in North America, Western-Europe and Japan.*

1.3.1 Biodegradation of VC and PCE

With oxygen, VC can be metabolized by e.g. *Pseudomonas* sp., *Mycobacterium* sp., and *Orchobactrum* sp. to CO_2, H_2O, and Cl^- using an epoxyalkane coenzyme M transferase as key enzyme (Danko *et al.,* 2006). Highly chlorinated ethenes, such as PCE, are aerobically recalcitrant, but can be degraded via an anaerobic pathway (Haston and McCarty, 1999; Middeldorp *et al.,* 2005). Nevertheless, co-metabolic PCE degradation by toluene monooxygenase has been shown under aerobic conditions (Ryoo *et al.,* 2000). In general, the more electron-withdrawing substitutions are present in a molecule (e.g. highly chlorinated molecules), the more easily the molecule is biodegraded under anaerobic conditions (Dijk, 2005).

Under anaerobic conditions, VC and PCE can be dechlorinated via an energy-yielding process of organohalide respiration. In this process, VC and PCE are used as terminal electron acceptor, in contrast to oxidative degradation pathways, where organic pollutants are used as electron donor. Organohalide respiration of chlorinated ethenes is a well-studied process, whereby PCE is dehalogenated sequentially with reductive dehalogenase enzymes via trichlorethene (TCE), dichloroethene (DCE), and VC, to ethene (Cheng *et al.,* 2010; McCarty, 1997) (Fig. 2). *Dehalococcoides* and *Dehalobacter* spp. are highly specialized bacteria, which exclusively use organohalide respiration for growth. Other genera that use reductive dehalogenation for growth, but can also use other metabolic processes, are for example *Desulfovibrio, Desulfitobacterium*, *Sulfurospirillum*, *Desulfomonile*, *Desulfuromonas*, *Geobacter* and *Trichlorobacter* (Smidt and de Vos, 2004). Different microorganisms can dehalogenate PCE to TCE, DCE or VC. However, only members of *Dehalococcoides* spp. are known to be able to reductively dechlorinate VC (He *et al.,* 2003).

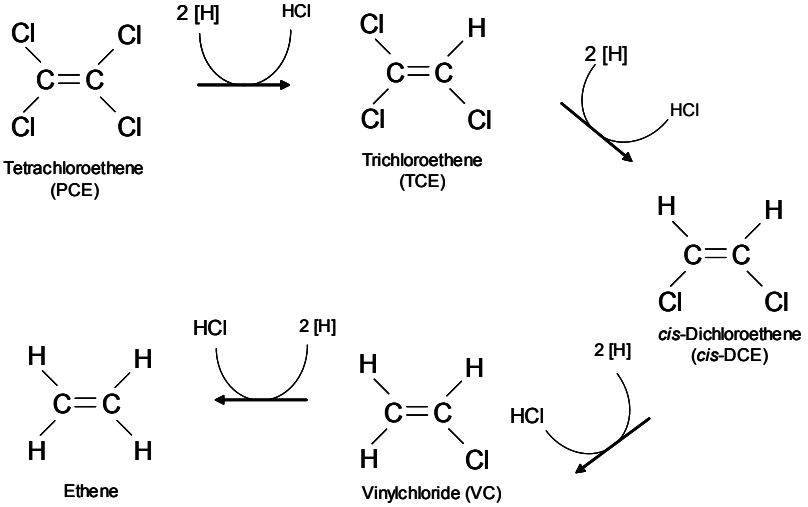


Figure 2: *Reductive dechlorination pathway of PCE, via TCE, cis-DCE, and VC to ethene.*

1.3.2 Biodegradation of 1,2-DCA

Under aerobic conditions, 1,2-DCA can be completely oxidized to CO_2, H_2O, and Cl^- by different microorganisms, including *Xanthobacter autotrophicus* and *Ancylobacter aquaticus* (Janssen *et al.,* 1985; van den Wijngaard *et al.,* 1992). The biodegradation is mediated by haloalkane dehalogenases and monooxygenases (Hage and Hartmans, 1999; Janssen *et al.,* 1994). Under anaerobic conditions, 4 different degradation pathways are described for biodegradation of 1,2-DCA. *Dehalococcoides ethenogenes* strain 195 and *Desulfitobacterium dichloroeliminans* strain DCA1 are bacterial isolates which can convert 1,2-DCA to ethane by organohalide respiration (de Wildeman *et al.,* 2003b; Maymo-Gatell *et al.,* 1997). Furthermore, some strains of methanogens are known to reductively dechlorinate 1,2-DCA to chloroethane and ethane, albeit co-metabolically (Holliger *et al.,* 1990). Anaerobic oxidation of 1,2-DCA with nitrate or iron as alternative electron acceptor has been reported by different research groups (Dinglasan-Panlilio *et al.,* 2006; Gerritse *et al.,* 1999b). Likely, 1,2-DCA can be in addition be degraded by a fermentative pathway (Dijk, 2005), however, this is not fully understood yet and therefore not indicated in Fig. 3, which schematically summarizes the biological and abiotic degradation pathways of 1,2-DCA.

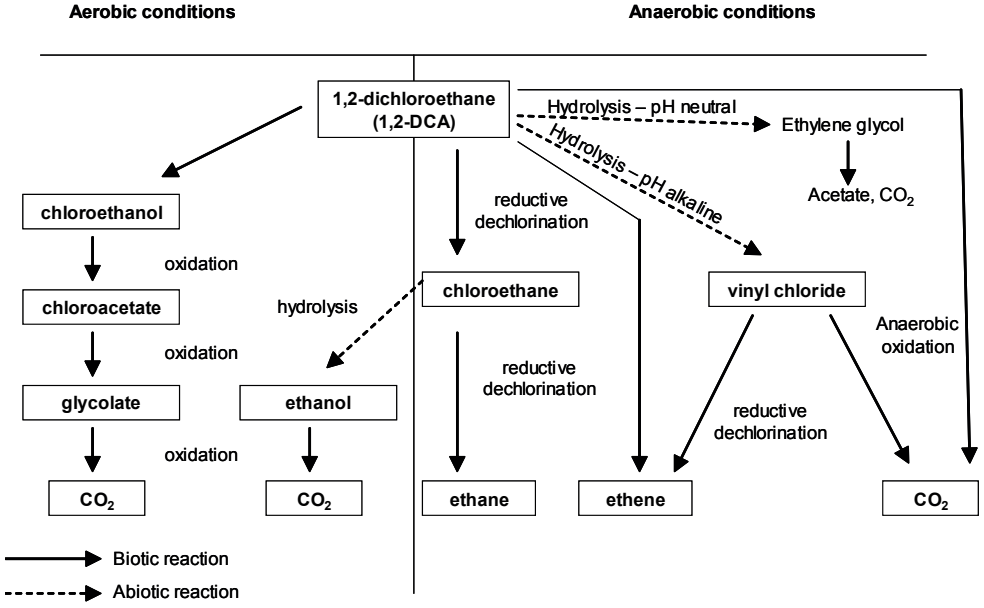


Figure 3: *Degradation pathways for 1,2-dichloroethane under aerobic and anaerobic conditions. (Adapted from (Cox et al., 2000)).*

1.3.3 Biodegradation of benzene

In the presence of oxygen, benzene can be readily biodegraded by different micro-organisms, for example by *Pseudomonas* sp. which use benzene as sole carbon and energy source (Gibson and Parales, 2000). During aerobic biodegradation of benzene, oxygen is not only used as electron acceptor, but is also involved in the first enzymatic activation of benzene. Mono- or dioxygenase enzymes catalyze the incorporation of oxygen into the aromatic ring-structure. After this crucial step, benzene can further be degraded to CO_2 and H_2O.

Although anaerobic biodegradation of benzene was thought for a long time to be impossible, anaerobic oxidation of benzene has now been demonstrated with nitrate, carbon dioxide, iron or sulfate as electron acceptor (Anderson *et al.,* 1998; Grbic-Galic and Vogel, 1987; Kazumi *et al.,* 1997; Lovley *et al.,* 1995; Rooney-Varga *et al.,* 1999). The biochemical pathways of anaerobic benzene degradation and involved enzymes are not well understood, but different possible mechanisms have been hypothesized, including carboxylation to benzoate, hydroxylation to phenol and methylation to toluene (Chakraborty and Coates, 2004; Coates *et al.,* 2002; Ulrich *et al.,* 2005). A few bacteria have been isolated that can degrade benzene with nitrate as electron acceptor, including two *Dechloromonas* strains and two *Azoarcus* strains (Coates *et al.,* 2001; Kasai *et al.,* 2006). *Alicycliphilus denitrificans* strain BC grows on benzene with chlorate as electron acceptor, however, this is a special case in anaerobic benzene degradation, as molecular oxygen is formed during chlorate reduction (Weelink *et al.,* 2008). Anaerobic benzene-degrading microorganisms using sulfate, iron or carbon dioxide as electron acceptors have not been isolated and identified yet.

1.4 Bioremediation strategies

Different bioremediation strategies are developed to clean-up environmental pollution by using microorganisms. Which bioremediation method is used at a specific location depends on local conditions, like type and scale of pollution, and geographical position of the pollution in relation to the urban environment. There are three main strategies for bioremediation: 1) monitoring the natural process of biodegradation ('intrinsic bioremediation'); 2) stimulating the natural biodegradation process ('biostimulation'); 3) addition of microorganisms able to biodegrade the pollution ('bioaugmentation'). These strategies can be applied at the polluted location itself (*in situ*), or the polluted soil or water is removed and treated elsewhere (*ex situ*). This paragraph focuses on *in situ* bioremediation.

1.4.1 Intrinsic bioremediation

During intrinsic bioremediation, or passive bioremediation, organic compounds are degraded by the microbial community intrinsically present at the contaminated location (Rittmann, 2004). This strategy is risk-based: it can be applied when it is likely that the pollutant is degraded to acceptable levels and when the pollutants do not form a direct and serious threat to the environment (Chen *et al.,* 2006; Kao *et al.,* 2010; Slenders *et al.,* 2005; Trigo *et al.,* 2009). Although the method is also referred to as "doing nothing", intensive monitoring of the pollution and corresponding biodegradation is essential to identify the progress of bioremediation, providing for example information as to whether the biodegradation process is complete, or whether biodegradation only takes place at parts of the polluted location (Wilson *et al.,* 2004). Further, the plume of the pollution might spread in time over a larger area, causing problems in the surrounding area. Therefore, monitoring should occur frequently and in a wide area in and outside the polluted location. Based on monitoring results, it can be judged if the actual situation can be maintained, or whether alternative (bio)remediation strategies should be implemented (Carey *et al.,* 2000) (Fig. 4).

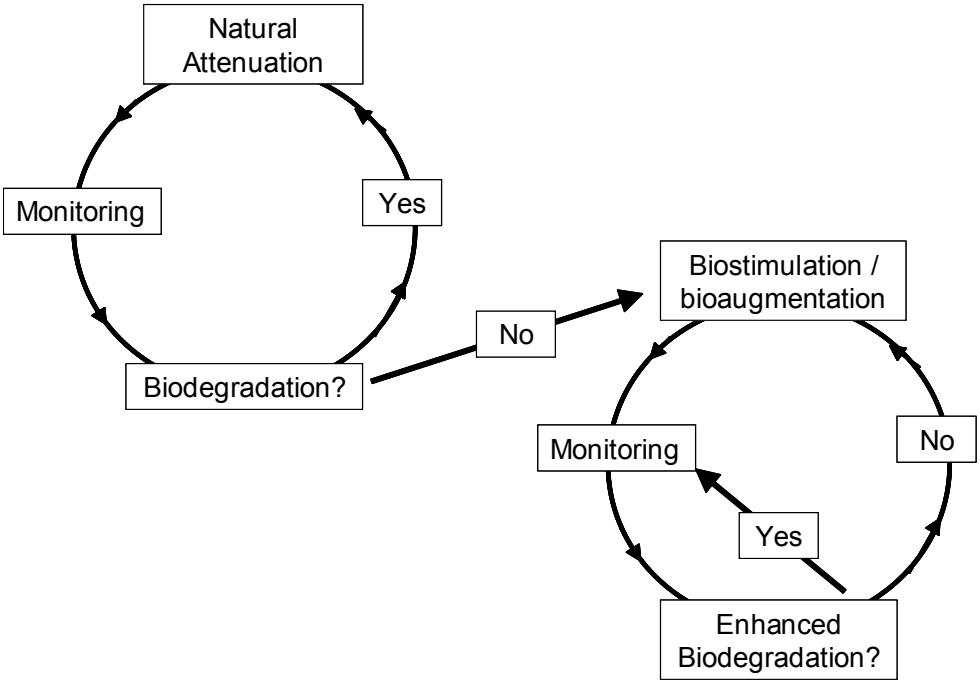


Figure 4: *The most suitable bioremediation strategy is selected based on monitoring of the biodegradation process.*

1.4.2 Biostimulation

By biostimulation, the natural process of biodegradation is enhanced by optimizing environmental conditions for the pollutant-degrading microorganisms, with the goal to stimulate their activity and growth (Major *et al.,* 2002). The environmental conditions can be optimized in various ways, for example by addition of electron donor or acceptor, addition of nutrients (organic carbon, nitrogen, phosphate, etc), adjusting the pH, or enhancing the bioavailability of the pollutant (Menendez-Vega *et al.,* 2007). Locations polluted with chlorinated ethenes are often limited for electron donor to biodegrade these pollutants via reductive dechlorination. In those cases, bioremediation can be stimulated by addition of a substrate like organic acids or alcohols (Christ *et al.,* 2005). Another common biostimulation activity is the introduction of oxygen as additional electron acceptor in a polluted area (Hickman and Reid, 2008). A serious risk in stimulated bioremediation is clogging of the stimulated soil system, since microorganisms may form biofilms, or compounds can precipitate when the processes are not fine-tuned (Da Silva *et al.,* 2005; Zhao *et al.,* 2009).

New technologies are in development to combine biostimulation of polluted locations with other environmental processes or techniques. One example is the simultaneous biodegradation of different pollutants, whereby one pollutant acts as electron donor and the other as electron acceptor, such as the integrated bioremediation of locations polluted with benzene or toluene and chlorinated solvents (Raynal and Pruden, 2008; Sewell and Gibson, 1991; Shen and Sewell, 2005; Weidhaas *et al.,* 2009). Also is proposed to combine bioremediation with the aboveground use of geothermal heat. Temporal storage of heat and mixing of groundwater in the subsurface can potentially enhance bioremediation processes.

Biostimulation selectively enriches dedicated degraders present within the intrinsic microbial community, however, when capable microorganisms for biodegradation of the pollutant are not present at significant concentrations, biostimulation is not effective. Adaptation of the microbial community can finally result in biodegradation of the pollutant, but this is often a slow process (Leahy and Colwell, 1990; van Beelen and Doelman, 1997). When biostimulation is not effective, it can be useful to introduce pollutant-degrading microorganisms, an approach that is also referred to as bioaugmentation.

1.4.3 Bioaugmentation

Bioaugmentation is stimulation of the biodegradation process by exogenous introduction of pollutant-degrading microorganisms (Major *et al.,* 2002). These microorganisms can originate from another polluted location, or be pre-cultivated in the laboratory. Bioaugmentation is often combined with biostimulation, to enlarge the chance of successful biodegradation, as shown for different organic pollutants, including PCE and chlorinated dioxins (Ahn *et al.,* 2008; Boon *et al.,*

2003; Major *et al.,* 2002; van Bemmel *et al.,* 2007). As for intrinsic bioremediation, monitoring the biodegradation process is essential during bioaugmentation to evaluate and control the effect of stimulation activities on pollutant degradation. Therefore, monitoring tools are required which can specifically identify and distinguish the biodegradation process among other biological or geochemical processes at the polluted location. In paragraph 1.5, different methods for the monitoring of bioremediation are described and discussed.

1.5 Monitoring biodegradation

Biodegradation processes must be monitored when they are applied as bioremediation strategy. For intrinsic bioremediation, authorities often require monitoring to demonstrate that the pollutant is biodegraded in the estimated timeframe (Rittmann, 2004). During biostimulation and bioaugmentation, monitoring is essential to investigate whether further intervention in the process is necessary. Many monitoring methods have been developed to identify and follow the biodegradation processes, but not all of them can specifically identify the responsible microorganisms and/or enzymes, or can be applied for large heterogeneous polluted locations. Currently, two categories of monitoring methods for bioremediation are used: geochemical and microbiological methods (Bombach *et al.,* 2010b). The microbiological methods include both culture-dependent and culture-independent methods (Fig. 5). The advantages and disadvantages of different monitoring methods are summarized in Table 2.

1.5.1 Geochemical methods

Sensitive chemical analysis methods are available to identify and quantify specifically a broad range of pollutants, even in a complex environmental sample that contains a mixture of different compounds. This offers the opportunity to monitor simultaneously concentrations of pollutants and their likely degradation products over time. The chemical analyses are generally relatively cheap and indicate whether degradation via a microbial process is likely. However, observed changes in concentrations can also be the result of abiotic processes, like dilution, volatilization or chemical transformation. Therefore, it is hard to identify biodegradation based on chemical analysis of the pollutant and its degradation products alone.

To obtain additional evidence that organic pollutants are biodegraded, geochemical conditions at polluted locations are monitored (Wiedemeier *et al.,* 1998). For example, monitoring available electron acceptors (NO^{3-}, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2) and their reduced redox species (NO_2^-, NH_4^+, Mn^{2+}, Fe^{2+}, S^{2-}, and CH_4) offers information with respect to the type of biological reactions that can occur (Christensen *et al.,* 2001; Vroblesky and Chapelle, 1994). However, also geochemical conditions provide no direct evidence for biological degradation, only which biological processes can theoretically occur.

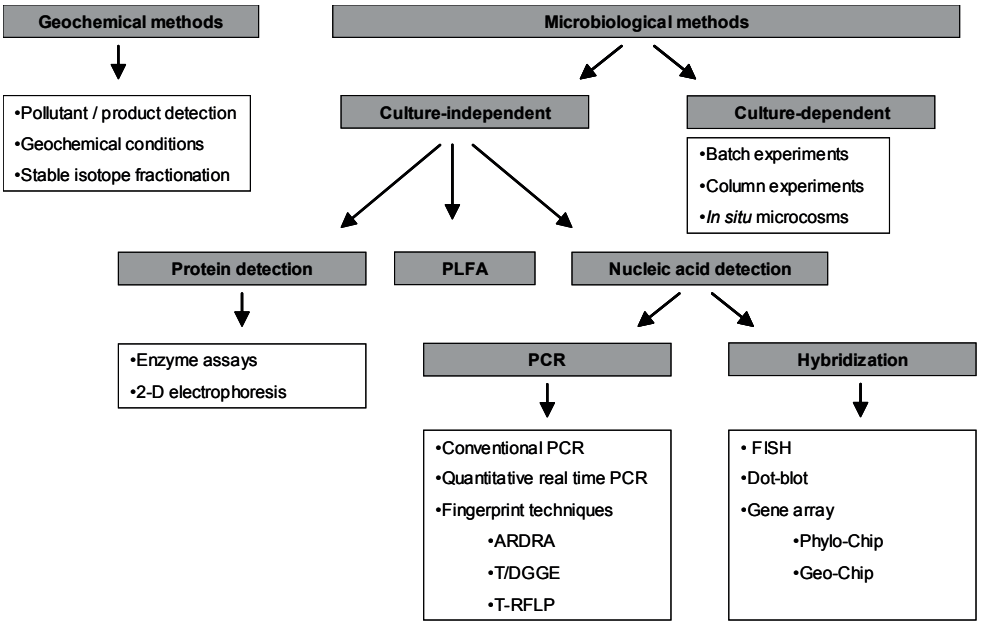


Figure 5: *Overview of methods actually used to monitor biodegradation processes in the environment.*

By the use of compound-specific stable isotope ratios (stable isotope fractionation), direct evidence for (bio)degradation of organic pollutants can be obtained (Meckenstock *et al.,* 2004). This method has already been used for chlorinated hydrocarbons, BTEX, alkanes, and MTBE (Bombach *et al.,* 2010b). Apart from qualification of the biological process, shifts in compound-specific isotope ratios can be used for quantification of the biodegradation rates with the Rayleigh equation-based approach (Meckenstock *et al.,* 2004). A limitation of stable isotope fractionation is that it is effective for small molecules, but not for large molecules like polycyclic aromatic hydrocarbons (PAHs), because the isotope ratio of intermediates and end-products of the degradation of these large compounds can not be distinguished from the isotope ratio of the un-transformed pollutant. Furthermore, isotope ratio shifts observed in the field can be the result of two or more microbiological processes, thus individual biodegradation processes are not easily identified. In addition to detection of current activity, stable isotope fractioning can also provide information with respect to historical biodegradation processes (Elsner *et al.,* 2005; Meckenstock *et al.,* 2004). Nevertheless, although repeated stable isotope measurements during bioremediation will show actual biodegradation, the method is not often used for routine bioremediation monitoring procedures, since isotope analyses are expensive.

Table 2: *Overview of the most important advantages and disadvantages of described methods to monitor biodegradation processes in the environment*

	Monitoring method	Advantage	Disadvantage
Geochemical methods	Pollutant / product detection	Specific, fast, cheap	No discrimination biotic and abiotic processes
	Geochemical conditions	Indication which biological processes can occur	No direct evidence that biological processes occur
	Stable Isotope Fractionation	Direct evidence for degradation	Detects historical degradation, and is no routine method
Microbiological (culture-dependent)	Batch experiments	Discriminate biotic and abiotic processes, biodegradation potential	Not representative for environmental situation
	Column experiments	As batch experiments, but under environmental conditions	Results not representative for whole polluted location
	In situ microcosms	As batch experiments, but under environmental conditions	Results not representative for whole polluted location
Microbiological (culture-independent)	Proteins	Actual expresses biological processes	
	- Enzyme assays	Fast, specific	Only available for known enzymes
	- 2-D Electrophoresis	Holistic, identify unknown but relevant proteins	Laborious, problematic protein separation for complex environmental samples
	PLFA	Phylogenatic identification	Discriminative capacity depends of phylogenetic group
Nucleic acids-hybridization	FISH	Fast, no cultivation or DNA/RNA extraction	Inefficiency detection depends on microbial specie and sample
	Dot blot	Specific detection of genes	Laborious, detection of one gene per analyses
	Gene array	Simultaneous detection of many phylogenetic or functional genes	Laborious, expensive, requires advanced biostatistics analyses
Nucleic acids-PCR	Conventional PCR	Specific, fast and sensitive detection of phylogenetic and functional genes	Not quantitative
	Quantitative real time PCR (qPCR)	As conventional PCR, but quantitative	Availability of target genes is limited
	Fingerprint techniques	Monitoring of changes within microbial community composition	Laborious, not useful as routine monitoring method

1.5.2 Culture-dependent microbiological methods

Culture-dependent methods are useful to distinguish between biotic and abiotic processes, and to isolate microorganisms responsible for specific processes occurring in the environment. This is useful to make a link between microbial activity and the biodegradation process (Pandey *et al.,* 2009). As a result, the current knowledge of microbial degradation processes is mainly based on cultivation-dependent methods. Batch studies give insight into the biodegradation potential, rates and efficiency, or toxic effects, of the tested pollutants in the sample material (Menendez-Vega *et al.,* 2007). To this end, material from a polluted location (e.g. groundwater, soil, or sediment), is incubated with a pollutant under defined conditions. MPN incubations enable quantification of the present microorganisms (Swanson *et al.,* 2001). However, defined laboratory conditions are often not representative of environmental conditions, like availability of nutrients or substrate fluxes. This limits possibilities to translate the results of batch experiments to the *in situ* situation (Bombach *et al.,* 2010b; Röling and van Verseveld, 2002). This is further supported by studies with pure cultures which show that generally less than 1% of all bacteria can be cultured, and that viable microorganisms which perform metabolic activity, can become temporally uncultivable (Amann *et al.,* 1995; Barer and Harwood, 1999). This last phenomenon is a physiological state of the microbial cell, known as the viable but not cultivable state (VBNC). Thus, laboratory batch experiments do not provide unequivocal information with respect to *in situ* biodegradation at polluted locations, but rather indicate the potential biodegradation capacity.

Column experiments (or mesocosms) and *in situ* microcosms (microcosms that are placed e.g. inside a well directly within an aquifer (e.g. BACTRAPs) are used to monitor biodegradation under environmental conditions (Stelzer *et al.,* 2006). Therefore, these experiments are more representative for the *in situ* biodegradation process than batch incubations. Nevertheless, also results of column experiments and *in situ* microcosms can not be translated without problems to the field situation. The samples represent only part of a polluted location, and the biogeochemical conditions might be different at other parts of the location due to heterogeneity (Bombach *et al.,* 2010b). To overcome limitations of culture-dependent methods to monitor biodegradation processes, culture-independent methods are used.

1.5.3 Culture-independent microbiological methods

By culture-independent methods, the presence or activity of microorganisms is detected based on molecules present in, or produced by, microbial cells (Lovley, 2003). The advantage of these molecular detection methods is that there is no need to cultivate microorganisms. The detected molecules can be proteins, like enzymes, or nucleic acids, like DNA and RNA, or lipids. Enzymes are produced by microorganisms and catalyze specific metabolic processes, for example haloalkane dehalogenase enzymes which catalyze the aerobic degradation of 1,2-DCA in *Xan-*

thobacter autotrophicus. The presence of specific enzymes can be identified by, for example, a colorimetric enzyme assay, and indicates the potential microbial activity for biodegradation (Holloway *et al.,* 1998). DNA contains unique hereditable genetic information of (micro)organisms on chromosomes and/or mobile genetic elements such as plasmids and transposons. The genetic code on genes contains information about the phylogenetic identity of the microorganism or the potential (metabolic) activities that these microorganisms can perform. When a specific gene is active (is expressed) in a microbial cell, RNA molecules of this gene are synthesized. Therefore, information about microbial activity is obtained by detection of RNA molecules.

Apart from detection of cell molecules, like proteins or nucleic acids, also complete microbial cells can be visualized with culture-independent methods, as microscopy. However, there are limited possibilities to identify microorganisms based on their morphology. Moreover, cell morphology is not related with cell function (Riley *et al.,* 2001).

1.5.3.1 Protein-based detection

Monitoring biodegradation processes in the environment based on proteins has the advantage over nucleic acid detection, that proteins represent the actual catalysts conferring a given activity of interest. Apart from colorimetric enzyme assays, 2-D electrophoresis can be used to monitor the expression of genes to proteins. By 2-D electrophoresis, all proteins present in a microbial community are extracted, separated, and can be identified (Berth *et al.,* 2008; Gonzalez-Fernandez *et al.,* 2008). Unfortunately, the 2-D electrophoresis method is laborious, and difficulties are known with isolation of different proteins from environmental samples. These limitations causes that 2-D electrophoresis is not commonly used as monitoring method for biodegradation processes, but mainly to identify the presence of specific proteins in laboratory studies on pure cultures. However, with the growing interest for methods to identify gene activity, protein detection methods (proteomic tools) are improved rapidly. By combining 2-D electrophoresis with mass-spectrometry (MS), isolated proteins can be identified immediately (Benndorf *et al.,* 2007; Jehmlich *et al.,* 2008; Kalyuzhnaya *et al.,* 2008; Morris *et al.,* 2006; Smith *et al.,* 2008). These proteomic method offers possibilities to identify microbial activity for biodegradation of organic pollutants, and has been shown valuable for insights into diversity of biodegradation enzymes for vinyl chloride (Chuang *et al.,* 2010).

1.5.3.2 Phospholipid fatty acids

Phospholipid fatty acids (PLFA) form an integral component of all microbial cell membranes. Since the composition of PLFAs varies per bacterial group, PLFA are used for phylogenetic identification of microbial species (Kohring *et al.,* 1994). The resolution for phylogenetic identification based on PLFA-analysis is lower than based on nucleic acids (parapraph 1.5.3), but can be useful in some specific situations when phylogenetic identification based on nucleic acids alone is not specific enough (Kohring *et al.,* 1994).

1.5.3.3 Nucleic acid-based detection

Nucleic acids are detected by polymerase chain reaction (PCR) based methods, or by hybridization. Other detection methods are available, like NASBA, but are not discussed here. Now also by direct shot gun sequencing, using next generation technology sequencing, nucleic acids can be detected relatively easily in environmental samples. PCR based methods are discussed in paragraph 1.6.

Hybridization methods are useful to interpret the survival of specific microorganisms, or presence of genes, under environmental conditions. Three different hybridization based methods are used to monitor biodegradation in the environment: Fluorescent *in situ* Hybridization (FISH), dot blot hybridization, and gene array technology. By FISH, the presence of specific microorganisms is identified based on sequence-specific binding of a fluorescent-labeled probe to target DNA (or RNA) in the microbial cell, without cultivation or DNA extraction (Wagner *et al.,* 1994). Therefore, the method is useful for rapid detection, however, FISH has an inefficient detection for some microorganisms or in relatively small sample volumes (Pandey *et al.,* 2009). FISH is also not easy to use for samples with a solid matrix, e.g. soil or sediment. By dot blot hybridization, microorganisms degrading pyrene or other organic pollutants were detected in *in situ* bioremediation studies, after DNA:DNA hybridization of a probe and DNA samples (Pandey *et al.,* 2009). Unfortunately, the method is laborious and only a single gene can be targeted per analysis. Therefore, it is not commonly used to monitor biodegradation in the environment. By "reverse dot blot", a sample is hybridized with a blot containing different probes. This is a low-cost version of a micro-array.

The gene array technology (micro-array) offers the possibility to analyze presence of thousands of genes simultaneously in a single sample. Phylogenetic gene arrays, like the PhyloChip, target thousands of rRNA gene sequences, to identify the microbial community composition (Yergeau *et al.,* 2009). These gene arrays have been used to identify the composition of microbial communities in soils, aquifers, fresh water, waste water, and air (Nyyssonen, 2009). Functional gene arrays, like the GeoChip, combine an enormous amount of target genes involved in nutrient cycling, metal resistance, or biodegradation (He *et al.,* 2007). Functional gene arrays are successfully used in different environments to profile degradation capacity of the microbial community (Berthrong *et al.,* 2009; van Nostrand *et al.,* 2009). For

bioremediation studies, genes arrays are not (yet) used commonly to monitor biodegradation processes. This is because this relatively new technology is expensive, and it brings an enormous amount of information that needs to be analyzed by advanced biostatistics analysis. Only a few laboratories are specialized for these gene array technology and corresponding biostatistics. Moreover, the functional gene arrays were initially developed to monitor nutrient cycling and metal resistance, and contained limited amount of genes to monitor biodegradation processes of organic pollutants. Recently, a new generation of GeoChips was developed, which contain more genes involved in biodegradation of organic pollution (He *et al.,* 2010). Likely, in near future this new GeoChip will be well used to monitor biodegradation of organic pollution.

1.6 Use of PCR-based techniques in bioremediation

To monitor bioremediation processes in the environment, it is important to use sensitive, specific and fast methods. PCR meets these requirements and is therefore commonly used to monitor biodegradation (Lovley, 2003; Röling and van Verseveld, 2002). Dependent on the gene targets used in PCR analyses, the presence of pollutant-degrading microorganisms or key enzyme-encoding genes involved in the degradation process can be determined (Erb and Wagner-Dobler, 1993; Whiteley and Bailey, 2000). For phylogenetic identification of microorganisms, the 16S ribosomal RNA (rRNA) gene is often used as target sequence. These 16S rRNA genes are highly conserved between different species of Bacteria and Archaea and therefore can function as a phylogenetic marker (Weisburg *et al.,* 1991). To monitor biodegradation, PCR targets can also be genes coding for key enzymes in the degradation process of a pollutant (functional genes).

1.6.1 Conventional and quantitative real time PCR

Conventional PCR provides qualitative data about a target gene, but no conclusions can be drawn on the dominance of the detected gene within the microbial community. Theoretically, one single gene copy can be detected with PCR, however, a single copy is not relevant from an ecological point of view (Gilliland *et al.,* 1990). By real-time measurement during the amplification process, and comparing to standards with a known amount of gene copies, the target gene can also be quantified (qPCR) (Mackay, 2004). This offers the opportunity to identify increases or decreases of a target gene in time and space during bioremediation studies. In different studies, both 16S rRNA or functional genes are monitored by qPCR to follow the biodegradation process of, for example, chlorinated ethenes and 1,2-DCA (Cupples, 2008; Raemdonck *et al.,* 2006). Quantification of 16S rRNA genes is relevant when specific (groups of) microorganisms are responsible for the biodegradation of the pollutant. For example, in a study of anaerobic oxidation of benzene with iron as electron acceptor, enrichment studies suggested that microorganisms closely related to *Geobacter* spp. were responsible for the observed benzene degradation (Rooney-Varga *et al.,* 1999). *Geobacter* spp. 16S rRNA genes were used as indicator for benzene-oxidation under iron-reducing conditions. Another example is the detection of 16S rRNA genes of *Desulfitobacterium* sp, *Dehalobacter* sp., and *Dehalococcoides* sp. for the monitoring of reductive dechlorination of chlorinated ethenes, because those are the most important microorganisms known to be responsible for this process.

Unfortunately, often phylogeny cannot be unequivocally related to functionality. Microorganisms with divergent phylogeny can perform the same specific process, like reductive dechlorination of PCE (Smidt and de Vos, 2004). Moreover, phylogenetic groups include strains which do not perform a specific process. An example is reductive dechlorination of vinyl chloride (VC), a process only known to be performed by specific strains within the genus *Dehalococcoides*. Therefore, quantification of *Dehalococcoides* spp. 16S rRNA genes will result in an overestimation of VC-dechlorinating microorganisms, since only three *Dehalococcoides* sp. strains (strain VS, strain BAV1, and strain GT) are currently known to metabolize VC (Krajmalnik-Brown *et al.,* 2004; Muller *et al.,* 2004; Sung *et al.,* 2006). To this end, functional gene targeted qPCR offers the opportunity to specifically monitor the potential for biodegradation of certain pollutants. A difficulty with functional gene targets is that development of primers and probes depends on availability and diversity of degrading strains (Röling and van Verseveld, 2002). Further, functional genes in phylogenetically different microorganisms are often similar, but not the same. This limits the possibilities for design of comprehensive functional gene targeting primers, even for microorganisms which are closely related (Ritalahti *et al.,* 2006). For this reason, the amount of available gene probes to detect key enzymes or microorganisms, involved in organic pollutant degradation, is still limited.

