Dehalococcoides spp. in River Sediments:

Insights in Functional Diversity and Dechlorination Activity

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Insights in Functional Diversity and Dechlorination Activity

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For my mother…

…biricik anneme

Abstract

Chlorinated pollutants are toxic and persistent organic compounds that, if left untreated, remain in the environment for decades. Even though their production and usage prohibited, they can still be detected in various environments. The discovery of microorganisms, which can transform chlorinated organics to non- or less toxic compounds, triggered the scientific community to undertake continued efforts towards understanding the functioning of these microorganisms in the environment, because they offer a great potential for cleansing of polluted environments. This thesis aimed to describe the diversity and functioning of a unique group of anaerobic bacteria, *Dehalococcoides* spp., which are specialized in and dedicated to the degradation of chlorinated compounds. Chlorinated compounds are used by *Dehalococcoides* spp. as terminal electron acceptors in an anaerobic respiration process, called halorespiration, resulting in the reductive transformation of the compound. This research explicitly focused on detection of diversity and activity of *Dehalococcoides* spp. in sediments and flood plain soils of several European river basins, which were shown to be sinks of many chlorinated organic pollutants, including hexachlorobenzene (HCB). By using state-of-the-art biomolecular techniques, integrated with controlled microcosm experiments and multivariate statistical analysis, environmental factors affecting reductive dechlorination by *Dehalococcoides* spp. were identified. Furthermore, correlations between environmental conditions and *Dehalococcoides* spp. activity and composition in the selected river basins were established. Seasonal variation, as well as spatial and temporal fluctuations in total organic carbon and/or nitrogen content and HCB contamination, were demonstrated to affect not only the abundance of *Dehalococcoides* spp. but also the composition of active populations and the diversity of genes involved in reductive dechlorination. HCB was transformed to less chlorinated compounds in the majority of sediment- and soil samples. *Dehalococcoides* spp., which were responsible for HCB transformation, were found to adapt to changes in environmental conditions, such as temperature and salinity, in a relatively fast manner unless the changes were extreme (for example, temperatures lower than 15°C, or high sea salt concentrations of 0.5M). Moreover, endogenous microorganisms present in European rivers' sediment demonstrated to possess a great potential to tackle several chlorinated compounds, such as chlorinated ethenes and HCB, simultaneously. Even with today's enhanced biomolecular techniques, it is difficult to understand the full extent of the dechlorination process since it is a part of a complex web of metabolic and regulatory interactions. Thus, it is proposed to study reductive dechlorination and the function of *Dehalococcoides* spp. in larger communities and within the environments where they belong. Along with the elucidation of functional properties of such communities, the true role and importance of *Dehalococcoides* spp. in the environment can be assessed.

Keywords: *Dehalococcoides* spp., reductive dechlorination, hexachlorobenzene (HCB), biogeography, river sediment

Contents

Aim and outline of the thesis

The changing global environment and pollution have a great impact on aquatic biota in river water, sediments and aquifers connected to river basins. To date, however, the effects of organic and inorganic pollutants in soils, sediments, ground- and surface water at catchments are poorly understood. The EU 6th Framework Integrated Project Aqua Terra was initiated to address these issues and to investigate the behavior of pollutants in several European river basins. Microbial communities play a crucial role in the global and local biogeochemical cycles, including degradation and subsequent detoxification of pollutants. As a part of the Aqua Terra project, the aim of the research described in this thesis therefore was to study the effect of changing environmental conditions and pollutants on the diversity and functioning of microbial communities. Biodegradation capacity of sediments and flood plain soils of European river basins was studied by using state-of-the-art biomolecular techniques integrated with controlled microcosm experiments. Chlorinated compounds, especially hexachlorobenzene (HCB), were the chosen pollutants of interest since they were previously detected at different locations within the river basins. Chlorinated pollutants are toxic and persistent organic compounds that, if left untreated, accumulate in the environment for decades. Therefore, the discovery of a unique group of anaerobic bacteria, *Dehalococcoides* spp., which are specialized and dedicated to the degradation of chlorinated compounds, was of great importance for the understanding and exploitation of their activities during the microbe-mediated cleaning of these pollutants from the environment. To this end, the three main goals of research described in this thesis were: i) the detection of *Dehalococcoides* spp. diversity and activity in selected European river basins, ii) establishing correlations between environmental factors and *Dehalococcoides* spp. functionality, and iii) the identification of environmental factors that affect the dechlorination activity of *Dehalococcoides* spp.

Chapter 1 provides information about the origin of chlorinated compounds and their microbial degradation via anaerobic reductive dechlorination. The physiological features of *Dehalococcoides* spp. are introduced and the current knowledge about their involvement in reductive dechlorination in the environment is summarized. Specific novel molecular research techniques, including microarray technology, and statistical analysis methods used in this research are described.

Soil and sediment samples from several different locations across Europe were screened for their capacity to degrade one of the most persistent chlorinated pollutants, HCB (**Chapter 2**). Combination of lab-scale transformation experiments and a culture-independent quantitative molecular detection method (qPCR) were applied to establish a link between the degradation of HCB and presence of *Dehalococcoides* spp. at different locations.

The following two chapters, **Chapters 3** and **4**, describe experiments towards characterizing the diversity of *Dehalococcoides* spp. and functional genes (genes that code for metabolic enzymes), respectively. The diversity and activity of *Dehalococcoides* spp. in the river basins was studied with 16S rRNA-targeted fingerprinting and quantification. Also microarrays were applied for the detection of genes involved in reductive dechlorination (**Chapter 3**). Several statistical analysis methods were used to correlate the variances in the *Dehalococcoides* spp. diversity and activity to spatial and temporal dynamics of environmental conditions. In-depth analysis of functional gene diversity of all microbial populations was covered in **Chapter 4**. Thanks to microarray technology, high-throughput detection of functional genes provided insight in the dynamics of microbial populations involved in biochemical processes, and the environmental factors significantly affecting their diversity in river sediments.

In **Chapters 5** and **6** the effects of environmental conditions on the reductive dechlorination of HCB were thoroughly investigated to identify the role of *Dehalococcoides* spp. in the process. The impact of changing temperature, sea-salt concentrations and co-existence of several chlorinated pollutants was studied. Dynamics in the functionality of *Dehalococcoides* spp. and their competence in response to the changing environmental conditions were demonstrated.

Finally, **Chapter 7** provides a summary and general discussion of the results of this thesis.

Chapter 01

General Introduction

Neslihan Taş, Miriam H.A. van Eekert, Willem M. de Vos

and Hauke Smidt

To be submitted with parts from **Chapter 7**

Introduction

The fate and persistence of hazardous chemicals in the environment have been a concern for the past 50 years. Past industrialization and extensive agricultural activities have led to their accumulation in the environment, while their adverse impact on various ecosystems and human health also became evident. The primary objective of the EU 6th Framework Integrated Project Aqua Terra was to provide a better understanding of the behavior of these pollutants and their fluxes with respect to climate and land use changes in European river basins. The study areas, located across Europe (Fig. 1), have their own environmental, climatic and demographic characteristics. Consequently, the goal was to provide information on pollutant dynamics in river basins, which will serve as a basis for the design of improved environmental management tools and conceptual models for environmental planning at larger scales (9).

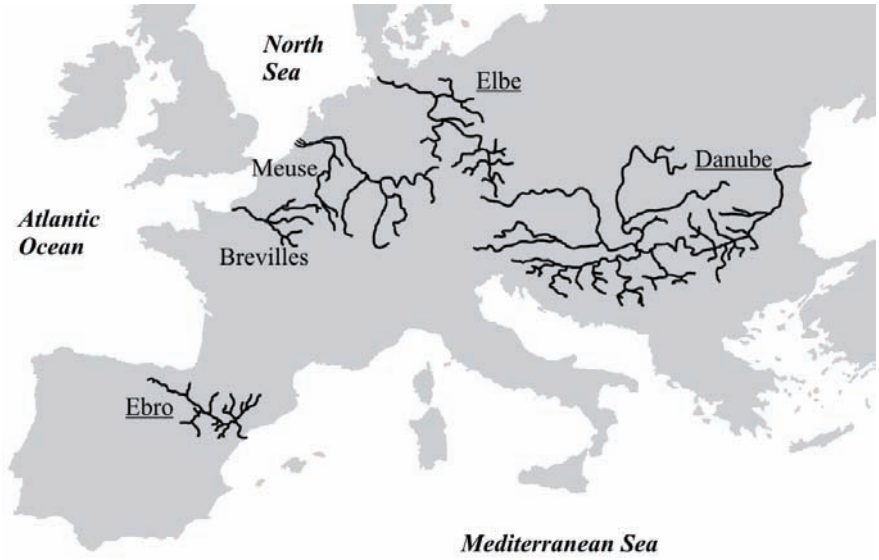


Figure 1. River basins studied within the framework of the Aqua Terra project. Map is re-drawn from Barth *et al.*(9). The names of the river basins studied in this thesis are underlined.

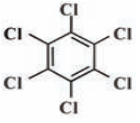
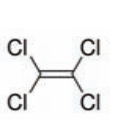
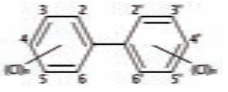
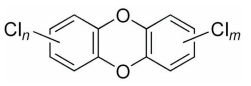
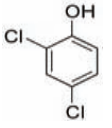
Recognition of the ability of microorganisms to degrade hazardous compounds opened up a new vista not only for the microbially-mediated remediation of polluted environments, but in addition it triggered the scientific community to undertake continued efforts towards the discovery, isolation and characterization of new microbial species. Among these, *Dehalococcoides* spp. represent dedicated degraders, which are specialized in the anaerobic detoxification of chlorinated organic contaminants that are otherwise persistent for decades.

The chlorinated compounds are used as the terminal electron acceptor in an anaerobic respiration, also termed halorespiration. It has been shown that several other bacteria belonging to the δ– and ε–Proteobacteria (*Anaeromyxobacter Desulfuromonas, Desulfomonile, Desulfovibrio, Geobacter, Sulfurospirillum*) or to the low-GC Gram-positive bacteria (*Desulfitobacterium*, *Dehalobacter*) are able to degrade chlorinated organic contaminants through halorespiration as well (Fig. 2) (70). However, with the exception of *Dehalobacter* spp., none of these species are as specialized as *Dehalococcoides*, and they are reported to grow as well e.g. by metal reduction, denitrification or fermentation. This introduction provides a summary of the present knowledge of *Dehalococcoides* spp. and their role in degradation of chlorinated organic contaminants.

Pollution of chlorinated compounds and bioremediation

Chlorine-containing organics (Table 1) are persistent pollutants in our environment. Even though it is often believed that chlorinated organic compounds originate exclusively from industrial pollution, many living organisms (e.g. marine sponges as a part of their defense mechanisms) and geological phenomena (i.e. volcanoes, forest fires, geothermal processes) also produce them naturally (31). Nevertheless, it is their extensive industrial (e.g. solvent, metal degreasing, rubber production) and agricultural (e.g. pesticide component) applications over the past 50 years that resulted in their deposition in various environments, especially in soils, groundwater aquifers and sediments (6, 8, 32, 56, 80). Due to their physicochemical properties, exposure to these compounds has carcinogenic and lethal effects on biota. Therefore, the production and application of most of these compounds is no longer allowed in 90 countries since the Stockholm convention in 2001 (20, 77). However finding the suitable clean-up techniques for the contaminated environments remains challenging. Remediation of soils and groundwater can be achieved via physicochemical methods such as thermal cleaning, chemical oxidation or adsorption on activated carbon (47), whereas there are no in-situ remediation technologies for sediments other than complete removal of the contaminated sediment (81). Moreover, the high ecological disturbance that these physicochemical treatment methods can cause in the environment makes them unsustainable solutions in the long term (81). Other than harsh physicochemical treatments, a far more preferable option is bioremediation. During bioremediation, chlorinated contaminants are largely degraded by microorganisms, even though, degradation by higher organisms has also been reported. Phytoremediation, where plants are employed to degrade, assimilate, metabolize, or detoxify chlorinated compounds, was reported to be an effective bioremediation method (72). For example, poplar trees were shown to assimilate and degrade trichloroethene (TCE) to 2,2,2-trichloroethanol, trichloroacetic acid, and dichloroacetic acid (59). In many ecosystems, fungi are among the major decomposers. Most fungi are robust organisms and are generally tolerant to high levels of pollution (68). Fungal lignocellulolytic enzymes have been

Table 1. Sources, biological impacts and physicochemical properties of chlorinated organic compounds that have been reported to be degraded by *Dehalococcoides* spp.

	HCB [a,b]	**PCE** [c] **/ TCE** [d]	**PCBs** [a,e]	**Dioxins** [a]	**CPs** [f]
		 [g]			
Natural Sources	Volcanic activity, minerals	Volcanic activity, barley	Volcanic activity	Forest fires	Metabolites of microbes, sponges
Anthropogenic Sources	Pesticide synthesis, waste incineration, dye production	Solvent (dry cleaning, metal cleansing), grain fumigation	Insulating fluid, microscope oil, stabilizing additive	Coal fired utilities, waste incineration, metal smelting, diesel truck, bleaching	Pesticides, bleaching wood pulp
Abiotic Degradation	Photolysis	none	Ultrasound	Photolysis	Photolysis
Effects	Immune system and liver damage, cancer	Liver and kidney damage, neurotoxicity, possibly cancer	Skin rashes, dizziness, liver damage, reproductive damage, possibly cancer	Cancer, hepatotoxicity birth defects, endocrine disruption	Cancer, birth defects
Molecular Weight	285	165 / 131	various	various (from 84-322)	various (from 128 - 266)
Water solubility (mg/L)	0.005	150 / 1280	$0.0027 - 0.42 \times 10^{-3}$	insoluble	10 - 905
Vapor pressure (kPa) [h]	0.1×10^{-3}	1.9 / 7.8	$1.1 \times 10^{-3} - 1.3 \times 10^{-7}$ (18)	NA [i]	$1 - 12.7 \times 10^{-3}$

[a](30); [b]Hexachlorobenzene (5); [c]Tetrachloroethene and [d]Trichloroethene (76); [e]Polychlorinated biphenyls,There are theoretically 209 different PCB congeners, although only about 130 of these were found in commercial PCB mixtures (78); [f]Chlorophenols (4);[g]only PCE is illustrated; [h]at 20°C; [i]not available

related to the degradation of various pollutants when used in combination with mediators and reactive radicals. Being the most commonly studied example, the white-rot fungi are able to detoxify a wide range of pollutants including chlorinated organics, with lignin and manganese peroxidases (25, 75).

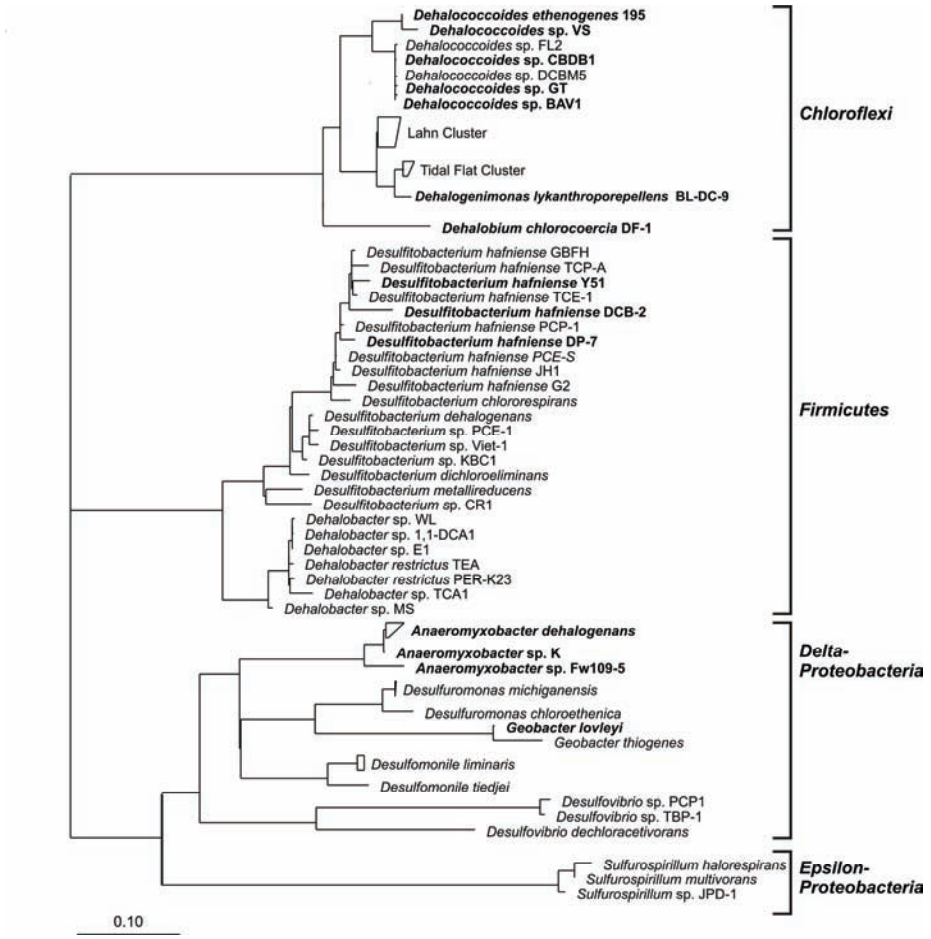


Figure 2. Phylogenetic tree of dechlorinating bacteria based on bacterial SSU rRNA sequences. Alignment and phylogenetic analysis were performed with the ARB software (62), and the tree was constructed using the neighbor joining method . The reference bar indicates the branch length that represents 10% sequence divergence. Bold face lettering indicates completed or ongoing genome sequencing.

The bacterial degradation of chlorinated pollutants can be either a result of fortuitous co-metabolic conversion, or can contribute to the energy metabolism of the degrading organism. During the latter metabolic processes, chlorinated compounds can be either used as

carbon source or as electron acceptors (coupled to the oxidation of an electron donor), depending on the oxidation state of the compound. Although many chlorinated compounds can also be degraded under aerobic conditions, the majority of polychlorinated compounds, such as those discussed in this review, are recalcitrant to aerobic degradation. Due to the electronegative nature of the chlorine atom, oxidation of the carbon backbone in the chlorinated compound becomes thermodynamically unfavorable (84), especially in polychlorinated compounds. As a result they serve as energetically favorable electron acceptors in microbial metabolism in anoxic environments such as sediments, subsurface soils and groundwater aquifers. Consequently, anaerobic bacteria, which can use these compounds as electron acceptors, are good candidates for bioremediation (79).

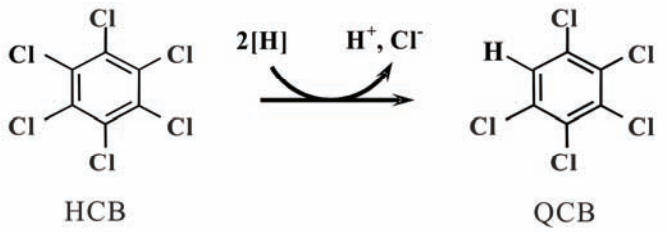


Figure 3. Reductive dechlorination of hexachlorobenzene (HCB) to pentachlorobenzene (QCB).

The genus *Dehalococcoides* and environmental relevance of reductive dechlorination

Dehalococcoides (Fig. 2) is a genus of strictly anaerobic gram-negative bacteria that is specialized on gaining energy from the reduction of chlorinated compounds by halorespiration. Cultured *Dehalococcoides* strains have an irregular, spherical shape often referred to as coccoid. These mesophilic (25 to 40°C) bacteria prefer neutral pH environments. Their growth on oxygen, nitrate, or sulfate as electron acceptors has never been reported (46, 67). Reductive dechlorination by *Dehalococcoides* occurs via the replacement of a chlorine atom in the chlorinated compound by hydrogen (hydrogenolysis) and results in a net input of one proton and two electrons (Fig. 3). *Dehalococcoides* is also capable of degrading chlorinated compounds via so-called dihaloelimination, a process where two neighboring chlorine atoms are concurrently being replaced via the formation of a double bond between the two carbon atoms. This reaction has a net input of two electrons. Because dihaloelimination requires less H_2 for the removal of more chlorine atoms than hydrogenolysis, its energy balance is more favorable under H_2 limited conditions (70). However, most of the biological processes reported so far show that degradation proceeds via reductive dechlorination.

Currently, more than 100 16S ribosomal RNA (16S rRNA) gene sequences of cultured and uncultured *Dehalococcoides* spp. have been deposited to the database of the National Center for Biotechnology Information (NCBI). With more than 99% sequence identity (Fig.

2), the 16S rRNA gene in *Dehalococcoides* spp. is highly conserved throughout the entire genus, however, various studies showed that this group is functionally very diverse (3, 22, 33, 36, 45, 54). Six *Dehalococcoides* strains have been isolated, mainly for their ability to degrade chlorinated ethenes. The first isolate of the genus, *Dehalococcoides ethenogenes* strain 195, is the only bacterium that can completely dechlorinate PCE (tetrachloroethene) to ethene, although degradation of vinyl chloride (VC) to ethene is cometabolic (54). *D. ethenogenes* strain *195* can dechlorinate HCB (hexachlorobenzene) to 1,3-DCB (dichlorobenzene), 1,4-DCB, 1,2-DCB and 1,3,5-TCB (trichlorobenzene) as well. Additionally, dechlorination of chlorophenols and polychlorinated dibenzo-*p*-dioxins to lesser-chlorinated derivatives has also been reported (24). Strain FL2 dechlorinates TCE , *cis*-DCE and *trans*-DCE but does not grow with 1,2-DCE or VC (34). Strains VS and BAV1 can dechlorinate dichloroethenes and VC to ethene, but can not use PCE or TCE (19, 33, 57). Strain GT can dechlorinate TCE, *cis*-DCE and VC to ethene (71). In contrast to the strains mentioned above, *Dehalococcoides* sp. CBDB1 was isolated for its ability to degrade chlorinated benzenes (3). This strain can degrade HCB to 1,3-DCB, 1,4-DCB, and 1,3,5-TCB. Dechlorination of chlorinated dioxin congeners (13), chlorophenols (1), and recently also transformation of PCE and TCE (trichloroethene) to *trans*-DCE (dichloroethene) was observed (1). Besides isolated strains several enrichment cultures that contain members of *Dehalococcoides* have been shown to degrade a variety of different chlorinated compounds. KB-1 enrichments dechlorinate PCE, TCE, *cis*-DCE or VC completely to ethene (23). Interestingly, while cultures enriched on PCE, TCE and *cis*-DCE contained three distinct members of *Dehalococcoides*, only one of these strains remained in VC fed enrichments after prolonged growth with only VC (22). Enrichment cultures from Housatonic River sediment containing *Dehalococcoides* have been shown to dechlorinate 64 commercial polychlorinated biphenyl (PCB) congeners simultaneously (10). Sediment enrichments from Saale River dechlorinated PCE, 1,2,3-TCB and 1,2,3,4-TCDD (tetrachlorodibenzo-*p*-dioxin) (7). In a similar study, several chlorinated compounds (PCE, TCE, 1,2,3-TCB and several polychlorinated dioxins) were dechlorinated in sediment enrichments of a Japanese river, which was polluted with polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F) (29, 87). The most recently obtained isolate within the genus is strain DCMB5 (14), which is most closely related to strains FL2 and CBDB1 based on the 16S rRNA gene sequence. Similar to CBDB1, also strain DCMB5 could be shown to transform 1,2,3-TCB to 1,3-DCB. In addition, this strain also could dechlorinate 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TCDD) to 2-monochlorodibenzo-*p*-dioxin. Besides sediment enrichments, dechlorination of chlorinated pollutants by *Dehalococcoides* was also reported in groundwater aquifers (11, 15, 38, 41, 48) and a denitrifying membrane-biofilm reactor (16). In addition to *Dehalococcoides* spp., two other distantly related isolates within the Chloroflexi have recently been obtained. The marine “Dehalobium chlorocoercia” DF-1(53) has been isolated for its ability to dechlorinate a variety of polychlorinated biphenyls

(PCBs). Most recently, “Dehalogenimonas lykanthroporepellens” (86) BL-DC-9 has been isolated from contaminated groundwater, and dechlorinates polychlorinated alkanes. Like *Dehalococcoides* spp., both isolates are strictly hydrogenotrophic.

Dehalococcoides spp. are difficult to maintain in pure culture (3, 33, 54); they are more easily maintained in a microbial community, on which they depend for H_2 supply, as long as ideal growth conditions are provided (22, 39). Very little is known about the diversity, distribution and functioning of *Dehalococcoides* in different environments despite their presence at several contaminated locations (36). A widely used ecological marker for qualitative and quantitative detection of *Dehalococcoides* spp. is the highly conserved 16S rRNA encoding gene. However, because there are considerable differences between dechlorination capabilities of the known *Dehalococcoides* strains despite 16S rRNA identities of >99%, their sole presence based on the detection of the 16S rRNA gene in an environment does not guarantee successful in-situ dechlorination of a specific pollutant. 16S rRNA is a more sensitive target than the 16S rRNA gene because not only there are more copies of rRNA in a cell than the single 16S rRNA gene, but also a strong positive correlation between growth and rRNA content has been shown for a variety of microorganisms (43, 44). Therefore it could be hypothesized that rRNA-based detection could help to assess the actively dechlorinating *Dehalococcoides* spp. in field conditions. Furthermore, due to the H_2 requirement of *Dehalococcoides* spp. in order to achieve reductive dechlorination, successful degradation of chlorinated compounds can not be solely related to presence or activity of *Dehalococcoides* spp. (64). Consequently, molecular tools that target metabolic activities of the entire microbial communities in the environment are needed to have a canonical assessment of the conditions.

Advanced molecular tools for environmental monitoring

Advances in molecular technologies like genome-wide analysis of DNA (genomics), mRNA expression (transcriptomics) and protein production (proteomics), create the opportunity to systemically study the molecular mechanisms (69) involved in the degradation of chlorinated pollutants. The development of various metagenomic approaches, which provide comprehensive catalogues of genetic material from microbial communities in a culture-independent fashion (66), has recently culminated in large scale community metagenome sequencing projects (82). These studies highlighted the great extent of the microbial genetic and functional diversity in various environments. So far, environmental microbial communities were mostly studied with small subunit rRNA (SSU rRNA; 16S rRNA in Bacteria and Archaea, 18S rRNA in Eukarya) targeted methods. With the aid of statistical analysis, conventional fingerprinting methods like denaturing gradient gel electrophoresis (DGGE) (58) or terminal restriction fragment length polymorphisms (T-RFLP) (50) and the more recently developed oligonucleotide microarrays (21, 51, 60) can be used to correlate

changes in the phylogenetic content of the microbial communities to environmental factors. With the availability of metagenomic data and the increasing number of complete individual genome sequences, it is now possible to use the mRNA and/or protein coding sequences (functional genes), which are dedicated to metabolic processes of interest to detect functional responses from the entire microbial community to changing environmental conditions (82). Functional genes are very useful molecular targets for monitoring the physiological status and functional activities of microbial populations. *In situ* degradation and transformation of pollutants can be assessed by studying the presence and expression of corresponding functional genes. Additionally, microarrays targeting functional genes, functional gene arrays (FGAs), allow fast and comprehensive analysis of metabolic potential and activity of microbial communities in the environment by targeting a large number of genes or their transcripts in one single experiment (85). Up to date the most extensive FGA platform is the GeoChip (35), which targets approximately 10,000 catabolic genes involved in major biogeochemical cycles, including those of carbon, nitrogen and sulfur. One of the applications of the GeoChip in a metal contamination site demonstrated the active involvement of metal- and sulfate reducing bacteria in the bioremediation process (35). Application of FGAs in the bioremediation of chlorinated compounds, however, has not yet been reported. Nevertheless, FGAs are potentially powerful tools for environmental monitoring of processes like reductive dechlorination, which not only depend on availability of reductively dechlorinating bacteria, but also require the presence of their synthrophic partners.

Table 2. Diversity indices used in molecular ecology studies

Diversity Indices
Alpha (α-) diversity : species diversity based on species richness and relative abundance
Simpson's reciprocal (37)
Shannon's H' (61)
Hill's N (37)
Fisher's alpha (26)
Rarefaction (65)
Beta (β-) diversity: species diversity between ecosystems or along environmental gradients.
Bray-Curtis (Sørensen) similarity index (12)
Whittaker's measure (83)
Gamma (γ-) diversity: the total of α- and β-diversities
Phylogenetic diversity: species diversity by incorporating the taxonomic differences between the species (17)

Understanding the structural and functional relationships between microbial communities and their environment is a starting point in the assessment of the field conditions. These physical, chemical, and biological variables may combine to influence the

Table 3. Multivariate statistical methods that are commonly used in microbial ecology (42, 63)

Methods		Application in microbial ecology
Exploratory analysis		
Cluster	Hierarchical clustering	to group similar objects (e.g. DGGE or TRFLP fingerprints) to each other and to separate objects from different groups
	K-means (partial) clustering	
PCA	Principal component analysis	to determine the maximum amount of variation from the original data set that can be depicted; environmental variables are projected only afterwards
Hypothesis-driven analysis		
Mantel's Test		to correlate corresponding positions in two matrices (e.g. community diversity to environmental heterogeneity) and to assess its significance
RDA	Redundancy analysis	to determine which environmental factors are the most significant to explain variation in microbial community composition (linear)
CCA	Canonical correspondence analysis	to determine most appropriate model to model species response to the environmental variation (unimodal)
LDA	Linear discriminant analysis	to identify linear combinations of additional environmental variables that best discriminate groups
ANOSIM	Analysis of similarities	to determine significant difference between two or more groups; based on any distance measure (e.g. Pearson correlation)
NMDS	Nonmetric multidimensional scaling	to identify gradients underlying the variation in the original dataset; can be based on various types of distance measures

abundance, diversity, and activity of microorganisms at many different spatial scales (27). Diversity indices provide information about the rarity and/or commonness of species so that the community structure in a studied environment can be defined (Table 2). Multivariate analysis has been widely used in ecological research (73). Because several variables can be considered simultaneously, a large number of microbial community descriptors and environmental factors can be evaluated together (42). There are several statistical procedures available for analysis of spatial patterns in environmental data such as cluster analysis or Mantel test (49) (Table 3). The aim of cluster analysis is to represent the (dis)similarity between samples/sites based on values of the variables associated with them, such that the similar objects are grouped near to each other, whereas the dissimilar objects are found further apart (63). Complex data sets like DGGE fingerprints and microarray hybridization patterns are mostly analyzed with principal component analysis (PCA), whereas hypothesis-driven techniques such as redundancy analysis (RDA) and canonical correspondence analysis (CCA) are more seldomly used (63). The significance of the relationships between species patterns and environmental variables can be assessed by permutation techniques like Monte Carlo permutation tests, which deduce statistical properties from the data itself (42). Results of these analyses are often visualized with ordination methods, which are based on presenting the (dis)similarities between individual species or samples based on the multiple variables, such that similar objects are represented near to each other and dissimilar objects are found further apart (63). With ecologically sound hypotheses and carefully chosen analysis methods, multivariate statistical procedures can lead to the identification of the causes of experimental observations or factors affecting the studied ecosystems.