1.6.2 Fingerprint techniques

Changes in the microbial community during biodegradation are caused by enrichment of degrading microorganisms, or by toxic effects of the pollutant or its degradation products. The composition of a microbial community can be characterized with molecular fingerprinting techniques, like amplified rDNA restriction analysis (ARDRA), denaturing / thermal gradient gel electrophoresis (D/TGGE), or terminal restriction fragment length polymorphism (T-RFLP) (Vaneechoutte *et al.,* 1992). These fingerprint techniques are based on PCR amplification of the 16S rRNA genes isolated from the microbial community, and can be selective for a single bacterial division or smaller group, or the entire bacterial community (Dunbar *et al.,* 2001). Although T-RFLP fingerprinting has been proven instrumental for detection of community dynamics during bioremediation (Pandey *et al.,* 2009), DGGE fingerprinting of total bacteria as well as of specific functional groups, including *Dehalococcoides* spp. and *Geobacter* spp., has been widely used for the characterization of bacterial community changes in polluted environments (Duhamel and Edwards, 2006; Imfeld *et al.,* 2010; Macnaughton *et al.,* 1999; Sleep *et al.,* 2006). Especially the application of advanced multivariate statistics has proven useful in the analysis of large datasets generated by these approaches, including the correlation with environmental factors mentioned above, such as pollutant concentrations, redox, pH, etc. (Tzeneva *et al.,* 2009; Viñas *et al.,* 2005). By next generation sequencing technology, 16S rRNA gene based fingerprinting is possible (Morozova and Marra, 2008; Shendure and Ji, 2008). However, no examples are available yet where this technology has been used to identify the biodiversity of pollutant-degrading microbial communities in environmental samples.

1.6.3 Detection of microbial activity

DNA-based monitoring of 16S rRNA, or functional genes, cannot easily be related to microbial activity, because observed target genes could origin from microorganisms, which have been inactive for many years. Although an increase of target genes over time, measured by qPCR, indicates microbial growth, this does not necessarily mean that the process of interest is performed. The link with activity can be made more directly when messenger RNA molecules (mRNA) are used as target in PCR based molecular detection, because these are produced when genes are expressed (Han and Semrau, 2004). Individual bacterial mRNA molecules survive in the cells only for a short period of time, and therefore microbial cells contain no mRNA of genes that are not (recently) expressed. Detection of RNA molecules is regularly used to identify microbial activity in laboratory studies, but are also applied to monitor bioremediation activity or other environmental studies (Revetta *et al.,* 2010; Tell *et al.,* 2007).

Problems associated with RNA-based PCR detection in biodegradation studies resulted from the instability of the RNA molecules. Often low RNA recoveries are observed from environmental samples, what is problematic when attempting quantification of biodegradation activity (Sessitsch *et al.,* 2002). This limits the possibilities to correlate observed mRNA gene copy numbers directly to microbial activity (Da Silva and Alvarez, 2008; Rahm and Richardson, 2008). Moreover, enzymes involved in biodegradation, and their corresponding mRNA, are often expressed constitutively, e.g. haloalkane dehalogenase of *Xanthobacter autotrophicus* GJ10 (Janssen *et al.,* 1989; Keuning *et al.,* 1985). A better understanding of how detected RNA gene copy numbers from environmental samples correspond to actual biodegradation activity will enlarge the relevance of RNA based detection in bioremediation studies.

1.6.4 Stable Isotope Probing

A pollutant-degrading microbial population can be identified from the microbial community by Stable Isotope Probing (SIP) studies (Chen *et al.,* 2010). With SIP, the microbial community is exposed to a substrate (pollutant) with enriched stable isotope $^{13}C_6$-concentration. Microorganisms that use this substrate as carbon source, incorporate the enriched $^{13}C_6$-concentration in their DNA. By density gradient centrifugation, this 'heavy' DNA can be separated of the other ($^{12}C_6$) DNA. The separated DNA fractions can be identified by cloning and sequencing, specific quantitative PCR or other molecular methods. SIP is a powerful tool to link microorganism identity to microbial function, and can also be combined with RNA-based detection (RNA-SIP) (Neufeld *et al.,* 2007). It has been used to identified microorganisms able to degrade toluene, benzene, PCB, phenol, and different other organic compounds (Bombach *et al.,* 2010a; DeRito *et al.,* 2005; Kasai *et al.,* 2006; Leigh *et al.,* 2007).

1.7 Aim and outline of this thesis

Over the last few decades, our understanding of biodegradation processes of organic pollutants has been improved greatly. This has resulted in a variety of strategies for bioremediation of polluted environments. However, monitoring and control biodegradation in the environment is challenging, because the exact biochemical pathways are often unknown or the available monitoring techniques are not specific enough. A better understanding of the interaction between biodegradation of organic pollutants and local geochemical conditions will result in more successful bioremediation strategies for the future. The aim of this thesis is to obtain better insight into biodegradation of organic pollutants in the environment. In particular, the thesis focuses on the relation between local geochemical conditions and biodegradation activity of chlorinated- and aromatic hydrocarbon-degrading microorganisms, and molecular, cultivation-independent methods to monitor these microorganisms and their metabolic functions in the environment.

In **Chapter 2**, the effect of environmental pollution on microbial communities is described. River sediment was incubated in laboratory mesocosms, and exposed to organic pollutants (1,2-DCA and PCE) or a high concentration of nutrients. Changes in phylogenetic diversity and abundance of various functional microbial groups caused by the applied pollution were investigated with culture-dependent and culture-independent methods. **Chapter 3** describes the degradation capacity of microbial communities in sediments from different European rivers (Ebro, Danube, and Elbe) for 1,2-DCA under various redox conditions. The river sediment was incubated in microcosms under aerobic, denitrifying, iron-reducing, sulfate reducing and methanogenic conditions. Biodegradation was monitored by chemical analysis of 1,2-DCA and its possible biotransformation products. Simultaneously, microorganisms known to biodegrade 1,2-DCA were quantified in the microcosms by real time PCR.

An extensive study to correlate geochemical conditions and reductive dechlorination capacity for vinyl chloride (VC) in polluted groundwater is described in **Chapter 4**. The VC dechlorination potential was identified by qPCR analysis of phylogenetic and functional genes in groundwater from 150 monitoring wells, spread over 11 chloroethene-polluted locations. Correlations between gene copy numbers and the local geochemical conditions were identified by multivariate statistical analyses.

Chapter 5 describes the physiological and phylogenetic characteristics of an anaerobic benzene-degrading microbial community. This microbial community was cultivated under nitrate-reducing conditions in a continuous culture for more than 8 years. Stable isotope probing (SIP) was used to identify the benzene-degrading microorganisms. In **Chapter 6,** the main conclusions of this thesis are summarized and discussed in a broader perspective.

Chapter 2

Stability of total and functional microbial communities in river sediment mesocosms exposed to anthropogenic disturbances

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This chapter has been published as:
Bas van der Zaan, Hauke Smidt, Willem M. de Vos, Huub Rijnaarts and Jan Gerritse (2010), Stability of total and functional microbial communities in river sediment mesocosms exposed to anthropogenic disturbances in FEMS Microbiology Ecology Vol. 74, p. 72-82

Abstract

River systems are exposed to anthropogenic disturbances, including chemical pollution and eutrophication. This may affect phylogenetic diversity as well as abundance of various functional groups within sediment-associated microbial communities. To address such potential effects, mesocosms filled with Ebro delta sediment covered with river water were exposed to chlorinated organic compounds or to a high nutrient concentration as used for fertilization. Changes in abundance of selected functional microbial groups, i.e. total aerobes, nitrate-, sulfate- and iron-reducers, organohalide-respiring microorganisms as well as methanogens, were examined by culture-dependent Most Probable Number (MPN) and culture-independent PCR methods targeting phylogenetic as well as functional gene markers. It was concluded that the abundance of functional groups was neither affected by pollution with 1,2-dichloroethane and tetrachloroethene nor by elevated nutrient loads, although changes in bacterial community composition were observed with 16S ribosomal RNA gene-targeted fingerprint techniques. This study reinforced the notion that complementary culture-dependent and molecular methods, focusing on different fractions of the microbial community (cultivable, active or total), should be used in combination for comprehensive description of phylogenetic diversity and functional potential.

2.1 Introduction

Human activities like spilling and disposal of industrial waste, mining activities and intensive agriculture, have resulted in pollution of many river basins worldwide (Schulz-Zunkel and Krueger, 2009; Skoulikidis, 2009; Weber *et al.,* 2008; Xing *et al.,* 2005). The adverse effects of pollutants, including heavy metals, chlorinated hydrocarbons, fecal pathogens or (in)organic fertilizers, on river ecosystem integrity have been studied extensively (Barceló, 2007; Mañosa *et al.,* 2001). Several studies focused on processes involved in removal of pollutants from river systems (D'Arcy *et al.,* 2007; Moss, 2008; Suehiro *et al.,* 2006). Microorganisms have been recognized to play a key role in nutrient cycling, (im)mobilization of metals, and dehalogenation of organohalide pollutants (Inubushi *et al.,* 2005; Marumoto, 1984; Wenhui *et al.,* 2007). In turn, microorganisms are affected by pollutants. Sensitive species can be replaced by more tolerant species and pollutant-degrading microbes may be enriched (Blanck, 2002). Thereby, the average microbial community tolerance to the pollutant is increased, but its diversity, as measured by the number and relative abundance of microbial species, can be reduced. On the other hand, perturbation can increase the diversity (Bressan *et al.,* 2008). Diversity is essential for stability in a microbial ecosystem (Torsvik and Øvreås, 2002), and a decrease in phylogenetic diversity can result in a loss of functional diversity (Cardinale *et al.,* 2002; Stefanowicz *et al.,* 2008; Wittebolle *et al.,* 2009). Most studies focused on the effect of pollutants on individual species or on changes in biodiversity in general, but little is known about the effect of pollution on specific functional groups within microbial communities. Some studies showed changes in microbial activity, but then unspecific parameters were tested, like biomass and respiration (Salminen *et al.,* 2001; Stefanowicz *et al.,* 2008). Recently, studies with the "Geo-Chip" (He *et al.,* 2007) in soil and groundwater systems demonstrated that the microbial (gene) diversity decreased as a result of pollution, and that key enzymes involved in pollutant degradation were specifically enriched (Liang *et al.,* 2009; Waldron *et al.,* 2009). However, quantitative comparison between various samples is challenging and the method can not easily be used for environmental RNA samples (Nyyssonen, 2009).

In the study presented here, the aim was to identify effects of environmental pollution on the total microbial community and abundance of specific groups in river sediment by combining culture-dependent and culture-independent methods. Sediment from Ebro river (Spain) was used, since different anthropogenic pollutants were detected in this river, and geochemical data were available from previous studies (Barth *et al.,* 2009). The sediment was incubated in mesocosms, and exposed to chlorinated organic compounds or an excess of nutrients, which represent industrial and agricultural contaminants, respectively (ATSDR, 1999; Gotz *et al.,* 1998; Randall and Mulla, 2001; Smith, 2003). Changes in microbial communities were identified based on molecular detection (DNA and RNA), and on cultivation by most probable number (MPN) incubations. Here, we used DNA to represent the total

microbial community, RNA to represent the active fraction of the total community, and MPN as the cultivable fraction of the total microbial community. For specific functional groups, the study focused on nitrate-reducing, iron-reducing, sulfate-reducing, dechlorinating and methanogenic micro-organisms.

2.2 Material and Methods

2.2.1 Mesocosms

A vertical sediment core of 20 cm depth and a diameter of 50 cm was taken from a floodplain in the southern part of the Ebro river delta (N: 40°41'11.8", E: 00°37'31.5") in February 2006. The floodplain area is used for intensive cultivation of rice and situated downstream of industrial plants along the Ebro river. After transport of the sealed sediment core at 10-15°C, it was incubated in the laboratory at 30°C and illuminated with an 80W electric lamp (Philips, white light) for 15 hours a day, to mimic native summer conditions (van der Zaan *et al.,* 2009). The sediment was covered with 1-2 cm Ebro river water that was also sampled in February 2006 in the Ebro delta and stored after sampling in a 5L glass bottle at 4°C. Demineralized water was added regularly to the sediment core to compensate for evaporation. In June 2008, three isolated mesocosms were defined in the core. To this end, 1L glass bottles (inner diameter 9 cm) without bottom were carefully pushed 20 cm into the sediment, to obtain physically undisturbed sediment in the mesocosms. The headspace (300 ml) was closed with a viton stopper (Fig. 1). The water phase was recirculated (900 ml/day) through viton tubing with a peristaltic pump. Mesocosm 1 was defined as reference system without additions. To mimic pollution with chlorinated hydrocarbon compounds, 200 µM 1,2-dichloroethane (1,2-DCA) and 25 µM tetrachloroethene (PCE) was added to the aqueous phase of mesocosm 2. Water in mesocosm 3 was enriched with urea (22.75 mM), NaH_2PO_4 (12.4 mM), Na_2HPO_4 (3 mM) and KCl (12.6 mM), corresponding to nitrogen, phosphate, potassium concentrations of 100 / 75 / 75 kg ha^{-1}, respectively, used for fertilization in agriculture (Lu *et al.,* 2006). The systems were incubated at 30°C for 6 months and exposed 15 hours per day to light, as described above. In mesocosm 3, nitrate, phosphate, and potassium were determined monthly. After 4 months, fresh nutrients (final concentrations in the water phase: N / P / K = 100 / 75 / 75 kg ha^{-1}) were added to mesocosm 3, to ensure that their concentration was above the level in mesocosms 1 and 2.

In situ measurement of pH, redox potential and oxygen level in a depth profile of the mesocosm sediments was done as described below in triplicate at the start and at the end of the 6 month incubation period. Samples for nucleic acid (DNA and RNA) extraction and chemical analysis were taken from the sediment core more

than 1 year before start of the mesocosm experiments (December 2006), and from the mesocosms at t = 0 and t = 6 months of incubation. Samples were taken in duplicate and defined as samples "A" and "B". Each individual sample (A and B) was homogenized and split in two parts to obtain duplicate samples for analysis.

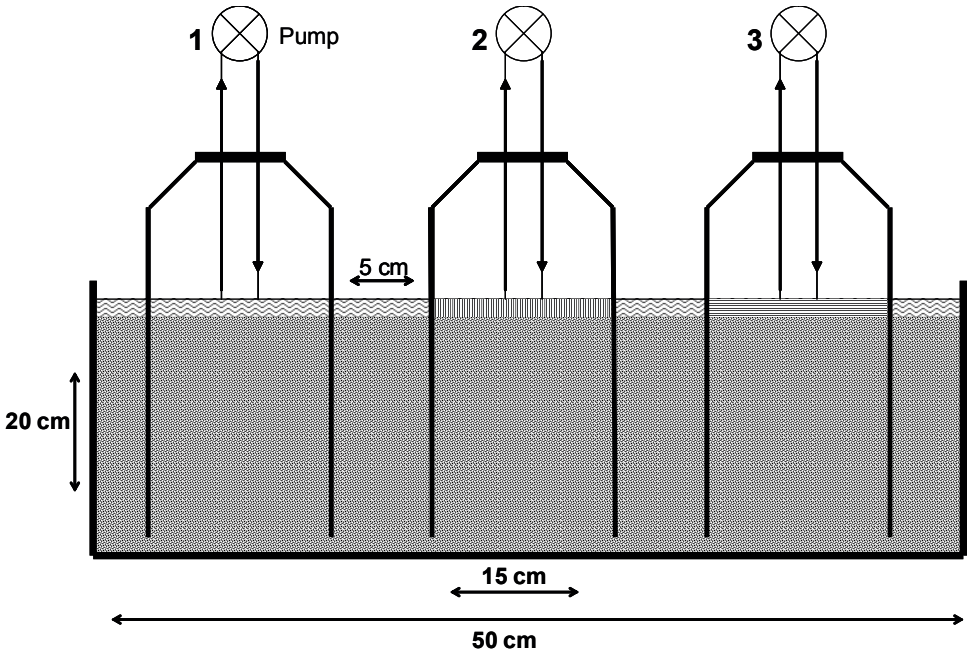

Figure 1: *Experimental setup of the mesocosms. Glass bottles without bottom were pushed 20 cm into the sediment, creating a headspace of about 300 ml closed with a viton stopper. Ebro river water (waved pattern) with 200 µM 1,2-DCA and 25 µM PCE (vertical striped pattern) or extra nutrients (horizontal striped pattern) was recirculated with a peristaltic pump.*

2.2.2 DNA extraction

DNA was extracted in duplicate from 0.5 gram homogenized top 2 cm mesocosm sediment, using the BIO101 Fast DNA Kit for Soil (Qbiogene, Inc, CA), as described in the manufacturer's protocol. Extracted DNA was checked for quantity on a Nanodrop ND-1000 spectrophotometer (Isogen, de Meern, the Netherlands) and for quality by agarose gel electrophoresis and stored for further analysis at -20°C.

2.2.3 RNA extraction

RNA was extracted in duplicate from 2 - 3 g homogenized top 2 cm mesocosm sediment using the RNA Power Soil Total RNA Isolation Kit (MoBio, CA, USA) as described in the manufacturer's protocol. Quantity and quality of the extracted RNA was checked on a Nanodrop ND-1000 spectrophotometer (Isogen, de Meern, the Netherlands). RNA was stored at -80°C.

2.2.4 cDNA synthesis

For downstream PCR analysis of the extracted RNA, cDNA was synthesized from 25 ng RNA with iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands), following the manufacturer's protocol. cDNA was stored at -20°C until further analysis.

2.2.5 DGGE

Diversity of bacterial communities was analysed by denaturing gradient gel electrophoresis (DGGE). Bacterial 16S rRNA gene fragments were amplified from extracted DNA and cDNA by a nested PCR. In the first PCR, complete 16S rRNA gene fragments were amplified with primer mixture (fD1/fD2 and rP1/rP2) (Weisburg *et al.,* 1991) on a C1000 PCR-system (Bio-Rad) using the following cycling program: 95°C for 3 min, 43 cycles 95°C for 30 s, 65°C for 30 s, 72°C for 1 min, a temperature decrease of 1°C for the annealing phase of the first 10 cycles and a time increment of 10 s added to the elongation phase of the last 33 cycles. The PCR product (1 µl) was used as template for a second PCR with forward primer 338GC and reverse primer 907 (Muyzer and Ramsing, 1995) to generate amplicons of about 560 bp with GC-clamp. The cycling program of this second PCR in an ICycler PCR-system (Bio-Rad) was 95°C for 3 min, 35 cycles 95°C for 30 s, 56°C for 30 s, 72°C for 1 min and a final elongation period of 7 min at 72°C. The amplified product (25 µl) was analysed on an 8% (w/v) polyacrylamide gel with a 30% - 70% denaturing gradient (40% acrylamide/bis solution, 37.5 : 1, Sigma-Aldrich) that was run for 16 hours at 60°C and 100V in a Dcode™ Universal Mutation Detection System (BioRad). After electrophoresis, the gel was stained for 45 min with SybrGold (Molecular Probes, Inc, Eugene, OR) in 20 ml 1xTAE. The DGGE profiles were visualized under UV light.

2.2.6 Quantification of functional groups

Bacteria, archaea and different functional groups (nitrate and nitrite-reducers, iron-reducers, sulfate-reducers, dechlorinators and methanogens) present within the total and active microbial communities of the mesocosms, were quantified by real time PCR using DNA or cDNA, respectively, as template. All PCR analyses, except for nitrate- and nitrite-reductase, were performed on an IQ5 Real-Time PCR System (Bio-Rad) with the following temperature program: 95°C for 3 min, followed by 35

cycles 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final elongation step of 5 min at 72°C. For detection of total bacteria, the 16S rRNA gene-targeted primers 519F (Lane, 1991) and 907R (Muyzer and Ramsing, 1995) were used. Primers Arch0025F and Arch344R (Vetriani *et al.,* 1999) were used for amplification of archaeal 16S rRNA genes. Nitrate- and nitrite-reducing microorganisms were quantified by detection of nitrate reductase gene *narG* and nitrite reductase gene *nirS*, with primer pairs narG 1960m2f / narG 2050m2r (López-Gutiérrez *et al.,* 2004) or nirS4QF / nirS6QR (Kandeler *et al.,* 2006), respectively. The PCR temperature program for these two assays was: 95°C for 3 min, followed by 45 cycles of 95°C for 15 s, 58°C for 30 s, 72°C for 30 s, and a final step of 5 min at 72°C. Iron-reducing bacteria were detected by targeting the iron(III)-reducing family *Geobacteraceae* 16S rRNA gene with the primers Geo564F and Geo840R (Holmes *et al.,* 2002). Sulfate-reducing bacteria (SRB) were detected by targeting the *dsrA* gene with primers dsr-1F (Wagner *et al.,* 1998) and dsr-500R (Dhillon *et al.,* 2003). *Dehalococcoides* spp., *Desulfitobacterium* spp. and *Dehalobacter* spp. 16S rRNA genes were selected as representatives of anaerobic reductively dechlorinating bacteria. *Dehalococcoides* spp. was quantified with 1200F and 1270R primers and a fluorescent probe (Ritalahti *et al.,* 2006). *Desulfitobacterium* spp. and *Dehalobacter* spp. were quantified as described by Smits *et al.,* 2004 (Smits *et al.,* 2004). Methyl co enzyme-M reductase (*mcrA*) gene was used as target for quantification of methanogenic archaea and was detected by primer pair ME1F / ME3R (Hales *et al.,* 1996).

2.2.7 Most Probable Number (MPN) incubation

Cultivable aerobic heterotrophic, anaerobic heterotrophic, nitrate-reducing, iron-reducing, sulfate-reducing, dechlorinating or methanogenic microorganisms were quantified by MPN. To this end, 0.5 g mesocosm sediment was suspended in triplicate in 4.5 ml liquid R2A-medium (DSMZ medium 830, www.dsmz.de). From these suspensions, up to 10^9-times diluted cultures in 5 ml specific medium were prepared for all conditions. Specific media for growth of the different functional groups were based on R2A medium. For aerobic heterotrophic microorganisms, the serial dilution in R2A medium was incubated in 18 ml test tubes for 2 weeks under air at 30°C and 150 rpm. Aerobic growth was indicated as increase in optical density (OD_{660} > 0.1) compared to non-inoculated control. All other specific serial dilutions were incubated in 20 ml vials with an N_2-atmosphere, capped with a viton stopper at 30°C and 150 rpm for 3 months. For anaerobic heterotrophic microorganisms, anaerobic R2A medium, reduced with 0.02% $Na_2S.9H_2O$, was used. Anaerobic growth was defined as increase of optical density (OD_{660} > 0.1) compared to a non-inoculated control. For nitrate-reducing bacteria, 2 mM KNO_3 was added to anaerobic R2A medium. Growth of these bacteria was defined as increase of optical density (OD_{660} > 0.1) compared to a non-inoculated control, in combination with a decrease (> 0.2 mM) of NO_3^-. For iron-reducing bacteria, 12 mM FeOOH (Lovley and Phillips, 1986) was added to anaerobic R2A medium and the medium was reduced

with 50 mM $Fe^{(II)}Cl$. Activity of iron-reducing bacteria was defined as a color change of the medium from red/brown to black, indicating that iron-oxides were reduced. Sulfate-reducing bacteria were cultivated in anaerobic R2A medium with 2 mM $Fe^{(II)}SO_4$ that was reduced with 0.1 g/l thioglycolic acid and ascorbic acid. Activity of sulfate-reducing bacteria was defined as formation of precipitate in the medium (FeS), in combination with a decrease (> 0.2 mM) of the SO_4^{2-} concentration. For dechlorinating microorganisms, 200 µM 1,2-DCA and 25 µM PCE were added to anaerobic R2A medium that was reduced with 0.02% $Na_2S.9H_2O$. To detect activity of dechlorinating microorganisms, concentrations of 1,2-DCA and PCE and their possible dechlorination products (chloroethane, vinyl chloride, ethene, and ethane), were analyzed. Activity of methanogens was detected by formation of methane in the anaerobic dilution series.

For the different functional groups, MPN numbers per gram sediment were calculated by comparing the results of the triplicates with an MPN-table (Swanson *et al.,* 2001).

2.2.8 Analytical procedures

1,2-DCA, chloroethane, vinyl chloride, ethene, ethane, and methane were identified and quantified in 500 µl headspace samples from the mesocosms, obtained by using a 1 ml Pressure-Lock gas syringe (Alltech, Breda, the Netherlands) with a sterile needle. The samples were injected on a Varian CP-3800 gas chromatographic system (GC) equipped with a flame ionization detector (FID) and a Porabond-Q column (0.32 mm x 25 m) (Varian, Middelburg, the Netherlands). GC settings were: injector temperature 200°C; detector temperature 300°C; oven temperature 3 min at 50°C, followed by an increase with 10°C/min to 250°C; carrier gas helium with a flow rate of 2 ml/min. External standards at 5 different concentrations in the range of 1 – 1000 µM were used for calibration. The lower detection limit was 1 µM for all analyzed compounds, except for 1,2-DCA, which had a detection limit of 10 µM. PCE and its potential dechlorination products TCE and DCE, were analyzed on a Varian 3800 gas chromatographic (GC) system equipped with a mass spectrometry detector (MS) and a Porabond-Q column (0.32 mm x 25 m) (Varian, Middelburg, the Netherlands). Headspace samples (50 µl) were taken from 1 ml water samples, which were diluted in MilliQ to a total volume of 8 ml in a closed (viton cap) 20 ml GC-vial. GC settings were: injector temperature 200°C; detector temperature 300°C; oven temperature 3 min at 40°C, followed by an increase of 10°C/min to 70°C; followed by an increase of 15°C/min to 250°C for 7 min; carrier gas helium with a flow rate of 2 ml/min. External standards at 6 different concentrations in the range between 0 – 30 µM were used for calibration.

Ion analysis was performed on 1 ml diluted mesocosm water samples on a Dionex ICS-1500 equipped with an IONPAC AS14 anion-exchange column and an A SRS*-Ultra 14 mm suppressor (Dionex Corporation, Sunnyvale, CA). The eluent (2.0 mM Na_2CO_3 and 0.75 mM $NaHCO_3$) flow rate was 1.3 ml/min. The injection needle was pre-flushed with 100 µl MilliQ-water and 50 µl samples were injected.

Profiles of oxygen concentration and pH in the sediment were determined from the water phase to 5 cm in the sediment with micro sensors OX500 and P100 (Unisense, Denmark), respectively, connected to a picoammeter (PA2000, Unisense). Redox potential in the sediment was measured with a platinum needle electrode (diameter 2 mm) and an external reference electrode. The sensors and needle electrode were carefully pushed into the sediment by hand.

Total organic carbon (TOC) content was determined in 0.2 g homogenized sediment by flash combustion followed by CO_2 analysis by an IR detector (Leco SC-632, Leco Mönchengladbach, Germany). Iron content of the samples was determined by ICP-OES (Inductively Coupled Plasma - Optical Emission Spectrometer) analysis (SPECTRO CIROS^{CCD} (Spectro, Kleve, Germany), after destruction with a mixture of $HF/HClO_4/HNO_3$.

2.2.9 Statistical analysis

DGGE bands were processed using Quantity-one version 4.6.2 image analysis software (Bio-Rad) and corrected manually when needed. After normalization of the gels, bands with relative peak area intensity above 1% compared to the most dominant band, were included in further analyses. Bacterial diversity was assessed by Shannon-Weaver diversity index ($H'=-\Sigma P_i \log P_i$) (Shannon and Weaver, 1949). P_i is the relative peak intensity of a DGGE band, calculated as $Pi= n_i / N$, where n_i is the peak area of the band and N the sum of all the peak areas in the DGGE lane. Significance of differences in diversity during the incubation period was analyzed using a Student's t-test. UPGMA dendrogram of the DGGE-patterns were based on Pearson correlation and constructed with Gelcompare II (Applied Maths, Belgium).

2.3 Results

2.3.1 Mesocosm systems

In the water phase and in the upper 5 cm of the mesocosms sediment, pH, redox and oxygen concentration were measured at start (t = 0) and end of the 6 months incubation period (Table 1). The pH was neutral (6.9 – 7.1) in all mesocosms at t = 0 and did not change significantly during incubation in mesocosms 1 and 3. In mesocosm 2, however, the pH in the sediment increased by 0.2 - 0.4 units during the experiment; from 7.1 to 7.3 at 1 cm, and from 7.0 to 7.4 at 2 cm depth. In all mesocosms, the redox potential decreased with depth during incubation. Moreover, differences between mesocosms were observed. The water phase at t = 0 had the highest redox potential (-10 mV) and during the experiment the redox potential decreased to below -150 mV in the first 2 cm of the sediments in the polluted mesocosms 2 and 3.

Table 1: *Overview of pH, redox potential and oxygen concentration at t = 0 and t = 6 months in the mesocosms. Numbers are based on triplicate measurements with a standard deviation of less than 0.1 for pH, less than 5 mV for redox potential and 0.1 mg/l for oxygen concentrations. "Water" = water phase at 2 cm above the sediment. "1 cm", "2 cm" and "5 cm" represents the depth in the sediment.*

	pH				Redox potential (mV)				Oxygen (mg/l)			
Mesocosm	Water	1 cm	2 cm	5 cm	Water	1 cm	2 cm	5 cm	Water	1 cm	2 cm	5 cm
Reference												
t = 0	7.1	7.1	7.0	7.0	-10	-28	-50	-61	3.4	0.0	0.0	0.0
t = 6 months	7.2	7.1	7.0	7.0	-50	-70	-72	-121	2.0	0.0	0.0	0.0
1,2-DCA / PCE												
t = 0	7.1	7.1	7.0	6.9	-10	-40	-85	-110	3.4	0.0	0.0	0.0
t = 6 months	7.1	7.3	7.4	7.2	-65	-120	-151	-164	2.6	0.0	0.0	0.0
Extra nutrients												
t = 0	7.1	7.1	7.0	7.0	-10	-90	-125	-140	3.4	0.0	0.0	0.0
t = 6 months	6.9	7.0	6.9	6.9	-80	-175	-193	-196	1.2	0.0	0.0	0.0

Oxygen was present in the recirculating water of all mesocosms, but the sediments were anoxic. This indicates that all oxygen was consumed in the top cm of the sediments. During the incubation period, the oxygen concentration in the water decreased by 1.4 ± 0.2 mg/l, 0.8 ± 0.1 mg/l, and 2.4 ± 0.2 mg/l in mesocosm 1, 2 and 3, respectively.

At t = 0, total organic carbon (TOC) in the upper 2 cm of the sediment of mesocosms 1, 2 and 3 was 25, 23 and 22 g/kg dry weight (dw) sediment, respectively. After 6 months, more TOC was recovered in the polluted mesocosms 2 and 3 (91 or 100%, respectively), compared to the reference mesocosm 1 (76% TOC recovered). The total amount of iron in the mesocosm sediments was 25 g/kg dw and did not change during incubation.

1,2-DCA and PCE concentrations in mesocosm 2 decreased from respectively 200 and 25 µM, to 10 and 1 µM within two months. Therefore, 200 µM 1,2-DCA and 25 µM PCE were re-added three times during the incubation period, to ensure continuous presence of these chlorinated pollutants. No dechlorination products like chloroethane, ethene, of vinyl chloride were observed in the water phase or headspace of the mesocosm and increase of chloride ions due to dechlorination could not be detected, because of the relatively high background of chloride in the mesocosm. In mesocosms 1 and 2, the phosphate concentrations varied from below detection limit to 3 µM. In mesocosm 3, the phosphate concentration varied from 0.1 – 15 mM. Nitrate concentrations in mesocosms 1 and 2 varied from below detection limit to 10 µM. In mesocosm 3, the nitrate concentration varied from below detection limit to 16 µM, with the lowest concentration at the end of the incubation period. Potassium was not monitored.

2.3.2 Phylogenetic diversity of the total bacterial community

Diversity of the bacterial communities was analyzed by DGGE on duplicate samples from the sediment core, taken one year before start of the mesocosms (December 2006), and from the mesocosms at t = 0 and t = 6 months. In a dendrogram, DNA-based DGGE-patterns of t = 0 samples from all three mesocosms clustered together (Fig. 2). This indicates the spatial similarity of the bacterial communities within the sediment core at the start of the experiment. Samples taken after 6 months from mesocosm 1 and 2 formed a second cluster. Corresponding t = 6 months samples from mesocosm 3 clustered separately from all other samples and were less related to t = 0 samples than to the December 2006 sample. As observed for DNA-based DGGE-analyses, also RNA-based DGGE-patterns of t = 0 from mesocosm 1, 2 and 3 clustered together (Fig. 3). The RNA DGGE-profiles of samples taken after 6 months formed separate clusters for the individual mesocosms, indicating divergent evolution of the active fraction of bacterial communities during incubation.

Shannon-Weaver indices (H') for sediment microbial diversity based on DNA, varied between 1.07 and 1.17 for all mesocosms at t = 0 (Table 2). During the experiment, diversity decreased in mesocosm 3 to H' = 0.90 ($p < 0.01$). In contrast, RNA based bacterial diversity increased in mesocosm 3 from H'= 1.26 to H'= 1.35 ± 0.3 ($p < 0.02$) during the experiment. In mesocosms 1 and 2, no biodiversity changes were observed based on DNA or RNA DGGE profiles.