Discoveries from *Dehalococcoides* spp. genomes

Our knowledge gap concerning the properties of *Dehalococcoides* spp. is closing rapidly with the developments in high-throughput sequencing technologies. Full-genome sequence analyses revealed that *D. ethenogenes* strain 195 (GenBank accession no. NC_002936) and strain CBDB1 (NC_007356) genomes are approximately 1.47 million base pairs (Mbp) and 1.39 Mbp, respectively. Both genomes comprise single circular chromosomes with 1591 predicted protein coding sequences (CDs) in strain 195 (67) and 1458 CDs in strain CBDB1 (Table 4). 1217 of the CDs from strain CBDB1 have orthologous genes in *D. ethenogenes* strain 195 (83.5%) (46). Strain BAV1 (NC_009455) has a genome of 1.34 Mbp with 1385 CDs based on information provided in the Integrated Microbial Genomes (IMG) database, release February 2009 (52). All of these genomes are among the smallest of free-living bacteria. Recently, however, whole genome sequencing of strain VS (NZ_ABFQ0000, unfinished draft, 2.39 Mbp) suggested that not all *Dehalococcoides* possess a small size genome. Nevertheless, different *Dehalococcoides* share many common properties. For example, one copy of each rRNA gene is present in all *Dehalococcoides* genomes (46, 67). In

Table 4. Comparison of whole-genome sequence statistics for reductively dechlorinating bacteria as presented in Integrated Microbial Genomes (IMG/M) database, March 2009 (52)

Genome Name	Phylum/Class/Genus	Bases (Mbp)	GC (%)	Genes	CDs	RNA	16S	Orthologs	Paralogs	*rdh* genes
	Proteobacteria									
Anaeromyxobacter dehalogenans 2CP-C	*Deltaproteobacteria*									
	Anaeromyxobacter	5.01	0.75	4419	4361	58	2	4290	2468	2
	Proteobacteria									
Geobacter lovleyi SZ	*Deltaproteobacteria*									
	Geobacter	3.87	0.55	3514	3476	38	1	3287	1858	2
	Firmicutes									
Desulfitobacterium hafniense DCB-2	*Clostridia*									
	Desulfitobacterium	5.28	0.48	4801	4712	89	5	4597	2921	7
	Firmicutes									
Desulfitobacterium hafniense Y51	*Clostridia*									
	Desulfitobacterium	5.73	0.47	5137	5060	77	6	4765	3200	4
	Chloroflexi									
Dehalococcoides ethenogenes strain *195*	*Dehalococcoidetes*									
	Dehalococcoides	1.47	0.49	1641	1591	51	1	1426	628	17
	Chloroflexi									
Dehalococcoides sp. BAV1	*Dehalococcoidetes*									
	Dehalococcoides	1.34	0.47	1436	1385	51	1	1327	488	10
	Chloroflexi									
Dehalococcoides sp. CBDB1	*Dehalococcoidetes*									
	Dehalococcoides	1.39	0.47	1516	1458	58	1	1378	541	32
	Chloroflexi									
Dehalococcoides sp. VS	*Dehalococcoidetes*									
	Dehalococcoides	2.39	0.55	2160	2096	64	1	2003	892	36

Abbreviations: Genes: total gene count; CDs: coding sequences; RNA: number of rRNA,tRNA and other RNA genes genes; 16S: number of 16S rRNA's, Orthologs : number of genes in orthologs; Paralogs: number of genes in paralogs

strains 195, CBDB1 and BAV1 the 16S rRNA gene is spatially separated from 5S and 23S rRNA genes. Comparative analysis of the four *Dehalococcoides* genomes showed that 70% of the all genes in these genomes have a high sequence and contextual conservation (55). However, one of the most important findings of the comparative genomic studies on *Dehalococcoides* genomes is the discovery of two high plasticity regions (HPRs). Different *Dehalococcoides* strains contain different numbers of reductive dehalogenase (*rdh*) genes that have been proven or predicted to catalyze the dechlorination reaction. The two HPRs contain 95% of the *rdh* genes (55).

When compared to the genomes of other dechlorinating bacteria, *Dehalococcoides* have the highest number of *rdh* genes in their genomes. Genomes of strains 195, CBDB1 and BAV1 have 17, 32 and 10 *rdh* genes respectively, whereas seven *rdh* genes were identified in the genome of *Desulfitobacterium hafniense* DCB-2, four *rdh* genes in *Desulfitobacterium hafniense* Y51 and two *rdh* genes in *Geobacter lovleyi SZ* and *Anaeromyxobacter dehalogenans* (74). The draft genome of strain VS contains the highest number of *rdh* genes (36 full-length genes), ever found in a single bacterial genome (55). Similarly, 14 *rdh* genes were detected via PCR amplification in *Dehalococcoides* sp. strain FL2 (40). However, the function of only a small number of these genes is known. Only two *rdh* genes from strain 195, DET0079 and DET0318, have been characterized as TCE (*tceA*) and PCE (*pceA*) reductive dehalogenases, respectively (28). Another *tceA* gene was identified in *Dehalococcoides* sp. strain FL2 (GenBank accession no. AY165309) (34). The cbdbA84 gene from strain CBDB1 was recently designated as a chlorobenzene reductive dehalogenase (*cbrA*), which is involved in dechlorination of 1,2,3,4-TeCB and 1,2,3-TCB (2). Additionally two VC reductase genes were identified from strain BAV1 (*bvcrA*, DehaBAV1_0847) (45) and strain VS (*vcrA*, GenBank accession no. AY322364) (57). Since metabolic function cannot be inferred from Dehalococcoides phylogeny, detection methods based on process-specific biomarkers are necessary to describe the bioremediation capacity and activity of *Dehalococcoides* in the environment. Therefore, genes like *rdh*'s that are specific to functions of interest can serve as useful biomarkers in monitoring of different *Dehalococcoides* activities.

Conclusions

Recent studies show that the ability of *Dehalococcoides* spp. to transform chlorinated compounds can be used for bioremediation of contaminated subsurface environments. Our increasing knowledge of physiological and genomic characteristics of the genus provides information for the development of novel tools and approaches to study and realize this potential. So far our knowledge about the diversity and activity of *Dehalococcoides* spp. in situ is limited to only a few contaminated sites. It is, however, also necessary to understand the functioning and capabilities of *Dehalococcoides* spp. in other environments to elucidate

the true potential of the genus in wider scale applications. Since *Dehalococcoides* spp. are phylogenetically very closely related and functionally diverse, it can be suggested that molecular detection tools need to focus on functional genes like *rdh* genes in addition to traditional 16S rRNA gene based methods.

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Chapter 02

Anaerobic Transformation of Hexachlorobenzene in European River Basins

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Hexachlorobenzene (HCB) was a commonly used compound in industry and agriculture until the beginning of the 1990's. This potentially toxic compound has been accumulating in the environment; especially in water bodies and agricultural areas, due to its low water solubility, high resistance to biological degradation and tendency to sorb to organic material. *Dehalococcoides* spp., highly specialized anaerobic bacteria that are capable of degrading chlorinated benzenes, were previously isolated from various environments including river sediments. In this study the biotransformation of HCB and the link between the HCB transforming capacity with the presence of *Dehalococcoides* spp. was evaluated in soils and sediments originating from several HCB contaminated river basins throughout Europe. The results showed that HCB could be transformed to tri- and dichlorobenzenes in the majority of the studied river basins. In most locations *Dehalococcoides* spp. could also be detected. However, the presence of *Dehaloccoides* spp. could not be correlated in an unequivocal way to the HCB transformation pattern or to *in situ* contamination levels. Nevertheless, a weak correlation could be established between the HCB transformation rates and the abundance of *Dehalococcoides* spp. in the samples. The widespread capability of sediments and soils to transform HCB is a promising find regarding the *in situ* bioremediation of this compound.

Introduction

Besides river morphology and eutrophication, industrial and agricultural pollution of the last century is believed to be one of the main reasons of deteriorating ecological health of European river systems (sediment, water, flood plain and catchments)(6). It is difficult to assess up to which level river systems can deal with pollution since little is known about pollutant loadings, mobility and turnover in these complex and heterogeneous systems. The EU project AquaTerra aims to study these issues and to provide the foundations for a better understanding of the behavior of environmental pollutants (5). One of the important factors in determining the fate of the river systems is the flexibility of the system towards pollutants found in the environment. Within the framework of the AquaTerra project hexachlorobenzene (HCB) was selected as a model compound. HCB is a commonly found pollutant in industrialized areas of Europe (4). HCB is among the priority compounds listed in the 76/464/CEE Council Directive (17). Chlorinated benzenes are present in several basins studied in the AquaTerra project.

Research in the past has been directed towards the reductive dechlorination of chlorinated aromatics like HCB and its lower chlorinated analogues (1). Reductive dechlorination is the only known transformation pathway from HCB to its lower chlorinated analogues. This anaerobic process has been observed in methanogenic and sulphate-reducing conditions (29). The Gibb's free energy of the reductive dechlorination of chlorinated benzenes decreases from –171.4 kJ/mol for HCB to –139.6 kJ/mol for monochlorobenzene, which is in the range of the energy obtained for the reduction of nitrate to nitrite (1). Only few bacteria (all belonging to or closely related to the genus *Dehalococcoides*) are known to perform this transformation in pure culture. Complete dechlorination of HCB to benzene by one single microorganism has never been observed.

In contrast, mixed anaerobic cultures have been described that are capable of complete dechlorination of HCB to monochlorobenzene (major product) and benzene (only a few percent) (23). Two major dechlorination patterns can be distinguished: firstly, a pattern where doubly flanked chlorine atoms are preferentially removed and secondly a pattern where singly flanked chlorine atoms are removed. The latter pattern proceeds via the thermodynamically less energy yielding dechlorination route, but the formation of 1,3,5-TCB, a dead-end product, is avoided (1). There is no clear relation between the dechlorination pattern observed and the origin of the culture or the chlorobenzene used for adaptation of the culture (1). Anaerobic transformation of dichlorinated benzenes has only been observed by mixed cultures (2), whereas complete anaerobic monochlorobenzene transformation has never been observed. *Dehalococcoides* strain CBDB1 was the first microorganism isolated that is able to grow using the energy conserved from the dechlorination of a variety of chlorobenzenes. This has been observed in a synthetic vitamin B_{12}-containing medium

amended with hydrogen as the electron donor and acetate as a carbon source (2). Strain CBDB1 is able to dechlorinate HCB, pentachlorobenzene (QCB), all tetrachlorobenzenes (TeCBs), 1,2,3-trichlorobenzene (1,2,3-TCB) and 1,2,4-TCB (2, 12) . In addition, strain CBDB1 is also able to dechlorinate dioxin congeners (7). A relative of the HCB dechlorinating cultures, *Dehalococcoides ethenogenes* strain 195, is able to transform tetrachloroethene (PCE) to ethene. This strain has been shown to be able to dechlorinate chlorobenzenes as well, albeit not always when provided as the sole terminal electron acceptor. *D.ethenogenes* strain 195 cannot grow with 1,2,3,5-TeCB or the TCBs. However, *D.ethenogenes* strain 195 is able to dechlorinate other aromatic compounds like dioxins, PCBs, chlorinated furans, chlorinated naphthalenes, and chlorophenols (9). *D.ethenogenes* strain 195 requires complex extracts of mixed microbial cultures in the medium besides hydrogen and acetate. "Dehalobium chlorocoercia" strain DF-1 that is a close relative of *Dehalococcoides* spp., was isolated from estuarine sediment. This strain was originally isolated because of its ability to dechlorinate PCB congeners with doubly flanked chlorine atoms. It requires formate or H_2/CO_2 as the electron donor and carbon source. Besides PCBs, strain DF-1 can transform HCB via reductive dechlorination to the dead-end product 1,3,5-TCB (31). *Dehalococcoides* spp. were previously detected in PCE contaminated soils and groundwater aquifers across Northern America and Europe (11). In the research described here the ability of sediments from three river systems in Europe (the Ebro, the Elbe and the Danube) to dechlorinate HCB was assessed. Marine sediments from two sites and a PCE contaminated soil were also included to the experimental set for comparison. From the present knowledge about PCE contaminated sites it was hypothesized that HCB contaminated sediments can also contain *Dehalococcoides* spp. which are capable of transforming chlorinated benzenes. Knowledge about the ability of the sediments to transform HCB may shed more light onto the capability of soils and sediments to deal with xenobiotic compounds. Also, we attempted to link the HCB transforming capacity with the presence of *Dehalococcoides* spp. and the presence of HCB as a pollutant at the specific locations.

Material and methods

Chemicals

All the chemicals used were of the highest quality available. Hexachlorobenzene (HCB) was purchased at Fluka. QCB (pentachlorobenzene), 1,2,3,4-TeCB, 1,2,4-TCB, 1,3-DCB and 1,4-DCB were purchased from Merck, Darmstadt, Germany. 1,2,4,5-TeCB (tetrachlorobenzene), 1,2,3,5-TeCB, 1,3,5-TCB (trichlorobenzene) and 1,2-DCB (dichlorobenzene) were obtained from Aldrich Chemicals Co. Ltd., Dorset, England. 1,2,3-TCB was from Janssen Chimica, Beerse, Belgium.

Sediment sampling

Sediment samples were taken anaerobically at the locations under study (Table 1). The sediments were immediately transferred into sterilized glass jars and kept under water. In some cases stainless steel sampling tubes were used which were tightly closed after sampling. All sediments were kept at 4°C until further use. After transfer to the laboratory the stainless steel sampling tubes were emptied in sterilized glass jars in the anaerobic hood under a N_2/H_2 (96/4) (vol-%/vol-%) atmosphere.