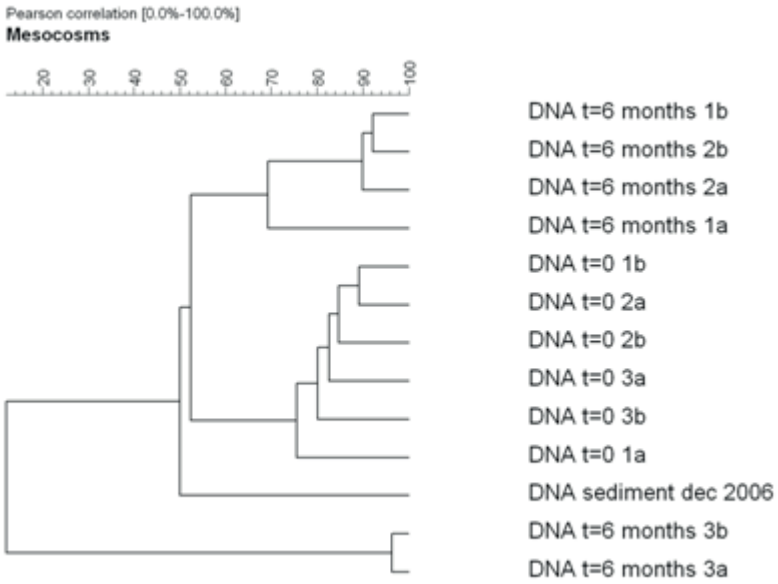

Figure 2: *UPGMA dendrogram of DGGE profiles of 16S rRNA gene PCR amplicons derived from duplicate DNA extracts obtained from the sediment core (December 2006), and from mesocosm samples at t = 0, t = 6 months.. Samples 1a and 1b are duplicate samples from mesocosm 1 (Reference), samples 2a and 2b are duplicate samples from mesocosm 2 (1,2-DCA / PCE), and samples 3a and 3b are duplicate samples from mesocosm 3 (Extra nutrients).*

Table 2: *Shannon-Weaver indexes (H') of bacterial communities in mesocosm samples, based on band patterns of the 16S rRNA gene based DGGE profiles. Significant differences between t = 0 and t = 6 months are indicated with "*".*

	DNA		RNA	
Mesocosm sample	t = 0	t = 6 months	t= 0	t = 6 months
Sediment core a	1.14			
Sediment core b	1.02			
1a - Reference	1.15	1.08	1.24	1.18
1b - Reference	1.10	1.08	1.24	1.28
2a - 1,2-DCA / PCE	1.10	1.11	1.22	1.22
2b - 1,2-DCA / PCE	1.16	1.14	1.25	1.28
3a - Extra nutrients	1.07 *	0.90 *	1.26 *	1.38 *
3b - Extra nutrients	1.17 *	0.91 *	1.26 *	1.32 *

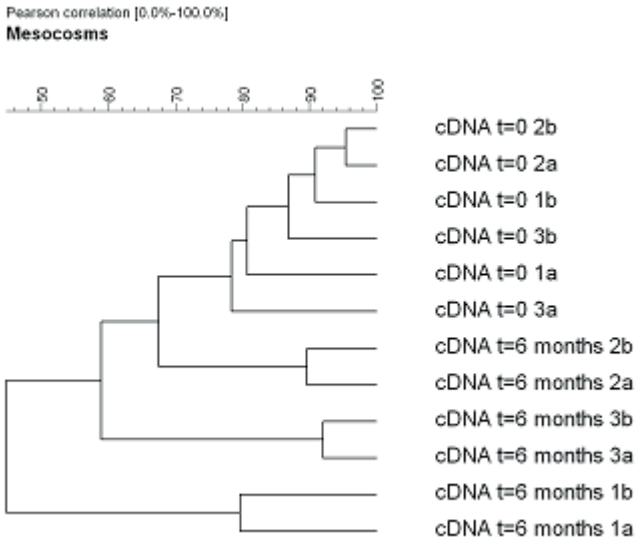

Figure 3: *UPGMA dendrogram of DGGE profiles of 16S rRNA RT-PCR amplicons derived from duplicate RNA extracts obtained from mesocosm samples from t = 0 and t = 6 months. Samples 1a and 1b are duplicate samples from mesocosm 1 (Reference), samples 2a and 2b are duplicate samples from mesocosm 2 (1,2-DCA / PCE), and samples 3a and 3b are duplicate samples from mesocosm 3 (Extra nutrients).*

2.3.3 Abundance of functional communities in the sediment

The abundance of various functional communities in the mesocosms was determined for the total amount of microorganisms (DNA), the active fraction (RNA), and the cultivable fraction of sediment microorganisms present (Fig. 4). In general, only small differences were observed in abundances of tested functional groups or 16S rRNA genes during the experiment. In all mesocosms, about 100 times more bacterial than archaeal genes were detected. In mesocosm 2, the active and cultivable fraction of dechlorinating bacteria (*Dehalococcoides* spp., *Desulfitobacterium* spp. or *Dehalobacter* spp.) increased during incubation. In contrast, the total amount of sulfate-reducing bacteria (DNA) was slightly decreased after 1,2-DCA and PCE pollution, although no significant effect was observed for the active (RNA) or cultivable (MPN) fractions. In mesocosm 3, the total amount of nitrate- and nitrite reductase genes (*narG* and *nirS*) increased. Remarkably, the active fraction (RNA) of these genes decreased in mesocosm 3. In contrast, an increase of *narG* and *nirS* genes was observed in the active fractions of mesocosms 1 and 2. *Geobacter* spp. 16S rRNA genes were enriched in all mesocosms during the experiment and therefore these changes were likely not induced by the compounds added to the mesocosms.

In all mesocosms, high variation of the cultivable fraction compared to the total amount of microorganisms was observed between the functional groups (Fig. 4). Assuming one 16S rRNA gene copy per cell, less than 0.0001% of the total amount of sulfate-reducers could be cultured. The cultivable fraction of methanogens was 0.08 ± 0.03%. The cultivable fraction of nitrate-reducing microorganisms increased during incubation from less than 0.0001% to 0.05 ± 0.03% in all mesocosms. Cultivable dechlorinators increased in mesocosm 2 from less than 0.0001% to 0.0064 ± 0.004%.

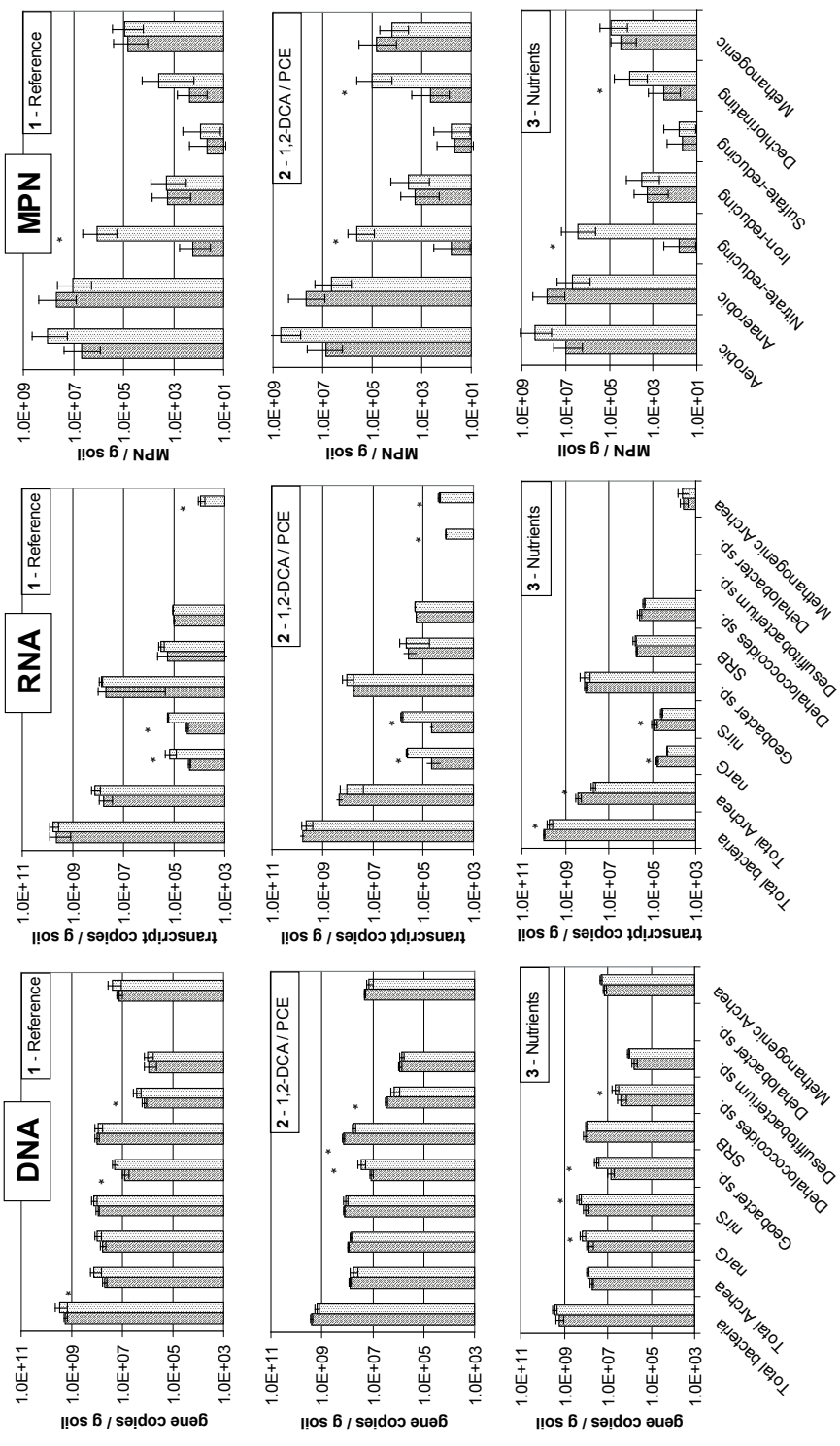

Figure 4: *Quantification of functional diversity in mesocosm 1 (Reference), mesocosm 2 (Chlorinated compounds) and mesocosm 3 (Extra nutrients) by real time PCR on DNA and RNA (cDNA) and by MPN at t = 0 (striped pattern) and at t = 6 months (dashed pattern). Error bars represent standard deviations based on duplicate (real time PCR) and triplicate (MPN) analyses of both A and B samples of the individual mesocosms. Significant differences between t = 0 and t = 6 months, are indicated with an "* " (Student's t-test, p<0.05).*

2.4 Discussion

The aim of this study was to identify the effect of environmental pollution on microbial communities. The phylogenetic diversity of bacterial communities and abundance of various functional groups within microbial communities in river sediment was studied. To this end, cultivation dependent and independent methods were used. Based on quantitative real-time PCR and MPN analyses, we concluded that the total abundance of functional groups in the tested sediment was not affected by pollution with 1,2-DCA and PCE or nutrients. The abundance of functional groups is not necessarily related to corresponding activity rates (Röling, 2007), but the data presented here suggest that the potential for different functional metabolic processes was not affected by the applied pollution. Our results are in agreement with studies of Taş *et al.* (2009) and Bowen *et al.* (2009), which also show the resistance of microbial communities to environmental stress. Sutton *et al.* showed that fertilization had a negative impact on microbial metabolic activity in soil, but this was examined with soil that was isolated from the reference soil for more than 10 years.

2.4.1 Phylogenetic diversity

Chemical pollution affected the composition of microbial communities in the tested sediments. From other studies it is known that enrichment with inorganic nutrients can lead to lower biodiversity (Allison *et al.,* 2008; Jangid *et al.,* 2008). In the present study, the phylogenetic diversity of bacterial communities also significantly decreased as a result of high nutrient concentration. The diversity indexes (H') of the reference and 1,2-DCA / PCE polluted mesocosms did not change. In these mesocosms, the biodiversity was comparable to values previously found for sediments from upstream locations of the Ebro river (de Weert *et al.,* 2010; Taş, 2009). An explanation for the unaffected biodiversity H', is that the relatively abundance of specialized dechlorinating bacteria present in environmental matrixes is normally low (Dong *et al.,* 2009). The specific PCR results of the present study showed that the cumulative relative abundance of *Dehalococcoides*, *Desulfitobacterium* and *Dehalobacter* 16S rRNA genes was 0.14 % for the total (DNA) and 0.03 – 0.05% for the active (RNA) fraction of the microbial community. To determine the diversity index, only species with a relative abundance >1% were taken into account. Thus, the H-index did not account for the full diversity potential (all species and genes present) which are important to consider, because the <1% exotic fractions may represent to a large extent the memory and capacity of natural biological systems to respond to and recover from geochemical impacts more severe than applied here. Knowledge about diversity within functional groups is relevant to indicate the robustness of a specific functional group (Wittebolle *et al.,* 2009). In the present study, diversity changes

within the different functional groups were not investigated. However, it could have provided additional information about the overall functioning of the microbial community, since different populations within function groups may have different kinetic properties of their enzymes.

2.4.2 Changes in abundance of functional genes and activity

In the 1,2-DCA / PCE polluted mesocosm, the active dechlorinating bacterial community increased. Remarkably, also nutrient addition stimulated the viability of dechlorinating species in the sediment. Other studies showed that nitrogen or phosphate addition can stimulate biodegradation of (chlorinated) pollutants (Ju *et al.,* 2007; Strong *et al.,* 2000). In the nutrient polluted mesocosm, *narG* and *nirS* genes were slightly enriched in the total microbial community. However, fewer nitrate reducers and denitrifiers seemed to be active after incubation with extra nutrients. This can be explained by temporal or special depletion of available nitrate and nitrite, as observed at the end of the incubation period. When nitrate became depleted, other microorganisms than nitrate-reducers could become active, due to a decrease of the redox potential. These changing conditions can also be the reason why the phylogenetic diversity in the active fraction (RNA) of the microbial community increased, while the total pool (DNA) of dominant species (nitrate-reducers) decreased in mesocosm 3.

After addition of urea to mesocosm 3, increased nitrate concentrations were expected, because urea can microbiologically be converted to ammonium, which can further be oxidized to nitrate (Hazell and Mendz, 1997). However, in all mesocosms, nitrate concentrations varied from <1 µM to 10-16 µM. Likely, urea was sequentially converted to ammonium, nitrate and nitrogen, as supported by the increase of nitrate and nitrite reductase genes (*narG* and *nirS)* during the incubation period.

The cultivability of aerobic heterotrophic microorganisms increased after pollutant exposure. To verify the specific enrichment of aerobes, specific qPCR analysis of aerobic microorganisms can be useful. However, to our knowledge a specific molecular marker for aerobes is not available. Until recently, also no marker was available for fermenting bacteria, although fermentation is an important microbial function in anaerobic environments. Now, a marker has been presented (Pereyra *et al.,* 2009) to monitor microbial fermentation, and can be included in future functionality studies.

2.4.3 Formation of reduced iron

The formation of reduced iron was used in MPN-incubations to identify the presence of dissimilatory iron-reducing bacteria. In theory, reduced iron might also be formed chemically by reduction with sulfide, produced after sulfate or sulfur reduction. However, in well mixed batches with ferrihydrate, reduction of iron and sulfate take place in a sequential way, since iron reduction is a preferential metabolic process over sulfate reduction (Garcia-Balboa *et al.,* 2009). Thus, chemically formed reduced iron is not likely in our MPN-incubations and we assumed that observed reduced iron was produced by dissimilatory iron-reducing bacteria.

2.4.4 Changes not induced by pollution

Geochemical conditions (e.g. redox potential and TOC concentration) in the sediments changed over time, but the spatial diversity was small at the start of the experiment, with the exception of redox-potential. DGGE analysis showed that the microbial community at four different locations in the sediment core was identical two months before start of the experiment (data not shown). Therefore, it is safe to assume that, although the influence of spatial heterogeneity in environmental studies can not be excluded totally, identified changes in phylogenetic diversity of the bacterial community, and changes in abundance of functional groups, were a result of pollutant addition. Nevertheless, changes in abundance of iron-reducing bacteria and archaea, and cultivability of nitrate-reducers, were observed in all three mesocosms. Therefore, we conclude that these changes did result from other conditions than pollutant exposure, for example by the constant incubation temperature.

2.4.5 Culture dependent and molecular methods

Both culture dependent and molecular methods showed that abundance of functional microbial groups in the sediments was resistant to pollution, although abundance, activity, and cultivability differed per functional group. Further, incubation conditions influenced the cultivability of specific groups (nitrate-reducing, aerobic, and dechlorinating bacteria) while the active fraction of these groups (RNA), did not change. Thus, the methods bring up complementary information on functional groups within microbial communities. A combination of cultivation dependent methods and molecular techniques resulted in a more reliable insight into the microbial community. This was also stated by others (Cheng and Foght, 2007; Larsen *et al.,* 2008).

The present study showed that predominant functional groups within microbial communities were resistant to applied pollution in Ebro river sediments, although individual microbial species were affected. Culture dependent and molecular methods are complementary and focus on different fractions of the microbial community (cultivable, active or total). Therefore, for future studies it is recommended to combine molecular detection of functional groups with identification of

specific microbial activity. This results in comprehensive description of phylogenetic as well as functional diversity and abundance. By using this method and increasing chemical pressures of various nature, new insight on ecological responses to varying pollution levels may be obtained.

Acknowledgments

This research was supported by the European Union through the Aquaterra Integrated project of the sixth framework program (FP6-SUSTDEV-2003-505428). We thank Stefan Jansen for kindly providing suggestions for the discussion.

Chapter 3

Degradation of 1,2-dichloroethane by microbial communities from river sediment at various redox conditions

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This chapter has been published as:
Bas van der Zaan, Jasperien de Weert, Huub Rijnaarts, Willem M. de Vos, Hauke Smidt, Jan Gerritse, Degradation of 1,2-dichloroethane by microbial communities from river sediment at various redox conditions, 2009, Water Research, Vol. 43, p. 3207-3216

Abstract

Insight into the pathways of biodegradation and external factors controlling their activity is essential in adequate environmental risk-assessment of chlorinated aliphatic hydrocarbon pollution. This study focuses on biodegradation of 1,2-dichloroethane (1,2-DCA) in microcosms containing sediment sourced from European rivers Ebro, Elbe and Danube. Biodegradation was studied under different redox conditions. Reductive dechlorination of 1,2-DCA was observed with Ebro and Danube sediment with chloroethane, or ethene, respectively, as the major dechlorination products. Different reductively dehalogenating micro-organisms (*Dehalococcoides* spp., *Dehalobacter* spp., *Desulfitobacterium* spp. and *Sulfurospirillum* spp.) were detected by 16S ribosomal RNA gene-targeted PCR and sequence analyses of 16S rRNA gene clone libraries showed that only 2 to 5 bacterial orders were represented in the microcosms. With Ebro and Danube sediment, indications for anaerobic oxidation of 1,2-DCA were obtained under denitrifying or iron-reducing conditions. No biodegradation of 1,2-DCA was observed in microcosms with Elbe sediment under the different tested redox conditions. This research shows that 1,2-DCA biodegradation capacity was present in different river sediments, but not in the water phase of the river systems and that biodegradation potential with associated microbial communities in river sediments vary with the geochemical properties of the sediments.

3.1 Introduction

Chlorinated aliphatic hydrocarbons (CAHs) like tetrachloroethene (PCE), 1,2-dichloroethane (1,2-DCA) and vinyl chloride (VC) are widely used in industries as solvents or as intermediates in chemical processes. Due to leakage and improper disposal, CAHs have often been detected in the environment (Westrick *et al.,* 1984). Here, they form a long-lasting danger to humans and the environment because of high water solubility, a long environmental half-life and carcinogenic effects (Vogel *et al.,* 1987).

Transformation of CAHs has been studied extensively, with specific attention for the microbiology (Mohn and Tiedje, 1992; Smidt and de Vos, 2004) , *in situ* biodegradation in soil systems (Maes *et al.,* 2006; Suchomel *et al.,* 2007; Takeuchi *et al.,* 2005) and enzymology (Bunge *et al.,* 2007). However, little information is available with respect to the variation of the microbial degradation potential and activity in river sediment systems (Bradley and Chapelle, 1998). This is in sharp contrast to the demands of regulatory bodies that require specific guidelines based on fundamental knowledge of the biodegradation of pollutants in river systems for adequate river management (Barth *et al.,* 2007; Gerzabek *et al.,* 2007).

Much attention has been given to 1,2-DCA as a model compound (de Wildeman *et al.,* 2003b; Falta *et al.,* 2005; Fishbein, 1979; Janssen *et al.,* 1994), since it can biologically be degraded under different geochemical conditions. Moreover, it has been produced in larger quantities than any other CAH (Pankow and Cherry, 1996). Currently, more than 17.5 million tons are produced annually in the United States, Western Europe and Japan (Field and Sierra-Alvarez, 2004). 1,2-DCA is frequently detected in river systems at several tens of micro molars (ATSDR, 1999; Gotz *et al.,* 1998; ICPS, 1995; Yamamoto *et al.,* 1997), which is above the natural background level of 5 micro mol in non-industrialized areas (de Rooij *et al.,* 1998). In Europe, the Water Framework Directive (WFD, Directive 2000/60/EC) classified 1,2-DCA as one of the 33 priority pollutants and it has been identified similarly by the U.S. Environmental Protection Agency. The 1,2-DCA pollution in rivers and estuaries is generally considered to be caused to a large extent by anthropogenic sources, however, 1,2-DCA can in low concentrations originate from natural sources (de Rooij *et al.,* 1998).

Previous studies have focused on the fate of 1,2-DCA in the environment and showed that it can be transformed through abiotic as well as biotic reactions. Abiotically, 1,2-DCA is transformed to ethylene glycol or VC. However, this is a very slow process (half live > 72 years) (Jeffers *et al.,* 1989), and the resulting products may even be more toxic than 1,2-DCA itself (Gallegos *et al.,* 2007). In contrast, microorganisms can transform 1,2-DCA rapidly to non-toxic end products via different pathways. In the presence of oxygen, 1,2-DCA can completely be oxidized to CO_2, H_2O and Cl^- by different bacterial species, including *Xanthobacter autotrophicus* (Janssen *et al.,* 1985) and *Ancylobacter aquaticus* (van den Wijngaard *et al.,* 1992).

However, in aquifers and sediments, oxygen is often not available. The oxidation of 1,2-DCA in the absence of oxygen has also been observed with nitrate as alternative electron acceptor (Gerritse *et al.,* 1999a). Recently, this process has been described in more detail by Dinglasan-Panlilio and co-workers (Dinglasan-Panlilio *et al.,* 2006).

Under methanogenic conditions, ethene is usually the main end product of 1,2-DCA dechlorination (Egli *et al.,* 1987) and is formed via either a cometabolic or halorespiration process. *Dehalococcoides ethenogenes* strain 195 (Maymo-Gatell *et al.,* 1999) and *Desulfitobacterium dichloroeliminans* strain DCA1 (de Wildeman *et al.,* 2003a) are bacterial isolates that can convert 1,2-DCA anaerobically into ethene by reductive dechlorination. Chloroethane and ethane can also be formed via reductive dechlorination of 1,2-DCA. However, this has scarcely been reported and only some strains of methanogens are known to be responsible for the process (Holliger *et al.,* 1990).

In some environmental studies, a thorough characterization of 1,2-DCA biodegradation potential was done resulting in insight in lag periods of microbial adaptation required for biotransformation of 1,2-DCA (Klecka *et al.,* 1998) and evidence that fermentative as well as anaerobic oxidative pathways can be involved in natural attenuation of 1,2-DCA (Gerritse *et al.,* 1999a). However, an investigation of the local biogeochemical conditions is required before it can be assessed to what extent and via which pathways 1,2-DCA is likely to be degraded in a specific sediment or groundwater system.

The aim of the present study was to asses the potential for biodegradation of 1,2-DCA by microbial communities of sediments originating from the rivers Ebro, Danube and Elbe at different redox conditions, and to identify biodegradation mechanisms and micro-organisms involved. To this end, microcosm and cultivation-independent approaches were combined to obtain insight to what extent the mechanisms and micro-organisms can be related to prevailing redox conditions and characteristics of the tested river inoculum material.

3.2 Material and Methods

3.2.1 Sampling of river sediments and river water

Sediments were sampled from the rivers Ebro, Elbe and Danube. Ebro sediment was sampled from a rice field in the southern part of the river delta (N: 40° 41’ 11.8”; E: 00° 37’ 31.5”) in July 2004 and had a clay-like structure. The rice field is flooded with water from the river, every year from April to September and has been formed by sedimentation. Elbe sediment was sampled near Schönberg (N: 52° 90’ 73.2”; E: 11° 87’ 21.9”) at the west bank of the river in October 2004. The sediment was clear and

sandy. Artificial reconstruction activities of the river bank were carried out about a year before sampling. Danube River sediment was sampled at the East bank near Budapest (47° 27' 25.8"; E: 19° 03' 46.8") in April 2005. This sediment had a clayey structure.

Sediment samples were taken as 20 cm long vertical cores, using sterilized PVC tubes (30 cm x 3,2 cm inner diameter). The PVC tube was completely pushed into the sediment and closed with butyl rubber stoppers on both sides when the tube was drawn back. The tubes were stored at 4°C for maximally 1 month until microcosms were set up. Water sampling was done using a 1-liter sterile glass jar, which was completely filled under water, closed with a viton stopper, and stored at 4°C.

Sediments and river water were characterized by partners in the Aquaterra project: Consejo Superior de Investigaciones Cient Ãficas (CSIC), Barcelona, Spain, for Ebro samples; Helmholtz-Zentrum für Umweltforschung (UFZ), Magdeburg, Germany, for Elbe samples; International Commission for the Protection of the Danube River (ICPDR) for Danube samples and additional analyses on the sediments were performed by Al-West B.V. (Deventer, the Netherlands) (Table 1). The concentration of most CAHs (vinyl chloride, 1-chloroethane, trans-1,1-dichloroethene, cis-1,1-dichloroethene, 1,1-dichloroethane, 1,1,1-trichloroethane, 1,2-dichloroethane, trichloroethene and tetrachloroethene) in river water samples was below detection limit (< 1 µg/l). In contrast, 1,2-DCA was detected in the river water of all locations and ranged from 1 to 2 µg/l, which is well above the natural background level reported for non-industrialized areas (de Rooij *et al.,* 1998). In Elbe river water, in addition to 1,2-DCA also trichloroethene and tetrachloroethene were present at 2 µg/l.

3.2.2 Microcosm experiments

Microcosms were prepared in an anaerobic glove box with 50 ml local river water, with and without 1-10% (w/w) sediment material and 100 µM 1,2-DCA. For Ebro river microcosms with sediment, artificial river water was used, which consisted of (in mM): Na_2HPO_4,0.3; KH_2PO_4, 0.05; NaCl,1.7; $NaSO_4$, 0.7; $NaHCO_3$, 1.2; $CaCl_2.2H_2O$, 0.44; NH_4Cl, 0.9; $MgCl_2.6H_2O$, 0.2 ; KCl, 0.13; $NaNO_3$, 0.06; Na-acetate, 0.06; humic acids (technical mix 50-60% as humic acids, Acros Organics, Geel, Belgium), 5; trace elements solution SL10 (DSMZ, medium 320), 0.1 ml/l and vitamin solution (Heijthuijsen and Hansen, 1986), 0.1 ml/l. The used 120 ml serum bottles were capped with viton stoppers to prevent evaporation and absorption of 1,2-DCA (Evans and Hardy, 2004).

Table 1: *Overview of river water and sediment characteristics of the sampled locations in Ebro, Elbe and Danube river.*

		Ebro		Elbe		Danube	
Physical parameters	Unit	Water	Sediment	Water	Sediment	Water	Sediment
Temperature	°C	23.4		15.0		14.1	
pH		8.2		7.9		8.2	
Redox potential	mV	66	125	200	55	144	25
Conductivity	µS/cm		560		150		370
Oxygen	mg/l	6.8		13.2		0.5	
Dry Matter (dm)	% (w/w)		68.2		59.3		80.4
Total Organic Carbon	% dm		2.6		1.1		0.1
Anions							
Chloride	mg/l	63		153		18	
Nitrite	mg/l	<3		<3		5	
Bromide	mg/l	<3		<3		<3	
Nitrate	mg/l	<3		13		5	
Phosphate	mg/l	<3		<3		<3	
Sulfate	mg/l	115		111		29	
Cations							
Lithium	mg/l	<3		<3		<3	
Sodium	mg/l	58		102		30	
Ammonium	mg/l	<3		<3		<3	
Potassium	mg/l	2		11		3	
Magnesium	mg/l	21		18		13	
Calcium	mg/l	84		11		54	

Biodegradation of 1,2-DCA was tested at the following redox conditions: aerobic (21% (v/v) O_2 in the gas phase), nitrate-reducing (2 mM $NaNO_3$ added), iron-reducing (10 mM FeOOH (Brock and O'Dea, 1977) added), sulfate-reducing (Na_2SO_4 added), and methanogenic (1-5 mM fatty acids added). For all conditions, duplicate microcosms were prepared as well as an autoclaved negative control. Elbe samples were not tested for nitrate-reducing, iron-reducing and sulfate-reducing conditions.

All anaerobic microcosms had a nitrogen atmosphere and the volumetric ratio headspace / liquid-sediment phase was 7:5. The pH was neutralized to 7.0 with NaOH. The microcosms were incubated at 30°C (Ebro) or 20°C (Danube and Elbe), shaken at 150 r.p.m., for at least 12 months. The incubation temperature for Ebro microcosms was higher than Elbe and Danube microcosms, corresponding to the natural environmental conditions.

Active, 1,2-DCA dechlorinating microcosms, were transferred (10%, v/v) to fresh medium (described above) containing 100-500 µM 1,2-DCA, to enrich the 1,2-DCA dechlorinating consortia. After three transfers, following incubation periods of 2 months and regular screening for 1,2-DCA dechlorination, these microcosms were sampled for DNA-based community analyses. Microcosms with sediment that did not show 1,2-DCA dechlorination, were also sampled for further DNA-based analyses.

3.2.3 Molecular analyses of microbial communities

3.2.3.1 DNA extraction

DNA was extracted from 5 ml of suspended microcosm material using the BIO101 fast DNA Kit for Soil (Qbiogene, Inc, CA). The only modification to the manufacturer's protocol was extension of the bead-beat step from 30 to 45 seconds. Extracted DNA was stored at -20°C.

3.2.3.2 Genus- and catabolic gene-specific PCR

Samples from 1,2-DCA dechlorinating microcosms were screened for the presence of known anaerobic dechlorinating bacteria, including the genera *Dehalococcoides*, *Dehalobacter*, *Desulfitobacterium*, *Sulfurospirillum*, using 16S ribosomal RNA (rRNA) gene-targeted assays, and the genes encoding the 1,2-DCA dechlorinating enzymes DCA reductase A (*dca*A) (Marzorati *et al.,* 2007) and haloalkane dehalogenase *dhl*A (Song *et al.,* 2004) by nested PCR. 16S rRNA gene fragments were amplified on an ICycler PCR system (Bio-Rad, Veenendaal, the Netherlands) with 6 µM bacterial primer mixture (fD1/fD2 and rP1/rP2) (Weisburg *et al.,* 1991) using the following cycling program: 94°C for 3 min, 43 cycles 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, with a temperature decrease of 1°C for the annealing phase of the first 10 cycles and a time increment of 10 seconds added to the elongation phase of the last 33 cycles. The resulting PCR product was purified with the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and used as template for subsequent nested PCR. Total DNA isolated from *Desulfitobacterium* strain DCA1 and *Xanthobatcer autotrophicus* strain GJ10 were used as positive control for *dcaA* and *dhlA* gene detection, respectively. Primers targeting the genera *Dehalobacter*, *Desulfitobacterium,* genera *Dehalococcoides*, and *Sulfurospirillum* were described before (Smits *et al.,* 2004). Amplification was achieved in a real time PCR system (IQ5, Bio-Rad Laboratories B.V., Veenendaal, the Netherlands) using 6 µM primer and 3 µl template DNA added to IQ SYBR Green Supermix (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands) with the following cycling program: 94°C for 3 min, 35 cycles 94°C for 30 s, 55°C or 58°C (55°C for *Dehalococcoides* spp., 58°C for *Dehalobacter* spp., *Desulfitobacterium* spp., and *Sulfurospirillum* spp.) for 20 s, 72°C for 30 s and a final extension at 72°C for 5 min. Relative abundance of bacteria from

the different genera was determined by normalizing the quantitative results of the specific genes to the total amount of bacterial 16S rRNA gene copies in the samples, which were quantified after the first PCR step by the same nested real time PCR procedure, with 6 µM Eub341F and Eub534R primers (Muyzer *et al.,* 1998).

3.2.3.3 Cloning and sequencing

To identify the most dominant bacterial species, clone libraries of 16S rRNA genes were constructed from 1,2-DCA transforming microcosms with Ebro sediment, incubated at methanogenic and denitrifying conditions, and from a 1,2-DCA transforming microcosm with Danube sediment, incubated at iron-reducing conditions,.

Almost complete 16S rRNA gene fragments, amplified by using the bacterial primer mixture fD1/fD2 and rP1/rP2 (Weisburg *et al.,* 1991), were cloned by using the pGEM-T Easy Vector System (Promega, Leiden, the Netherlands) according to the manufacturer's protocol. Cloned inserts were sequenced (MWG-Biotech, Germany) and the obtained sequences were initially classified using the tools available at Greenegenes (http://greengenes.lbl.gov) (DeSantis *et al.,* 2006). Furthermore, sequences subjected to phylogenetic analysis using the ARB software package (version December 2007) and the most recent release of the SILVA SSURef 96 database (Ludwig *et al.,* 2004; Pruesse *et al.,* 2007). Sequences obtained in this study were aligned using the SINA aligner at http://www.arb-silva.de, and the alignment was manually refined using the ARB-editor and appropriate reference sequences. Tree reconstruction was performed using the maximum parsimony method as implemented in ARB (Ludwig *et al.,* 2004).

3.2.4 Analytical procedures

1,2-DCA and its potential dechlorination products chloroethane, vinyl chloride, ethene, ethane, and methane were identified and quantified in 500 µl headspace samples from microcosms, obtained by using a 1 ml Pressure-Lock gas syringe (Alltech, Breda, the Netherlands) with a sterile needle. The samples were injected onto a Varian 9001 gas chromatographic system (GC) equipped with a flame ionization detector (FID) and a Porabond-Q column (0.32 mm x 25 m) (Varian, Middelburg, the Netherlands). GC settings were: injector temperature 200°C; detector temperature 300°C; oven temperature 3 minutes at 50°C, followed by an increase with 10°C/min to 250°C; carrier gas helium with a flow rate of 2 ml/min. External standards at 5 different concentrations were used for calibration. The lower detection limit was 1 µM for all analyzed compounds, except for 1,2-DCA, which had a detection limit of 10 µM.

CO_2 was analyzed on an Interscience GC-8000 gas chromatograph with a thermal conductivity detector (TCD) and a Poraplot Q column (Varian, Middelburg, the Netherlands). The injector temperature was set at 100°C and the detector temperature at 200°C, with a constant oven temperature of 90°C. Helium was used as carrier gas at a flow rate of 30 ml/min. External standards were used for calibration.

Liquid phase samples (5 ml) were taken for ion analyses on a Dionex DX-100 ion-chromatograph with Dionex IONPAC AS9-SC ion-column (Dionex Benelux B.V., Amsterdam, the Netherlands).

Fe^2 and Fe^3 concentrations in the iron-reducing microcosms were measured by the spectrophotometric method described before (Viollier *et al.,* 2000).

Half-live times ($t_{1/2}$) were calculated for transformation of 1,2-DCA in the microcosms according to $t_{1/2} = \ln 2 / k$, where $k = (\ln c_1 - \ln c_2) / (t_2 - t_1)$ (day^{-1}). c_1 is the concentration of 1,2-DCA at time t_1 and c_2 is the concentration of 1,2-DCA at time t_2, where $t_2 > t_1$ and $c_1 > c_2$.

3.3 Results

3.3.1 Degradation of 1,2-DCA in microcosms

Transformation of 1,2-DCA was observed in microcosms prepared with sediment, but not in microcosms with river water without sediment or in sterilized negative controls. This indicated that the observed transformation was biologically derived. Additionally, the results showed that the observed biodegradation capacity for 1,2-DCA of the different river systems was related to the sediment and not to the water phase. Therefore, the study further focused on sediment-inoculated microcosms.

3.3.1.1 Ebro River sediment

Four microcosms with Ebro sediment, incubated under methanogenic conditions, transformed 100 µM 1,2-DCA completely within 100 days. In these microcosms acetate or lactate was supplied as electron donor. Initially, dechlorination of 1,2-DCA yielded chloroethane as the major dechlorination product (80-90% of 1,2-DCA removed), and little amounts of ethene and ethane (< 5%) were produced (Fig. 1A). However, the relative amounts of the different degradation product shifted over time. After three transfers, most of the 1,2-DCA (> 64%) was dechlorinated to ethene and only traces of chloroethane were detected (< 2%) (Fig. 1B). The dechlorination rate of 1,2-DCA in the methanogenic microcosms did not change with the transfers and corresponded to a half-live of: 46 ± 14 days.

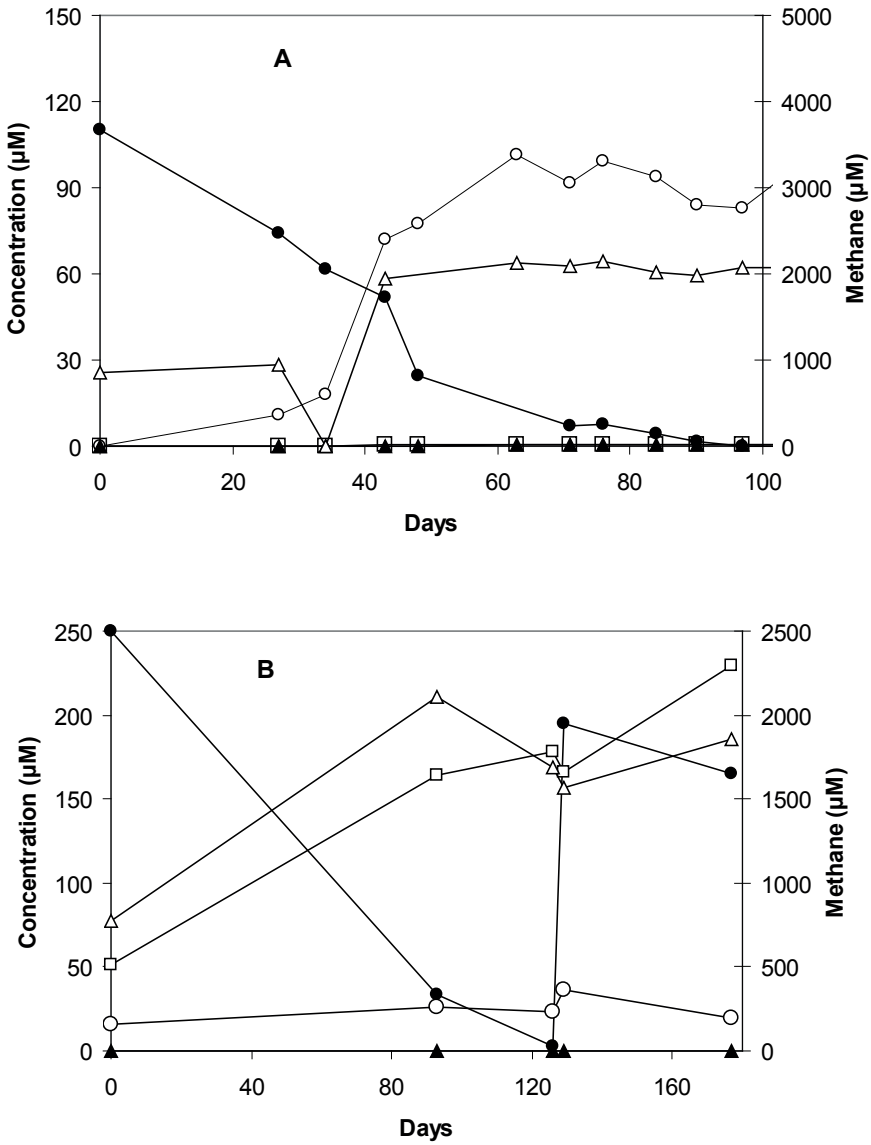

Figure 1: *Concentrations of 1,2-DCA and transformation products (A) in a methanogenic microcosm with Ebro sediment and (B) in a methanogenic sediment-free microcosm after three sequential transfers in artificial river water. Symbols left Y-axis: 1,2-DCA (closed circle), chloroethane (open circle), ethene (open square), ethane (closed triangle); right Y-axis: methane (open triangle).*

Under nitrate-reducing conditions, 100 µM 1,2-DCA was degraded within 90 days in microcosms with Ebro sediment, in parallel with the removal of 1.9 mM nitrate. Per mol of 1,2-DCA degraded, 1.9 ± 0.1 mol of chloride ions were produced. No degradation products of a reductive dechlorination pathway were observed. Half-lives for 1,2-DCA in these denitrifying microcosms were 12 ± 6 days, about 4 times shorter than under methanogenic conditions.

At the other tested redox conditions, 1,2-DCA was not transformed in Ebro sediment microcosms within the incubation period of 12 months, although the electron acceptors present (sulfate, iron or oxygen) were consumed over time .

3.3.1.2 Danube River sediment

Microcosms with sediment from Danube river dechlorinated 1,2-DCA completely to ethene under methanogenic conditions. In contrast to microcosms with Ebro sediment, chloroethane or ethane formation was not observed. The half-lives of 1,2-DCA under methanogenic conditions were 38 ± 6 days, which was similar to those observed in Ebro microcosms.

Danube sediment microcosms, incubated under iron-reducing conditions transformed 1,2-DCA over time (Fig. 2), with a half-live of 15 ± 3 days. This degradation continued in transfers (10% v/v) into artificial river water with 10 mM FeOOH and 1,2-DCA concentrations of 100 µM or 500 µM (not shown), respectively. Per mol of 1,2-DCA degraded, 12 mol of Fe^{2+} was produced. Further enrichment of the iron-reducing culture was done by re-addition of 1,2-DCA. This did not result in higher degradation rates, suggesting that the process may have been limited by the availability of iron, supplied as amorphous iron oxide (data not shown).

Under aerobic, sulfate-reducing and denitrifying conditions, the concentration of the respective electron acceptors decreased over time in Danube sediment microcosms, but 1,2-DCA transformation was not observed.

3.3.1.3 Elbe River sediment

No 1,2-DCA transformation was observed in microcosms incubated with Elbe sediment at any of the tested redox conditions.

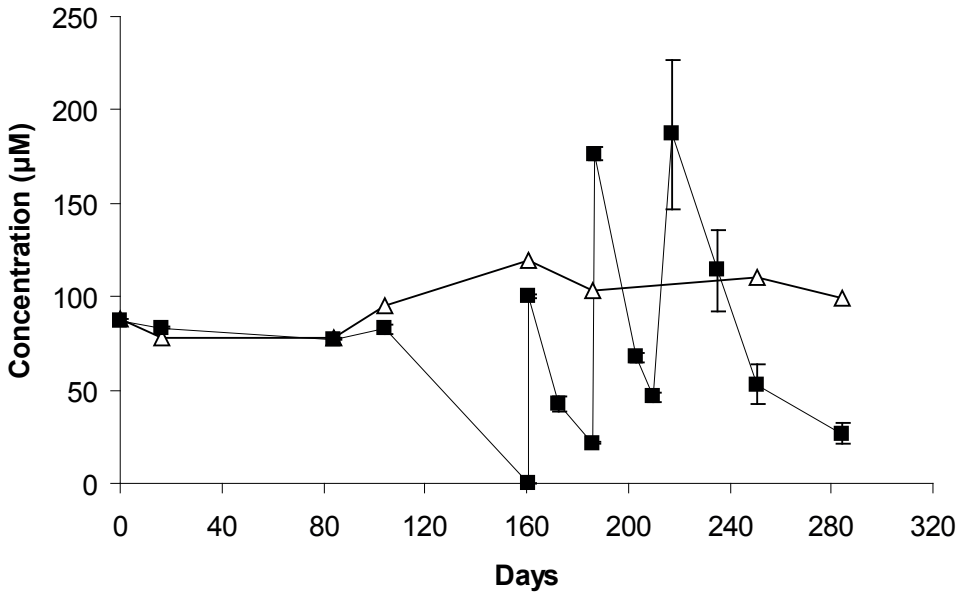

Figure 2: *Concentrations of 1,2-DCA in duplicate iron-reducing microcosms with Danube river sediment (square) and an autoclaved negative control (triangle). Error bars show variation of two duplicate samples and microcosms were spiked with 1,2-DCA at days 161, 187 and 217.*

3.3.2 Molecular identification of the dechlorinating population

Molecular detection by PCR amplification showed that 16S rRNA genes from known reductive dehalogenating micro-organism were present in Ebro and Danube microcosms (Table 2). In all microcosms, the *dca*A and *dhl*A genes were below detection limit (100 gene copies/ml) and in microcosms with Elbe sediment, none of the tested micro-organisms could be detected. Although dehalogenating micro-organisms were detected in both Ebro and Danube river sediment, differences between the river sediments and the different redox conditions used for incubation, were observed.

Table 2: *Dechlorination and relative abundance (‰ of total bacterial 16S rRNA genes) of 16S rRNA genes of known reductive dechlorinating bacteria in microcosms. Presence or absence of Sulfurospirillum 16S rRNA genes is indicated with "+" (= detected) or "-" (= not detected), respectively. << indicates below detection limit (1 x 10^{-6}).*

Redox condition	1,2-DCA dechlorination	*Dehalococcoides* spp.	*Dehalobacter* spp.	*Desulfitobacterium* spp.	*Sulfurospirillum* spp.
Ebro					
Methanogenic	+	3.2×10^{-5}	4.0×10^{-4}	5.5×10^{-4}	+
Denitrifying	+	<<	<<	<<	-
Iron-reducing	-	<<	3.0×10^{-2}	4.8×10^{-5}	+
Sulfate-reducing	-	<<	8.1×10^{-3}	4.7×10^{-5}	+
Aerobic	-	1.4×10^{-3}	<<	<<	+
Danube					
Methanogenic	+	1.41	<<	<<	+
Denitrifying	-	1.3×10^{-4}	<<	<<	-
Iron-reducing	+	4.3×10^{-4}	<<	<<	-
Sulfate-reducing	-	1.5×10^{-4}	<<	1.3×10^{-5}	+
Aerobic	-	<<	<<	1.7×10^{-3}	+

Dehalobacter spp. could not be detected in Danube microcosms, indicating that those micro-organisms were absent in the tested sediment. In contrast, all tested dechlorinating species (*Dehalococcoides* spp., *Dehalobacter* spp., *Desulfitobacterium* spp. and *Sulfurospirillum* spp.) were present in Ebro sediment, albeit at largely varying relative abundance depending on the conditions (Table 2). At methanogenic conditions, *Dehalococcoides* spp. were present in relatively high concentrations, indicating enrichment of members of this genus. This was also observed for Ebro microcosms that were initially incubated aerobically. However, oxygen in these microcosms was depleted during the incubation period, after which 1,2-DCA was transformed to ethene (data not shown). At denitrifying conditions, none of the dechlorinating bacteria could be detected in active and transferred microcosms. In 1,2-DCA transforming microcosms at iron-reducing conditions, only *Dehalococcoides* spp. 16S rRNA genes were detected, but at concentrations that were not higher than in denitrifying and sulfate reducing microcosms with the same sediment that did not show 1,2-DCA degradation.

A taxonomic classification of sequenced clones (deposited under Genbank accession numbers FJ802135 - FJ802386), derived from 1,2-DCA transforming microcosms incubated under methanogenic, denitrifying and iron-reducing conditions, is presented in table 3. All clones were positioned in a phylogenetic tree of bacterial 16S rRNA sequences, using the ARB software environment (Ludwig *et al.,* 2004; Pruesse *et al.,* 2007)

In the dominant bacterial communities of the three enriched microcosms, only a few Phyla were represented. Characteristic for the methanogenic microcosm is the high number of sequences that were most closely related to bacteria within the *Syntrophobacterales*, including the genera *Pelotomaculum*, *Smithella* and *Synthrophus*. These organisms are often involved in synthrophic consortia that degrade organic compounds. Sequences affiliated with the genus *Dehalococcoides* were also found at high relative abundance (6%) in the methanogenic microcosm. The sequence identity of these 16S rRNA genes with known *Dehalococcoides* spp., was relatively low (91%), and clustered separately from the know *Dehalococcoides* spp., indicating a specific phylogenetic position.

Table 3: *Phylum- and order-level classification of isolated 16S rRNA gene clones from 1,2-DCA transformating microcosms at methanogenic, denitrifying and iron-reducing conditions. Numbers indicate relative abundance of clones grouped by order in % of the total number of clones.*

Phylum	Order	Methanogenic	Iron-reducing	Denitrifying
Proteobacteria	*Rhodocyclales*		7	38
	Burkholderiales		28	10
	Desulfobacterales			6
	Desulfuromonales	4		5
	Neisseriales			4
	Desulfovibrionales			2
	Hydrogenophilales		10	2
	Rhizobiales		10	
	Methylophilales		3	
	Syntrophobacterales	28		
Chloroflexi	*Dehalococcoides*	6		
	Caldilineales			10
	Anaerolineales			3
Bacteroidetes	*Bacteroidales*	6		2
	Flaviobacteriales			1
Firmicutes	*Clostridiales*	55	42	
Chlorobi	Unclassified *Chlorobi*			18

In the iron-reducing microcosm only two Phyla were represented in the corresponding clone library. Most of the detected sequences were closely related to *Burkholderiales* spp. and *Clostridiales* spp., which includes the genera *Thiobacillus*, *Acidovorax* or *Clostridium*. For those taxa, one or more iron-reducing species have previously been described (Finneran *et al.,* 2003; Johnson *et al.,* 2001; Lin *et al.,* 2007; Ottow, 1971; Sugio *et al.,* 1985; Vu *et al.,* 2004).

In the denitrifying microcosm a relatively high abundance of *Rhodocyclales* spp. was found, including the denitrifying genera *Azoarcus* and *Thauera*. These organisms are known to degrade aromatic pollutants. In the study of Dinglasan-Panlilio *et al* (2006), *Thauera*-like bacteria were found to be responsible for 1,2-DCA degradation under denitrifying conditions. A total of 4% of the clones shared highest similarity with sulfate-reducing *Desulfuromonas* spp. or *Desulfovibrio* spp., some of which are known as dehalogenating bacteria (Smidt and de Vos, 2004).

3.4 Discussion

The aim of this study was to assess the potential for biodegradation of 1,2-dichloroethane by microbial communities of sediments originating from European rivers at different redox conditions, and to identify biodegradation mechanisms and micro-organisms involved.

1,2-DCA was detected in the water phase of all sampled rivers (Ebro, Danube and Elbe). However, biodegradation capacity of 1,2-DCA was only detected in the sediments, whereas it appeared not present in the water phase. This indicates that the relevant microbial community is present in the sediments, which therefore act as an important sink of 1,2-DCA from the water phase.

Although 1,2-DCA can in principle biologically be degraded under aerobic conditions (Janssen *et al.,* 1985; van den Wijngaard *et al.,* 1992), the present microcosm study showed that in the river systems, transformation of 1,2-DCA occurred only under anaerobic conditions. Anaerobic 1,2-DCA transformation was observed under three different redox conditions: methanogenic, denitrifying and iron-reducing. Under methanogenic conditions, the major process of 1,2-DCA transformation was reductive dechlorination. Initially, the reductively dechlorination products chloroethane and ethene (Holliger and Zehnder, 1996) were detected. The observed half-lives of about 7 weeks were relatively fast compared to half-lives of 1,2-DCA in the environment, estimated at 64-165 days (Belay and Daniels, 1987; Egli *et al.,* 1987) or 1-30 years (Bosma *et al.,* 1998), but lower than those previously reported from highly enriched cultures (1.2 days) (Gerritse *et al.,* 1999a). Specialized reductive dehalogenating bacteria, such as *Dehalococcoides* spp. were enriched in the methanogenic microcosms and identified as one of the relatively dominant populations in the clone library, albeit at only moderate 16S rRNA gene sequence similarity with known *Dehalococcoides* spp.. As the observed high relative abundance of *Dehalococcoides* spp. based on clone library analysis was in disagreement with that based on real time PCR, we tested and found that the cloned *Dehalococcoides* spp. - like 16S rRNA gene sequences were inefficient amplified (recovery of 0.01-0.1 %) by the method used in this study and by many others. This indicates that the diversity of dehalogenating *Dehalococcoides*-like bacteria is broader than initially anticipated and is in line with previous reports of dehalogenating populations within the *Chloroflexi* spp., which are only distantly related to the genus *Dehalococcoides,* including the DF-1/o-17 clade of marine PCB-dehalogenating bacteria, as well as the Lahn- and Tidal Flat-clusters of PCE-degrading bacteria (Fagervold *et al.,* 2007; Kittelmann and Friedrich, 2008a, b; Watts *et al.,* 2005).

Transformation of 1,2-DCA under denitrifying or iron-reducing conditions has been reported rarely (Cox *et al.,* 2000; Dijk *et al.,* 2005; Dinglasan-Panlilio *et al.,* 2006; Gerritse *et al.,* 1999a). Although it could not be unequivocally concluded, which bacteria or degradation pathways were responsible for the observed 1,2-DCA transformation, this study provided further evidence for nitrate-coupled oxidation

of 1,2-DCA. Transformation of 1,2-DCA did not proceed via a reductive dechlorination pathway, since no dechlorination products or reductively dechlorinating micro-organisms were detected. The observed 1,2-DCA dechlorination, nitrate reduction and chloride formation were in accordance with the stoichiometry of nitrate-coupled 1,2-DCA oxidation as previously proposed by Dijk, et al. (2005): $C_2H_4Cl_2 + 2\ NO_3^- \rightarrow 2\ CO_2 + 2\ Cl^- + N_2 + 2\ H_2O$, whereby it is assumed that some NO_3^- was reduced with other electron-donors in the microcosm.

For iron-reducing conditions, also no evidence for a reductive pathway was obtained. An iron-coupled anaerobic 1,2-DCA oxidative process is likely, since 1,2-DCA was biologically transformated in parallel with reduction of a stoichiometric amount of iron required for complete oxidation of 1,2-DCA: $C_2H_4Cl_2 + 10\ Fe(OH)_3 + 18\ H^+ \rightarrow 2\ CO_2 + 2\ Cl^- + 10\ Fe^{2+} + 26\ H_2O$. Of the clones from this microcosm, 40% clustered with taxa for which one or more iron-reducing species have been described, while these organisms were not detected in the clone libraries from the methanogenic or denitrifying microcosm.

In contrast to Ebro and Danube sediment, no degradation of 1,2-DCA was observed in microcosms with Elbe river sediment. The apparent absence of 1,2-DCA in Elbe sediment may be caused by a higher oxygen concentration in the water phase and a much lower amount of organic material compared to the other two river sediments (Table 1). These conditions may have constrained the development of anaerobic niches for 1,2-DCA dechlorinating bacteria, as they were not detected in the sediment. The artificial reconstruction activities of the Elbe river bank one year before the sampling campaign may have disturbed the 1,2-DCA degradation capacity. Thus a significant period of time may be required to (re)establish an anaerobic dechlorinating potential after physical perturbations of river sediment. More generally, enrichment of different microbial populations can be linked to different origin, geochemical conditions or pollution. This may explain the observed variation in degradation capacity in microcosms derived from different rivers under the selected redox conditions.

In conclusion, this study showed that biodegradation capacity of 1,2-DCA was present in the sediments, but not in the water phase, indicating that the sediments act as a sink for 1,2-DCA. Under methanogenic conditions, 1,2-DCA can be reductively dechlorinated to chloroethane and ethene in sediment from Ebro river, or to ethene in sediment from Danube river. Under denitrifying or iron-reducing conditions, 1,2-DCA can be degraded by oxidation in sediment from Ebro river or Danube river, respectively. For further description, modeling and forecasting pollutant biodegradation in river systems, the variability in microbial communities, geochemical conditions and associated oxidative and reductive bioconversion processes need to be taken into account.

Acknowledgments

This research was supported by the European Union through the Aquaterra Integrated project of the sixth framework program (FP6-SUSTDEV-2003-505428). We thank Ingo Lobe (UFZ, Magdeburg, Germany), Alain Hildebrandt and Alicia Navarro (CSIC, Barcelona, Spain), and Neslihan Taş (WU, Wageningen, the Netherlands) for providing data of characterized sediment and their assistance during sampling.

Chapter 4

Correlation of *Dehalococcoides* 16S rRNA and chloroethene-reductive dehalogenase genes with geochemical conditions in chloroethene-contaminated groundwater

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This chapter has been published as:
Bas van der Zaan, Fredericke Hannes, Nanne Hoekstra, Huub Rijnaarts, Willem M. de Vos, Hauke Smidt and Jan Gerritse, 2010, Correlation of *Dehalococcoides* 16S rRNA and chloroethene-reductive dehalogenase genes with geochemical conditions in chloroethene-contaminated groundwater in Applied and Environmental Microbiology, Vol. 76, (3), p. 843-850

Abstract

Quantitative analysis of genes that code for *Dehalococcoides* 16S rRNA and chloroethene reductive dehalogenases TceA, VcrA and BvcA was done on groundwater sampled from 150 monitoring wells spread over 11 chlorinated ethene polluted European locations. Redundancy analysis (RDA) was used to relate molecular data to geochemical conditions. *Dehalococcoides* spp. 16S rRNA- and VC-reductase genes were present at all tested locations in concentrations up to 10^6 gene copies per ml groundwater. However, differences between and also within locations were observed. Variation in *Dehalococcoides* spp. 16S rRNA gene copy numbers were most strongly correlated to dissolved organic carbon concentration (DOC) in groundwater and to conditions appropriate for biodegradation of chlorinated ethenes (EPA-score). In contrast, *vcrA* gene copy numbers correlated most significantly to VC and chlorinated ethene concentrations. Interestingly, *bvcA* and especially *tce*A were more correlated with oxidizing conditions. In groundwater microcosms, dechlorination of 1 mM VC was correlated to an increase of *vcrA* and/or *bvcA* gene copies by 2-4 orders of magnitude. Interestingly, in 34% of the monitoring wells and in 40% of the active microcosms, the amount of individual VC-reductase gene copies exceeded that of *Dehalococcoides* spp. 16S rRNA gene copies. It is concluded that the geographical distribution of the genes was not homogeneous, depending on the geochemical conditions, whereby *tceA* and *bvcA* correlated to more oxidized conditions than *Dehalococcoides* spp. 16S rRNA and *vcrA*. Because variation in VC-reductase gene numbers was not directly correlated to variation in *Dehalococcoides* spp., VC-reductase genes are better monitoring parameters for VC dechlorination capacity than *Dehalococcoides* spp.

4.1 Introduction

Chlorinated ethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE) are persistent groundwater pollutants (McCarty, 1997; Westrick *et al.,* 1984). Because these compounds are toxic and mobile in groundwater systems, they form a serious risk for human health and the environment. PCE and TCE can be dechlorinated by microorganisms under anaerobic conditions by reductive dehalogenation to dichloroethene (DCE), vinyl chloride (VC) and ethene (Smidt and de Vos, 2004). Bioremediation strategies for chloroethene-contaminated sites are often based on (stimulation of) reductive dechlorination of the chlorinated ethenes to ethene (Gerritse *et al.,* 1999b; Lendvay, 2003; Major *et al.,* 2002). In practice, reductive dechlorination of PCE and TCE can be incomplete, resulting in accumulation of DCE or VC. Since VC is much more mobile, toxic and carcinogenic than PCE and TCE (Henschler, 1994), monitoring and stimulation of VC dechlorination are essential steps in bioremediation strategies.

Only members of *Dehalococcoides* spp. are known to be able to reductively dechlorinate VC. Therefore, 16S rRNA genes of these species are often used as molecular target to indicate and monitor DCE and VC dechlorination capacity at contaminated sites. However, previous studies showed different dechlorination capacities for individual *Dehalococcoides* species, and only a few strains are known to metabolically dechlorinate VC (Duhamel *et al.,* 2004; He *et al.,* 2003; Krajmalnik-Brown *et al.,* 2004; Muller *et al.,* 2004; Sung *et al.,* 2006). As a consequence, 16S rRNA gene-based detection can lead to overestimation of VC dechlorination capacity. In contrast, although metabolic reductive dechlorination of VC has mostly been linked to *Dehalococcoides* spp., it can not be excluded that other microbial species, which perform this dechlorination, exist. Genes coding for DCE and VC reductases may be exchangeable between different microbial species via horizontal gene transfer. This is plausible since it has been shown that the metabolic genes for VC dechlorination, *vcrA* and *bvcA*, have a different evolutionary history than most other *Dehalococcoides* genes (McMurdie *et al.,* 2007). Consequently, *Dehalococcoides* 16S rRNA gene-based detection can also lead to underestimation of VC dechlorination capacity.

To more precisely determine VC dechlorination capacity, genes directly involved in reductive dechlorination of VC should be used as a molecular target, in addition to *Dehalococcoides* spp. 16S rRNA genes. A quantitative method was described to detect genes coding for VC-reductases VcrA and BvcA identified in *Dehalococcoides* sp. strains VS and GT and in *Dehalococcoides* sp. strain BAV1, respectively (Krajmalnik-Brown *et al.,* 2004; Muller *et al.,* 2004; Sung *et al.,* 2006). Different studies showed direct correlation of *vcrA* and *bvcA* gene copy numbers with reductive dechlorination of VC in batch cultures, soil columns and contaminated sites (Behrens *et al.,* 2008; Lee *et al.,* 2008; Scheutz *et al.,* 2008).

Quantification of genes that encode VC-reductases can be a useful method to monitor reductive dechlorination of VC in chloroethene-contaminated groundwater during enhanced natural attenuation activities (Cupples, 2008; Scheutz *et al.,* 2008). However, little is known about the presence, dispersion and importance of specific dehalogenase genes in chlorinated ethene polluted groundwater and their correlation to biogeochemical conditions and reductive dechlorination.

The objective of this study was therefore to identify the relative importance of TCE-reductase gene *tceA* and VC-reductase genes *vcrA* and *bvcA* in chloroethene-polluted groundwater, and to identify geochemical parameters that contribute to variation in copy numbers of these genes. To this end, groundwater of 150 monitoring wells from 11 European polluted sites was analyzed. Furthermore, microcosms with groundwater from 6 locations were started to test whether VC dechlorination is directly correlated to an increase of *vcrA* or *bvcA* genes.

4.2 Materials and Methods

4.2.1 Sample locations

Eleven sample locations (Table 1) were selected based on availability of groundwater for this study, presence of chlorinated organic pollutants, presence of different monitoring wells at the same location, and availability of geochemical data from the monitoring wells. All locations are situated in the Netherlands, except location H, which is situated in the United Kingdom. Pollution with chlorinated ethenes originated from (former) industrial activities and was present for several decades. At locations A, C, F, and H, other pollutants like 1,2-dichloroethane, pesticides and mineral oil were also present. The natural bioremediation process of chlorinated ethenes was enhanced at locations H, I, J and K by addition of electron donor into the groundwater. For all locations, monitoring wells within and outside the polluted zone were included in the study.

Table 1: *Overview of amount of analyzed monitoring wells, redox conditions, vinyl chloride concentration, presence of other pollutants, and performed remediation activities for the 11 sample locations. The values represent the lower and upper extremes of the individual monitoring wells at the locations. N.A. indicates that the information was not available.*

Location	# of wells	pH	Eh (mV)	DOC (mg/l)	VC (µg/l)	Other pollutants present	Remediation actions
A	27	6.5 - 6.9	-166 - -70	8 - 110	<1 - 434	1,2-dichloroethane	Natural attenuation
B	12	6.6 - 7.2	-130 - -59	9 - 47	2 - 14000		Natural attenuation
C	9	5.8 - 6.3	-43 - +31	n.a.	<1 - 150	Mineral oil, cresol, heavy metals	Natural attenuation
D	11	5.8 - 6.4	-22 - +95	10 - 41	<1 - 870		Natural attenuation
E	12	6.7 - 7.3	-182 - -86	4 - 41	<1 - 4978		Natural attenuation
F	12	5.1 - 6.6	-139 - +208	3 - 15	<1 - 19	Mineral oil, aromatics, heavy metals, pesticides	Natural attenuation
G	10	n.a.	n.a.	<1 - 48	<1 - 5		Natural attenuation
H	16	n.a.	n.a.	n.a.	74 - 160417	1,2-dichloroethane	Substrate addition
I	15	n.a.	n.a.	n.a.	<1 - 27		Substrate addition
J	16	5.5 - 6.6	-110 - +170	n.a.	<1 - 2742		Substrate addition
K	10	6.7 - 7.2	-114 - +295	n.a.	<1 - 1671		Substrate addition

4.2.2 Groundwater sampling

Groundwater was collected between 2007 and 2008 from monitoring wells, using a peristaltic pump and Marprene tubing. Before duplicate samples were taken, three water volumes of the monitoring well were discarded to refresh the well water. Sterilized 1L glass bottles were filled completely and stored at 4°C. The samples were used within one week for DNA extraction and within one month for chemical analyses and preparation of microcosms.

4.2.3 Microcosm incubation

Anaerobic microcosms B_{mw421}, C_{mwdb2}, E_{mw403}, E_{mw100}, F_{mw401}, F_{mw54}, G_{mwRH05}, G_{mwRH48}, H_{mw68} and H_{mw267} were prepared with groundwater from monitoring wells pb-421 (location B); pb-db2-23,5 (location C); pb-403 and pb-100-61 (location E); pb-401 and pb-54 (location F); pb-RH05-22 and pb-RH48 (location G); pb-mwsa68 and pb-BH267 (location H), respectively. Monitoring wells with both high or low VC concentration, high or low concentration of vinyl chloride reductase genes, and with high, low or unknown biodegradation potential (EPA-score) were selected, to minimize a potential bias of site selection criteria. Samples of 200 ml groundwater were incubated at 20°C and 150 rpm for 4 months in 220ml serum bottles, closed with a viton stopper. Protamylasses, which is a product from the potato-industry, (1 ml, Avebe, Veendam, the Netherlands) was added as electron donor. The protamylasses solution contained 180 mg/l total organic carbon (TOC) per liter. This corresponded to 75 mM organic carbon added to the bottles. Assuming an average composition as sugar $[CH_2O]_n$, this accounts for 300 mM of added "electron equivalents". For each monitoring well two microcosms were started, one of which received 1.5 mM of VC. Headspace analyses (500 µl) for VC concentration and liquid samples (5 ml) for DNA extraction were taken monthly under sterile and anaerobic conditions with a syringe .

4.2.4 DNA isolation

Biomass from 50-250 ml groundwater samples was concentrated by vacuum filtration in triplicate on 0.2 µm pore-size filters (Schleicher & Schuell, ME 24 membrane filters, 25 mm diameter, Den Bosch, the Netherlands). Biomass was concentrated from 5 ml microcosm samples in duplicate by centrifugation (20 minutes at 4000 rpm) and the supernatant was filtrated through 0.2 µm pore-size filters (Schleicher & Schuell, ME 24 membrane filters, 25 mm diameter). All filters were crushed with a sterilized wooden tooth pick. Total DNA was extracted from the concentrated biomass with BIO101 Fast DNA Kit for Soil Kit (Qbiogene, Inc, CA). Besides addition of one extra 1/4" ceramic sphere (Qbiogene) to Lysing Matrix E of the DNA isolation kit, no modifications to the manufacturer's protocol were applied. Quality and quantity of extracted DNA was determined using a Nanodrop ND-1000 spectrophotometer (Isogen, de Meern, the Netherlands) and by electrophoresis on a 1% agarose-gel. DNA was stored at -20°C.

4.2.5 Quantitative PCR analysis

Copy numbers of VC-reductase genes *vcrA* and *bvcA, tceA,* and *Dehalococcoides* spp. 16S rRNA genes were quantified in 25 µl quantitative PCR (qPCR) assays, using IQ-Supermix (Bio-Rad, Veenendaal, the Netherlands) and 3 µl template DNA isolated from the filters. For all filters, 10- and 100- times diluted DNA extracts were analyzed in duplicate. Sterilized MilliQ water and an environmental sample without

target genes were used as negative controls. As positive controls, genes of interest cloned into a pGEM-T vector (Promega) were used. The coefficient of variance was below 5% for all qPCR assays. Specific primers and probes for detection of *vcrA, bvcA, tceA* and *Dehalococcoides* spp. 16S rRNA genes were described by (Ritalahti *et al.,* 2006). The temperature program for all PCR assays on an IQ5 Real-Time PCR System (Bio-Rad) was as follows: 3 minutes at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 58°C and 1 min at 72°C, and a final elongation step of 5 minutes at 72°C. The number of gene copies was calculated by comparing the C_t-values of the individual samples with the C_t-values of a calibration curve. For *Dehalococcoides* spp. 16S rRNA gene, *vcrA* gene, *bvcA* gene and *tceA* gene calibration was done using 10-fold serial dilutions of 10^1-10^7 genes/ml of pGEM-T easy vectors (Promega, Madison, USA) containing a single copy of the gene of interest. The amount of gene copies in the DNA samples was extrapolated to the amount of genes copies per ml groundwater or microcosm culture. The lower detection limit in groundwater of *Dehalococcoide*s 16S rRNA and reductase genes differed because of different PCR efficiencies and were 10 and 100 gene copies/ml groundwater, respectively. The detection limits were determined based on the gene concentration present in the most strongly diluted standard of the calibration curve, which gave a positive signal in the qPCR analysis. This concentration was divided by the amount of groundwater used for DNA extraction. Every single qPCR run had its own calibration curve. The detection limits were 100-fold higher for microcosm samples, due to a lower sample volume used for DNA extraction. Regularly, the size of the obtained amplicons were checked on a 2% agarose gel. The standard error for quantification the individual genes, differed from 0.52 to 0.75 order of magnitude for the *Dehalococcoides* spp. 16S rRNA and VC or TCE reductase genes.