Table 1. List of sediment and soil samples used in this study

Location	Nature [a]	Sampling time	Coordinates	
The River Ebro				
Reinosa	RS	Summer 2006	N 42º35'32.6"	W 4º09'10.8"
Conchas de Haro	RS	Summer 2005	N 42º35'20.4"	W 2º50'31.2"
Zaragoza	RS	Summer 2005	N 41º37'23.0"	E 0º54'28.0"
Monzon	RS	Winter 2004	N 41º43'30.3"	E 0º08'09.4"
Lerida	RS	Summer 2004	N 41º32'10.8"	E 0º30'44.6"
Flix	RS	Summer 2004	N 41º13'43.6"	E 0º33'09.0"
Tortosa	RS	Winter 2004	N 40º44'58.3"	E 0º34'03.6"
Delta-Rice field	RS	Summer 2004	N 40º41'11.8''	E 0º37'31.5"
Delta-Estuary	RS	Summer 2004	N 40º38'22.2"	E 0º42'39.6"
The River Elbe				
Schönberg	RS	Autumn 2004	N 52°90'73.2"	E 11°87'21.9"
Schönberg- 42	S	Autumn 2004	N 52º54'20.1"	E 11º52'15.3"
Schönberg- 45	S	Autumn 2004	N 52º54'21.8"	E 11º52'15.4"
Roßlau	RS	Autumn 2004	N 51º52'35.4"	E 12º16'57.8"
The River Danube				
Budapest	RS	Spring 2005	N 47°27'37.2"	E 19°04'19.8"
Other sampling locations				
Waddenzee	MS	Summer 2003	N 53°19'52.9"	E 05°09'06.1"
Baltic Sea	MS	Summer 2004	N 56°12'05.6"	E 11°38'01.7"
Rhine river	RS	Winter 2003	N 51°57'36.8"	E 05°46'54.4"
PCE contaminated soil	S	Autumn 2002	N 51°49'07.8"	E 04°42'37.3"

[a] RS:river sediment, S:soil, MS:marine sediment

Batch transformation experiments

The ability of the sediments was assessed in batch experiments. Batch experiments were conducted within the timeframe 2004 to 2006. Basal medium (36 ml) described previously by Luijten et al.(19), was transferred anaerobically to 120 ml serum flasks. The bottles were closed with viton stoppers (Rubber BV, The Netherlands) and aluminium crimp caps. The gas phase was exchanged with N_2/CO_2 (80/20) (vol-%/vol-%) to a 0.2 bar overpressure). After sterilization the medium bottles were opened in an anaerobic hood and approximately 3 grams of the sediment was transferred aseptically to the bottles. Afterwards the headspace in the bottles was changed again to N_2/CO_2 (80/20) (vol-%/vol-%, 0.2 bar) or H_2/CO_2 (80/20) (vol-

%/vol-%, 0.7 bar). Additional medium components (19) were added from sterile anaerobic stock solutions. In some cases lactate (final concentration 5mM) was used as electron donor. In the case of H_2 as electron donor acetate was added as the carbon source (final concentration 5 mM). The bottles were incubated statically in the dark at 30±2°C. In some cases tetrachloroethene (PCE) and/or 1,2-dichloroethane (12DCA) dissolved in medium were added to the incubations at a level of 10 mg/l. Sulfate (1 mM) was added to the marine sediment bottles. The chlorinated benzenes were added dissolved in acetone (8mM stock solutions, final concentration in the medium 25-50 µM). The chemical or abiotic transformation was also checked in blanks without sediment or blanks containing sediment that had been autoclaved twice for 20 minutes with a three day interval. Correlations analyses were performed in R software (25).

Analysis of contaminants

Samples for chlorobenzene analysis were taken at regular time intervals. All chlorobenzenes, except monochlorobenzene, were analyzed in the liquid phase. 2 ml of medium was taken from the bottles, divided into two 1 ml portions and transferred to extraction tubes. To each of the duplicate tubes 1 ml of a hexane/acetone (80/20, vol/vol) solution containing either 50 µM 1,2-DCB or 50 µM HCB as the internal standard was added. The extraction tubes were closed with aluminium crimp caps with a teflon-lined butyl rubber septum. The extraction tubes were sonified in water bath for 30 minutes followed by the extraction of at least 24 hrs in an end-over-end shaker.

Hexa-, penta, tetra-, tri- and dichlorinated benzenes were analyzed according to the method described earlier by Middeldorp et al. (20). At given time intervals 1 ml sample was extracted with 1 ml hexane containing 1,2-DCB or HCB as internal standard for at least 24 hrs after 15 minutes sonification. The hexane extract was analyzed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a mass selective detector (series 5197A), an automatic sampler (series 7673A) and a fused silica analytical column (HP5, 30m x 0.25 mm i.d.). The oven operating conditions were: 60°C for 3 minutes, followed by a 5°C $minute^{-1}$ increase to 180°C. Injector and detector temperatures are 250 and 280°C, respectively. Helium was used as a carrier gas. Mass spectra were detected using a SIM (selected ion monitoring mode) procedure.

Chlorobenzene and benzene were detected injecting 0.2 ml of headspace gas into a 436 Chrompack gas chromatograph equipped with a flame ionization detector connected to a Sil 5CB column (25 m x 0.32mm X 1.2 µm) and with split injection. Operating temperatures of the injector and detector were 250 and 300°C, respectively. The oven temperature condition was 50°C (5 minutes) followed by 10°C $minute^{-1}$ to 110°C. N_2 was used as the carrier gas with a inlet pressure of 100 kPa. The peak areas are determined with a Shimadzu C-3A integrator. In all cases external standards were used for quantification. PCE, and lower

Table 2. Summary of results from batch biotransformation experiments, combined with molecular ecological data

Location	Biotransformation Experiments				Quantitative Molecular Analysis	
		HCB		PCE		
	Nature [a]	T½ (n)[c] (days)	Lag phase [b] (days)	Products [d]	Products [d]	*Dehaloccoides* spp. [e] *10^4	Eubacteria [e] *10^8
					(16S rRNA gene copies/g)	
The River Ebro						
Reinosa	RS	∞ (3)	n.d. [f]	-	n.a [g]	BDL	0.5 ± 0.0
Conchas de Haro	RS	7-30 (3)	5-48	1,3,5-TCB, 1,3/1,4-DCB	n.a [g]	BDL	59.5 ± 23.5
Zaragoza	RS	3-27 (3)	5-19	1,3,5-TCB, 1,3/1,4-DCB	*trans*DCE[h]	1.9 ± 0.5	18.3 ± 0.4
Monzon	RS	5-6 (2)	<28	1,3,5-TCB, 1,3/1,4-DCB	TCE[i]/*cis*DCE	2.6 ± 0.9	8.2 ± 0.6
Lerida	RS	14-30 (2)	<28	1,3,5-TCB, 1,3/1,4-DCB	TCE/*cis*DCE	8.1 ± 3.9	50.0 ± 8.7
Flix	RS	2-47 (4)	<28	1,3,5-TCB, 1,3/1,4-DCB	TCE	4.1 ± 2.1	5.5 ± 0.7
Tortosa	RS	6-12 (2)	<28	1,3,5-TCB, 1,3/1,4-DCB	*cis*DCE	7.0 ± 2.8	21.8 ± 6.6
Delta-Rice field	RS	5-62 (4)	<36	1,3,5-TCB, 1,3/1,4-DCB	*cis*DCE	0.3-0.6 ± 0.2-0.4	29.5 ± 3.6
Delta-Estuary	RS	8-11 (2)	<28	1,3/1,4-DCB	*cis*DCE/*trans*DCE	1.8 ± 0.4	24.3 ± 11.2
The River Elbe							
Schönberg	RS	6-17 (3)	<62	1,3,5-TCB, 1,3/1,4-DCB	TCE	BDL-2.8 ± 0.4	25.5 ± 1.6
Schönberg- 42	S	∞ (2)	n.d. [f]	-	TCE/cisDCE	BDL	69.3 ± 4.8
Schönberg- 45	S	6-140 (3)	28-62	1,2,4/1,3,5-TCB, 1,2-DCB	TCE	BDL	70.8 ± 11.5
Roßlau	RS	∞ (4)	n.d. [f]	-	-	1.6 ± 1.2	4.6 ± 2.0
The River Danube							
Budapest	RS	4-64 (3)	5-19	1,3,5-TCB, 1,3/1,4-DCB	n.a [g]	2.5 ± 1.5	17.5 ± 2.0
Other sampling locations							
Waddenzee	MS	∞ (4)	n.d. [f]	-	*trans*DCE	1.2 ± 0.2	6.5 ± 3.8
Baltic Sea	MS	∞ (4)	n.d. [f]	-	*trans*DCE	0.2 ± 0.0	1.3 ± 0.1
Rhine river	RS	6-14 (2)	<28	1,3,5-TCB, 1,2/1,4-DCB	TCE/*cis*DCE	2.7 ± 0.7	13.1 ± 0.8
PCE contaminated soil	S	260-316 (2)	62-153	1,2,4/1,3,5-TCB, 1,3-DCB	*cis*DCE, VC, Ethene	77.8 ± 55.7	5.0 ± 0.4

[a] RS:river sediment, S:soil, MS:marine sediment; [b] Observed lag phase before dechlorination occurred; [c] Calculated half life (via first order rate constants) (n=number of bottles used for the calculation); [d] Major chlorinated benzene products after 5 months or major chlorinated etheene products after 2 months; [e] Mean value and standard deviation of (at least) triplicate analysis.; [f] n.d.: no transformation could be detected in the experimental period of 153 days; [g] n.a.: data not available; [h] DCE, dichloroethene; [i] TCE, trichloroethene

chlorinated ethenes were analyzed via head-space analysis with a method described previously (8).

DNA isolation

DNA was isolated directly from soils (0.5 g) using the Fast DNASPIN®Kit For Soil (Qbiogene, Carlsbad, CA) according to the manufacturers' instructions. The DNA extraction yield was measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Real-time PCR

Real-time quantitative PCR (qPCR) was performed using an iQ5 iCycler (BioRad, Veenendaal, Netherlands) with the thermocycling program as previously described (27) for 16S rRNA genes of *Dehalococcoides* and Universal Bacteria using SYBR Green Dye. PCR reactions were prepared in 25 µl total reaction volume containing 5µl template DNA, 1× BioRad SYBR Green PCR master mix (BioRad, Veenendaal, Netherlands), 0.2µM of each primer (Dco728F and Dco944R for *Dehalococcoides* spp. (28) and 968F (24) and 1401R (18) for Universal Bacteria) and 6.5µl sterilized MQ. Samples were analyzed in quadruplicate, and no-template controls were included. Standard curves were generated from triplicate dilution series. Real-time PCR standards were prepared by cloning PCR-amplified 16S rRNA genes of *Dehalococcoides* into the pGEM-T Easy plasmid vector (Promega, Madison, WI). PCR-products amplified from plasmid vectors using T7- and SP6-promotor targeted primers (Promega) were used as real-time PCR standards.

Results

Transformation of HCB and lower chlorinated benzenes by the sediments and soils

Nineteen different locations across the European continent were screened for their ability to degrade HCB in laboratory scale transformation experiments and *Dehalococcoides* spp. content with 16S rRNA gene targeted qPCR. Most of the sediments were able to transform HCB to lower chlorinated benzenes (Table 2). A typical example of this transformation is illustrated by the sediment from Flix in the Ebro River basin in Fig. 1. HCB (21.2± 3.4 µM) was rapidly transformed to lower chlorinated benzenes in the presence of either H_2 or lactate as the electron donor. HCB was not degraded in autoclaved sediment controls or in blanks without sediment, indicate that microbial transformation reactions are responsible for the removal of chlorinated benzenes. This was confirmed in another experiment described in **Chapter 5** in which the growth and increased activity (based on 16S rRNA copies) of *Dehalococcoides* spp. in the sediment was shown during the transformation of HCB

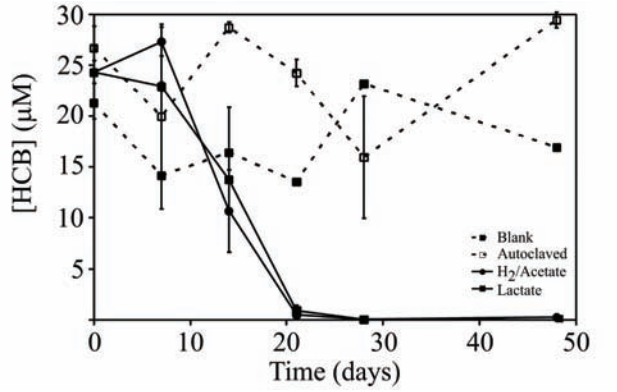

Figure 1. Transformation of HCB by the sediment from Flix, The River Ebro

Lower chlorinated benzenes were formed after the transformation of HCB (Fig. 2). The microorganisms in this sediment transformed HCB predominantly to trichlorobenzenes in the case of H_2 as the electron donor while mainly dichlorobenzenes were formed with lactate. However, it was not possible to relate the product formation to the nature or the amount of electron donor dosed to the sediments. Penta- or tetrachlorobenzenes were always below the detection limit of the method indicating that these intermediates are dechlorinated rapidly as well. Monochlorobenzene formation was not observed even after longer (over 150 days) periods of incubation. The electron donor was completely converted to methane (data not shown). The acetone added as a solvent for the chlorobenzenes was previously shown to be non-toxic for the methanogenic archaea (30). In a follow-up experiment, the Flix sediment was exposed to different chlorobenzene congeners to gain additional insight into pathway of HCB transformation. Results confirmed that the bacteria present in the sediment were able to reductively dechlorinate the chlorobenzene congeners to their lower chlorinated analogues (Fig. 3). Again, as illustrated earlier in Figure 1, no transformation was observed in autoclaved sediment controls and blanks without sediment (data not shown). 1,3,5-TCB and 1,3- and 1,4-DCB (or a mixture of these compounds) were the end products observed in all the experiments.

In most cases the other sediments that were exposed to HCB in the batch experiments showed a similar dechlorination pattern to Flix sediment (Table 2). 1,3,5-TCB and 1,3- and 1,4-DCB were the end products observed after (up to five months) incubation irrespective of the addition of H_2 or lactate as the electron donor. In some cases (Schönberg-45 soil in the Elbe basin and Rhine sediment) 1,2-DCB was the end product of the transformation of HCB (Table 2). In this case 1,2,3-TCB formation was often observed as a transient intermediate (data not shown). HCB transformation was also observed in soil that was originally contaminated with PCE (Table 2). Unlike any other samples, HCB was transformed only to

1,3-DCB, whereas 1,2- or 1,4-DCB production was not observed. Transformation was completed in a longer period than observed for sediments contaminated with HCB; however this could be the result of the long term storage of this sample at 4°C. This sample did contain *Dehalococcoides* spp. prior to HCB exposure; however HCB transformation was not expected given the history with chloroethene contamination. Once again, none of the soils transformed HCB after inactivation by autoclaving (data not shown).

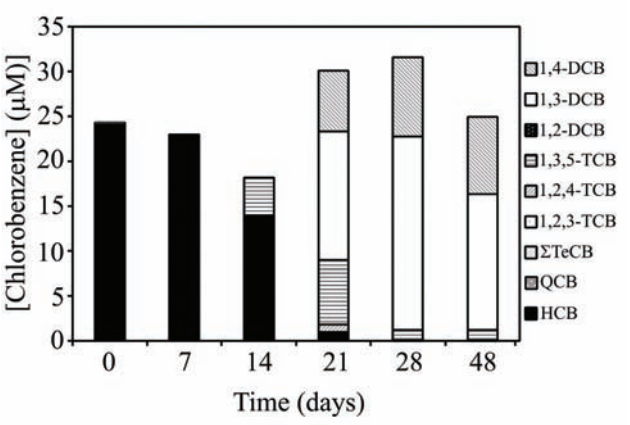

Figure 2. Formation of lower chlorinated benzenes during the transformation of HCB in sediment from Flix, the River Ebro.