4.2.6 Chemical analyses

Chlorinated hydrocarbons (PCE, TCE, DCE, 1,2-dichloroethane) were analyzed in 8 ml diluted groundwater samples, on a Varian 3800 gas chromatographic (GC) system equipped with a mass spectrometry detector (MS) and a Porabond-Q column (0.32 mm x 25 m) (Varian, Middelburg, the Netherlands). GC settings were: injector temperature 200°C; detector temperature 300°C; oven temperature 3 minutes at 40°C, followed by an increase of 10°C/min to 70°C; followed by an increase of 15°C/min to 250°C for 7 minutes; carrier gas helium with a flow rate of 2 ml/min. External standards at 6 different concentrations in the range between 0 – 30 µM were used for calibration.

VC, ethene, ethane, and methane in groundwater samples were quantified in 1 ml samples on a Varian 3800 GC equipped with a flame ionizing detector (FID) and a Porabond-Q column (0.32 mm x 25 m) (Varian). GC settings were: injector temperature 200°C; detector temperature 300°C; oven temperature 3 minutes at 40°C, followed by an increase of 10°C/min to 70°C; followed by an increase of 15°C/min to 250°C for 7 minutes; a flow rate of 2 ml/min. For microcosm analyses, 500

µl headspace samples, obtained by using a 1 ml Pressure-Lock gas syringe (Alltech, Breda, the Netherlands) with a sterile needle, were analyzed on the GC-FID described above. External standards at 6 different concentrations in the range between 0 – 3 mM were used for calibration. The lower detection limit was 1 µM for all analyzed compounds.

Chloride, nitrate and sulfate ion analyses were done on 1 ml diluted groundwater and microcosm samples on a Dionex ICS-1500 equipped with an IONPAC AS14 anion-exchange column and an A SRS®-Ultra 14 mm suppressor (Dionex Corporation, Sunnyvale, CA). The eluent (2.0 mM Na_2CO_3 and 0.75 mM $NaHCO_3$) flow rate was 1.3 ml/min. The injection needle was pre-flushed with 100 µl MilliQ-water and 50 µl samples were injected. External standards at 6 different concentrations in the range between 0 – 250 µM, were used for calibration. Groundwater and microcosm samples were diluted 10, 100 and 500 times, before analysis.

Dissolved organic carbon (DOC) content was determined in 0.5 ml groundwater by flash combustion followed by CO_2 analysis by an IR detector (Leco SC-632, Leco Mönchengladbach, Germany). Iron content of the samples was determined by ICP-OES (Inductively Coupled Plasma - Optical Emission Spectrometer) analysis (SPECTRO CIROS^{CCD} (Spectro, Kleve, Germany), after destruction with a mixture of HF/$HClO_4$/HNO_3.

Hydrogen concentration in the groundwater was analyzed on-site, using a gas chromatograph with a reduction gas detector (Trace Analytical, Bester, Amstelveen, the Netherlands), which was equipped with a Carbosieve II column. The carrier gas was N_2 with a flow rate of 20 ml/min. The column and detector temperatures were 104°C and 265°C, respectively.

Temperature, pH, redox potential, and salinity was measured on groundwater that was pumped through a flow-cell (Eijkelkamp B.V., Groesbeek, the Netherlands) on-site, with a pH/mV/EC/Sal/TDS/T/O_2 multiparameter (Eijkelkamp) and corresponding probes (Eijkelkamp).

4.2.7 Biodegradation potential

Biodegradation potential of the individual monitoring wells was quantified by the EPA-score, based on 9-10 critical geochemical parameters, as described by the United States Environmental Protection Agency (EPA) in "Protocol for evaluating natural attenuation" (Wiedemeier *et al.,* 1998). For the EPA-score, positive or negative values are provided tot geochemical parameters which are presumed critical for reductive dechlorination of chlorinated ethenes (e.g. an oxygen concentration above 0.5 mg/l is provided with a score of -3 and a sulfate-concentration below 20 mg/l is provided with score 2). The sum of the scores for the individual parameters results in the EPA-score. In groundwater with an EPA-score above 15, reductive dehalogenation of chloroethenes is likely.

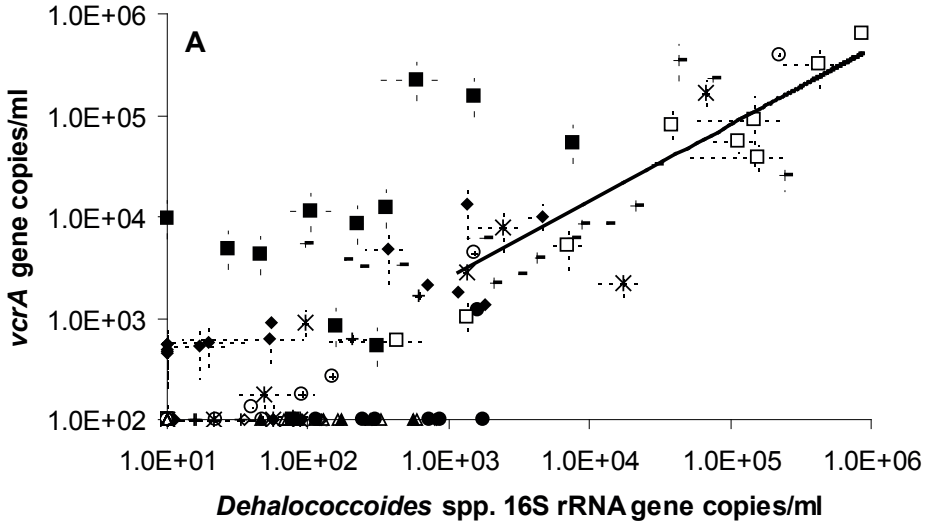
4.2.8 Redundancy analysis

Redundancy analysis (RDA) is a multivariate regression analysis, whereby variation in one set of variables is explained by another set of variables. In this study, gene copy numbers were selected as one set of variables (species data) and geochemical data were selected as the explaining set of variables (environmental data). The RDA was performed with Canoco 4.5 software (Microcomputer Power, Ithaca, NY, USA) on data of all monitoring wells from locations A, B, E, F and K and monitoring wells pb103, pb104, pb105 and pb1022 from location C, since for these monitoring wells an almost complete dataset was available. For the redundancy analysis, a linear distribution of the "species" (in this case, gene copy numbers) was assumed to explain their variation by environmental variables. Data were log-transformed (Y=Log(AY+B), A=1 and B=1), for parameters with more than 10-fold variation within the data. In the analysis, data below the detection limit were substituted by values of 70% of the detection limit. To test the significance of the relationship of gene copy number variance with geochemical parameters, unrestricted Monte Carlo permutation tests were performed with 499 random permutations and a significance level (p) of 0.05.

4.3 Results

4.3.1 Molecular detection of chloroethene reductive dehalogenase and *Dehalococcoides* spp. 16S rRNA genes

Vinyl chloride reductase genes (*vcrA* and *bvcA*), TCE reductase gene (*tceA*), and *Dehalococcoides* spp. 16S rRNA genes were detected in 150 groundwater samples obtained from 11 contaminated locations. At all tested locations, *Dehalococcoides* spp. 16S rRNA genes and VC-reductase genes were present in the groundwater, albeit not in all monitoring wells. Moreover, differences between and within locations were observed with respect to the ratio of *vcrA* and *bvcA* with *Dehalococcoides* spp. 16S rRNA genes in the individual monitoring wells (Fig. 1). The concentration of *Dehalococcoides* 16S rRNA genes ranged for most monitoring wells from below detection limit (10^1 gene copies/ml) up to 10^4 gene copies/ml, but exceeded 10^5 gene copies/ml at locations H, I and K, where enhanced bioremediation techniques were applied.


A
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1.0E+05
1.0E+04
1.0E+03
1.0E+02
vcrA gene copies/ml
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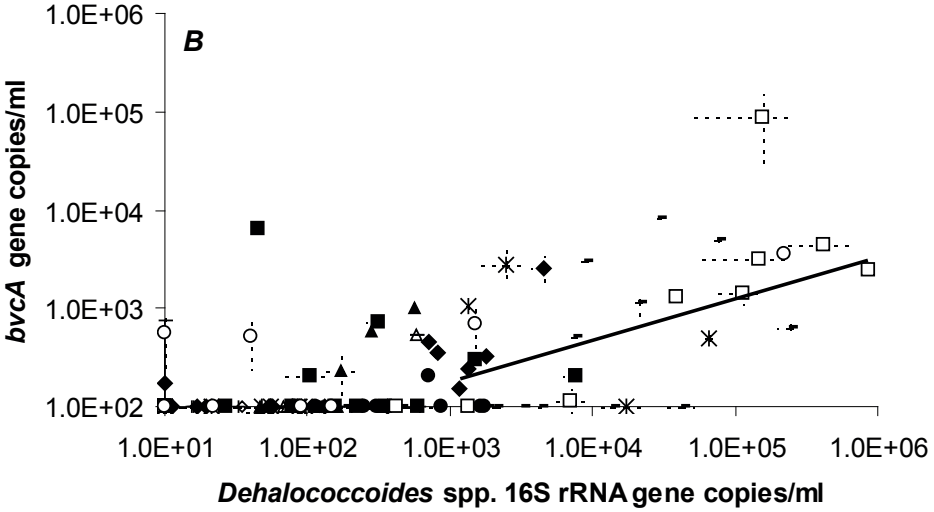
1.0E+06
Dehalococcoides spp. 16S rRNA gene copies/ml


B
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1.0E+03
1.0E+02
bvcA gene copies/ml
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1.0E+04
1.0E+05
1.0E+06
Dehalococcoides spp. 16S rRNA gene copies/ml

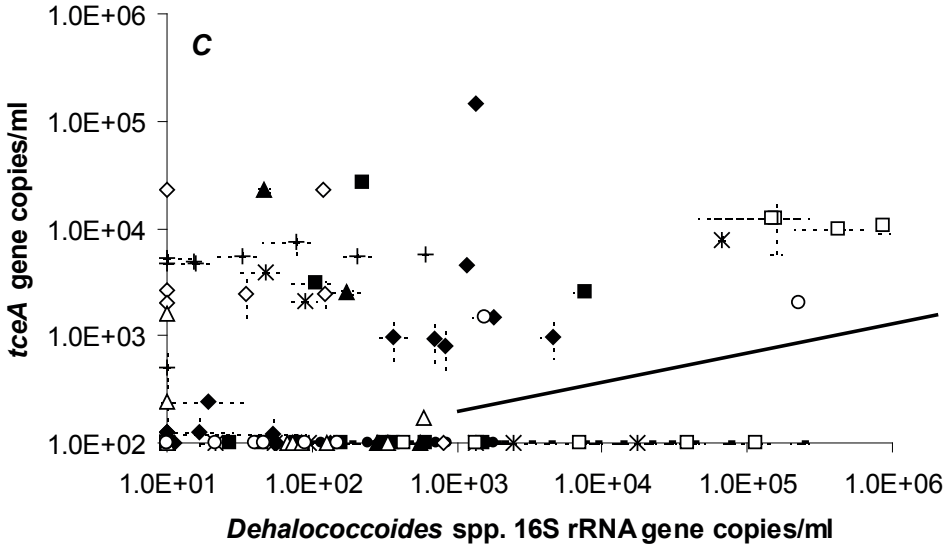

Figure 1: *Total amount of Dehalococcoides spp. 16S rRNA gene and specific reductase vcrA **(A)**, bvcA **(B)** or tceA (**C**) gene copies, respectively, in groundwater of the individual monitoring wells. Symbols represent data of monitoring wells from the different locations (♦=location A; ▪=location B; ◄=location C; •=location D; *=location E; ◊=location F; +=location G; -=location H; ☐=location I; Δ=location J; o=location K). X- and Y-Error bars indicate standard deviations of the Dehalococcoides spp. 16S rRNA or reductase genes, respectively, and were only visualized when larger than the symbols. Regressions lines (**A**: $y=13.19x^{0.7526}$ ($R^2=0.62$); **B**: $y=5.64x^{0.4977}$ ($R^2=0.33$); **C**: $y=31.29x^{0.2777}$ ($R^2=0.06$)) were given for monitoring wells with > 10^3 16S rRNA gene copies / ml groundwater.*

Of the two VC-reductase genes, *vcrA* was usually more abundant than *bvcA*. At 5 different locations, the *vcrA* concentration was above 105 gene copies/ml, although for most locations concentrations up to 104 gene copies/ml were found. Except for monitoring wells pb-307 (location D) and pb-605 (location J), in all samples *vcrA* genes were detected when the concentration of *Dehalococcoides* spp. 16S rRNA gene copies was above 103 per ml. For samples with more than 103 *Dehalococcoides* spp. 16S rRNA gene copies, the average ratio of *vcrA* to *Dehalococcoides* spp. 16S rRNA gene copies was found to be 1.3 ± 0.5 with a correlation coefficient of 0.62. For monitoring wells with *Dehalococcoides* 16S rRNA concentrations below 103 gene copies/ml, *vcrA* gene copy numbers were 11.5 ± 6.9 times higher than *Dehalococcoides* spp. 16S rRNA genes. However, the difference between both genes is smaller than the standard error for quantification the individual genes, which differed from 0.52 to 0.75 order of magnitude for the *Dehalococcoides* spp. 16S rRNA and VC or TCE reductase genes. Remarkably, at location B, the amount of *vcrA* gene copies was about 10-100 times above the detected amount of *Dehalococcoides* spp. 16S rRNA genes ($p<0.05$).

BvcA genes could not be detected in 71% of the analyzed monitoring wells. In most other monitoring wells the *bvcA* concentration was lower than that of *Dehalococcoides* spp. 16S rRNA genes ($p<0.05$). At locations G and J, *bvcA* was below detection limit in all monitoring wells. Only in 15 monitoring wells, *bvcA* copy numbers were above 10^3 copies/ml. Of those 15 wells, 11 were from locations where bioremediation was stimulated by addition of electron donor. At locations C, F and J, *bvcA* but no *vcrA* was detected. At locations A, B, E, H, I and K, *vcrA* and *bvcA* were simultaneously present. Here, the *vcrA* concentration was always 1-2 orders of magnitude higher than the *bvcA* concentration ($p<0.01$).

*Tce*A genes were most abundant in monitoring wells of locations F and G at concentrations of above 10^3 copies/ml. For the other locations, *tce*A genes were present in one or more monitoring wells at concentrations up to 10^4 copies/ml, however the genes were not detected at locations D and H. No correlation was observed between *tce*A and *Dehalococcoides* spp. 16S rRNA or VC-reductase genes.

4.3.2 Geochemical parameters

Redundancy analyses (RDA) showed that variation in *Dehalococcoides* spp. 16S rRNA, VC-reductase, and TCE reductase gene copy numbers was explained by different geochemical parameters (Fig. 2), since the vectors representing the different genes show different angles with the vectors which represent specific geochemical conditions. In general, the smaller the angle between vectors in the RDA-plot, the better the correlation between the parameters these vectors represent. Vectors that point in opposite direction indicate that corresponding variables are negatively correlated. Along the first canonical axis (i.e. the x-axis in Fig. 2), *Dehalococcoides* spp. 16S rRNA and *vcrA* genes were both positively correlated with pH, EPA-score and methane, ethene and VC concentrations. Along the second canonical axis

(y-axis) both VC-reductase genes correlated negatively with *Dehalococcoides* spp. 16S rRNA. *Dehalococcoides* spp. 16S rRNA correlated best with Fe^{2+}, NO_3, DOC and biodegradation potential. Variation in VC and CAH concentrations and conductivity of the groundwater had the highest correlation with *vcrA* genes. B*vcA* genes were on the Y-axis correlated to the same environmental parameters as *vcrA* genes, but on the X-axis *bvcA* genes were more positively correlated to Fe^{2+}, NO_3 and Eh (high redox potential). Sulfate concentration was strongly negatively correlated to *Dehalococcoides* spp. 16S rRNA and *vcrA* genes. *TceA* genes correlated to different, more oxidized, geochemical parameters than the other analyzed genes and even opposite to *vcrA* genes.

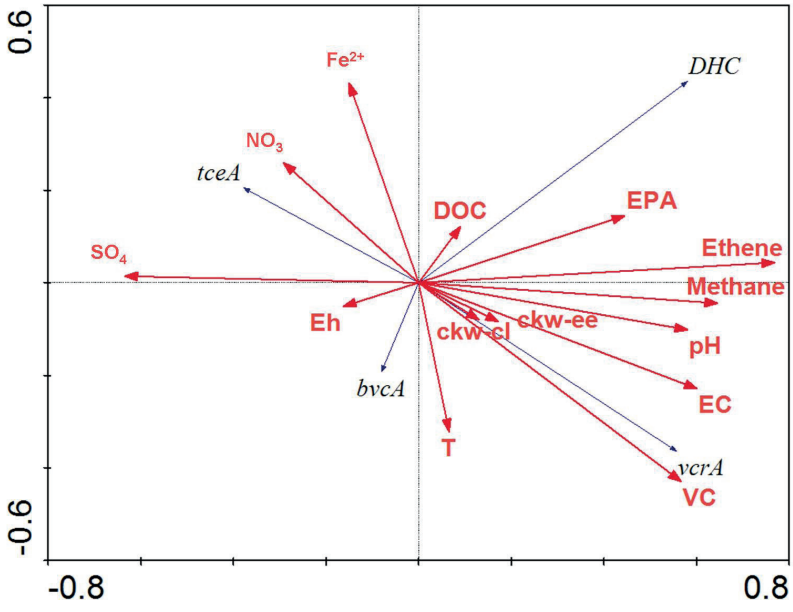

Figure 2: *Redundancy analysis of geochemical parameters and gene copy numbers. Multivariate analysis was used to explain correlation between geochemical parameters on concentration of Dehalococcoides spp. 16S rRNA (DHC 16S), vcrA, bvcA and tceA gene copy numbers. Data are presented as vectors, whereby the bolt arrows represent the geochemical parameters (EPA= EPA score; methane = methane concentration; T = temperature; pH = pH; EC = conductivity; VC = vinyl chloride concentration; ethene = ethene concentration; CAH = total concentration of PCE, TCE, DCE and VC, in units of potential available ethene; SO_4 = sulfate; NO_3 = nitrate; DOC = Dissolved organic carbon; Eh = redox potential; Fe^{2+}= concentration of Fe^{2+}). The angle between vectors represents correlation of those vectors, whereby vectors pointing in the same direction (angle < 90°) are positively correlated while vectors in opposite direction are negatively correlated. The Eigenvalues of the first (X) and second (Y) canonical axes are 0.238 and 0.114, respectively.*

4.3.3 Microcosms

In microcosms with groundwater from locations B, C and E, incubated with protamylasses and VC, dechlorination of VC to ethene was observed (Table 2). No VC dechlorination was observed in groundwater from locations F and G during the incubation period of 4 months. In microcosm H_{MW68}, VC was dechlorinated to ethene, whereas in microcosm H_{MW267} no dechlorination occurred. In the absence of VC-dechlorination, no increase of *Dehalococcoides* spp. 16S rRNA, *vcrA* or *bvcA* gene copy numbers was found. In contrast, an increase of 2-4 orders of magnitude in the gene concentrations was observed in VC-dechlorinating microcosms during the incubation period. After incubation, total gene concentrations for both *Dehalococcoides* spp. 16S rRNA and VC-reductase genes above 10^7 copies/ml were observed. In the VC-dechlorinating microcosms, *vcrA* gene copy numbers were 1 to 2 orders of magnitude above *bvcA* gene copy numbers, as in the groundwater. However, in microcosm C_{mwdb2} a significant increase of *bvcA* occurred, whereas no *vcrA* was detected. This corresponded to absence of *vcrA* and presence of *bvcA* in groundwater of location C. After incubation, the *vcrA* / *Dehalococcoides* 16S rRNA gene ratio in dechlorinating microcosms was 0.49±0.06 and thus lower than observed in the groundwater.

Table 2: *VC dechlorination activity and gene copy numbers of Dehalococcoides 16S rRNA, vcrA and bvcA genes detected in the microcosms before and after incubation with protamylasses and 1.5 mM VC.*

			Gene copies/ml					
			Before incubation			After incubation		
Microcosm	Monitoring well	VC dechlorination	16S-DHC	vcrA	bvcA	16S-DHC	vcrA	bvcA
B_1	pb-421	+	$1.7\pm0.2 \times 10^5$	$6.5\pm2.5 \times 10^3$	$5.3\pm1.6 \times 10^2$	$1.4\pm1.8 \times 10^7$	$6.5\pm1.3 \times 10^6$	$1.7\pm0.7 \times 10^4$
C_1	pb-DB2-23,5	+	$2.9\pm0.3 \times 10^2$	<100	$3.1\pm0.0 \times 10^2$	$7.3\pm4.6 \times 10^6$	<10000	$3.3\pm0.4 \times 10^6$
E_1	pb-403	+	$1.8\pm3.5 \times 10^4$	$2.2\pm1.2 \times 10^3$	<100	$1.7\pm0.3 \times 10^7$	$7.8\pm5.5 \times 10^7$	$9.7\pm3.9 \times 10^5$
E_2	pb-100-61	+	$2.4\pm1.8 \times 10^3$	$7.9\pm6.8 \times 10^3$	$2.8\pm2.1 \times 10^3$	$1.7\pm0.3 \times 10^6$	$4.8\pm0.6 \times 10^6$	$2.4\pm0.1 \times 10^5$
F_1	pb-401	-	<10	<100	<100	<1000	<10000	<10000
F_2	pb-54	-	<10	<100	<100	<1000	<10000	<10000
G_1	pb-RH05-22	-	<10	<100	<100	<1000	<10000	<10000
G_2	pb-RH48	-	$6.1\pm1.1 \times 10^2$	$1.7\pm0.2 \times 10^3$	<100	<1000	<10000	<10000
H_1	pb-mwsa68	+	$3.5\pm0.6 \times 10^2$	<100	<100	$3.8\pm0.5 \times 10^6$	$2.1\pm0.4 \times 10^6$	$5.2\pm1.0 \times 10^4$
H_2	pb-BH267	-	$6.3\pm1.5 \times 10^2$	$1.2\pm1.8 \times 10^3$	$4.8\pm0.1 \times 10^2$	<1000	<10000	<10000

4.4 Discussion & Conclusion

The aim of this study was to identify the relative importance of *Dehalococcoides*, TCE-reductase TceA and VC-reductases VcrA and BvcA in chlorinated ethene polluted groundwater, and to identify geochemical parameters that most significantly contribute to variation in corresponding 16S rRNA, *tceA*, *vcrA* and *bvcA* genes. Although a few environmental studies reported the presence of *tceA*, *vcrA* or *bvcA* genes in combination with *Dehalococcoides* spp. for one or two locations (Behrens *et al.,* 2008; Carreon-Diazconti *et al.,* 2009; Lee *et al.,* 2008), this is the first study that described quantities of all those genes at multiple locations, and in combination with geochemical data, allowing for robust multivariate analysis. At all tested locations, VC-reductase-encoding genes were detected, and highest gene copy numbers were observed at locations where enhanced bioremediation techniques were applied. In general, *vcrA* genes were detected in more monitoring wells and at higher concentrations than *bvcA* genes. This is in accordance with the results of Carreon-Diazcont and co-workers (Carreon-Diazconti *et al.,* 2009). Only at one of the locations investigated in this study (location C), *bvcA* was present but *vcrA* apparently not. This contrasts to other environmental studies (Behrens *et al.,* 2008; Lee *et al.,* 2008), where the amount of *bvcA* gene copies numerically exceeded the amount of *vcrA* genes. Thus, as our results showed that the geographical distribution of *vcrA* and *bvcA* is heterogeneous, it can be concluded in combination with other studies that at different locations, the dominant VC-reductase need not be the same. Therefore, both *vcrA* and *bvcA* genes should be monitored when VC-reductase-encoding genes are used to identify VC dechlorination capacity.

In monitoring wells with more than 10^3 *Dehalococcoides* spp. 16S rRNA gene copies per ml, the correlation coefficient with *vcrA* gene copy numbers was 0.62 (Fig. 1a). This indicates that both genes are correlated to each other in the tested monitoring wells. Different factors can be the reason for this relatively low correlation coefficient, e.g. variation in numbers of gene copies per *Dehalococcoides* cell. This should be addressed in future studies, e.g. by assessing 16S rRNA/VC reductive dehalogenase gene ratios at single cell level. The relative amount of VC-reductase genes to *Dehalococcoides* spp. 16S rRNA genes is relatively high (>1) compared to the studies of Lee *et al.* (2008) and Scheutz *et al.* (2008), which reported ratios below 1.

The amount of *Dehalococcoides* spp. 16S rRNA genes, correlated well with the EPA-score for biodegradation potential of chlorinated ethenes (Fig. 2). The EPA-score was developed to identify for a polluted location whether it is likely that chlorinated ethenes are transformed via reductive dechlorination, for example by *Dehalococcoides* spp.. Our study confirms that for predicting the presence or absence of *Dehalococcoides* spp. at a location, EPA-score is a robust and better parameter than individual geochemical parameters, such as total concentration of CAH's, and sulfate or nitrate concentrations. In contrast, TCE- and VC-reductase

genes appeared to have a less strong correlation with the EPA-score than *Dehalococcoides* spp. 16S rRNA genes. This indicates that at favorable conditions for reductive dechlorinating bacteria, *Dehalococcoides* strains with genes encoding for *vcrA* or *bvcA*, like strains VS, BAV1 and GT, are not necessarily enriched. Thus, capacity for TCE and VC dechlorination are not necessarily high when EPA-score is high, or high numbers of *Dehalococcoides* spp. are present. *Dehalococcoides* spp. 16S rRNA genes are an effective predictor of overall reductive dechlorination capacity, however, these phylogenetic marker genes can not predict the biodegradation potential for a specific chlorinated ethene, like VC. Rather, the specific reductase genes are more direct predictors of respectively TCE and VC dechlorination capacity. Although, it should always be kept in mind that groundwater samples might not fully represent the *in-situ* conditions, since bacteria can have different affinities for the groundwater or soil matrix.

Variation in presence and abundance of VC-reductase *vcrA* appeared to be more strongly related with concentrations of VC, CAH and methane than *Dehalococcoides* 16S rRNA genes. However both genes had a positive correlation with ethene. Carreon-Diazconti *et al.,* (2009) also found a positive relation between high concentrations of ethene and *vcrA*, but not with *bvcA*. However, since ethene can be produced from other contaminants than VC in the groundwater, it can not be concluded that ethene alone is a good indicator for VC dechlorination. *Dehalococcoides* spp. and *vcrA* correlated to low redox potential. In contrast, *bvcA* and *tceA* correlated to high redox potential, sulfate, nitrate and iron and least with methane and EPA score. Especially *tceA* had a more positive correlation with more oxidized conditions than *Dehalococcoides* 16S rRNA genes and *vcrA*. Possibly, *Dehalococcoides* strains containing *tceA,* which dechlorinate higher chlorinated ethenes (PCE and TCE), tolerate more oxidized conditions than VC dechlorinating *Dehalococcoides* strains. This is in agreement with results of Amos *et al.* (2008) where it was shown that *Dehalococcoides* strains respond differently to oxygen exposure: strains with VC-reductase genes are more susceptible to oxygen inhibition than others (Amos *et al.,* 2008). Lu *et al.* (2001) showed that for dechlorination of VC, hydrogen threshold values were in the range of methanogenesis (2 - 24 nM), while these threshold values for PCE and TCE were in the range of denitrification and ferric iron reduction (0.1 – 0.4 nM) (Lu *et al.,* 2001). Unfortunately, hydrogen data were only available for locations A, I and J and could therefore not be included in the redundancy analysis. However, also these data showed that in monitoring wells with a hydrogen concentration above 1 nM, VC-reductase gene concentrations above 10^3 / ml were observed.

In the monitoring wells, a relatively low correlation between concentrations of *vcrA* and *bvcA* genes was found. Redundancy analysis suggested that micro-organisms that contain *vcrA* or *bvcA* genes have different niches, where *Dehalococcoides* sp. with *bvcA* genes prefer higher redox potential. Niche differentiation of these genes was also observed in soil column experiments, where specific enrichment of *vcrA* or *bvcA* genes was observed after addition of different carbon-source (unpublished data).

In line with previous studies (Lee *et al.,* 2008; Scheutz *et al.,* 2008), VC-dechlorination in microcosms was found to be correlated to an increase of *vcrA* and *bvcA* gene copies. The mere presence of VC reductase- or *Dehalococcoides* 16S rRNA genes in low concentrations (10^3 gene copies / ml), was not in all cases related to occurrence of dechlorination of VC. Rather, an increase of the VC reductase gene copy numbers with an order of magnitude or more during microcosm incubation, correlated with biodegradation. In the current study, *in situ* monitoring of VC dechlorination over time was only possible at one location. In this case, VC dechlorination also correlated with increase of *vcrA* and *bvcA* genes. As robust indicator for VC dechlorination, an increase of VC reductase gene concentrations with an order of magnitude should be observed. In combination with other *in situ* parameters, such as ethene formation, redox potential or decrease of VC concentration, the confidence in *in situ* VC dechlorination can be reinforced.

In groundwater and VC-dechlorinating microcosms, gene copy numbers for *vcrA* were structurally 10 to 100 times higher than for *bvcA*. This difference is significantly higher than the standard error within the replicate analyses. Thus, incubation in the presence of VC under anaerobic conditions did not influence the average ratio between *vcrA* and *bvcA* gene copies. In groundwater from location C, where *vcrA* genes appeared to be absent, *bvcA* genes were enriched to the same levels as *vcrA* in other microcosms. These results suggest that bacteria containing VC-reductase *vcrA* are in competition with bacteria containing VC-reductase *bvcA*, whereby species with *vcrA* are more efficient under tested groundwater conditions and therefore more readily enriched. In literature similar doubling times for *Dehalococcoides* strains VC and BAV1 have been reported (Cupples *et al.,* 2003; He *et al.,* 2003). It should be noted, however, that these values were determined under artificial laboratory conditions (e.g. culture medium), whereas in our study the populations were enriched in the original groundwater. This supports the idea of niche differentiation between *vcrA* and *bvcA* gene containing *Dehalococcoides*, as proposed above.

In the present study, we demonstrated that *Dehalococcoides* spp. 16S rRNA and the specific reductase genes *tceA*, *vcrA* and *bvcA* are widespread within different groundwater systems. The geographical distribution of the genes was not homogeneous, depending on the geochemical conditions, whereby *tceA* and *bvcA* correlated more to oxidized conditions than *Dehalococcoides* spp. 16S rRNA and *vcrA*. Because variation in VC-reductase gene numbers was not directly correlated to variation

in *Dehalococcoides* spp., VC-reductase genes are better monitoring parameters for VC dechlorination capacity than *Dehalococcoides* spp. 16S rRNA genes. To indicate VC dechlorination at contaminated locations, high concentrations (>10^3 copies / ml) of *vcrA* or *bvcA*, and an increase of more than an order of magnitude should be detected over time.

Acknowledgement

This study was supported by funding of Stichting Kennisontwikkeling Kennisoverdracht Bodem (SKB) and by the European Union through the Aquaterra Integrated project of the sixth framework program (FP6-SUSTDEV-2003-505428). We thank Cajo ter Braak for assistance with statistical analysis, and we thank Arian Koeken for practical assistance with qPCR analyses.

Chapter 5

Anaerobic benzene degradation under denitrifying conditions: Identification of *Peptococcaceae* as dominant benzene degraders and indications for a syntrophic process

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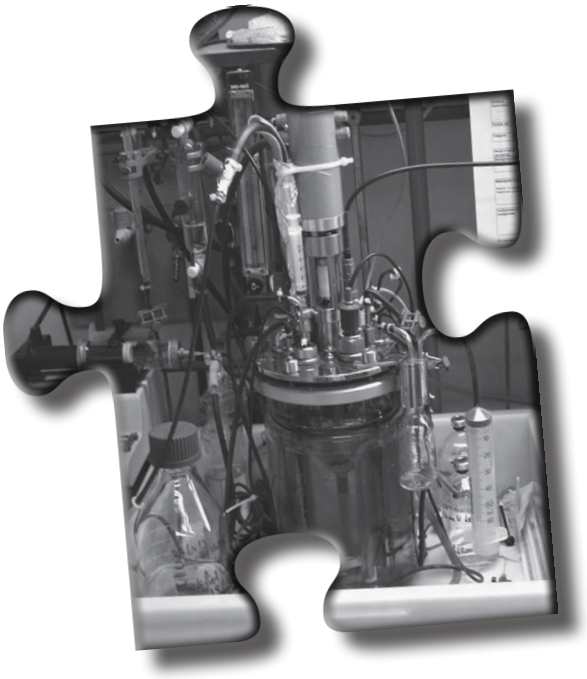

Manuscript in preparation

Abstract

A microbial community was enriched on benzene in an anaerobic continuous culture for more than 8 years with nitrate as electron acceptor. By Stable Isotope Probing with $^{13}C_6$-labelled benzene, the benzene-degrading microorganisms were identified and the effect of different electron donors and acceptors on the degradation process was investigated. Apart from nitrate, the microbial community was able to use sulfate or ferric iron as electron acceptor, and exogenous hydrogen inhibited degradation. The obtained degradation-rate constants were 0.7 day^{-1}, 0.1 day^{-1} and 0.29 day^{-1} with nitrate, sulfate or ferric iron as electron acceptor, respectively. The presence of a biofilm appeared to be important for the benzene degradation process in the continuous culture. Members of *Peptococcaceae* were identified as dominant benzene consumers, but also *Rhodocyclaceae*, *Burkholderiaceae* and *Gemmatimonadetes* were involved in the anaerobic benzene degradation process. The importance of a biofilm, the inhibiting effect of hydrogen, and the ability to degrade benzene with different electron acceptors suggests that benzene was degraded in a syntrophic process.

5.1 Introduction

Contamination of soils and groundwater with mono-aromatic hydrocarbons, such as benzene, toluene, ethyl-benzene and xylenes (BTEX), has become a worldwide problem (Coates *et al.,* 2002). Because of their high water solubility and toxicity, BTEX compounds form a serious risk for human health and the environment. Benzene is of major concern because it is carcinogenic and recalcitrant in anoxic environments (Agency, 2002). As a result, much attention is paid on removal of benzene from the environment, e.g. by biodegradation (Lovley, 2000).

Under aerobic conditions, benzene can be readily biodegraded (Gibson and Parales, 2000). However, in polluted groundwater, oxygen is often not available (Lovley, 1997). The first observation that benzene can be biodegraded under anaerobic conditions dates from 1987 (Grbic-Galic and Vogel, 1987). Nowadays this process has been shown under denitrifying (Burland and Edwards, 1999), iron-reducing (Anderson *et al.,* 1998; Rooney-Varga *et al.,* 1999), sulfate-reducing (Kazumi *et al.,* 1997; Lovley *et al.,* 1995), chlorate-reducing (Tan *et al.,* 2006; Weelink *et al.,* 2007) and methanogenic conditions (Grbic-Galic and Vogel, 1987). The biochemical pathways of anaerobic benzene degradation, however, are not well understood. Possible mechanisms include carboxylation to benzoate, hydroxylation to phenol and methylation to toluene (Chakraborty and Coates, 2004; Coates *et al.,* 2002; Ulrich *et al.,* 2005). It has been proposed that benzene is degraded under anaerobic conditions via a general process where benzene is first hydroxylated to phenol, then carboxylated, and subsequently dehydroxylated to benzoate (Chakraborty and Coates, 2005). However, Ulrich *et al.* (2005) presented data that support the initial methylation of benzene to toluene under denitrifying conditions (Ulrich *et al.,* 2005). Recently, direct carboxylation of benzene to benzoate was reported as the most likely initial activation mechanism under sulfate-reducing conditions (Abu Laban *et al.,* 2009; Kunapuli *et al.,* 2008).

To date only 5 bacteria have been described that degrade benzene under anaerobic conditions: *Dechloromonas* strains RCB and JJ, *Azoracus* strains DN11 and DN9 degrade benzene with nitrate (Coates *et al.,* 2001), and *Alicycliphilus denitrificans* strain BC grows on benzene with chlorate as electron acceptor (Weelink *et al.,* 2008). Benzene degradation with chlorate is a special case as molecular oxygen is formed during chlorate reduction. Phylogenetic analysis of enrichment cultures showed that the dominant benzene-degrading microorganisms belonged to different bacterial groups. Organisms of the family *Rhodocycleae* were enriched in benzene-degrading nitrate-reducing cultures, and were closely related to *Azoarcus* strains DN11 and AN9 and *Dechloromonas* strains RCB and JJ (Ulrich *et al.,* 2005; Ulrich and Edwards, 2003). However, members of the families *Geobacteraceae* and *Peptococcaceae,* were enriched on benzene with iron as electron acceptor (Kunapuli *et al.,* 2007; Rooney-Varga *et al.,* 1999). These *Peptococcaceae* were also were identified as numerically dominant organisms in several benzene-degrading cultures

under sulfate-reducing conditions (Da Silva and Alvarez, 2007; Kunapuli *et al.,* 2007; Musat and Widdel, 2008; Oka *et al.,* 2008; Phelps *et al.,* 1998). Representatives of the *Desulfobacteraceae* were identified as dominant microorganisms in benzene-enrichments under denitrifying, sulfate-reducing and methanogenic conditions (Chang *et al.,* 2005; Ulrich and Edwards, 2003). To identify the role of various microorganisms in benzene degradation under iron-reducing conditions, Kanapuli *et al*. (2007) proposed a syntrophic interaction of bacteria involved in anaerobic benzene degradation. The electrons released by oxidation of benzene by members of *Peptococcaceae* may be transferred to *Desulfobulbaceae*, which reduce the ferric iron. Kleinsteuber *et al*. (2008) hypothesized a similar process in a sulfate-reducing culture, where *Pelotomaculum* sp. converted benzene to acetate and hydrogen, which could be used by δ-proteobacteria to reduce sulfate. Sakai *et al*. (2009) showed that such a mechanism may occur also under methanogenic conditions (Sakai *et al.,* 2009).

In the present study, we report the physiological and phylogenetic characterization of a benzene-degrading microbial community. The community was enriched on benzene in an anaerobic continuous culture for more than 8 years with nitrate as electron acceptor. In continuous culture and batch experiments, the effect of changes in electron donors and acceptors was tested, and microorganisms involved in anaerobic benzene degradation under denitrifying conditions were identified by DNA-based Stable Isotope Probing (DNA-SIP).

5.2 Material & Methods

5.2.1 Operation of the continuous culture

An anaerobic continuous culture (Applikon, Schiedam, the Netherlands) was operated with benzene as electron donor and nitrate as electron acceptor for more than 5 years. Originally, the continuous culture was inoculated with soil from a benzene-polluted industrial location situated in the northern part of the Netherlands, that was enriched on 100 µM benzene and 5 mM chlorate. After successful degradation of 100 µM benzene under chlorate-reducing conditions with acetate as co-substrate, the continuous culture was switched to nitrate-reducing conditions. The volume of the glass reactor vessel was 3 liters, with a liquid phase of 2 liters and a gas phase (80% N_2 and 20% CO_2) of 1 liter. Temperature, redox potential, and pH were measured continuously with corresponding probes (Applikon) The influent mineral medium (KH_2PO_4 (1 gl^{-1}); $CaCl_2$ (1 gl^{-1}); $MgCl_2$ (0.1 gl^{-1}); $(NH_4)_2SO_4$ (0.5 gl^{-1}); $NaHCO_3$ (2.5 gl^{-1}), $NaNO_3$ (4 gl^{-1} = 2.5 mM), 2 ml l^{-1} vitamin solution (Heijthuijsen and Hansen, 1986), 1 ml l^{-1} trace elements solution (SL-10, DSMZ, (Sleat *et al.,* 1984)) was pumped via a Maprene pump tube and viton tubing (Rubber BV, Hilversum, the Netherlands) into the reactor. Humic acids (Sigma Alldrich, St. Louis, MO, USA) were supplied via syringe pump from 100 $g.l^{-1}$ stock solution. The dilution

rate (D) was 0.01 d^{-1}. Benzene (100 µM) was injected directly in the liquid phase of the continuous culture via a syringe pump (SP2001, WPI, Berlin, Germany). The temperature was maintained at 20°C and the pH was 7.2. The liquid phase was continuously mixed at 400 rpm and the headspace was flushed (20 $ml.h^{-1}$) with N_2 / CO_2 gas (80% / 20%), which was passed over hot (250°C) cupper frizzles to remove traces of oxygen. The effect of different electron donors (lactate, benzoate, naphthalene, phenol) and electron acceptors (nitrate, sulfate, oxygen, ferric iron, humic acids) were tested on anaerobic benzene degradation.

5.2.2 Microcosm incubations

Biofilm samples (50 ml) from the glass wall of the benzene degrading continuous culture were incubated in 120 ml glass bottles, closed with viton stoppers (Rubber BV, Hilversum, the Netherlands) and an anaerobic (80% N_2 and 20% CO_2) or aerobic (air; 78% N_2, 20% oxygen, 2% CO_2) atmosphere at 20°C or 30°C and ambient pressure. Benzene was added at various concentrations in the range of 10 µM – 100 µM. Benzene concentrations were measured monthly in the headspace of the cultures to monitor benzene degradation.

Biofilm cells were sampled anaerobically with a long syringe from the inner wall of the benzene degrading continuous culture, and were incubated (approx. 500 mg biomass) in 120-ml glass bottles filled with 40 mL of anaerobic phosphate and bicarbonate buffered AW-1 medium (Weelink *et al.,* 2007) and closed with Viton stoppers. The headspace contained 80% N_2 and 20% CO_2 and pH was 7.3. Sodium nitrate (8 mM) was added as electron acceptor from a sterile, anaerobic stock solution. For Stable Isotope Probing (SIP) experiments, $^{13}C_6$-benzene (99% $^{13}C_6$, Cambridge Isotope Laboratories, Andover, Massachusetts, USA) or $^{12}C_6$-benzene (≥ 99%; Sigma, Saint Louis, MO) was added from water-saturated anoxic stock solutions (2 mM) to a final benzene concentration of approximately 20 µM. Benzene was re-added to the microcosms when it was depleted, approx. every 7-12 days. Replicate bottles, amended with $^{13}C_6$-benzene or $^{12}C_6$-benzene, were incubated at 25°C in the dark without shaking and subsequently sacrificed for nucleic acid extraction after 0, 5, 16, 40 and 77 days. Benzene-concentration in the headspace was analyzed twice a week. Nitrate was periodically analyzed.

From the continuous culture and denitrifying benzene-degrading microcosms, 6 colonies were isolated on R2A-agar plates which were incubated under anaerobic conditions. The colonies were transferred to mineral medium as used in the continuous culture (see abobe) with nitrate (2.5 mM), plus acetate (5 mM), lactate (5 mM) or benzene (25 µM) as sole carbon source.

5.2.3 Nucleic acid extraction

From the continuous culture, total DNA was extracted of the liquid phase (60 ml) or biofilm (approx. 0.5 g) from the inner reactor wall, using BIO101 Fast DNA Kit for Soil Kit (Qbiogene, Inc, CA), as described in the manufacturer´s protocol. Prior to DNA extraction, the liquid culture (40 ml) was concentrated by filtration over a 0.2 µM pore-size ME 24 membrane filter (Schleicher & Schuell, Den Bosch, the Netherlands).

From the microcosms, Total DNA was extracted from 20 ml cell-suspension by using the CTAB – Doe Joint Genome Institute protocol (http://my.jgi.doe.gov/general/protocols/DNA_Isolation_Bacterial_CTAB_Protocol.doc, 2010). This protocol resulted in higher DNA quality than the BIO101 Fast DNA Kit protocol, which was important for further analysis of the microcosms samples. The integrity of DNA was checked by standard agarose gel electrophoresis and ethidium-bromide staining and quantified using a NanoDrop ND1000 Spectrophotometer (Intas, Göttingen, Germany). Isolated DNA was stored at -20°C for maximal 1 month until analysis.

5.2.4 Isopycnic centrifugation

Equilibrium (isopycnic) density gradient centrifugation and fractionation were adapted for DNA-SIP from methods for RNA-SIP (Manefield *et al.,* 2002) using cesium trifluoroacetate (CsTFA) solution without addition of formamide.

Isopycnic density gradient centrifugation was performed in 11 ml polyallomer UltraCrimp tubes (Sorvall) using a Kontron TVF 65.13 vertical rotor in a Centrikon T-1065 centrifuge (Kontron Instruments). Centrifugation conditions were 20°C at 37,000 rpm (130,000 g_{av}) during 70 h. DNA was resolved in CsTFA gradients with an average density of 1.6200 g ml^{-1}. Solutions were prepared by mixing 2.00 $g.ml^{-1}$ CsTFA stock solution (Amersham Pharmacia Biotech, Roosendaal, the Netherlands), gradient buffer (0.1 M Tris-HCl, pH 8; 0.1 M KCl; 1 mM EDTA), and 2.2 µg DNA to a final volume of 11 ml. Prior to centrifugation, the average density of all prepared gradients was checked by weighing of defined volumes, and an AR200 digital refractometer (Reichert Inc., Depew, NY, USA). When necessary, the average density was adjusted by adding small aliquots of CsTFA solution or gradient buffer.

5.2.5 Gradient fractionation and evaluation of DNA concentration

Centrifuged gradients were fractionated from bottom to top into 16 equal fractions of exactly 690 µl, using a peristaltic pump (Watson Marlow, Rotterdam, the Netherlands) with a flow rate of 0.69 ml min^{-1} to displace the gradient medium with water containing 0.01% (w/v) resazurin from the top of the centrifuge tube. Buoyant density of CsTFA gradient fractions containing $^{12}C_6$-DNA and $^{13}C_6$-DNA was determined by measuring refraction index as described above, and comparing the results to a calibration curve of a CsTFA gradient mixture with water. From the CsTFA

gradient fractions, nucleic acids were precipitated overnight after addition of 750 µl cold iso-propanol at -20°C, followed by centrifugation (13,200 rpm, 30 min, 4°C). Precipitates were washed with 70% cold ethanol (0.5 ml) and re-dissolved in 30 µl elution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Total DNA was determined using the *PicoGreen® DNA Quantitation Kit* (Molecular Probes, Leiden, the Netherlands), according to the manufacturer's instructions.

Amplification with primers 519F (Lane, 1991) and 907R (Muyzer and Ramsing, 1995) were used to quantify 16S rRNA gene copies in the different fractions by quantitative real-time PCR on an IQ5 PCR System (Bio-Rad, Veenendaal, the Netherlands). The following PCR temperature program was used: 95˚C for 3 minutes, followed by 35 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final elongation step of 5 minutes at 72°C.

5.2.6 Denaturing Gradient Gel Electrophoresis

Phylogenetic diversity of the bacterial communities was analysed by denaturing gradient gel electrophoresis (DGGE). Bacterial 16S rRNA gene fragments were amplified from 1 µl extracted DNA with forward primer 338GC and reverse primer 907 (Muyzer and Smalla, 1998) to generate amplicons of about 560 bp with GC-clamp. The temperature program in an ICycler PCR-system (Bio-Rad) was 95°C for 3 min, 35 cycles 95°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final elongation period of 7 minutes at 72°C. The amplified product (25 µl) was analysed on an 8% polyacrylamide gel with a 30% - 70% denaturing gradient (40% acrylamide/bis solution, 37.5 : 1, Sigma-Aldrich and 242 g/l urea) that was run for 16 hours at 60°C and 100V in a Dcode™ Universal Mutation Detection System (BioRad). After electrophoresis, the gel was stained for 45 minutes with SybrGold (Molecular Probes, Inc, Eugene, OR) in 20 ml 1xTAE. The DGGE profiles were visualized under UV light.

5.2.7 Clone library and phylogenetic analysis

To identify the dominant bacterial species involved in the benzene degradation process, clone libraries of 16S rRNA genes were constructed from total DNA of the continuous culture biofilm, and from a heavy and a light DNA fraction (7 and 11,respectively), obtained after isopycnic centrifugation of DNA isolated from a $^{13}C_6$-benzene amended microcosm at t = 40 days. These three clone libraries were named: "total biofilm library", "heavy fraction library" and "light fraction library", respectively. Almost complete 16S rRNA gene fragments, amplified with primers FD1/2 and RP1/2 (Weisburg *et al.,* 1991), were cloned into pCR2.1 TOPO vector by using the TOPO TA cloning kit (Invitrogen, Breda, the Netherlands) according to the manufacturer's protocol. Cloned inserts were sequenced (MWG-Biotech, Germany) and aligned with reference sequences using the online alignment tool SINA available at http://www.arb-silva.de (Pruesse *et al.,* 2007). The aligned sequences were imported into the Silva98 release of the ARB-Silva reference

database, and the alignment was manually refined using tools available in the ARB software package (Ludwig *et al.,* 2004). A phylogenetic tree was constructed using the Neighbor Joining method as implemented in ARB. Operational taxonomic units (OTU) were defined based on a threshold of 97% sequence identity with most closely related full-length reference sequences available in the Silva database.

5.2.8 Analytical procedures

5.2.8.1 Benzene

Benzene was measured by headspace analysis using a gas chromatograph (GC) equipped with a flame ionization detector as described previously (Tan *et al.,* 2006). The nominal benzene concentration in the liquid phase of the culture was calculated by comparing the obtained area from the headspace GC-analysis to headspace analysis of external standards with known benzene concentration. The benzene concentrations in the external standards ranged between 0 – 250 μM, and had the same liquid phase / gas phase ratio as the continuous culture.

5.2.8.2 Ion analysis

Nitrate, nitrite and sulfate ion analysis was performed on 1 ml diluted culture samples on a Dionex ICS-1500 equipped with an IONPAC AS14 anion-exchange column and an A SRS®-Ultra 14 mm suppressor (Dionex Corporation, Sunnyvale, CA). The eluent (2.0 mM Na_2CO_3 and 0.75 mM $NaHCO_3$) flow rate was 1.3 $ml.min^{-1}$. The injection needle was rinsed with 100 μl MilliQ-water and 50 μl samples were injected. External standards at 6 different concentrations in the range between 0 – 250 μM were used for calibration.

5.2.8.3 O_2 and CO_2

Oxygen and carbon dioxide were analyzed on an Interscience GC-8000 gas chromatograph with a thermal conductivity detector (TCD) and a Poraplot Q column (Varian, Middelburg, the Netherlands). The injector temperature was set at 100°C and the detector temperature at 200°C, with a constant oven temperature of 90°C. Helium was used as carrier gas at a flow rate of 30 $ml.min^{-1}$. External standards were used for calibration.

5.2.8.4 Hydrogen

Hydrogen concentration in the headspace of the continuous culture was analyzed by injecting 500 μl gas samples in a gas chromatograph with a reduction gas detector (Trace Analytical, Bester, Amstelveen, the Netherlands), which was equipped with a Carbosieve II column. The carrier gas was N_2 with a flow rate of 20 $ml.min^{-1}$. The column and detector temperatures were 104°C and 265˚C, respectively.

5.2.9 Calculations

The first-order degradation constants were calculated according to equation 1:

Equation 1: $k = (\log(x_1)-\log(x_2) / (t_2 - t_1)) + D$

$t_2 - t_1$ is the time-interval in days. x_1 is the benzene concentration at time t_1, and x_2 is the benzene concentration at time t_2. D is the dilution rate of the continuous culture per day.

5.3 Results

5.3.1 Biodegradation of benzene

The continuous culture was routinely operated at a dilution rate of 0.01 hr^{-1}, pH 7.2 at 20°C with 2.5 mM nitrate in the reservoir medium. Initially, 100 µM of benzene was degraded under nitrate-reducing conditions with humic acids (1 $g.l^{-1}$) to mimic soil conditions. After one year, stable benzene degradation under anaerobic conditions was achieved without humic acids, and with nitrate as sole electron acceptor. Per 100 µM benzene, 660 µM nitrate was reduced. This is close to the theoretically amount of 600 µM nitrate needed for complete oxidation of 100 µM benzene to CO_2, according to equation 2 (Burland and Edwards, 1999):

Equation 2: $C_6H_6 + 6NO_3^- + 6H^+ \rightarrow 6CO_2 + 3N_2 + 6H_2O$.

The addition of 15 µM naphthalene as co-substrate, did not influence the benzene degradation. Naphthalene was degraded in parallel with benzene. However, 5 mM lactate in the liquid phase or 2.5 mM hydrogen in the gas phase, inhibited benzene degradation (Fig. 1). Benzene degradation was also not observed in the absence of CO_2 in the headspace of the continuous culture.

Benzene degradation stopped when nitrate was omitted from the medium. Apart from nitrate, also chlorate, oxygen, sulfate and ferric iron were successfully used as electron acceptor for benzene degradation. Based on the observed kinetics, the maximal obtained degradation-rate constants (k_{max}) with different electron acceptors were determined (Table 1). When oxygen was added as electron acceptor, initially a relatively high degradation-rate constant was observed. After two days, when oxygen was depleted, benzene degradation stpped, even in the presence of nitrate. This indicated that the anaerobic degradation process was inhibited by oxygen.

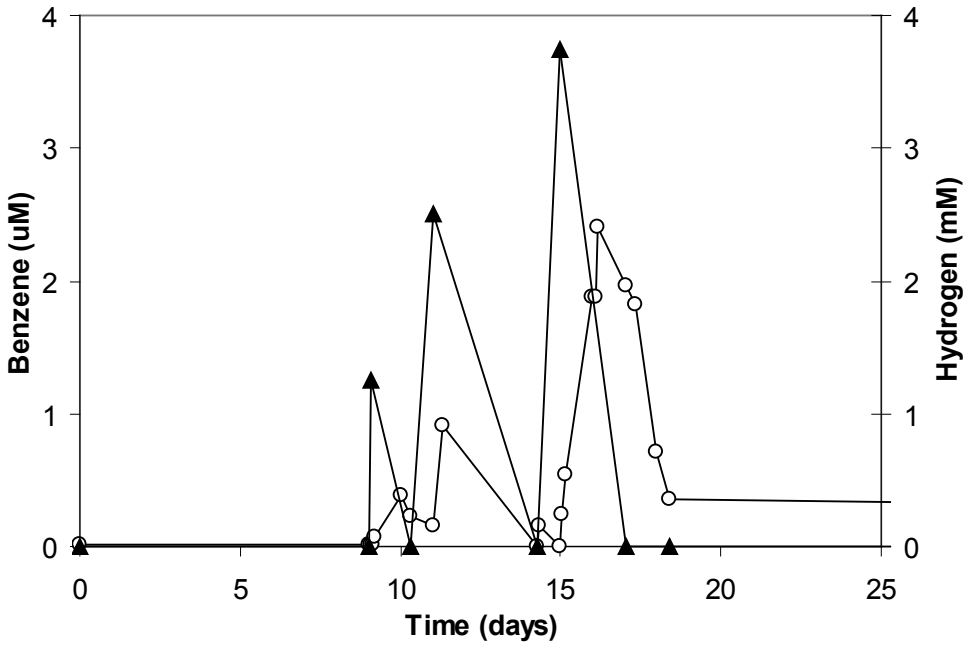

Figure 1: *Benzene (open circles) and hydrogen (black triangles) concentration in the continuous culture with a dilution rate of 0.01 h^{-1}. After addition of hydrogen to the headspace of the continuous culture, the benzene concentration increased.*

Table 1: *Maximal 1st order degradation-rate constants obtained with different electron acceptors.*

Electron acceptor	K_{max} (d^{-1})
Chlorate	0.26
Humic acids + nitrate	0.42 ± 0.05
Nitrate	0.70 ± 0.12
Oxygen (direct)	3.97 ± 3.60
Oxygen (after 2 days)	0.0
Sulfate	0.10
Ferric iron	0.29 ± 0.04

Sulfate and ferric iron were tested at fed-batch conditions, since observed degradation rates for benzene at those conditions were below the dilution rate of 0.01 h^{-1}. Sulfate was tested as electron acceptor three year after start of the continuous culture. Eight years after the start-up, the experiment with sulfate as electron acceptor was repeated, however, at that time no benzene degradation was observed. In contrast, ferric iron was successfully used as electron acceptor after eight years of operation of the continuous culture (Fig. 2).

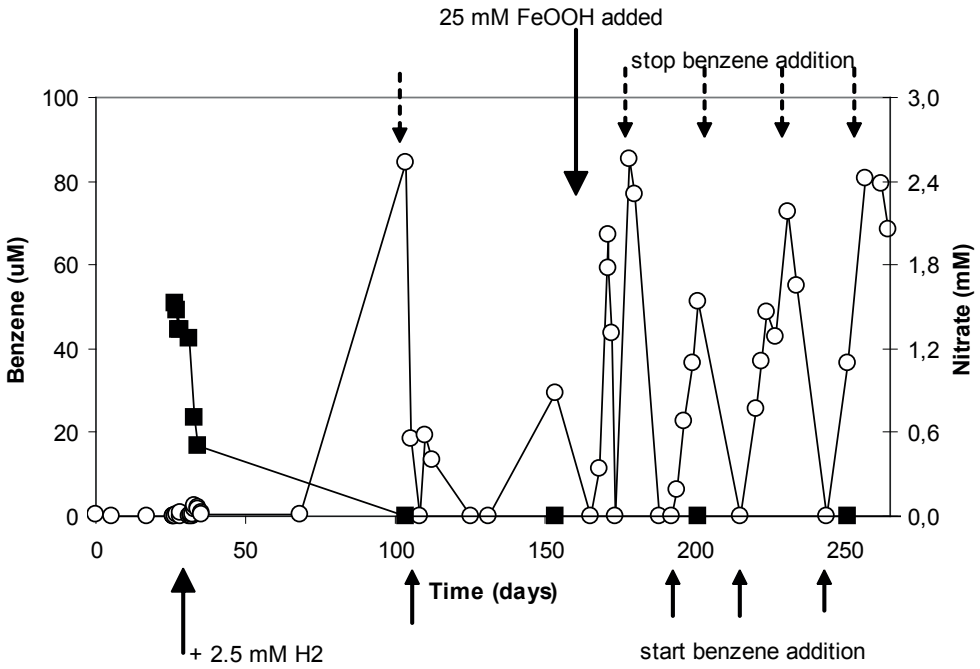

Figure 2: *Benzene (open circles) and nitrate (black squares) concentration in the continuous culture with a dilution rate of 0.01 h^{-1}, after nitrate-addition was stopped. When nitrate was depleted, benzene concentration increased. Arrows at the bottom of the figure indicate when benzene and medium addition was started, and dashed arrows at the top of the figure indicate when benzene and medium addition was stopped. At day 161, 25 mM FeOOH was added as electron acceptor.*

5.3.2 Microcosm analysis

We failed to transfer benzene degradation capacity from the continuous culture liquid to microcosms. However, when the biofilm material from the walls of the continuous culture was collected and transferred to microcosms, benzene was successfully degraded in microcosms. During this sampling process, the biofilm was partly removed and as a result the benzene degradation capacity in the continuous culture was negatively influenced, by a lower benzene degradation rate. The recovery of the benzene degradation activity took some days to weeks, likely dependent on sampling time and the amount of biofilm sampled. In the microcosms prepared with biofilm samples, no difference in degradation rate between $^{12}C_6$- and $^{13}C_6$-benzene was observed (Fig. 3). The maximal degradation-rate constant was 0.52 ± 0.03 day^{-1}.

From the continuous culture and denitrifying benzene-degrading microcosms, 6 colonies were isolated and transferred to mineral medium with nitrate, plus acetate, lactate or benzene as sole carbon source. Although the isolated microorganisms grew on acetate and lactate, benzene degradation was observed by none of these isolates.

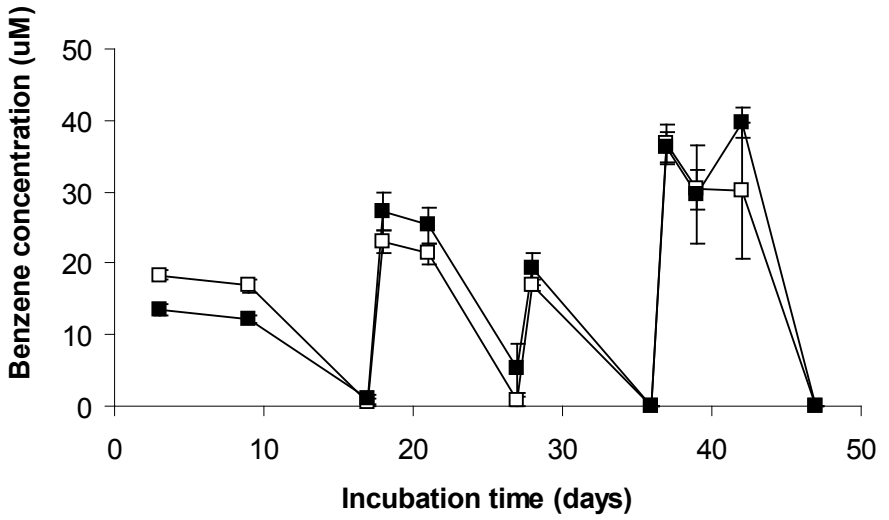

Figure 3: *Benzene concentration in $^{12}C_6$-benzene (open symbols) and $^{13}C_6$-benzene (closed symbols) amended microcosms, incubated with biofilm of the continuous culture and incubated at denitrifying conditions. Error bars represent the standard deviation (n=4).*

5.3.3 Stable isotope enrichment & identification of benzene degraders

To identify the microbes that were involved in the benzene degradation, the culture was amended with $^{13}C_6$-benzene and following 5, 16, 40, and 77 days of incubation, DNA was extracted from the microcosms and subject to isopycnic density gradient centrifugation. Subsequently, the DNA and 16S rRNA gene concentrations of the obtained fractions were quantified after 5, 16, 40 and 77 days of incubation. While some shifts were observed after 5 and 16 days of incubation, a significant shift of the bulk DNA and 16S rRNA genes from fractions with a buoyant density (BD) of 1.590 g/ml to fractions with a BD of 1.610 g/ml was observed after 40 days in the $^{13}C_6$-benzene microcosm compared to the $^{12}C_6$-benzene microcosm (Fig. 4). After 77 days, a comparable shift as after 40 days was identified (data not shown). However, to minimize the influence of cross-feeding, it was decided to use for further analyses DNA samples of the 40 days incubated microcosms.

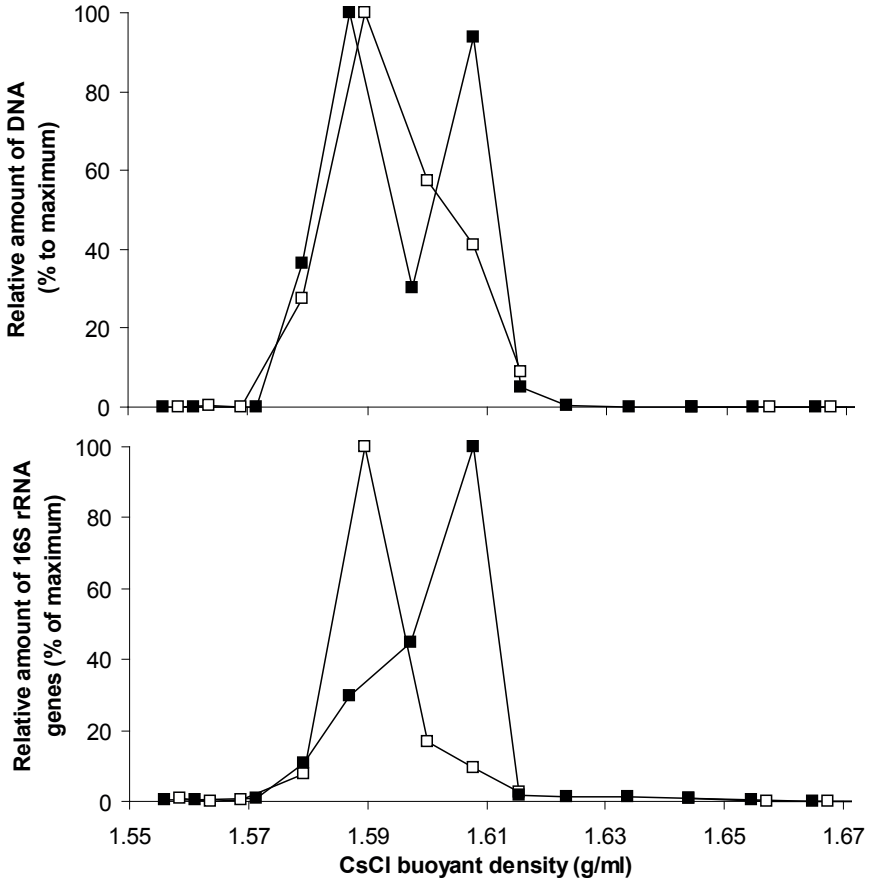

Figure 4: *Relative amount of DNA and 16S rRNA genes in $^{12}C_6$-benzene (open symbols) and $^{13}C_6$-benzene (closed symbols) microcosms isopycnic centrifugation fractions after 40 days of incubation.*

PCR-based 16S rRNA gene DGGE analysis was done on the $^{12}C_6$- and $^{13}C_6$-enriched fractions of the benzene microcosms after 40 days of incubation (Fig. 5). The differences in the DGGE profiles were mainly observed in fractions 6, 7, 8, 9, and 10 with the corresponding buoyant densities of 1.597 – 1.633 g/ml. This corresponds to the fractions that show an increase of total abundance of DNA and 16S RNA after ^{13}C-benzene addition. At the start of the incubations (t = 0 days), no differences were observed in the DGGE patterns between $^{12}C_6$- and $^{13}C_6$-benzene amended microcosms (data not shown).

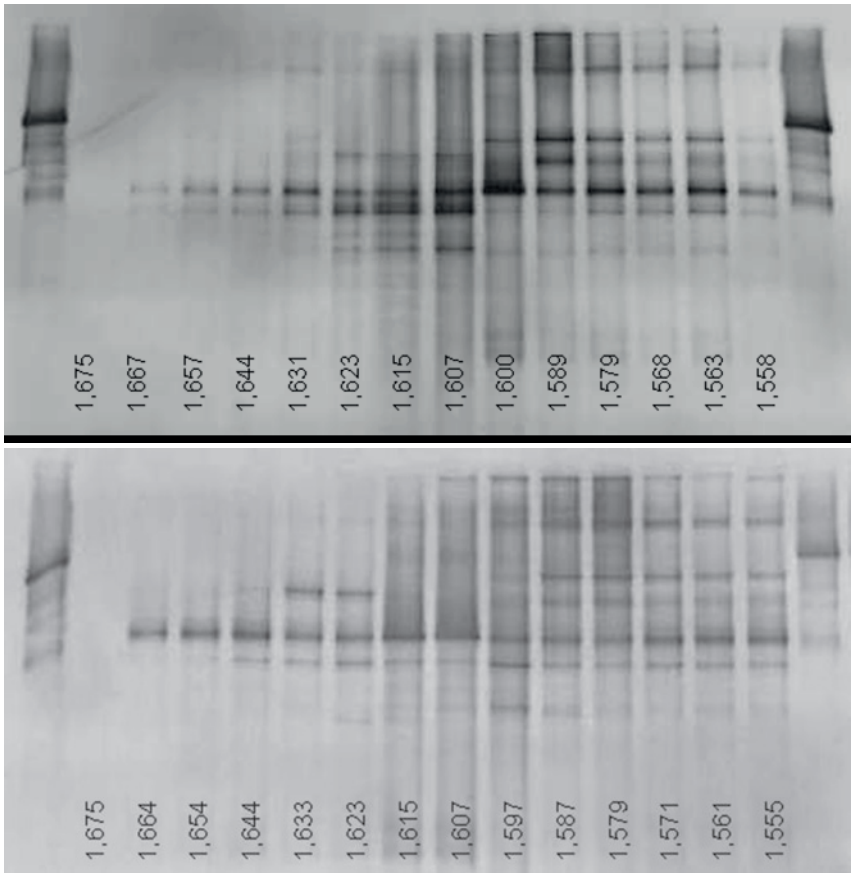

Figure 5: *DGGE profiles of the isopycnic centrifugation fractions (3 – 15) of the ^{12}C-benzene incubated microcosm (A) and ^{13}C-benzene incubated microcosm (B) after 40 days of benzene degradation. The buoyant density corresponding to the different fractions is indicated in the figure. A DGGE Marker was added at the far right and left of the DGGE-gels.*

Clone libraries were constructed of total DNA of the inoculation (biofilm) material at t=0, and of fractions 7 (BD = 1.623 g/ml) and 11 (BD = 1.587 g/ml) of the $^{13}C_6$-benzene amended microcosm (Fig. 6), to phylogenetically identify bacterial species which are involved in benzene degradation. From the "total biofilm", "heavy fraction", and "light fraction" clone libraries 93, 79, and 88 clones, respectively, were identified.