Several sediments were not capable of HCB dechlorination. HCB was not dechlorinated in the two marine sediments (Baltic Sea and Waddenzee). The Baltic Sea sediment did not produce any methane whereas the Waddenzee was methanogenically active. A reason for the lack of HCB dechlorination may be that the *Dehalococcoides* spp. present in the sediments are not capable of HCB dechlorination. Also, the medium may have been lacking essential nutrients, or the sulfate may have inhibited dechlorination. The sediment from Reinosa (in the Ebro basin) and Roßlau (in the Elbe basin) were also inactive regarding HCB, although methanogenic activity was detected in the sediments.

***Dehalococcoides* in the soils and sediments**

Dehalococcoides was shown to be present in most but not all of the samples studied here (Fig. 4). In most of the samples exhibiting HCB-dechlorinating activity *Dehalococcoides* was present, but in some cases where HCB dechlorination was observed (Conchas de Haro, Schönberg soils), *Dehalococcoides* could not be detected in the sediments prior to the experiments. The relative abundance of *Dehalococcoides* spp. rRNA gene copies was in general not very high when compared to the concentration of total Bacteria rRNA gene copies (mostly below 0.1%). In contrast, the PCE-contaminated soil contained more than 1% of

Dehalococcoides spp. rRNA copies as compared to total Bacteria and thus appears to be highly enriched for this specific group of dechlorinators.

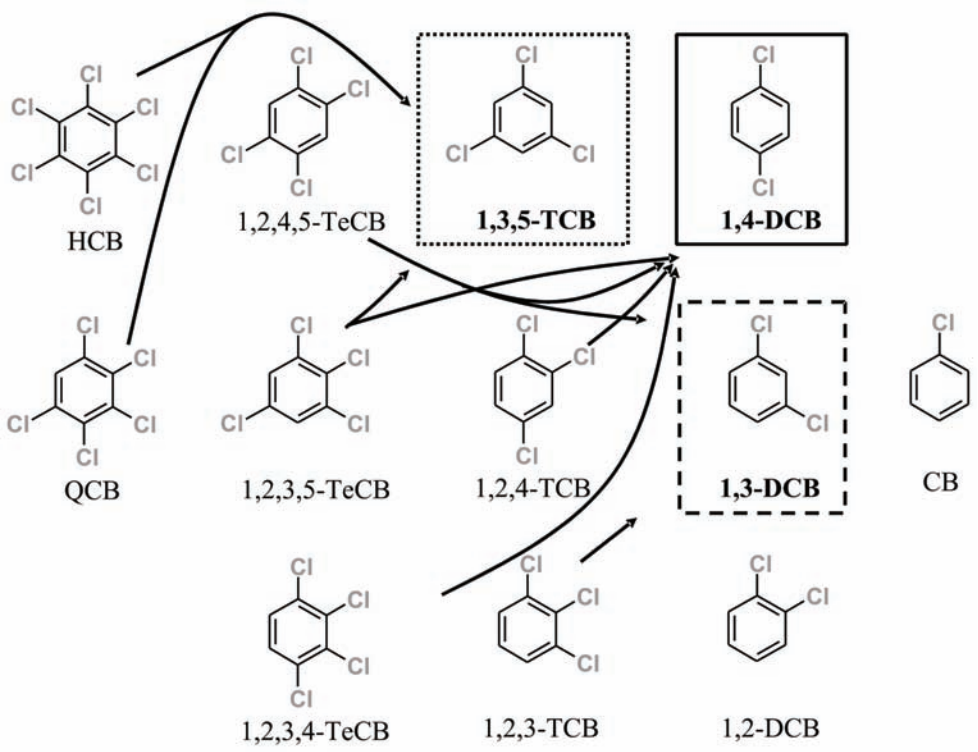

Figure 3. End products formed after the transformation of HCB and lower chlorinated benzenes in sediment from the location Flix, the River Ebro. Arrows indicate observed end products of dechlorination.

Dechlorination of chlorinated ethenes

The detection of *Dehalococcoides* spp. in most of the sediments that were used for the HCB screening experiment suggested that there exists an extensive endogenous dechlorinating potential. The different members of the *Dehalococcoides* genus are known for their capability to dechlorinate chlorobenzenes, chlorinated ethenes and 1,2-dichloroethane. Therefore, the sediments that were previously exposed to HCB received a dose of PCE (around 10 mg/l) to investigate whether the bacteria present are also capable to dechlorinate this compound. The formation TCE was indeed observed in all of the sediments, accept the ones from Reinosa, Conchas de Haro (in the Ebro basin) and Budapest (in the Danube), after a lag phase of one to two weeks. Dichloroethene was the end product most commonly observed after two months of incubation. The only sample capable of dechlorinating PCE to VC and ethene originated

from a site contaminated with PCE (Table 2). In none of the batches 1,2-dichloroethane (12DCA) was dechlorinated (data not shown).

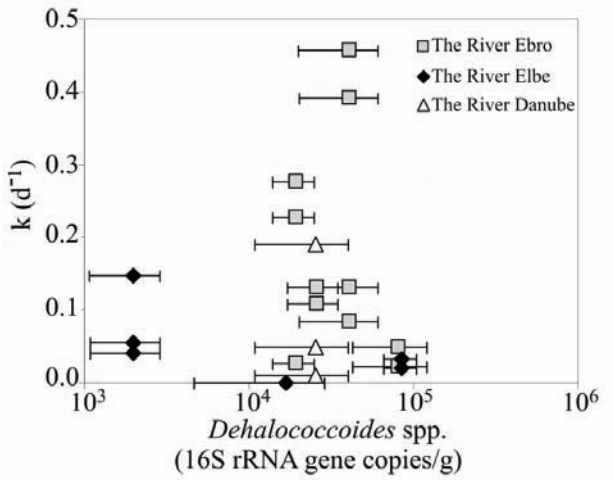

Figure 4. First order rate constant of HCB dechlorination as a function of number of *Dehalococcoides* spp. 16S rRNA copies in the studied river basins

Dechlorination rate and abundance of *Dehalococcoides*

Most of the sediments exhibited the ability to dechlorinate HCB once the sediment was transferred into medium that supports for reductive dechlorination by anaerobic microorganisms in the sediments. The data from the transformation experiments were used to determine the HCB dechlorination rates under these in vitro conditions (Table 2). The transformation rates depicted as half lives were calculated for each location and are grouped for each basin (Ebro/Elbe/Danube) or experimental group (marine sediments, contaminated) (Table 2). The first order rate constants were calculated from the HCB transformation curves. A weak negative correlation (Spearman's rho= -0.40, p=0.075) was observed between the HCB transformation rates and the number of *Dehalococcoides* (Table 2) in the samples. Although extremely short half lives of only a few days were observed for sediments at some locations, it is clear that a large variation exists in degradation rates among the samples from each basin. Unfortunately, not all the results of the experiments carried out in the screening experiment could be used for the determination of the rates. In some experiments, data of specific time points could not be obtained due to analytical problems. Also, it cannot be excluded that for some sediments the extraction procedure may have been suboptimal, not quantitatively extracting all chlorobenzenes, leading to an overestimation of the HCB removal rate. Nevertheless, in the batches that were used for rate determination, the HCB removal coincided with the production of lower chlorinated benzenes indicating that the major part of the HCB removal was indeed of microbiological origin and not due to sorption processes.

Discussion

The majority of the samples tested in this study exhibited the capacity to dechlorinate HCB irrespective of the amount of HCB present *in situ*. As a result it can be concluded that similar to PCE contaminated groundwater and soil, HCB contaminated sediments from different origins can also contain *Dehalococcoides* spp. which are capable of transforming this pollutant once they are exposed to the appropriate conditions for reductive dechlorination. However several exceptions to this conclusion could also be demonstrated. The only river sediments that did not exhibit the ability to transform HCB in the laboratory were from Reinosa in the Ebro basin, and Roßlau in the Elbe basin. Also, HCB was not transformed by the bacteria in the marine sediment-associated microbiota. The number of *Dehalococcoides* spp. per gram of Reinosa sediment was below the detection limit, which may be the explanation for the lack of transformation by this particular sediment. Lack of dechlorination activity in the Roßlau (Elbe) river samples could be due to the high amount of heavy metals and especially arsenic deposition in this river which is toxic to entire biota (15). Also, and particularly in the case of the other sediments, environmental parameters like temperature, pH, salinity and the presence of other (toxic) compounds, may have played an important role in absence of transformation in the laboratory and in the field. Although Reinosa in the Ebro basin lies only a few kilometers from the origin of the Ebro river, compounds like DTT degradation products (several tens of ppb's) and polyaromatic hydrocarbons (up to 1500 ppb in total) have been detected (22). These may have inhibited the development of a HCB transforming population. On the other hand, HCB was not found as a pollutant at this specific location. The relatively widespread capability of sediments and soils to transform HCB is remarkable, but not entirely unexpected. Although the amount of HCB in the environment has been decreasing over the past three to four decades there is still a substantial amount (between 10000 and 26000 tones worldwide) present in the environment due to the global turnover of HCB. Mass balance calculations predict that the soils and sediments may be the most important sinks for HCB (4).

The number of *Dehaloccoides spp.* present in the soils and sediments could not be correlated in an unequivocal way to the dechlorination pattern, or HCB contamination levels. For example Flix is the only location in the Ebro river basin where substantial amounts (between 120 and 280 μg/kg) of HCB were found. The only other locations with detectable concentrations of HCB were downstream of Tortosa (but not in the rice fields) where levels reached 10 to 75 μg/kg (22). However, in most locations other chlorinated compounds, more specifically hexachlorocyclohexane or DDT-like compounds, were present. HCB levels similar to those of Flix (of up to 340 μg/kg) were found in the soils of the Elbe basin, but levels in the sediment were 30 μg/kg or lower (**Chapter 3**). We observed a weak correlation

of *Dehalococcoides* spp. abundance with the rate at which the dechlorination reactions took place. This weak correlation could be a result of involvement of populations closely related to, but not within the genus *Dehalococcoides*, in HCB transformation. For example "Dehalobium chlorocoercia" strain DF-1, as well as PCE-degrading members of the Lahn- and Tidal Flat clusters (13, 14) were shown to be involved in reductive dehalogenation of chlorinated aliphatic and aromatic pollutants. Moreover, it can not be excluded that the extraction efficiency may be different for different soils and sediments. Finally, it should be noted that, even though we have screened 19 samples for their HCB transformation capacities, this data set is relatively small from a statistical point of view and may be too small to observe significant and/or strong correlations.

Microorganisms that are known for their dechlorinating capabilities, *Dehalococcoides* (-like) spp., are found in most of the sediments under study (Table 2). Despite their presence (sometimes in relatively high numbers, e.g. Flix) HCB is still present in the sediments. Apparently, the dechlorination processes in the sediment proceed very slowly or not at all *in situ*. Several factors could limit the *in situ* transformation of HCB. The bioavailability of HCB is limited due to the extreme low solubility of HCB in water (around 5 µg/l) (3). Furthermore, the dechlorinating activity of the bacteria could be inhibited because of a limited availability of suitable electron donors and/or other nutrients. Also, other (abiotic) factors could play a role like e.g., temperature, redox condition, and/or water activity.

The end products that were formed in most sediments (1,3,5-TCB, and 1,3-and 1,4-DCB) are the dechlorination products that are expected when the microorganisms dechlorinate via the thermodynamically most favourable route (1). A few sediments show a thermodynamically less favourable route that involves the removal of chlorine atoms that are only singly flanked. 1,2,3,4-TeCB was found to inhibit the dechlorination of other chlorobenzenes (10). Consequently, once 1,2,3,4-TeCB is formed the "normal" dechlorinating pathway may be blocked. This could lead to the subsequent formation of 1,2,3-DCB and 1,2-DCB (Fig. 3). So far, *D. ethenogenes* strain 195 has been found capable of dechlorinating HCB to 1,2,3,5-TeCB and 1,3,5-TCB, but only in the presence of PCE. The major end products of HCB transformation by strain CBDB1 are 1,3,5-TCB, 1,3-DCB and 1,4-DCB (12). The PCB degrading microorganism strain DF-1, which can dechlorinate HCB to 1,3,5-TCB, was also found capable to dechlorinate PCE to *trans*- and *cis*-dichloroethene (21).

It remains unclear how *Dehalococcoides* spp. maintain themselves in the field. As far as we know, *Dehalococcoides* spp. can only use chlorinated compounds as their electron acceptor. Analysis of the genome sequence of two different strains (*D. ethenogenes* strain 195 and *Dehalococcoides* strain CBDB1) has shown no indication of the presence of any genes encoding for enzymes involved in pathways for denitrification, iron or sulfate reduction or the use of any of the other "normal" electron acceptors (16, 26). One of the drawbacks of the molecular tool used for the determination of the number of *Dehalococcoides* is that it is based

on the detection of the 16S rRNA gene. Species of *Dehalococcoides* may exist that are unable to dechlorinate, and these will also be detected by the methods used. Also, part of the *Dehalococcoides* spp. may be unable to degrade HCB but able to reduce other (halo)organic or inorganic compounds.

However, the sequenced species were maintained in the laboratory for a long time solely on chlorinated compounds and may have lost their ability to use other (non-) halogenated compounds. Another possibility is that these bacteria are able to use other naturally produced chloro-organic compounds, such as humic acids as the electron acceptor. This, however, cannot be substantiated with evidence in the laboratory until we are either able to detect *Dehalococcoides* activity in the field or until there is a *Dehalococcoides* isolate that is able to use other inorganic compounds. Finally, as discussed above, non-*Dehalococcoides* spp. might be involved, which are not detected by the assay used in this study.

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Chapter 03

Tracking Functional Guilds: *Dehalococcoides* spp. in European River Basins Contaminated with Hexachlorobenzene

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Hexachlorobenzene (HCB) has been widely used in chemical manufacturing processes and as pesticide. Due to its resistance to biological degradation, HCB accumulated mainly in fresh water bodies and agricultural soils. *Dehalococcoides* spp., anaerobic dechlorinating bacteria that are capable of degrading HCB, were previously isolated from river sediments. Yet there is limited knowledge about the abundance, diversity and activity of this genus in the environment. This study focused on the molecular analysis of the composition and abundance of active *Dehalococcoides* spp. in HCB-contaminated European river basins. 16S ribosomal RNA-based real-time quantitative PCR and denaturing gradient gel electrophoresis in combination with multivariate statistics were applied. Moreover, a functional gene array was used to determine reductive dehalogenase (*rdh*) gene diversity. Spatial and temporal fluctuations were demonstrated not only in the abundance of *Dehalococcoides* spp. but also in the composition of active populations and *rdh* gene diversity. Multivariate statistics revealed that *Dehalococcoides* spp. abundance is primarily affected by spatial differences, whereas species composition is under the influence of several environmental parameters, such as seasonal changes, total organic carbon and/or nitrogen content and HCB contamination. This study provides new insight in the natural occurrence and dynamics of active *Dehalococcoides* spp. in HCB contaminated river basins.

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Introduction

Halogenated organic compounds are among the most widespread environmental pollutants. Previously believed to be only anthropogenic, a large number of these compounds, including aliphatic, aromatic and heterocyclic derivatives, are introduced into the environment via biogenic and geogenic sources (9, 21). Hexachlorobenzene (HCB) is believed to be persistent in the environment (22) due to its chemical stability and its resistance to biodegradation. HCB is a hydrophobic and bio-accumulative compound and is listed in the EC-Directive (14) as "priority hazardous substance". At its peak production in the early 1980s, thousands of tons of HCB were produced to be used as fungicide, wood-preservative, porosity-control agent, or in the manufacturing of dyes. The usage of HCB is no longer allowed in most countries because of its toxicity and carcinogenity towards fish and mammals. Nevertheless, it is still being released to the environment as a by-product of various chemical processes, as a result of incomplete combustion or from old landfills (4, 6, 7). HCB contamination has been reported in different environments. Compared to rivers in sparsely populated regions, lakes and sea (32, 42), significantly higher amounts of HCB could be found in river water in agricultural areas, and in densely populated or highly industrialized areas. HCB concentrations were shown to positively correlate with organic matter content of sediments and soils, and European soils were observed to have the highest HCB concentrations globally (38). Several authors reported on the fate and behavior of HCB in the environment at regional or global scales. Nevertheless, our knowledge about microbial degradation of this compound in natural environments remains limited. It has been shown that HCB from air and water bodies can be removed via physical processes like volatilization and photolysis (6, 43). Adsorption also plays an important role in the removal of HCB from aquatic environments, but in turn results in deposition in sediments. In these light scarce environments biodegradation offers a great potential of transforming this persistent organic pollutant (7, 29). The only known pathway for microbial dehalogenation of HCB is the reductive dechlorination under anaerobic conditions, which results in formation of less chlorinated benzenes (1).

The reductively dechlorinating bacteria isolated up to now belong to the δ– and ε–*Proteobacteria* (*Geobacter*, *Sulfurospirillum*, *Desulfuromonas*, *Desulfomonile*), the *Firmicutes* (*Desulfitobacterium* and *Dehalobacter*), or to the *Chloroflexi* (*Dehalococcoides* and related groups) (52). So far, however, *Dehalococcoides* is the only bacterial genus whose members are known to transform HCB. Several *Dehalococcoides* strains were isolated that could grow with a broad variety of chlorinated aliphatic and aromatic compounds, including chlorinated benzenes and phenols, biphenyls, chloroethenes and dioxins. Nevertheless, until now only two strains, *Dehalococcoides sp.* strain CBDB1 (3) and *Dehalococcoides ethenogenes* strain 195 (18), which can transform HCB to tri- and dichlorobenzenes and use

the energy conserved in the process for growth, could be isolated. Besides HCB, *Dehalococcoides sp.* strain CBDB1 can also reductively dechlorinate chlorinated dioxins (11) and chlorophenols (2), whereas *Dehalococcoides ethenogenes* strain 195 can dechlorinate various chlorinated ethenes, 1,2-dichloroethane (1,2-DCA) and vinyl chloride (VC) (37).

Until now microbial community analyses of *Dehalococcoides* spp. largely focused on chlorinated ethene-contaminated aquifers or soils. The presence of *Dehalococcoides* spp. in uncontaminated and contaminated (PCE, TCE or VC) sites from North America, Europe, and Japan was reported (24, 26, 30, 34, 60). Furthermore, quantitative analyses targeting the *Dehalococcoides* spp. 16S ribosomal RNA (rRNA) gene in chlorinated ethene bioremediation sites showed that $8.6x10^3$-$2.5x10^6$ copies/ g aquifer material (33) and $1.9x10^2$-$1.1x10^7$ copies/ g soil (51) could be detected depending on the type of treatment applied. Although reductive dechlorination by *Dehalococcoides* spp. is an energy yielding process, microcosm studies conducted under controlled environmental conditions showed that growth of the organisms is relatively slow (28). Moreover, the presence of other halorespiring species may result in competition for chlorinated compounds or electron donors. This may adversely affect the success of the reductive dechlorination of HCB in natural environments. Hence, monitoring the indigenous dechlorinating species is needed to understand their diversity and activity in contaminated sites.

The aim of this study was to assess the diversity of active *Dehalococcoides* spp. in HCB polluted river basins and to reveal the links between species composition and abundance with changing environmental parameters, using 16S rRNA- and reductive dehalogenase-encoding gene-targeted molecular analyses, in combination with multivariate statistics. Even though our knowledge on the diversity of the *Dehalococcoides* spp. in river sediments is very limited it can be hypothesized that several environmental factors could be selective in the composition and activity of the genus. As they are mesophilic bacteria (see detailed review in **Chapter 1**) *Dehalococcoides* spp. population composition and activity is expected to decrease with decreasing water temperature. Additionally, being a specialist dedicated to reductive dechlorination, it can be expected that changes in HCB concentrations may effect the activities of *Dehalococcoides* spp.. This study attempts to provide new insights on natural occurrence and dynamics of reductively dechlorinating bacteria in light of changing environmental conditions.

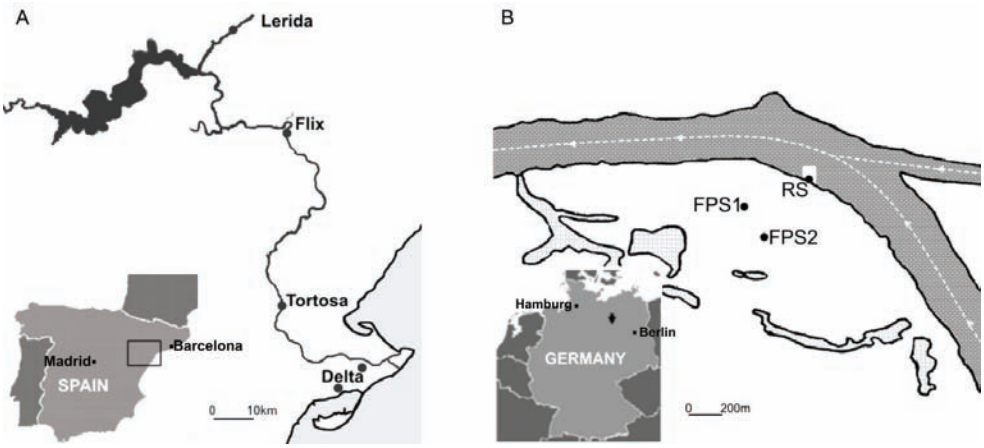

Figure 1. Sampling locations in the Ebro (**A**) and the Elbe (**B**) Rivers. Maps were re-drawn from Google ® Maps. Black squares represent approximate locations of the major cities closest to the sampling sites. Both maps are drawn according to scale as mentioned. (**A**) The Ebro River is located in the north east of the Iberian peninsula. Sampling was done in the downstream area of the River over a distance of approximately 200 km. Black dots represent sampling locations. (**B**) The Elbe River sampling points were river sediment (RS), flood plain soil 1 (FPS1) and flood plain soil 2 (FPS2). The black arrow represents the location of the sampling site in Germany. Light trimmed areas represent ponds created during flooding events. The dotted white line with arrow heads represents the flow direction of the Elbe.

Materials and Methods

Study sites and sampling procedure

Samples from two European rivers, the Ebro in Spain and the Elbe in Germany, were collected at several locations (Fig. 1; for exact coordinates, see Appendix I Table A1). The Ebro River (928 km) is located in the northeast of Spain (Fig. 1A). The Ebro River delta (330 km^2) contains rice fields (210 km^2) and wetlands (80 km^2). Samples from the Ebro River were taken in July 2004, February 2005 and February 2006. During the last sampling campaign additional samples were taken from one of the upstream locations (Flix, Tarragona), which has a chlor-alkali plant with more than one hundred years of activity. At this location HCB concentrations in the river sediment are higher than elsewhere in the Ebro River (20, 32). The Elbe River (1091 km) is one of the longest rivers in Central Europe flowing from Czech Republic to its mouth at the North Sea, Germany. Samples were taken from the Elbe River in October 2004, April 2005 and October 2005. The river Elbe was sampled at only one location, Schönberg-Deich (Fig. 1B), and samples were taken from river sediment and flood plain soil. The sampling site was located in the middle reach of the Elbe River, downstream of the Bitterfeld-Wolfen industrial area. All samples were taken in duplicate. River sediment

samples were taken approximately 1.5 m away from the river shore. At each location, sterilized PVC tubes (25 cm, internal diameter four cm) were inserted vertically into sediment or soil, retracted, and immediately sealed from the top and the bottom with rubber caps. Cores were frozen in liquid nitrogen, transported on dry ice, and stored at -80°C. Under sterile laboratory conditions frozen soil and sediment cores were cut into four slices (five cm thick, approximately 25 gram material), and homogenized by mixing with a spoon. This resulted in samples representing 0-5cm, 5-10cm, 10-15cm and 15-20cm of depth in sediment or soil. The slices were transferred into 50ml falcon tubes and stored at -80°C until use. Samples were analyzed by AGROLAB (Al-West B.V., Deventer, The Netherlands) for detection of geochemical parameters according to standardized methods. HCB concentrations were measured according to ISO 10382 protocol.

Nucleic Acid Extraction

RNA was extracted using the FastRNA® Pro Soil-Direct Kit (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions with minor modifications. Briefly, 0.5 gram soil or sediment sample taken from the frozen stock was subjected to bead beating with a Fastprep Cell Disruptor (Qbiogene, Carlsbad, CA), which was followed by phenol/chloroform extraction and incubation at -20°C for one hour. Total RNA was eluted with DEPC-treated distilled H_2O, which was supplied by the manufacturer. RNA purity was checked by electrophoresis in 1.0% (wt/vol) low-melt agarose gels. In case of DNA contamination RNA samples were treated with amplification-grade DNase I (Promega, Madison, WI) as specified by the manufacturer. DNA was isolated directly from soils (0.5 g) using the Fast DNASPIN®Kit For Soil (Qbiogene, Carlsbad, CA) according to the manufacturers' instructions. The RNA and DNA extraction yield was measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Reverse transcription (RT) and PCR amplification

DGGE-PCR for *Dehalococcoides* spp. A nested RT-PCR approach was used to specifically amplify *Dehalococcoides spp.* 16S rRNA fragments. Reverse transcription of 16S rRNA and subsequent PCR amplifications were performed in the same tube by using the Access RT-PCR System® (Promega, Madison, WI). The reaction mix (50 μL total volume) consisted of 10-15 ng of total RNA, 0.8×AMV/Tfl Reaction Buffer, 1 mM $MgSO_4$, 0.1 mM dNTP mix, 0.2 μM of each primer (DeF and DeR, Appendix I, Table A2), 4U of AMV polymerase, 4U Tfl polymerase, 0.5 μL BSA (20mg/mL, Roche). Reverse transcription and further PCR amplification was carried out at 45°C for 45 min, 94°C at 2 min, 35 cycles of 94°C at 30 sec, 55°C at 30 sec, 68°C at 1 min and final elongation at 68°C for 10 min. The products of RT-PCR were then used as template for PCR for the generation of amplicons suitable for analysis by denaturing gradient gel electrophoresis (DGGE) using previously described conditions

(54), with primers 968F -introducing a GC-clamp (40)- and DHC1350R (Appendix I, Table A2).

Reverse transcription of RNA templates for quantitative PCR. Reverse transcription (RT) of 16S rRNA was performed as described above, but with primers 27F and 1492R (Appendix I, Table A2) with second strand synthesis to produce double stranded cDNA fragments.

DGGE

DGGE was performed according to the protocol of Muyzer et al.(41) using the Bio-Rad gene detection system (BioRad, Hercules, CA) with denaturing gradients ranging from 35% to 58%. The gels were stained with $AgNO_3$ (49) and analyzed with BioNumerics 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). The Pearson product-moment correlation (23) was used to determine the similarity between DGGE fingerprints by calculating the similarity indices of the densitometric curves of the fingerprints. Bands were identified using the band search algorithm as implemented in BioNumerics, and manually checked by comparison to the corresponding densitometric curves. The Jaccard correlation coefficient was used to compare fingerprints based on the presence or absence of individual bands in the DGGE gels.

Real-Time Quantitative PCR

Real-time quantitative PCR (qPCR) was performed using an iQ5 iCycler (BioRad, Veenendaal, Netherlands) with the thermocycling program as previously described (53) for 16S rRNA genes of dehalogenating bacteria (*Dehalococcoides*, *Desulfitobacterium*, *Dehalobacter*) and total Bacteria using SYBR Green Dye. PCR reactions were prepared in 25 µl total reaction volume containing 5 µl template cDNA or DNA, 1× BioRad SYBR Green PCR master mix (BioRad, Veenendaal, Netherlands), 0.2 µM of each primer (Appendix I, Table A2) and 6.5 µl sterilized milli Q. Samples were analyzed in duplicate, and no-template controls were included. Standard curves were generated from triplicate dilution series. qPCR standards were prepared by cloning PCR-amplified 16S rRNA genes of targeted dehalogenating bacteria into the pGEM-T Easy plasmid vector (Promega, Madison, WI). PCR-products amplified from plasmid vectors using T7- and SP6-promotor targeted primers (Appendix I, Table A2) were used as real-time PCR standards.

Cloning and Sequencing

For the construction of clone libraries, 16S rRNA fragments were amplified by nested (RT-) PCR with primers DeF and DeR in the first PCR, which was followed by DeF and DHC1350R in the second reaction. The clone library for the Ebro River was prepared from a sample taken in winter 2005, from location Flix. For Elbe River the clone library was constructed from a sediment sample obtained in spring 2005. Both libraries were prepared from samples taken at a depth of 0-5 cm. The PCR products were cloned using the pGEM-T

Easy plasmid vector (Promega, Madison, WI), and *E. coli* XL1 blue cells (Stratagene, La Jolla, CA) according to the manufacturers' instructions. To assess the diversity of cloned fragments, the 1.3-kb PCR products were digested with the restriction enzyme MspI or/and AluI at 37°C for 3 hrs. Digestion mixtures (20 µl) contained 5µl of the PCR product, 0.25 U of the respective restriction endonuclease (Promega, Madison, WI), 0.1 mg of acetyl-bovine serum albumin (Promega, Madison, WI) and 1× restriction buffer (Promega, Madison, WI). The resulting fragments were separated by electrophoresis for 1hr at 125V in 12% (wt/vol) pre-cast Poly(NAT)® gels (Elchrom, Cham, Switzerland), using the Elchrom Submerged Gel Electrophoresis System. Representative clones containing inserts with different restriction patterns were selected and sequenced completely. The CHECK_CHIMERA program of the Ribosomal Database Project (36), BLAST searches and phylogenetic analyses of separate sequence domains identified one potential chimeric artifact, which was excluded from further phylogenetic analyses. Sequences belonging to two operational taxonomic units (OTUs) from Ebro river and eight OTUs from Elbe river were deposited to NCBI database. Sequences EU700499 and EU700500 originate from the Ebro River. Sequences EU700494-EU700497 and EU700502- EU700505 are from the Elbe River. Sequences obtained in this study were aligned with reference sequences using the online alignment tool SINA available at http://www.arb-silva.de (44). The aligned sequences were imported into the latest release of the ARB-Silva reference database (Silva96), and the alignment was manually refined using tools available in the ARB software package (35). A phylogenetic tree was constructed using the Neighbor Joining method as implemented in ARB (35).