The microbial community of the biofilm was dominated by members of *Firmicute*s (35 clones, 37% of total) and *Planctomycetes* (26 clones, 28% of total) (Fig. 6). Also members of *beta-Proteobacteria, Chloroflexi*, *alpha-Proteobacteria* and *Chlorobi* were detected in the "total biofilm library". The majority (>90%) of the *Firmicutes* clones in this library, shared 98-99% sequence identity to uncultured bacteria from the family *Peptococcaceae* (Kunapuli *et al.,* 2007).

The heavy and light fraction libraries were partly similar. However, in the heavy fraction library, 50 clones (63% of total) were similar to uncultured *Peptococcaceae* bacteria, whereas these *Firmicutes* clones contributed only for 13% (15 clones) in the light fraction library. Other phylogenetic types, which were more dominant in the heavy fraction library compared to the total biofilm library and not enriched in the light fraction library, were members of *beta-Proteobacteria*. Within this phylum, 8 clones had 98% sequence identity with uncultured *Rhodocyclaceae*, and 3 clones shared 96% sequence identity with uncultured *Burkholderiaceae*. In the library of the heavy fraction, 10 clones (13%) were identified as members of *Gemmatimonadetes*, while those clones were not identified in the other two clone libraries. All clones that were more enriched in the light fraction library and not in the heavy fraction library, compared to the total biofilm library, were classified as *Chlorobia*.

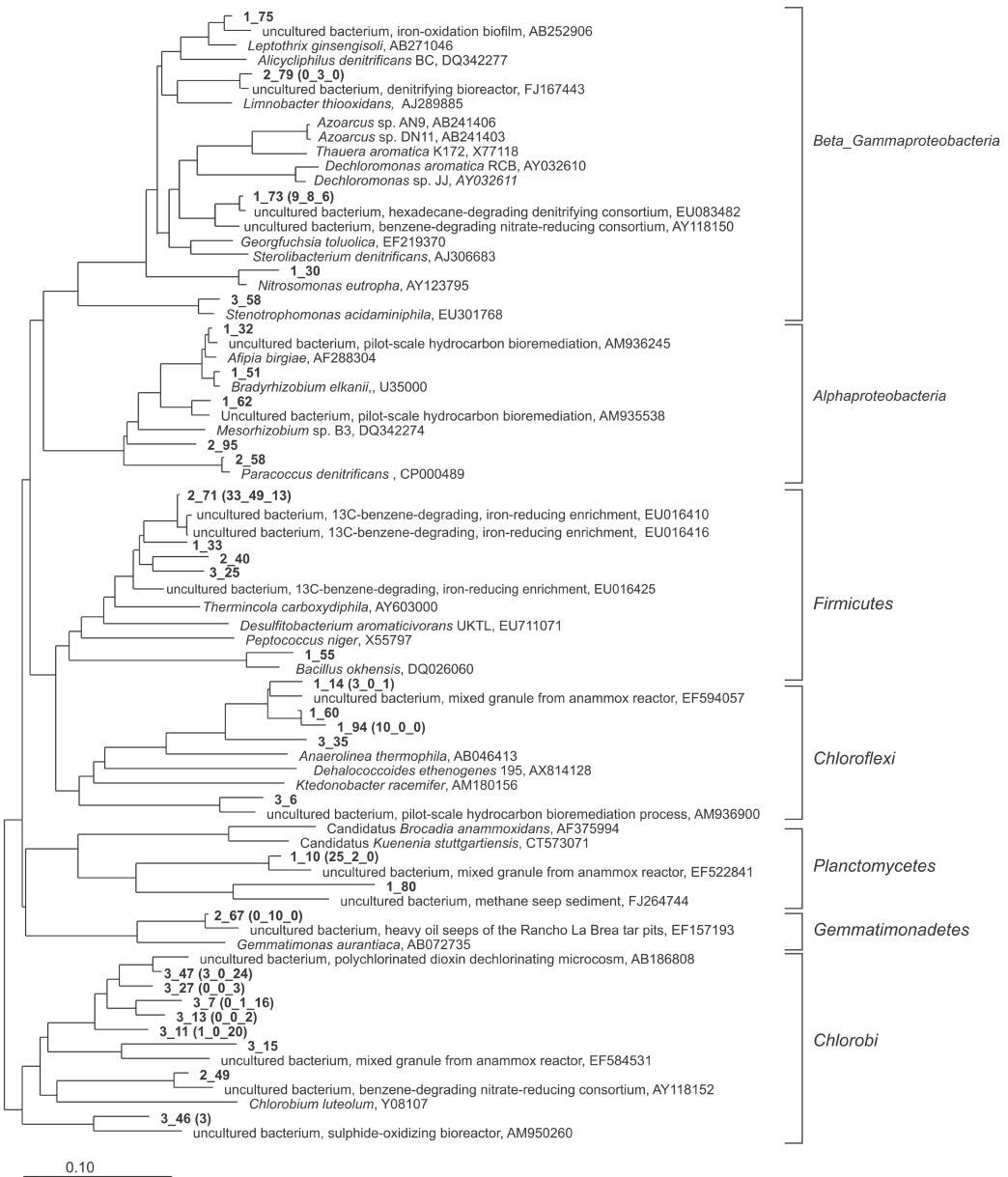

Figure 6: *Phylogenetic tree of clones (names in bold) from the three clone libraries. For each Operational Taxonomic Unit (OTU), one representative sequence was included in the tree (1; total biofilm, 2; heavy fraction, 3; light fraction). The numbers in brackets behind the clone names, represent: first number = # sequences of the OTU which belong to "total biofilm", second number = # sequences of the OTU which belong to "heavy fraction" and third number = # sequences of the OTU which belong to "light fraction".*

5.3.4 Dominance of clones in benzene-degrading enrichments

Clones representative of unique Operational Taxonomic Units (OTU's) (all in Fig. 6) of the heavy fraction library were compared by DGGE with DNA isolated from the continuous culture and the microcosms (Fig. 7). A DGGE band observed in the sample of a OTU that shared 98% sequence identity with uncultured *Peptococcaceae* bacteria, appeared at the same vertical position as the most dominant band observed in benzene-degrading enrichment microcosm inoculated with biofilm. Also in the continuous culture samples, one of the five most dominant bands appeared at this position. Two other dominant bands in the continuous culture samples appeared at the same vertical position as bands in samples from OTU's identified as *Rhodocyclaceae* or *Burkholderiaceae*, which were enriched in the heavy fraction library after incubation on $^{13}C_6$-benzene.

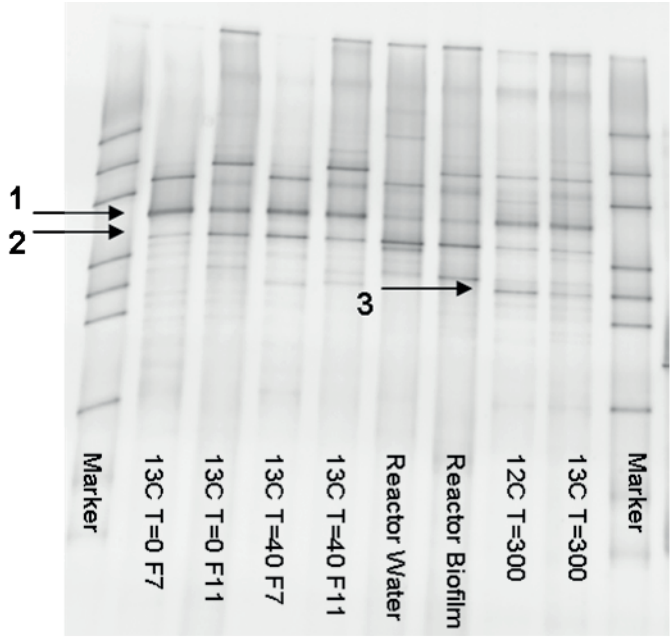

Figure 7: *DGGE profiles of continuous culture water and biofilm samples, from heavy (F7) and light (F11) fractions of the $^{13}C_6$-enriched microcosms after 0 and 40 days, and from a $^{12}C_6$- and $^{13}C_6$-amended microcosm after 300 days of incubation. The arrow indicate the positions of bands obtained in DGGE profiles of clone samples with closest sequence similarity with 1) Peptococcoceae, 2) Rhodocyclaceae, 3) Burkholderiaceae.*

5.4 Discussion & Conclusion

Benzene was degraded in an anaerobic continuous culture for more than 8 years under denitrifying conditions. The biofilm covering the surfaces in the continuous culture system appeared to be essential for benzene degradation, associated with nitrate reduction. Maximum observed benzene degradation-rate constants were about three times higher in the continuous culture (Table 1), and about two times higher in the microcosms inoculated with biofilm. Degradation rates could not be related to biomass concentrations, as quantification of biomass present as biofilm in the continuous culture was not possible. Therefore, degradation-rate constants (k_{max}) were calculated (varying from 0.1 to 0.7 under anaerobic conditions) to compare our results with benzene biodegradation rates reported by others. Previously reported k_{max} values observed under denitrifying conditions varied from 0.04 day^{-1} to 0.14 day^{-1} and were thus lower than those observed in the present study (Edwards and Grbic-Galic, 1992; Kasai *et al.,* 2006; Ulrich *et al.,* 2005; Ulrich and Edwards, 2003).

In absence of nitrate, benzene was degraded with chlorate, sulfate or ferric iron as electron acceptor. The maximum degradation-rate constants under iron-reducing conditions (0.29 day^{-1}) were about two times higher than found by Kunapuli, *et al*, 2008. Under sulfate-reducing conditions, we found a k_{max} of 0.09 day^{-1}, which is comparable to k_{max}-values of 0.03 – 0.1 day^{-1} found by others under sulfate-reducing conditions (Abu Laban *et al.,* 2009; Herrmann *et al.,* 2010; Kleinsteuber *et al.,* 2008). Chlorate-coupled benzene degradation in pure culture of *Alicycliphilus denitrificans* strain BC was reported by Weelink et al (2008) with a k_{max}-value of 1.4 day^{-1}. This is higher that the k_{max} of 0.26 day^{-1} found in the present study (Weelink *et al.,* 2008).

The inhibition of benzene degradation some days after oxygen addition indicates that the observed nitrate-coupled benzene degradation is a strict anaerobic process. Remarkably, an increased benzene degradation rate was observed directly after oxygen addition. A plausible explanation for this observation is that a population of aerobic benzene degrading bacteria was present in the culture, which temporally degraded benzene with oxygen.

Attempts to obtain benzene-degrading isolates from the continuous culture failed. Therefore, Stable Isotope Probing (SIP) was used to identify the benzene degraders in microcosms incubated with benzene-degrading biofilm samples. After 40 days of incubation, the bulk DNA and 16S rRNA genes shifted 0.02 $g.ml^{-1}$ in buoyant density (BD) in the $^{13}C_6$-benzene amended microcosm. Such a shift was also observed in the SIP-study by Kunapuli *et al.* (2007) to identify iron-reducing microorganisms involved in anaerobic benzene degradation, and corresponds to

50% of the expected density shift by complete label incorporation (Tillmann *et al.,* 2004). The BD shift indicated that the DNA became $^{13}C_6$-labelled, with $^{13}C_6$ originating from benzene. Thus, DNA enriched in the heavy fraction, originates from microorganisms involved in benzene degradation.

The majority of clones (50 clones, 63%) identified in the library from the heavy fraction clustered in one OTU and shared closest sequence identity (98%) with uncultured *Peptococcaceae*. This OTU was already abundant in the clone library of total DNA isolated from the biofilm at start of the incubation with $^{13}C_6$-labelled benzene, but was further enriched in the clone library of the heavy fraction and decreased in the clone library of the light fraction.Therefore, we conclude that the identified *Peptococcaceae* were involved in benzene degradation. This conclusion is supported by results of the DGGE analysis, where the band of the uncultured *Peptococcaceae* clone appeared at the same vertical position as the band that was most dominant in the DGGE profile of the benzene-enriched microcosm. Uncultured *Rhodocyclaceae* bacteria, uncultured *Burkholderiaceae* bacteria, and members of *Gemmatimonadetes* were also enriched in the heavy fraction clone library, and less abundant in the light fraction clone library. Therefore, it is likely that these microorganisms were also involved in benzene degradation. As for *Peptococcaceae*, DGGE profiles of *Rhodocyclaceae* and *Burkholderiaceae* clones supported the role of these microorganisms in benzene degradation.

The *Peptococcaceae* clones identified in this study were most closely related to the BF1-cluster described by Kunapuli *et al.* (2007). These Gram-positive fermenting bacteria were involved in the initial step of benzene degradation under anaerobic iron-reducing conditions. More recently, closely related *Peptococcaceae* were also identified in anaerobic benzene degrading cultures under sulfate-reducing conditions, being responsible for the initial attack of benzene (Abu Laban *et al.,* 2009; Herrmann *et al.,* 2010; Kleinsteuber *et al.,* 2008). In the present study, it was shown that *Peptococcaceae* are also involved in anaerobic benzene degradation under denitrifying conditions, providing new insight of the role of these bacteria in benzene degradation in environmental settings.

In the characterized benzene-degrading microbial community, *Rhodocyclaceae* were likely involved in nitrate reduction, since our clones were relatively closely related with *Azoarcus* sp. DN11, *Azoarcus* sp. AN9, *Dechloromonas* strain JJ and *Dechloromonas aromatica* RCB. These strains were described previously to be responsible for anaerobic benzene degradation under denitrifying conditions (Coates *et al.,* 2001; Kasai *et al.,* 2007). Most likely, benzene or degradation products of benzene (e.g. H_2 or organic acids) were used as electron source by these bacteria in our cultures. *Burkholderiaceae* are known PCB (polychlorinated biphenyl) and benzene degraders. *Gemmatimonadetes* clones were related to uncultured bacteria

in heavy oil seep of Rancho La Brea Tar Pits (Kim and Crowley, 2007) and to *Gemmatimonas aurantiaca*, an aerobic, polyphosphate accumulating microorganism, which is the first cultured representative of the new bacterial phylum *Gemmatimonadetes* phyl. nov (Zhang *et al.,* 2003).

After 8 years of enrichment in a continuous culture, a consortium of various bacteria were involved in the benzene degradation process. Therefore, it is possible that benzene is degraded by a cooperation of two or more species. The exact role of these microorganisms was not identified, but bacteria involved in biofilm formation could have prevented the wash-out of others. Others already suggested that benzene can be degraded by a syntrophic interaction of *Peptococcaceae* species together with hydrogen-consuming species (Abu Laban *et al.,* 2009; Herrmann *et al.,* 2010; Kleinsteuber *et al.,* 2008; Kunapuli *et al.,* 2007). In the present study, *Peptococcaceae* were involved in benzene degradation, and therefore it is possible that benzene was degraded via such a syntrophic interaction. Other observations that support this hypothesis are:

1) The biofilm was essential for benzene degradation, indicating the relevance of close (physical) cooperation of multiple microbial species in the benzene degradation process;

2) Hydrogen inhibited benzene degradation. In many cases, syntrophic species require low hydrogen levels (McInerney *et al.,* 1992), maintained by hydrogen-consuming microorganisms;

3) No pure cultures able to degrade benzene were obtained, despite several attempts;

4) *Peptococcaceae* were involved in anaerobic benzene degradation under iron-reducing, sulfate-reducing and denitrifying conditions (Herrmann *et al.,* 2010; Kunapuli *et al.,* 2007). Here we showed that the enrichment culture was able use nitrate, sulfate as well as ferric iron, as electron acceptor. This indicates that benzene degradation process could be coupled to different types of terminal electron accepting processes. These results suggest that in the environment, *Peptococcaceae* can be involved in anaerobic benzene degradation under a variety of geochemical conditions

Chapter 6

General Discussion and Future Perspectives

6.1 Introduction

Chlorinated aliphatic hydrocarbons and aromatic compounds, like 1,2-dichloroethane (1,2-DCA), vinyl chloride (VC) and benzene, are organic pollutants that entered the environment in excess by human activities. In the environment, these compounds are persistent, and cause toxic effects (Vogel *et al.,* 1987). This forms a danger for ecosystem functioning and human health. Therefore, it is important that these pollutants are removed from the environment. Yearly, more than 300 million euros are spent on soil remediation in the Netherlands, whereby at about 60% of the locations the polluted soil is excavated (>3000 ton/year) (EPA, 2010; RIVM, 2008). However, by these activities pollutants are removed, but still contain their toxic properties, and hence excavated material has to be stored and/or treated off-site. An important and cost-effective alternative method to clean-up the environment, is bioremediation. Hereby, pollutants are converted to safe products by the natural process of biodegradation, capitalizing on the broad degradation capacity of soil microorganisms (Lovley, 2003; Rittmann, 2004; Röling and van Verseveld, 2002).

To identify, monitor, or enhance biodegradation, it is important to observe the biological degradation process accurately. **Chapter 1** of this thesis discusses available methods to monitor biodegradation, including their advantages and disadvantages for application in bioremediation studies. An important advantage of culture-independent and molecular microbiological monitoring methods over geochemical methods and cultivation-based approaches is that *in situ* biological processes can be identified and quantified relatively easily. Unfortunately, for various organic pollutants, the biodegrading microorganisms or biochemical degradation pathways are unknown (Totsche *et al.,* 2010). For example, benzene is biodegraded under sulfate-, or iron-reducing conditions, but no microbial strains responsible for this process have been isolated and identified yet. This deficiency of knowledge, specifically the. lack of appropriate target sequences, limits possibilities for development and application of molecular monitoring methods in bioremediation studies. Furthermore, biodegradation pathways in the environment are difficult to predict because local geochemical conditions can be variable or unknown (Abe *et al.,* 2009). To improve bioremediation and monitoring strategies, it is necessary to have a better understanding of biodegradation processes and activity at local geochemical conditions.

Hence, the **aim of this thesis** was to obtain an improved insight in the relation between local geochemical conditions and occurrence and activity of chlorinated- and aromatic hydrocarbon-degrading microorganisms This was realized by applying a complementary set of molecular methods to monitor these microorganisms in the environment. As representatives for a wide range of relevant pollutants, the widely spread and persistent chlorinated aliphatic hydrocarbons 1,2-dichloroethane (1,2-DCA), tetrachloroethene (PCE), vinyl chloride (VC), and benzene, were used as model compounds.

6.2 Monitoring biodegradation in the environment

Changes in environmental conditions that microbial communities are facing, such as accumulation of polluting chemicals or depletion of available nutrients, may alter their composition, and thus their functional repertoire (Kassen and Rainey, 2004). These dynamics within microbial communities can influence biodegradation potential and activity, and are therefore important factors that need to be considered in the framework of *in situ* bioremediation strategies (Viñas *et al.,* 2005). The biodegradation experiments described in **Chapter 3** of this thesis reinforced this notion, as it was shown that under defined methanogenic conditions, the biodegradation pathways of 1,2-DCA changed over time. Microbial communities from Ebro river sediment initially converted 1,2-DCA to chloroethane, but after enrichment cultivation, ethene became the major degradation product. Chloroethane and ethene both resulted from a reductive dechlorination pathway (see **Chapter 1**). Different microorganisms are known to be able to form these products (de Wildeman *et al.,* 2003b; Holliger *et al.,* 1990; Maymo-Gatell *et al.,* 1997). Chloroethane-formation from 1,2-DCA is known to be a co-metabolic process, whereas ethene is formed in energy-yielding organohalide respiration. Thus, a shift in the microbial community caused by enrichment of specific species able to use 1,2-DCA metabolically, resulted in different degradation products. The shift of chloroethane to ethene as major dechlorination product of 1,2-DCA is an important for monitoring bioremediation processes. The monitoring should not focus on a single degradation product (e.g. chloroethane), because this can result in misinterpretation of the actual biodegradation process, even under apparently stable geochemical conditions. It is recommended to monitor indicators (e.g. degradation products, microorganisms, and key enzymes) for the various biodegradation pathways, which are likely to occur under the local geochemical conditions.

Empirical protocols are developed that describe geochemical conditions which are favorable for reductive dechlorination of chlorinated solvents (Wiedemeier *et al.,* 1998). The "EPA-protocol for evaluating natural attenuation" combines 15-20 geochemical parameters, like organic matter content, sulfate concentration, or pH, to an EPA-score. Above a certain threshold value of this EPA-score, chlorinated ethenes are likely biodegraded by microbial species such as *Dehalococcoides* and *Dehalobacter.* **Chapter 4**, however**,** shows that the EPA-score for different groundwaters correlates well with the distribution of *Dehalococcoides* 16S rRNA genes, but not with the amount of VC-reductase genes present. This is remarkable, since VC-reductases are currently only known to be present in *Dehalococcoides* strains (Krajmalnik-Brown *et al.,* 2004; Muller *et al.,* 2004; Sung *et al.,* 2006). Hence, a good indication for the biodegradation capacity of chlorinated ethenes can be obtained based on geochemical survey, however not for individual compounds (for example VC). By specific monitoring of functional genes, however, it was possible to identify the exact dechlorination process of vinyl chloride (**Chapter 4**). In microcosm experi-

ments, it was even possible to correlate an increased gene copy number to microbial metabolic activity. More specifically, it was found that microbial dechlorination of VC to ethene could be detected when an increase of VC-reductase gene copies by an order of magnitude or more is observed at absolute gene concentrations above 10^3 gene copies / ml groundwater.

Although the importance of functional gene monitoring to identify biodegradation processes in the environment is shown in **Chapter 4**, no functional gene assays are available for anaerobic oxidative biodegradation processes as observed in **Chapter 3**. Under denitrifying and iron-reducing conditions, oxidation of 1,2-DCA was rarely reported before, and microorganisms responsible for anaerobic oxidation of 1,2-DCA have neither been isolated nor identified yet (Cox *et al.,* 2000; Dijk *et al.,* 2005; Dinglasan-Panlilio *et al.,* 2006; Gerritse *et al.,* 1999b). Nevertheless, the ecological relevance of this process may be underestimated, since this capacity was observed in the present study in sediments of different European rivers (Ebro and Danube). Additional cultivation experiments are required to identify and characterize anaerobic 1,2-DCA oxidation, but it is difficult to translate these results to *in situ* field conditions (Amann *et al.,* 1995; Bombach *et al.,* 2010b). Only by intensive studies to identify microorganisms or key enzymes involved in anaerobic oxidation of 1,2-DCA, gene assays can be developed to monitor this process, for example by qPCR or micro-array technology. **Chapter 5** describes such a study aimed to identify microorganisms responsible for anaerobic oxidation of benzene at denitrifying conditions. Stable Isotope Probing (SIP) of community DNA was used to identify the benzene-degrading microorganisms, which were grown for more than 8 years in a continuous culture. No gene assays are available for the specific detection of anaerobic benzene-oxidation with nitrate as electron acceptor. Only 4 bacterial strains have been described that can perform this process, and the exact degradation pathway is unknown (Coates *et al.,* 2001; Kasai *et al.,* 2006). Bacteria that belong to *Peptococcaceae*, *Rhodocyclaceae*, *Burkholderiaceae*, and *Gemmatimonadetes* were shown here to be involved in the anaerobic benzene degradation process. Also under iron-reducing and sulfate-reducing conditions, *Peptococcaceae* species were shown to be associated with anaerobic benzene-oxidation (Herrmann *et al.,* 2010; Kleinsteuber *et al.,* 2008; Kunapuli *et al.,* 2007). Since *Peptococcaceae* appeared to be involved in benzene-oxidation under various redox conditions, it is suggested here to develop a phylogenetic gene assay as molecular monitoring tool for anaerobic benzene-oxidation in the environment. Such gene assays can reveal the presence of phylogenetically related microorganisms based on their 16S rRNA gene. When groups of microorganisms perform unique metabolic processes, these phylogenetic markers can be used as indicators for this process. For example, only a few microorganisms are known to reductively dechlorinate PCE and trichloroethane (TCE) and therefore *Desulfitobacterium* sp, *Dehalobacter* sp., and *Dehalococcoides* sp. 16S rRNA genes are often used to monitor reductive dechlorination of PCE and TCE. Another example is the use of *Geobacter* spp. 16S rRNA genes as indicator

for benzene-oxidation under iron-reducing conditions (Rooney-Varga *et al.,* 1999). A drawback of using a phylogenetic marker for *Peptococcaceae* as indicator for anaerobic benzene-oxidation, is that these bacteria can also use other substrates than benzene as carbon source, like propionate (Imachi *et al.,* 2007). Therefore, such phylogenetic gene assay might be not specific enough to identify anaerobic benzene degradation capacity.

By functional gene assays, genes that code for enzymes involved in specific metabolic pathways can be detected, for example for anaerobic dechlorination of VC. However, such specific enzymes are not known for anaerobic benzene degradation, limiting the possibilities to develop a functional gene assay. For other compounds, like 1,2-DCA, VC, phenol, and toluene, functional genes assays have already been developed. There are several dozens of qPCR targets and primers currently available for the amplification of functional genes involved in the biodegradation of chlorinated hydrocarbons and aromatic compounds (Table 1).

A phylogenetic gene assay for anaerobic benzene degradation can be made more specifically by combining a *Peptococcaceae*-target (16S rRNA gene) with targets of microorganisms that cooperate in a syntrophy. In **Chapter 5** evidence for such a syntrophy was obtained, because: 1) *Peptococcaceae* were involved in benzene degradation, 2) the presence of a biofilm in the continuous culture vessel was important for benzene degradation, 3) hydrogen inhibited the degradation process, and 4) benzene was degraded with different electron acceptors. Hence, future studies should aim at the identification of syntrophic partners of anaerobic benzene degraders, including the elucidation of key functions and corresponding gene sequences that can be used as biomarkers.

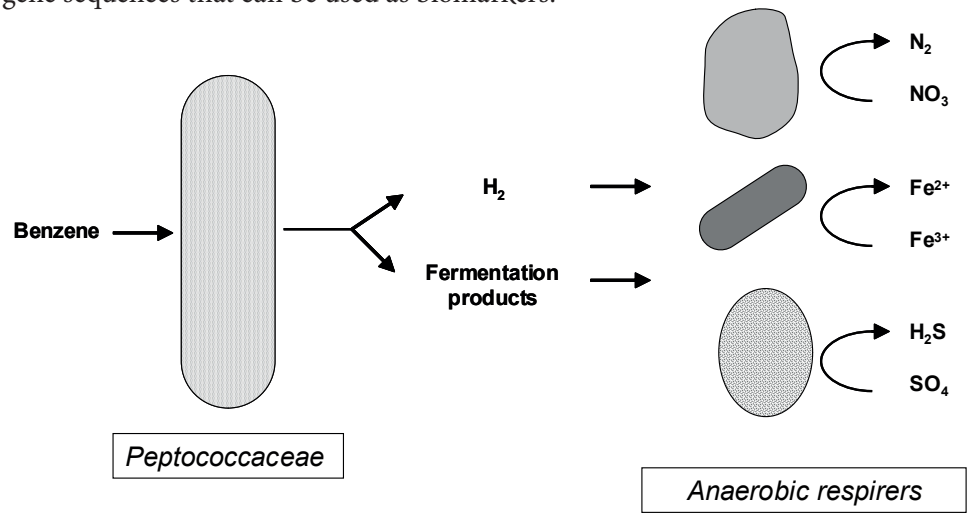

Figure 1: *Hypothetical syntrophic interaction for anaerobic benzene degradation by Peptococcaceae and various anaerobic respirers.*

Phylogenetic gene assays are regularly used in fundamental studies to identify the composition of microbial communities, and for applied studies to identify the presence of relevant microorganisms (Freeborn *et al.,* 2005; Nikolausz *et al.,* 2009). For some years now, phylogenetic gene assays are also used in routine bioremediation processes to monitor biodegradation, and based on quantitative real time PCR (qPCR) detection, because it is relatively simple, fast and cheap. The *Dehalococcoides* spp. 16S rRNA gene is often used as indicator for the biotransformation of chlorinated ethenes. Because this phylogenetic gene assay is not specific for reductive dechlorination of VC (**Chapter 4**), functional gene assays targeting VC-reductase genes have already been used successfully in a few bioremediation studies (Carreon-Diazconti *et al.,* 2009; Maphosa *et al.,* 2010b; Scheutz *et al.,* 2008). These functional gene assays are available now to be applied in routine bioremediation. Other functional gene assays that have been developed (Table 1), are still in their infancy to be applied, and for many pollutants, like benzene, specific functional gene assays are not available yet. For anaerobic toluene degradation, the benzylsuccinate synthase A (*bssA*) genes can be used as functional gene assay. However, developed qPCR primers on *bssA* genes (Beller *et al.,* 2002) are only specific for *bssA* gene-containing *Thauera* and *Azoarcus* strains, but not for other *bssA* gene-containing strains, like *Geobacter grbiciae* and *Desulfobacula toluolicado* (Winderl *et al.,* 2007). Therefore, many gene assays should be analyzed simultaneously during routine bioremediations processes. This is time consuming when analyzed by conventional or quantitative real time PCR techniques, and it would be more efficient to use multiple (functional) gene assay techniques, like those based on micro-arrays or naliter scale PCR systems which allow high throuput quantification (Maphosa, 2010).

6.3 Biodegradation capacity in the environment

By molecular monitoring methods the diversity of microbial communities and spatial distribution of microorganisms responsible for biodegradation of chlorinated aliphatic hydrocarbons or aromatic compounds can relatively easily be identified (Angel *et al.,* 2010; Fierer and Jackson, 2006; Martiny *et al.,* 2006). Thereby, the potential biodegradation capacity for these compounds can be localized. For example, the biotransformation capacity of hexachlorobenzene (HCB) was found widely spread over soils and European river basins (Taş *et al.,* 2009). Remarkably, in this study biotransformation capacity could not be correlated to HCB contamination levels. The capacity to biodegrade 1,2-DCA or VC is also widespread in the environment, but does not correlate clearly with pollution levels (**Chapters 3 and 4**). VC-reductase genes were identified in unpolluted groundwater and were sometimes not observed in groundwater with high concentrations of VC. Since pollution appeared not to relate to biodegradation capacity, the origin and existence of the widespread capacity of biodegradation has been discussed intensively in literature

(Kassen and Rainey, 2004; Rodriguez-Valera *et al.,* 2009). The influence of pollution on microbial communities is described in **Chapter 2**. Sediments polluted with 1,2-DCA and PCE, or an excess of nutrients, indeed affected individual species of the sediment microbial communities. Some species were enriched, while the relative abundance of others decreased. Nutrient addition resulted in a reduction of the microbial diversity within the sediment. However, important functions of the microbial community, like denitrification, sulfate-reduction, or dechlorination, were robust in spite of the applied nutrient pollution, and even stimulated enrichment of dechlorination capacity of the microbial community for 1,2-DCA and PCE. Environmental microbial communities appeared resistant to changes in geochemical conditions, e.g. pollution. This was shown before by others by means of general parameters like total biomass, respiration, and diversity, but extended here for specific microbial functions, including dechlorination, denitrification, sulfate-reduction and methanogenesis, in **Chapter 2** (Salminen *et al.,* 2001; Stefanowicz *et al.,* 2008; Tobor-Kaplon *et al.,* 2005; Yagi *et al.,* 2010).

In **Chapter 3** new evidence was obtained indicating that the diversity of reductively dechlorinating *Dehalococcoides* spp. is broader than detected by most commonly used molecular monitoring methods (Fagervold *et al.,* 2007; Kittelmann and Friedrich, 2008b; Ritalahti *et al.,* 2006). *Dehalococcoides* spp. clones were dominant in a clone library constructed of an 1,2-DCA degrading microbial community, but were inefficiently amplified by quantitative real time PCR, because the clones were only moderately related to other *Dehalococcoides* spp. (Fig. 2). Additionally, **Chapter 4** shows that in groundwater polluted with chlorinated ethenes, VC-reductase genes were 10-100 times more abundant than *Dehalococcoides* spp. 16S rRNA genes. This is in line with observations previously made in a chloroethene-dehalogenating bioreactor (Maphosa *et al.,* 2010b). Based on current knowledge from genomes of VC-dehalogenating *Dehalococcoides* spp., individual cells contain only one gene copy of the 16S rRNA and VC-reductase genes, while VC-reductase genes are only known to be present in *Dehalococcoides* spp. (Krajmalnik-Brown *et al.,* 2004; McMurdie *et al.,* 2009; Muller *et al.,* 2004; Sung *et al.,* 2006). Thus, it is likely that not all *Dehalococcoides* spp. present could be detected by the 16S rRNA gene based method. It is concluded that the actual biodegradation capacity for chlorinated ethenes by *Dehalococcoides* spp., is higher than expected based on actual available *Dehalococcoides* spp. 16S rRNA gene analysis methods. Additionally, VC-reductases might be present in microorganisms that do not fall in the *Dehalococcoides* genus, but belongs to clusters that are closely related. This implies that at polluted locations where the biodegradation capacity for VC was identified by *Dehalococcoides* 16S rRNA gene analysis, the biodegradation process of VC might develop more rapidly than expected, if environmental conditions are favorable.