Microarray Analysis

The GeoChip (25) was used to detect functional genes in sediment- and agricultural soil samples in the Ebro River. Since the current version of the GeoChip does not include all the reductive dehalogenase (*rdh*) genes sequences currently deposited in public databases, new probes were designed and added to the microarray to have comprehensive coverage of these genes. Oligonucleotide probe design, synthesis and fabrication was performed as described previously (25). A list of all *rdh* gene sequences for which additional probes were designed is given in Appendix II. Samples from two locations, upstream (Flix) and downstream (Rice Fields), and two depths (0-5 cm and 10-15 cm) were analyzed with the GeoChip. High molecular weight DNA extraction was performed by lysis in a CTAB buffer at 60°C using a phenol-chloroform purification protocol (61). Rolling circle amplification, which has been shown to amplify total DNA from low biomass microbial communities prior to microarray hybridization (58), was carried out using the TempliPhi kit (Amersham, Piscataway, NJ) following manufacturer's instructions. Spermidine (0.1 µg/µL) and single-strand binding protein (0.04 mM) were added to the reaction to aid amplification. Reactions were incubated

Table 1. Sediment and soil characteristics for Elbe and Ebro Rivers (0-5cm depth)

		DM %	TKN g kg^{-1} DM	TOC % DM	TP g kg^{-1} DM	Total DDT mg kg^{-1} DM	Total Drins mg kg^{-1} DM	Total HCH mg kg^{-1} DM	HCB mg kg^{-1} DM
River Ebro									
2004	Lerida	60.7	1.40	2.50	560	nd	nd	nd	<0.003
	Flix	58.2	0.46	0.49	260	0.990	nd	nd	<0.040
	Rice Fields	63.0	2.40	2.40	470	0.080	0.004	0.002	0.006
	Delta Estr.	13.2	8.30	3.00	680	nd	nd	nd	<0.002
2005	Lerida	45.4	3.30	2.40	na	0.003	nd	nd	<0.001
	Flix	63.6	0.97	1.30	370	0.250	nd	nd	0.009
	Tortosa	50.9	1.90	2.00	510	0.220	nd	0.02	<0.005
	Rice Fields	68.8	2.60	2.20	530	0.110	0.008	nd	<0.003
	Delta Estr.	70.4	0.82	0.89	360	0.002	nd	nd	<0.001
2006	Lerida	12.2	9.00	10.0	2400	0.024	nd	nd	<0.002
	Flix	62.4	0.90	0.88	300	0.097	nd	nd	0.120
	Tortosa	36.8	2.60	6.00	2100	0.034	nd	nd	<0.002
	Rice Fields	59.5	2.50	2.90	380	0.046	0.004	0.009	<0.001
	Delta Estr.	51.5	0.82	0.70	300	nd	nd	nd	<0.001
River Elbe									
2004	River Sed.	10.6	4.80	3.90	1400	0.078	nd	nd	0.018
	FPS1	77.2	1.70	2.50	1000	0.110	nd	0.026	0.047
	FPS2	75.7	4.60	7.50	1400	0.092	nd	0.006	0.170
2005a	River Sed.	77.0	0.45	0.68	340	0.035	nd	0.009	<0.030
	FPS1	47.7	6.70	10.0	2800	0.160	nd	0.031	0.190
	FPS2	60.6	6.40	8.90	1900	0.170	nd	nd	0.160
2005b	River Sed.	66.9	0.18	0.34	280	0.005	nd	nd	0.004
	FPS1	65.6	5.90	11.0	2600	0.220	nd	0.066	0.240
	FPS2	78.3	4.70	7.10	1500	0.140	nd	0.009	0.320

Abbreviations: DM: dry matter; TKN: total Kjeldahl nitrogen; TOC: total organic carbon; TP: total phosphorus; Total Drins: sum of aldrin, dieldrin and endrin; Total HCH: sum of alpha, beta and gamma derivatives of hexachlorocyclohexane; HCB: Hexachlorobenzene; nd: not detected; na: measurement is not available; 2005a: samples from April 2005; 2005b: samples from October 2005; River Sed.: river sediment; Delta Estr.: delta estuary

at 30°C for 3 hrs and the enzyme was then inactivated by incubation at 60°C for 10 min. The amplification products were labeled with Cystidine-5 (Cy-5) dye (Amersham, Piscataway, NJ). Hybridizations were performed in a HS4800 Hybridization Station (TECAN US, Durham, NC) as previously described (59) with following modifications. The first wash was carried out at 50°C for 1 min with a pre-hybridization solution (5X SSC, 0.1% SDS and 0.1% BSA) followed by a 45 min pre-hybridization. The slides were then washed four times with water at 23°C for 5 min with 30 sec soaking. Labeled DNA dissolved in the hybridization solution was then injected at 60°C and hybridization was carried out at 42°C for 10 hrs with high agitation. Slides were then washed and dried under a flow of nitrogen gas. Arrays were scanned using a ProScanArray microarray scanner (PerkinElmer, Boston, MA) at 633 nm using a laser power of 95% and a PMT gain of 80%. Images were processed by ImaGene 6.0 (BioDiscovery, El Segundo, CA), where a grid of individual circles defining the position of each DNA spot on the array was used to locate each fluorescent spot to be quantified. Spot calling was based on the following parameters; (signal to noise ratio) SNR<1.2 and (cumulative variance of background signal) CV<30% were chosen due to highly variable total hybridization signal between the different samples. Spots with two times higher signal intensity than the rest of the designated gene probes were accepted as an outlier and removed from the analysis.

Multivariate Analysis

In order to relate the changes in the *Dehalococcoides* spp. community composition and *rdh* gene variations to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands). Presence/absence and relative abundance (peak areas) of DGGE bands as well as normalized signal intensities of *rdh* genes were used as predictors. The environmental variables tested were time of sampling, distance between the sampling locations, sediment depth from which samples were taken, *Dehalococcoides spp.* 16S rRNA copies/g sediment, hexachlorobenzene concentration , water temperature and pH, total organic carbon (TOC), total Kjeldahl nitrogen (TKN) and total phosphorus (TP) measurements that were made on soil and sediment samples. All of the environmental data except for pH data were also transformed as log (1 + x). A Monte-Carlo permutation test based on 999 random permutations was used to test the null hypothesis of "*Dehalococcoides* spp. fingerprints are not related to environmental variables". Community structure was visualized via ordination triplots with scaling focused on inter-sample differences. Multivariate analysis of microarray data was performed using calculated relative intensity (abundance) values for each hybridization signal, which were normalized with Box-Cox transformation (10) prior to analysis. Redundancy analysis (CANOCO 4.5) was used to test the null hypothesis of "Variances in reductive dehalogenase genes are not related to environmental variables". Gene

distributions were plotted with scaling focused on inter-sample differences. Hierarchical cluster analysis of genes was generated using the pairwise complete-linkage clustering algorithm in CLUSTER software (15) and visualized with TREEVIEW (http://rana.stanford.edu). For all statistical analyses, correlations were considered highly significant at $p<0.05$ and significant at $p<0.10$ unless mentioned otherwise. All ANOVA and correlations analyses were performed in R software (45).

Results

Sediment and soil geochemistry

The geochemistry of sediment and soils samples varied significantly between different sampling locations in the Ebro River (Fig. 1A, Appendix I Table A1, Table 1 and 2). Correlation between sediment (soil) TOC, TKN and TP content and sampling time was not significant. Pollutant concentrations were highest in the upstream locations (mainly in Flix), with dichlorodiphenyltrichloroethane (DDT) and HCB being the main contaminants. The presence of contaminants other than HCB indicates that there may be a selective pressure for dechlorination bacteria. Major differences were observed between sediment and soil samples for TOC, TKN and TP in the Elbe River. In most of the cases, river sediments had lower concentrations of these compounds than floodplain soils. Moreover, the contamination was significantly higher in floodplain soils than river sediments. In Elbe, HCB was found to be the main contaminant, which was followed by DDT and hexachlorocyclohexane (HCH).

Table 2. Redundancy analysis results for the effect of sampling time, location and depth on measured geochemical parameters **(A)** River Ebro **(B)** River Elbe

A

	T (°C)	pH	DM	TKN	TOC	TP	HCB	DDT
Sediment/Soil	ns	**	ns	ns	ns	ns	ns	ns
Distance	ns	*	*	*	**	**	ns	ns
Time	*	ns	ns	ns	ns	ns	ns	*
Depth	ns	ns	ns	ns	ns	ns	ns	*

B

	T (°C)	pH	DM	TKN	TOC	TP	HCB	DDT
Sediment/Soil	na	na	ns	**	**	**	**	**
Distance	na	na	ns	*	*	ns	*	ns
Time	na	na	ns	ns	ns	ns	ns	ns
Depth	na	na	*	*	*	ns	ns	ns

Abbreviations: T: water temperature, pH: water pH, DM: dry matter; TKN: total Kjeldahl nitrogen; TOC: total organic carbon; TP: total phosphorus; HCB: Hexachlorobenzene; DDT: total DTT isomers; ns: not significant; na: not applicable; +: P≤0.10; *P≤0.05; **: P≤0.01; ***: P≤0.001

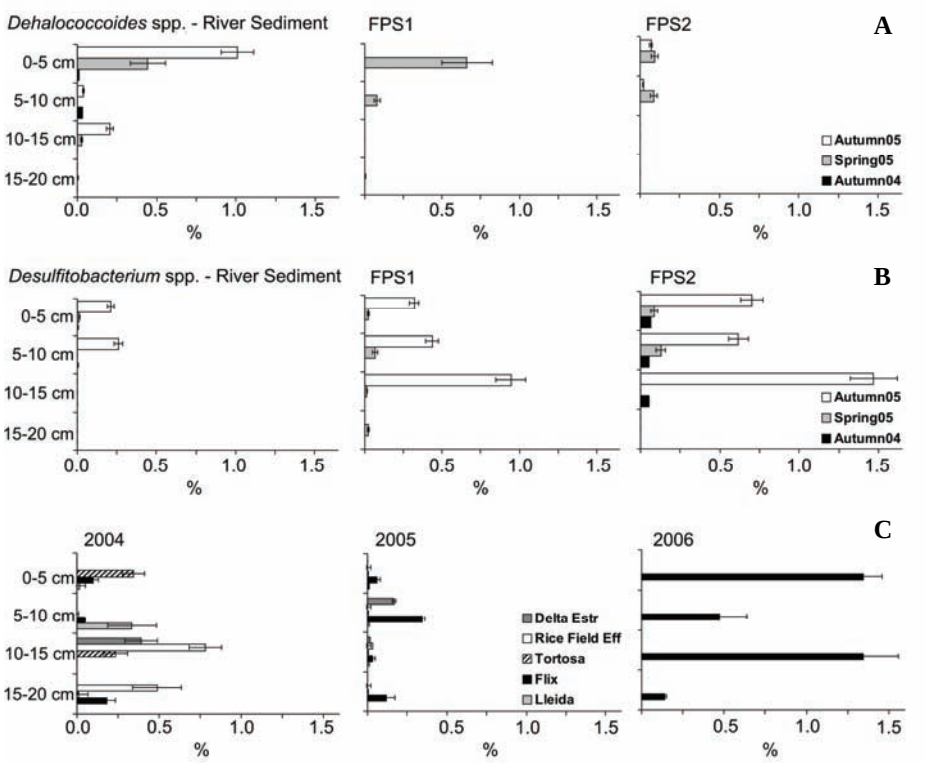

Figure 2. Changes in the relative abundance of 16S rRNA copies of dechlorinating bacteria in percentage of all bacterial 16S rRNA copies. The error bars represent the standard deviation of duplicate measurements. **(A-B)** Changes in the relative abundance of *Dehalococcoides spp.* and *Desulfitobacterium spp.* 16S rRNA copies in the Elbe River during sampling period of 1.5 years. The horizontal axis shows relative abundance in percentages. The vertical axis represents sample depth. Locations of the floodplain soil 1 and 2 are indicated in Figure 1. (**C**) *Dehalococcoides spp.* in Ebro River during sampling period in years 2004, 2005, 2006. The horizontal axis shows relative abundance in percentages. The vertical axis represents sample depth.

The impact of sediment (soil) geochemistry, spatial and temporal gradients on 16S rRNA abundance of dechlorinating bacteria

16S rRNA-targeted reverse transcription RT-qPCR assays were used for quantitative detection of several dechlorinating genera in the river basins. *Dehalococcoides* spp. and *Desulfitobacterium* spp. 16S rRNA could be detected in different quantities in the sampling locations (Appendix III), whereas *Dehalobacter* spp. rRNA could not be detected. Both absolute rRNA copy numbers and the relative abundances (i.e. *Dehalococcoides* spp. 16S rRNA copies / total Bacteria 16S rRNA copies) were used to calculate pairwise correlations (Spearman's correlation coefficient-r_s) of each genus with environmental variables (Table 3). In river Ebro, total bacterial rRNA copy numbers were significantly higher in samples with

Table 3. Spearman's rank correlations (r_s) between sediment (soil) parameters and 16S rRNA abundance of dechlorinating bacteria. **(A)** River Ebro **(B)** River Elbe

A

	Depth	Time	Distance	TKN	TOC	TP	HCB	Sediment/Soil	Temperature	pH
All Bacteria	-0.30	0.26	-0.35+	0.44+	0.53**	0.67•	-0.02	-0.30	0.08	-0.10
Dehalococcoides spp.	0.10	-0.01	-0.77•	0.09	0.06	0.39	0.19	-0.33	-0.20	0.07
Desulfitobacterium spp.	-0.29	0.20	-0.40+	-0.08	-0.03	0.26	-0.05	-0.45+	-0.16	0.09
Dehalococcoides spp./ All Bacteria	0.18	-0.15	-0.45+	-0.18	-0.23	-0.05	0.26	-0.09	-0.28	0.08
Desulfitobacterium spp./ All Bacteria	-0.20	-0.01	-0.26	-0.45+	-0.43+	-0.16	0.01	-0.48	-0.07	0.26

B

	Depth	Time	Distance	TKN	TOC	TP	HCB	Sediment/Soil
All Bacteria	-0.03	-0.81•	-0.23	-0.10	-0.29	-0.31	-0.31	-0.29
Dehalococcoides spp.	-0.43+	0.31	-0.21	0.17	0.07	0.03	-0.31	-0.35
Desulfitobacterium spp.	-0.37	-0.23	0.24	0.10	0.05	0.14	-0.01	0.05
Dehalococcoides spp./ All Bacteria	-0.40+	0.44+	-0.03	0.21	0.14	0.17	-0.21	-0.16
Desulfitobacterium spp./ All Bacteria	-0.34	0.81•	0.54+	0.17	0.30	0.45*	0.33	0.37+

Abbreviations: TKN: total Kjeldahl nitrogen; TOC: total organic carbon; TP: total phosphorus; +: P≤0.10; *P≤0.05; **: P≤0.01; •: P≤0.001

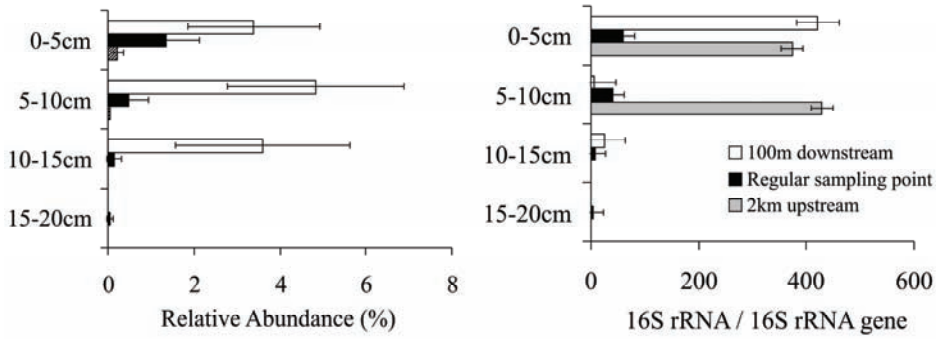

Figure 3. Changes in the rRNA relative abundance **(A)** and 16SrRNA copies/16SrRNA gene ratio **(B)** of *Dehalococcoides* spp. in three locations (regular sampling point, 2km upstream of regular sampling point and 100m downstream of regular sampling point) in the highly contaminated location Flix, in February 2006. The relative abundance of 16S rRNA copies was calculated as *Dehalococcoides* spp. 16SrRNA copies / Total Bacteria 16SrRNA copies. The vertical axis shows the sediment depth. Error bars represent standard deviation of duplicate measurements.

high TOC, TKN and TP content. Correlation between total bacterial rRNA copy numbers and spatial and temporal gradients were not significant. In river Elbe, total bacterial rRNA copy numbers were found to be decreasing (r_s=-0.81, $p \leq 0.001$) during the sampling period. No significant correlations were found with geochemical parameters. There was no significant correlation between HCB pollution levels and total bacterial rRNA copy numbers in both rivers.