Primer	Sequence (5' --> 3')	Target gene	Degradation	Pathway	Reference
Chlorinated aliphatic hydrocarbons					
dcaA F	GTTAAAAAGGCAGCCTGTT	Reductive dehalogenase *(dcaA)*	1,2-DCA	Anaerobic	(Marzorati *et al.*, 2007b)
dcaA R	GGCAAATCCCATGGCATTA	Reductive dehalogenase *(dcaA)*	1,2-DCA	Anaerobic	(Marzorati *et al.*, 2007b)
	To be developed	Haloalkane dehalogenase *(dhlA)*	1,2-DCA	Aerobic	
	To be developed	Haloalkane dehalogenase *(dhlA*	1,2-DCA	Aerobic	
vcrA1022F	GAATAGTCCGTGCCCTTCCTC	Vinyl chloride reductase *(vcrA)*	VC	Anaerobic	(Ritalahti *et al.*, 2006)
vcr1093R	CGGGCGGATGCACTATTTT	Vinyl chloride reductase *(vcrA)*	VC	Anaerobic	(Ritalahti *et al.*, 2006)
vcr1042Probe	CGCAGTAACTCAACCATT-TCCTGGTAGTGG	Vinyl chloride reductase *(vcrA)*	VC	Anaerobic	(Ritalahti *et al.*, 2006)
bvc925F	AAAAGCACTTGGCTATCAAGGAC	Vinyl chloride reductase *(bvcA)*	VC	Anaerobic	(Ritalahti *et al.*, 2006)
bvc1017R	CCAAAAGCACCACCAGGTC	Vinyl chloride reductase *(bvcA)*	VC	Anaerobic	(Ritalahti *et al.*, 2006)
bvc977probe	TGGTGGCGACGTGGCTATGTGG	Vinyl chloride reductase *(bvcA)*	VC	Anaerobic	(Ritalahti *et al.*, 2006)
	In development	Epoxyalkane:coenzyme M transferase	VC	Aerobic	Deltares
	In development	Epoxyalkane:coenzyme M transferase	VC	Aerobic	Deltares
tceA1272F	ATCCAGATTATGACCCTGGTGAA	Trichloroethene reductase *(tceA)*	TCE	Anaerobic	(Ritalahti *et al.*, 2006)
tceA1336R	GCGGCATATTAGGGCATCTT	Trichloroethene reductase *(tceA)*	TCE	Anaerobic	(Ritalahti *et al.*, 2006)
tceA1294probe	TGGGCTATGGCGACCGCAGG	Trichloroethene reductase *(tceA)*	TCE	Anaerobic	(Ritalahti *et al.*, 2006)

Aliphatic and aromatic hydrocarbons					
23CAT-F	CGACCTGATCTCCATGACCGA	Cathechol-2,3-dioxygenase	BTEX / PAH	Aerobic	(Mesarch *et al.*, 2000)
23CAT-R	TCAGGTCAGCACGGTCA	Cathechol-2,3-dioxygenase	BTEX / PAH	Aerobic	(Mesarch *et al.*, 2000)
	To be developed	*unknown*	Benzene	Anaerobic	
	To be developed	*unknown*	Benzene	Anaerobic	
PAH-RHDα GP F	CGG CGC CGA CAA YTT YGT NGG	PAH ring hydroxylating dioxygenase *(pah-rhd)*	PAH	Aerobic	(Cebron *et al.*, 2008)
PAH-RHDα GP R	GGG GAA CAC GGT GCC RTG DAT RAA	PAH ring hydroxylating dioxygenase *(pah-rhd)*	PAH	Aerobic	(Cebron *et al.*, 2008)
SP9 F	CAGTACAAYTCCTACACVACBG	6-OCH-CoA hydrolase *(bamA)*	aromatic compounds	Anaerobic	(Kuntze *et al.*, 2008)
ASP1 R	CMATGCCGATYTCCTGRC	6-OCH-CoA hydrolase *(bamA)*	aromatic compounds	Anaerobic	(Kuntze *et al.*, 2008)
nahAcF	CAGAGCGTYCCRTTYGAAAA	Naphthalene dioxygenase *(nahAc)*	naphtalene	Aerobic	(Debruyn *et al.*, 2007)
nahAcR	TCGAAGCAACCRTARATGAA	Naphthalene dioxygenase *(nahAc)*	naphtalene	Aerobic	(Debruyn *et al.*, 2007)
nahAcProbe	TGGGGTTGAAAGAAGTCGCTCG	Naphthalene dioxygenase *(nahAc)*	naphtalene	Aerobic	(Debruyn *et al.*, 2007)
pheF	GTGCTGACSAAYCTGYTGTTC	Phenol monooxygenase *(phe)*	phenol	Aerobic	(Baldwin *et al.*, 2003)
pheR	CGCCAGAACCAYTTRTC	Phenol monooxygenase *(phe)*	phenol	Aerobic	(Baldwin *et al.*, 2003)
bssaF	ACGACGGYGGCATTTCTC	Benzylsuccinate synthase A *(bssA)*	toluene	Anaerobic	(Beller *et al.*, 2002)
bssaR	GCATGATSGGYACCGACA	Benzylsuccinate synthase A *(bssA)*	toluene	Anaerobic	(Beller *et al.*, 2002)

Table 1: *Available and qPCR primers to be developed for functional genes involved in biodegradation of chlorinated aliphatic hydrocarbons and aromatic compounds*

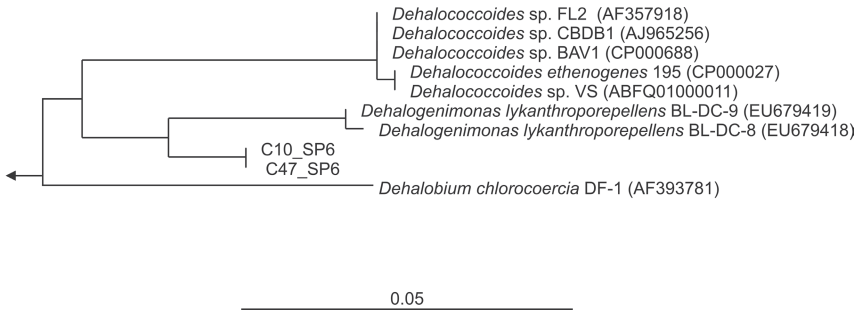

Figure 2: *Phylogenetic position of clones retrieved from 1,2-DCA degrading microcosms, based on 16S rRNA gene sequences. The tree was constructed using the AxML procedure as implemented in ARB (Ludwig et al., 2004), using E.coli positions 582 to 952. Accession numbers of reference sequences are shown in parentheses. The reference bar indicates 5 nucleotide substitutions in 100 positions.*

6.4 Environmental conditions to forecast biodegradation

Microbial activity is dependent on the local geochemical conditions, and therefore it is important that geochemistry is investigated during bioremediation studies. Unfortunately, it is difficult to identify biodegradation based on geochemical data only, since biotic processes also influence biodegradation (**Chapter 1**). When various geochemical conditions are studied simultaneously, biodegradation of chlorinated ethenes can be predicted based on geochemical data alone. This was demonstrated in **Chapter 4**, where the combination of different geochemical conditions (the EPA-score), showed the best correlation with presence of *Dehalococcoides* spp.: organohalide respiring microorganisms able to dechlorinate these chlorinated ethenes. Nevertheless, biodegradation activity can sometimes be excluded based on single geochemical parameters. For example, the presence of oxygen restricts anaerobic biodegradation pathways. In **Chapter 4** it was demonstrated that sulfate has a strong negative influence on reductive dechlorination of vinyl chloride, even more than any other geochemical parameter, like nitrate or pH. No oxidative biodegradation was reported for chlorinated ethenes at sulfate-reducing conditions. In **Chapter 3**, anaerobic oxidation of 1,2-DCA was tested under various redox conditions. Under denitrifying, iron-reducing, and methanogenic conditions, 1,2-DCA was dechlorinated. However, under sulfate-reducing conditions, no dechlorination activity was observed. The negative influence of sulfate on biodegradation of 1,2-DCA and chlorinated ethenes was previously observed and it may have important consequences for bioremediation in area's with high sulfate concentrations in the groundwater (van Eekert *et al.,* 2000). Sulfate concentration in groundwater of coastal area's, like the Netherlands, is influenced by marine water. Therefore, groundwater in these

area's often contains high sulfate concentrations (van der Welle *et al.,* 2006). To assess the risks of chlorinated ethenes, 1,2-DCA and other chlorinated aliphatic hydrocarbons, it is suggested here to consider sulfate as an important geochemical parameter that negatively influences the biodegradation of these compounds.

Chapters 3 and 5 show that the biodegradation capacity for 1,2-DCA and benzene was not associated with the water phase, but rather with the solid phase of river sediment or biofilm, respectively. In the river sediment, geochemical conditions were more favorable for 1,2-DCA-degrading microorganisms than in the water phase. This can explain the sediment-associated biodegradation capacity. However, another explanation is that different microorganisms prefer to grow on solid surfaces (e.g. sediment particles), or live in biofilms to cope with environmental stress like pollution or high flow rates, or transfer of degradation products (e.g. H_2) between syntrophic microorganisms (Battin *et al.,* 2003; Costerton *et al.,* 1995). It was observed in **Chapter 5**, that the formation of a biofilm in the reactor vessel appeared to be essential for benzene degradation. The benzene-degrading microorganisms were found to be maintained within the biofilm, preventing wash out from the continuous culture. Moreover, benzene was likely degraded via a syntrophic interaction, as described above. The biofilm facilitated the opportunity for a close interaction of hydrogen-consuming, anaerobic respiring, microorganisms and benzene-consuming *Peptococcaceae* (see Fig 1). Other studies already indicated that growth in biofilms is important for biodegradation of organic pollutants, like benzene and MTBE (Jechalke *et al.,* 2010; Singh *et al.,* 2006). The actual biodegradation capacity may be underestimated, when only the water phase is investigated, as in the study described in **Chapter 4**. Therefore, it is important to investigate biodegradation capacity in the environment on both liquid and solid phase, for example on surface water and sediment for river systems, and on groundwater and soil particles for soil systems.

6.5 Future perspectives for monitoring bioremediation

Molecular monitoring of phylogenetic or functional genes (see above and Table 1) of microorganisms involved in biodegradation processes, combined with survey of the geochemical situation will give an accurate overview of biodegradation potential. For some biodegradation processes, like reductive dechlorination of VC, specific molecular monitoring tools and knowledge about relevant geochemical conditions are available now (Maphosa *et al.,* 2010a). However, for many organic pollutants biodegrading microorganisms or biochemical degradation pathways are unknown. For these compounds, it is important that molecular targets are identified by monitoring specifically the microbial degradation process and link microbial activity to the responsible microorganisms. New metagenomic approaches offers now possibilities to relate microbial activity to unknown microorganisms or biochemical pathways (Kalyuzhnaya *et al.,* 2008; Smith *et al.,* 2008). As also presented in **Chapter 5** for

anaerobic benzene degradation, SIP-analysis allow to couple metabolic processes, to specific populations within the microbial community, which perform this metabolic process (Uhlík *et al.,* 2009). Until now, SIP-studies are mainly executed on laboratory cultures (pure cultures or microcosm enrichments). However, SIP can also be applied in the environment, for example by adding ^{13}C-labelled substrate to groundwater or *in situ* microcosms (e.g. BACTRAPs) (Stelzer *et al.,* 2006). This enables us to overcome selective growth under laboratory conditions, and to identify unknown pollutant-degrading microorganisms under local geochemical conditions. Rapid developments in next-generation sequencing (NGS) will enable us in near future to identify unknown pollutant-degrading microorganisms, or genes coding for key enzymes in biodegradation, from complex microbial ecosystems like soil or groundwater systems (MacLean *et al.,* 2009). By pyrosequencing or application of other NGS tools, complete complex metagenomes can be identified in a time- and cost-efficient way (Ghai *et al.,* 2010; Qin *et al.,* 2010; Wommack *et al.,* 2008). Therefore, metagenomic research can focus much 'deeper' into microbial communities as mainly done till now, by cloning, sequencing, and identification of only the most dominant species (>1% relative abundance) by classic technologies. Combining NGS with (*in situ*) biodegradation experiments will link specific microbial groups, or genes involved in biochemical processes, to biodegradation processes, and have therefore the potential to identify new phylogenetic and functional gene assays for monitoring biodegradation.

Once phylogenetic and functional genes are identified as specific biomarkers for a given activity, these can relatively easily be monitored in the environment by qPCR during bioremediation processes. However, as mentioned above, there are various examples or gene assays that are not specific enough, or do not cover all genes of interest, and should therefore be combined with other gene assays. Although qPCR is relatively fast and cheap, multiple gene-detection by qPCR is inefficient. Multiple gene-detection technologies (e.g. micro-arrays) offers then important advantages, because all genes can be monitored in one analysis. Phylogenetic and functional micro-arrays, like the PhyloChip and the GeoChip, are continuously improved (He *et al.,* 2010; He *et al.,* 2007; Loy *et al.,* 2002). Moreover, micro-arrays can be produced specifically for a selected set of genes. Alternatively, there are several nanotechnology developments that allow multiple parallel qPCR analyses to be performed efficiently and cost-effective. For common bioremediation processes, these advanced technologies are actually not applied, since the technology is expensive and often specific knowledge is not available. However, when new phylogenetic and functional gene assays are developed with upcoming SIP and NGS technologies, the insight in microorganisms and genes involved in biodegradation will increase rapidly. With this, also the need to combine different gene assays to monitor specifically biodegradation processes will increase. Therefore, the application of multi-gene detection technology for bioremediation processes will become more important.

Detection of phylogenetic or functional genes can reveal biodegradation capacity under characterized geochemical conditions. However, it is difficult to translate gene copy numbers to actual biodegradation activity. This may be overcome partly by transcriptomic analysis: quantitative detection of mRNA copies in relation to microbial activity. However, this relation varies between microorganisms or under various geochemical conditions. In addition, it is technically difficult to quantify mRNA concentrations in the environment (Da Silva and Alvarez, 2008; Morris *et al.,* 2006; Rahm and Richardson, 2008; Sessitsch *et al.,* 2002). New sophisticated proteomic technologies, like non-gel based proteomics, are promising for quantitative identification of biomarkers, with high specificity and sensitivity (Benndorf *et al.,* 2007; Jehmlich *et al.,* 2008; Morris *et al.,* 2007; Werner *et al.,* 2009). Further, proteomics will be important for characterization of unknown expressed enzymes, and therefore improve our insight on biochemical processes (Singh, 2006).

To verify the relation between actual biodegradation activity, geochemistry, and phylogenetic and functional gene targets, (*in situ*) biodegradation experiments are important. A combined approach of geochemical survey, molecular identification of specific biodegradation targets (DNA, RNA or proteins), and (*in situ*) biodegradation experiments, is essential to identify and understand the exact involved microbial processes of pollutant degradation. Only by such an integrated approach, we can develop and apply efficient bioremediation strategies to clean-up the environment.

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Summary

Microorganisms are able to transform organic pollutants into products with reduced toxicity. Therefore, biodegradation is an important process to decontaminate polluted environments that form a serious risk for human health and ecosystem functioning. For policymakers, land-users and land-owners, it is crucial that biodegradation processes are monitored and controlled at polluted locations. However, since various physical, geochemical and biotic factors influence the microbial activity in the environment, detailed knowledge of the biodegradation process is required for monitoring. This study describes the relation between geochemical conditions and biodegradation activity of microorganisms, and methods to monitor their activity and metabolic functions in the environment. The widely spread and persistent chlorinated aliphatic hydrocarbons 1,2-dichloroethane (1,2-DCA), tetrachloroethene (PCE), vinyl chloride (VC), and benzene, were used as model compounds.

Insight is provided into the effect of pollution on the composition of microbial communities in river sediment. Mesocosms with river sediment were exposed to chlorinated hydrocarbons or extra nutrients, and the composition of the microbial communities was monitored by culture-dependent and molecular techniques. Although individual microbial species were influenced by the organic pollutants, a considerable stability of the total microbial communities as well as specific functional subgroups was observed.

The biodegradation capacity for 1,2-DCA in river systems was tested at various redox conditions by microcosm experiments and molecular detection of 1,2-DCA-dechlorinating microorganisms. In the river sediment, but not in the water phase, 1,2-DCA disappeared due to microbial activity, under denitrifying, iron-reducing and methanogenic conditions. The 1,2-DCA-dechlorinating microorganisms were widespread over the river systems, but only showed dechlorinating activity at suitable geochemical conditions. This emphasizes the need to monitor simultaneously both geochemical and microbiological conditions.

The biodegradation capacity for VC was found to be widely present in chloroethene-polluted groundwater as deduced from the presence of *Dehalococcoides* spp. and the VC-reductase genes *vcrA* and *bvcA*. The latter VC-reductase genes were shown to be better indicators for VC dechlorination capacity than *Dehalococcoides* spp. 16S rRNA genes, since various *Dehalococcoides* strains were found to dehalogenate other chlorinated compounds than VC.

Members of the *Peptococcaceae* were identified by DNA Stable Isotope Probing as dominant benzene-consumers in an anaerobic continuous culture under denitrifying conditions. Other microorganisms identified to be involved in the benzene degradation process were members of *Rhodocyclaceae*, *Burkholderiaceae*, and *Gemmatimonadetes*. Furthermore, evidence was found for the existence of benzene degradation via a syntrophic process.

Summary

Overall, these findings contribute to a better understanding of biodegradation processes in the environment. Moreover, the results indicate that a combined approach of geochemical survey, molecular identification of specific biodegradation targets, and (*in situ*) biodegradation experiments, is essential to identify and understand the microbial processes involved in pollutant degradation. Such an integrated approach allows for developing efficient bioremediation strategies that include monitoring pollution degradation and clean-up of the environment.

Samenvatting

De aanwezigheid van organische verontreiniging in het milieu vormt een belangrijk risico voor de gezondheid van mensen en het functioneren van ecosystemen. Micro-organismen zijn in staat om organische verbindingen om te zetten in minder schadelijke stoffen en spelen daarom een belangrijke rol bij het opruimen van milieuvervuiling. Voor beleidsmakers, landgebruikers en landeigenaren is het belangrijk dat er bij milieuverontreiniging goed toezicht wordt gehouden op de biologische afbraakprocessen en dat deze goed worden beheerst. Aangezien microbiële activiteit in het milieu wordt beïnvloed door diverse fysische, geochemische en biologische factoren, is nauwkeurige kennis van het afbraakproces hierbij noodzakelijk. In dit promotieonderzoek wordt de relatie tussen geochemische condities en de biologische afbraakcapaciteit van micro-organismen in het milieu beschreven, inclusief methoden om de activiteit en metabole functies van deze micro-organismen in het milieu te meten. De moeilijk afbreekbare en wijdverspreide gechloreerde koolwaterstoffen 1,2-dichloorethaan (1,2-DCA), tetrachlooretheen (PCE), vinylchloride (VC) en benzeen zijn als modelverbindingen voor het onderzoek gebruikt.

Wat de invloed is van organische vervuiling op de samenstelling van microbiële gemeenschappen in riviersediment wordt beschreven in hoofdstuk 2. Voor dit onderzoek zijn bakken met onverstoord riviersediment naar het laboratorium gebracht en daar blootgesteld aan gechloreerde koolwaterstoffen of extra nutriënten. Veranderingen in de samenstelling van de microbiële gemeenschappen zijn met zowel kweekafhankelijke als met moleculaire technieken onderzocht. Het bleek dat individuele microbiële soorten wel werden beïnvloed door de organische vervuiling, maar dat de samenstelling van de totale microbiële gemeenschap en de functionele subgroepen stabiel was.

De biologische afbraakcapaciteit van 1,2-DCA in riviersystemen is onderzocht voor verschillende redox condities (hoofdstuk 3). Hiervoor zijn afbraakexperimenten uitgevoerd en 1,2-DCA-afbrekende micro-organismen geïdentificeerd met behulp van moleculaire technieken. In het sediment van de Donau en de Ebro werd 1,2-DCA door micro-organismen omgezet onder denitrificerende, ijzer-reducerende of methanogene condities. In het water van deze rivieren werd 1,2-DCA echter niet afgebroken. Micro-organismen die 1,2-DCA kunnen afbreken, bleken aanwezig te zijn in verschillende riviersysteem, maar ze vertoonden alleen afbraakactiviteit onder geschikte geochemische condities. Dit benadrukt het belang om bij microbiologische afbraakstudies ook de geochemische condities in acht te nemen.

Hoofdstuk 4 laat zien dat in grondwater dat verontreinigd is met chloorethenen, de dechlorerende *Dehalococcoides*-bacteriën en de VC-reductase genen *vcrA* en *bvcA* wijdverspreid aanwezig zijn. Het grondwater voor dit onderzoek is verzameld uit 150 peilbuizen verspreidt over 11 (voormalig) industriële locaties in Nederland en Groot-Brittannië. Ondanks dat *Dehalococcoides* spp. 16S rRNA genen veelal

worden gebruikt als indicator voor VC dechloreringscapaciteit, blijken de twee VC-reductase genen *vcrA* en *bvcA* hiervoor een betere indicator te zijn. Dit kan worden verklaard doordat verschillende *Dehalococcoides*-stammen geen VC, maar andere gechloreerde verbindingen dehalogeneren.

In hoofdstuk 5 is aangetoond dat bacteriën die behoren tot de Familie *Peptococcaceae* benzeen kunnen consumeren in een anaerobe continue-culture onder denitrificerende condities. In deze continue-culture werd gedurende 8 jaar microbiologische benzeenafbraak geobserveerd, maar de verantwoordelijke micro-organismen konden ondanks meerdere pogingen niet worden geïdentificeerd. Met behulp van stabiele isotoop labelling zijn de benzeenafbrekende micro-organismen nu geïdentificeerd. Andere micro-organismen die betrokken zijn bij de benzeenafbraak in deze continue-culture, behoren tot de *Rhodocyclaceae*, *Burkholderiaceae* en *Gemmatimonadetes*. In dit hoofdstuk wordt ook beschreven dat er bewijs is gevonden voor benzeenafbraak via een syntrofe samenwerking.

De bevindingen van dit promotieonderzoek dragen bij aan een beter begrip van microbiologische afbraakprocessen in het milieu. Een gecombineerde aanpak van geochemische karakterisatie, moleculaire detectie van specifieke microbiologische afbraakindicatoren, en (*in situ*) afbraakexperimenten zijn essentieel om de afbraakprocessen in het milieu te identificeren en te begrijpen. Een dergelijke integrale aanpak maakt de ontwikkeling van efficiënte bioremediatie mogelijk, om te komen tot een schoner leefmilieu.

Curriculum Vitae

Bastian Mattijs van der Zaan was born on the 2nd of September 1980 in Emmeloord, the Netherlands. In 1998 he obtained his "Athenaeum" diploma at the Ichthus College in Kampen. In that same year, he started his study Biology at the Rijks*universiteit* Groningen, where he specialized in microbial ecology and environmental biotechnology. He did his first MSc-project in the department of Microbial Ecology, where he studied anaerobic biodegradation of BTEX. He wrote a master-thesis about CO_2-storage in the oceans by iron addition and did a colloquium about the anammox-process. In his second MSc-project, at TNO in Apeldoorn, he worked on the reduction of biofouling in industrial cooling towers.

After his graduation in 2003, he started working at "TNO Environment, Energy, and Process Innovation" as junior researcher. There he got experienced with various molecular techniques to identify microbial communities. In August 2004 he started with his PhD project at TNO and Wageningen University. His PhD project was part of the European 6th Framework project titled Aquaterra. The results of his PhD research project are presented in this thesis.

Since September 2009, he is working as a researcher at Deltares in the Geo Environmental Research Laboratory on detection of microbial processes in soil, surface water and groundwater systems.

List of Publications

Barth, A.C., Grathwohl, P., Fowler, H.J., Bellin, A., Gerzabek, M.H., Lair, G.J. Barcelo, D., Petrovic, M., Navarro, A., Negrel, Ph., Petelet-Giraud, E., Darmendrail, D., Rijnaarts, H., Langenhoff, A., de Weert, J., Slob, A., van der Zaan, B., Gerritse, J., Frank, E., Gutierrez, A., Kretzschmar, R., Gocht, T., Steidle, D., Garrido, F., Jones, K., Meijer, S., Moeckel, C., Marsman, A., Klaver, G., Vogel, T., Burger, C., Kolditz, O., Broers, H.P., Baran, N., Joziasse, J., von Tumpling, W., van Gaans, P., Merly, C., Chapman, A., Brouyere, S., Batlle Aguilar, J., Orban, Ph., Tas, N., Smidt, H., **2008**, Mobility, turnover and storage of pollutants in soils, sediments and waters: achievements and results of the EU project AquaTerra. A review. Agronomy for Sustainable Development, vol. 29, 2008, p. 161-173

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Dankwoord

Na zo'n vijf jaar experimenteren, bemonstering, labwerk, schrijf- en denkwerk is mijn boekje eindelijk af. Ik ben blij dat ik het heb kunnen doen en heb erg veel geleerd in deze periode. Ook al is het doen van promotieonderzoek veelal zelfstandig werk, veel mensen hebben mij geholpen en gesteund om de eindstreep te halen. Deze mensen wil ik hier graag bedanken.

Allereerst Jan. Als dagelijkse begeleider en copromotor heb je een belangrijke rol gehad bij de totstandkoming van dit boekje. Tijdens mijn stage bij TNO heb jij je ingezet om mij bij TNO in dienst te nemen en later nogmaals om mij aan te nemen als AIO binnen Aquaterra. Ik heb veel van je geleerd: inhoudelijke microbiologische kennis, maar ook over het maken van een goede experimentele opzet, discipline tijdens het uitvoeren van onderzoek, en het schrijven van artikelen. Vooral dit laatste onderdeel heeft jou de eerste 3 a 4 jaar van mijn onderzoek vast heel wat kopzorgen opgeleverd. Toch heb je altijd vertrouwen in mij gehad en daar ben ik je dankbaar voor. Uiteindelijk is het ook met die publicaties wel goed gekomen.

Hauke, als copromotor heb je me wegwijs gemaakt binnen het Wageningse. Je stond altijd meteen klaar om mijn vragen te beantwoorden of om te helpen als iets vastliep. Vooral op het gebied van moleculaire ecologie heb je me de actuele kennis bijgebracht, wat zeker een belangrijke bijdrage heeft geleverd aan de gepubliceerde artikelen en de hoofdstukken uit dit boekje. Je vriendelijkheid en positieve benadering waardeer ik erg.

Willem, bedankt dat je mijn promotor wilde zijn. Ondanks dat je mij als "externe" AIO niet veel hebt zien rondlopen bij Microbiologie, was je toch altijd goed op de hoogte hoe het er met mijn onderzoek voorstond. Het was erg nuttig dat je me tijdens de PhD/PostDoc-meetings wist te wijzen op juist die dingen die inderdaad nog niet goed in mijn hoofd zaten of nog niet voldoende waren uitgezocht. Ik vond het super dat je alle stukken zo vlak voor de deadline nog kritisch hebt kunnen bekijken, waardoor het op tijd naar de leescommissie kon worden gestuurd.

Jasperien, als mede-AIO binnen Aquaterra heb ik veel met jou mogen samenwerken. De monsternamerondes van de Ebro en de Elbe waren dankzij jou erg geslaagd en we vulden elkaar goed aan tijdens de werkzaamheden. Door jouw oplettendheid zijn we uiteindelijk toch nog in Apeldoorn beland en niet in Lille…. Verder heb ik veel van je geleerd over biobeschikbaarheid en sediment, maar ook om meer assertief te zijn als mens en onderzoeker. Bovendien heb je me vaak geholpen om praktische "regel dingen" uit te leggen over cursussen, het submitten van een manuscript, of voor de promotie. Daarom is het niet meer dan logisch dat ik het leuk vind dat je mijn paranimf bent.

Dankwoord

Als initiator van het Aquaterra project en later als afdelingshoofd is Huub altijd nauw betrokken geweest bij mijn onderzoek. De inhoudelijke overleggen die we samen hebben gehad waren erg waardevol, omdat je binnen een half uur alle kleine losse eindjes weer aan elkaar wist te knopen tot een rode draad. Dit hielp mij om de experimenten in een breder perspectief te plaatsen.

Tijdens de afgelopen 5 a 6 jaar heb ik erg fijne collega's om me heen gehad bij TNO in Apeldoorn en TNO/Deltares in Utrecht. Enkele (ex-) collega's wil ik speciaal bedanken. Alette, jij bent altijd een goede vraagbaak voor praktische, maar ook inhoudelijke dingen. Jouw koeriersdiensten van de "Alette-Fons-Express" waren onmisbaar. Hendrik, van jou heb ik veel geleerd over moleculaire detectie methoden. Graag liet je me ook alle voordelen en beperkingen van de verschillende technieken weten. Ik vind het nog steeds jammer dat we sinds de verhuizing in 2007 naar Utrecht en Delft geen directe collega's meer zijn. Gelukkig kan ik nog wel iedere dag samenwerken met Fredericke, Andre, Habiba, Paul en Sara. Door jullie als behulpzame en gezellige collega's blijf ik met plezier binnen de afdeling werken. Nanne, de Catan-avonden zijn mede dankzij jou altijd erg gezellig, maar ook nuttig omdat er tijdens de treinreis vaak te weinig tijd is om bij te praten over van alles en nog wat. Ook vind ik het erg leuk om Arjan en Sjef als mede-kolonisten nog regelmatig te spreken. Nu mijn boekje helemaal af is, zal ik niet zo vaak meer een afspraak afzeggen. Veel gezelligheid in de labs heb ik ook gehad met Arjan M., Suzanne, Karin, Nick, Rita, Frank, Jean-Paul, Luaine, Koen, Maaike, Hugo, Peter E., Maurice, Marc, Vesela en Ivo.

Tijdens mijn promotieonderzoek heb ik Arian Koeken mogen begeleiden tijdens zijn afstudeerstage. Ik vond het leuk om te doen en volgens mij is het voor ons allebei erg nuttig geweest. Veel praktisch werk dat in hoofdstuk 4 beschreven staat heb jij uitgevoerd.

Als "externe" AIO was ik bij Microbiologie in Wageningen misschien een vreemde eend in de bijt. Toch heb ik me er altijd welkom gevoeld en dat was mede dankzij een aantal mensen. Neslihan, it was nice that we started together as PhD-student in the Aquaterra project. I learned a lot from you about *Dehalococcoides* and reductive dechlorination genes. You did a great job in Oklahoma trying to get anything and everything out of our DNA-samples. I am still grateful that you saved my wallet out of the hands of the "highway scooter pickpocket" in Barcelona.

Miriam, Gosse en Tim bedankt voor de samenwerking binnen onze gezamenlijke Aquaterra werkpakket en voor het meedenken met mijn onderzoek. Daarnaast wil ik Tim bedanken voor het kritisch lezen van mijn proefschrift. Je hebt er in elk geval een foutje uit weten te halen..... Fons en Caroline wil ik bedanken voor onze samenwerking op het gebied van benzeenafbraak. Het is fantastisch dat alle faciliteiten ter beschikking stonden en de besprekingen met jullie waren erg verhelderend. Ik zal mijn uiterste best doen om van het 5e hoofdstuk een mooie gezamenlijke publicatie te maken. Flávia, it was great to work with you. Your enthusiasm and cheerfulness definitely contributed to the nice results we obtained.

During the sampling campaigns and general Assemblies of the Aquaterra project, I worked together with many kind people from all over Europe. I would like to thank Alain, Alicia, Miriam, Christian, Edda, David, Dietmar, Brit, Ingo, Frank, and all others, for the cooperation and the nice times when we met each other.

Mijn promotieonderzoek heeft veel tijd en energie gekost, vooral de laatste 2 jaren. Ik ben heel blij dat ik in deze tijd fijne vrienden en lieve familie om me heen had staan. Ook al snapten jullie er geen snars van waarom ik nu per se in Spanje een flesje water en een schepje grond moest halen, of wat een PCR-reactie is. Jullie waren altijd nieuwsgierig naar hoe het met mijn onderzoek ging. Ook was het juist fijn om met jullie over heel andere dingen dan onderzoek te praten of te doen.

Met Maarten en Sander heb ik regelmatig ervaringen en frustraties over AIO-zijn kunnen delen. Dat gaf weer nieuwe energie om door te gaan, omdat het altijd maar weer blijkt dat het overal hetzelfde is. En wie stond er ook al weer bovenaan in het rijtje "grootste lab-blunders?" Ilja, jij kent de inhoud van mijn proefschrift inmiddels beter dan wie dan ook van mijn vrienden of familie. Als paranimf zal je de verdediging zo over kunnen nemen….Ik vind het echt geweldig dat je me geholpen hebt met de lay-out en er zo veel tijd aan hebt besteed.

Speciaal wil ik mijn ouders bedanken voor wat ze mij hebben meegegeven. Pa en ma, jullie hebben me de kans gegeven te gaan studeren en me altijd vrij gelaten om mijn eigen keuzes te maken en eigen ideeën te hebben. Ook hebben jullie me geleerd naar anderen om te zien en mensen in hun waarde te laten. Hierdoor heb ik me kunnen ontwikkelen tot wie ik nu ben.

Thijs en Anne, ik geniet zo van jullie, elke dag weer. Jullie vrolijkheid, jullie onbezorgdheid, jullie levendigheid, jullie onschuld. De kleinste details die jullie opvallen, de simpelste oplossingen die jullie bedenken, de kleinste dingen waar jullie al blij van worden, ……ik leer heel veel moois van jullie. Ik hoop dat ik net zo veel van jullie broertje of zusje zal kunnen gaan genieten, als van jullie.

Lieve Lisette, als allerlaatste, maar ook het allermeest wil ik jou bedanken. Jij hebt me steeds weer het duwtje gegeven dat ik nodig had om door te gaan als het onderzoek vast liep. Als ik ergens niet meer uit kwam, wist jij me weer op te vrolijken door juist te laten zien wat er wel goed ging. Bedankt voor je geduld en al die uren, avonden, dagen en weken dat je me in alle rust hebt laten doorwerken. Het is voor jou ook zeker niet altijd makkelijk geweest. Ik ben erg blij dat het boekje nu af is, maar het meest gelukkig ben ik met jou en ons gezin.

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- PhD scientific writing
- Workshop "Electron transfer processes at biogeochemical gradients", 2008, UFZ Leipzig, Germany

Management and research skills

- Organize and execute sampling campaign to various European rivers
- "Verantwoordelijk Medewerker" – Laboratory of GeoBiotechnology TNO/Deltares, Utrecht, The Netherlands
- Planning of infrastructure new molecular microbial laboratory of TNO, Utrecht, The Netherlands

Oral Presentations

- AquaTerra workshop: "Long-term fate of pollutants in soils: Mobility, stability, and transformation", 15 March 2007, Tuebingen, Germany
- Soil & Water Symposium, 7 June 2007, Zeist, The Netherlands
- Soil & Water Symposium, 10 June 2008, Zeist, The Netherlands
- SENSE symposium "Innovative Techniques for a Sustainable Environment", 19 February 2009, Wageningen, The Netherlands

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This thesis was financially supported by the European Union, as part of the integrated research project AquaTerra (project number 505428 GOCE) within the 6e Framework Program, as well as TNO Built Environment and Geosciences and Deltares.

Layout: Ilja Wieselmann
Printed by: Ipskamp Drukkers B.V., Enschede