In the Ebro River *Dehalococcoides* spp. comprised on average 0.2% of the bacterial 16S rRNA pool (up to 0.91%). *Dehalococcoides* spp. rRNA was consistently and significantly more abundant in the upstream locations (Lleida and Flix) than in downstream locations (Tortosa, Rice Fields and Estuary of the Ebro Delta) (r_s=-0.77, $p \leq 0.001$) (Fig. 2C, Table 2). Other environmental variables, including HCB pollution, did not significantly contribute to explain the changes in the relative abundance of *Dehalococcoides* spp. 16S rRNA copies. To further investigate the effect of sampling location, additional samples were taken from upstream and downstream of the regular sampling point in Flix in February 2006. Samples taken from 2 km upstream and 100 m downstream of the regular sampling point were analyzed to assess the spatial variation in the relative abundance of *Dehalococcoides* spp. rRNA copies as well as the ratio of 16S rRNA copies to 16S rRNA gene copies (rRNA/DNA) around the regular sampling point (Fig. 3). Results showed that relative abundance and rRNA/DNA of *Dehalococcoides* spp. could vary remarkably. For example, in the upper 5cm, four to five-fold differences in the rRNA/DNA ratio and two to 16-fold differences in the relative abundance between the locations could be detected. There were no clear correlations between rRNA/DNA ratio and relative abundance.

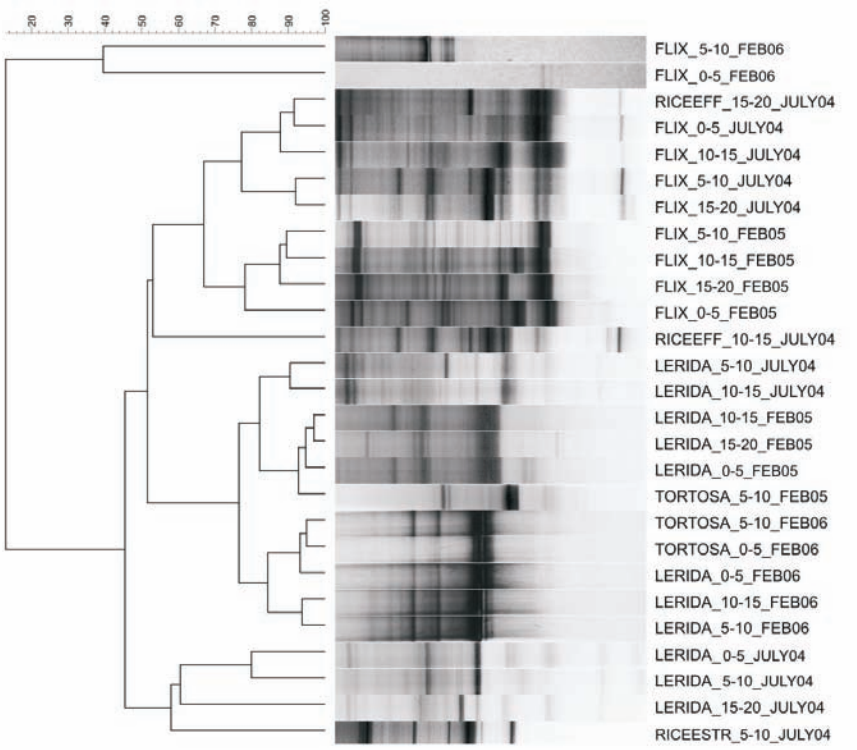

Figure 4. Clustering of *Dehalococcoides* spp. profiles in the Ebro River. RICEEFF; Delta – Rice Fields, RICEESTR; Delta-Estuaria. Samples were grouped by applying the unweighted pair group method with average linkages (UPGMA), the scale shows the percentage Pearson correlation between the samples. Numbers correspond to sample depth (i.e.0-5 being 0-5cm of the sediment/soil) followed by the sampling date (month and year, i.e. FEB05 being February 2005)

In the Elbe River, *Dehalococcoides* spp. had similar relative abundances as observed for the Ebro, comprising on average 0.26% of the bacterial 16S rRNA pool. However, for the flood plain soils rRNA abundances were considerably lower (0.05-0.12%; Fig. 2A). During the sampling period species abundances tended to increase (r_s=0.44, $p \leq 0.10$). *Dehalococcoides* spp. rRNA, when detected, had its highest abundances - as high as 1% - in the upper layers of sediments (r_s=-0.40, $p \leq 0.10$). Geochemical parameters, including HCB concentrations had no significant correlation to *Dehalococcoides* spp. rRNA abundances.

The abundance of *Desulfitobacterium* spp. rRNA was similar in Ebro River in samples taken at different times and depths, but there were differences between different sampling locations (r_s=-0.40, $p \leq 0.10$). Relative rRNA abundances were in general 10-fold lower than for *Dehalococcoides* spp. (data not shown). Throughout the sampling period, the relative abundance of *Desulfitobacterium* spp. rRNA decreased in upstream locations and increased in

the downstream locations in the river delta. Furthermore, in the Elbe River significant increases in *Desulfitobacterium* spp. rRNA relative abundances (r_s=0.88, p≤0.001) were detected during the sampling period. In contrast to *Dehalococcoides* spp., relative abundances of *Desulfitobacterium* spp. rRNA were higher in flood plain soils compared to river sediment throughout the sampling period (r_s=0.37, p≤0.1). The highest relative abundances were detected in deeper (5-15cm) layers of the soils (Fig. 2B).

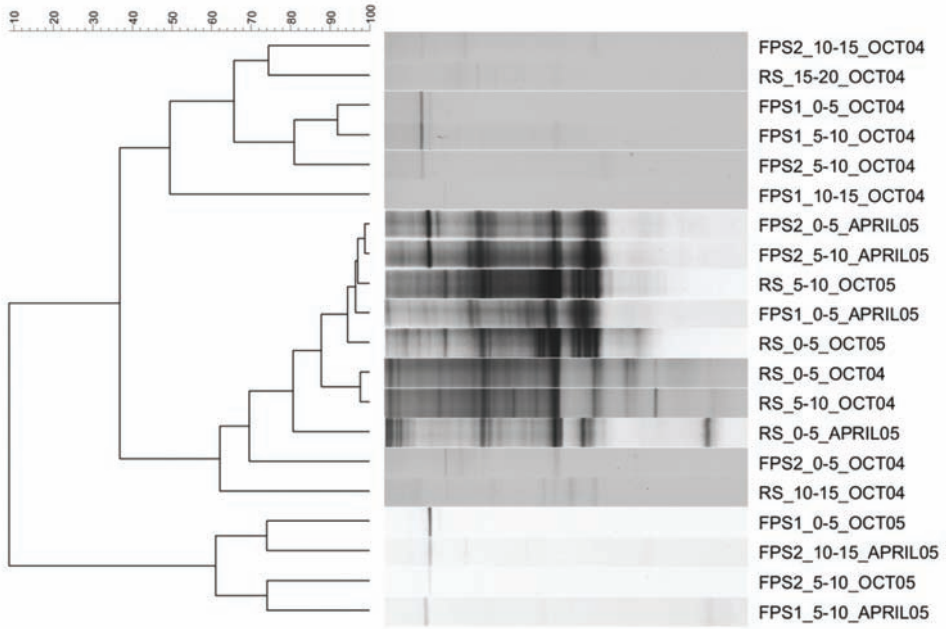

Figure 5. Clustering of *Dehalococcoides* spp. profiles in the Elbe River. RS; river sediment, FPS; floodplain soil. Samples were grouped by applying the unweighted pair group method with average linkages (UPGMA) to the matrix of similarities obtained from 16S rRNA based *Dehalococcoides*-specific DGGE profiles after Pearson correlation of densitometric curves from the profiles. The scale shows the percentage Pearson correlation between the samples. Numbers correspond to sample depth (i.e.0-5 being 0-5cm of the sediment/soil) followed by the sampling date (month and year, i.e. OCT05 being October 2005)

16S rRNA composition of *Dehalococcoides* spp. in the river basins

Changes in the composition of *Dehalococcoides* spp. were followed by DGGE of *Dehalococcoides*-specific 16S rRNA RT-PCR amplicons. The Pearson product-moment correlation (23) was used to compare DGGE fingerprints. In the Ebro River, Pearson correlation between all locations decreased from 68% in June 2004 to 13% in February 2006 (Fig. 4). Pearson correlation within the sampling locations decreased most drastically in Flix, namely from 93% to 19% during the sampling period. Similarity indices among the Elbe

River sediment and flood plain soils were as low as 9% (Fig. 5). Except for samples taken in October 2004, *Dehalococcoides* spp. 16S rRNA fingerprints could only be generated for samples from the top 10cm of the river sediment. *Dehalococcoides* spp. fingerprints showed 77% correlation in this fraction of the sediment throughout the sampling period (data not shown). Variations observed between DGGE fingerprints concerned differences in *Dehalococcoides* spp. composition rather than variation in the intensity of the bands. This was supported by the fact that pair-wise similarities and clustering based on either Jaccard or Pearson correlation coefficients did not differ significantly (data not shown).

Clone libraries of the most diverse DGGE fingerprints were constructed from 16S rRNA fragments amplified by RT-PCR to confirm that all bands indeed correspond to *Dehalococcoides* spp. related populations. Blast analysis (5) was conducted for sequences from 10 different OTUs, as defined by RFLP analysis. All the sequences from both rivers were affiliated with *Dehalococcoides* and close phylogenetic relatives. In the Ebro River sequences had 91-98% identity to 16S rRNA sequences of known *Dehalococcoides* spp. whereas the Elbe River sequences had 95-99% identity (Fig. 6).

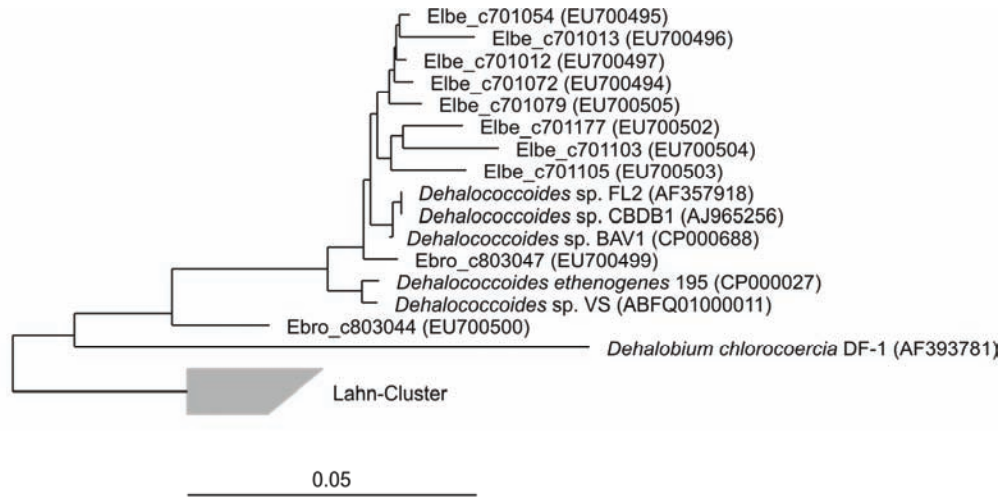

Figure 6. Neighbour-joining tree of aligned *Dehalococcoides* spp. 16S rRNA sequences derived from sediment samples taken from Ebro and Elbe Rivers, and appropriate reference sequences within the *Dehalococcoidetes*. Definition of Lahn-Cluster sequences was according to (30).Sequences EU700499 and EU700500 originate from the Ebro River. Sequences EU700494-EU700497 and EU700502- EU700505 are from the Elbe River. The tree was constructed based on partial sequences (*E. coli* positions 184 – 926) of the 16S rRNA-encoding gene. Scale bar represents 5% dissimilarity.

Redundancy Analysis (RDA) of *Dehalococcoides* spp. 16S rRNA abundance and composition

Multivariate statistics were used to determine to what extent environmental parameters (i.e. spatial and temporal gradients, and sediment (or soil) geochemistry) and 16S rRNA abundance contributed to the differences in the *Dehalococcoides*-specific DGGE fingerprints. The analysis was conducted on band positions (i.e. presence/absence). In the Ebro River the distribution of *Dehalococcoides* spp. in the ordination space was most significantly correlated with the gradient "time" (sampling period, $p \leq 0.001$) (Table 4). In addition, a Monte-Carlo significance test revealed that also the geographical distances (sampling location) had a significant effect on *Dehalococcoides* spp. composition. The model formed by the significant environmental parameters could explain 37.7% of the variation in *Dehalococcoides* spp. composition (p=0.061). When samples were grouped based on the sampling period, samples from 2004 and 2006 did not intersect, indicating a significant change in the community composition (Fig. 7A). A smaller number of species positively correlated with increasing TOC content as compared to the effect of water temperature and pH. Moreover most of the populations negatively correlated with thesampling period, indicating a decrease in richness in time. Correlations to depth (sampling depth), 16S rRNA copy numbers, TKN, TP and HCB concentrations were found to be insignificant.

Table 4. Summary of the results obtained for the RDA test for the significance of environmental variables in explaining the variance in *Dehalococcoides* spp. 16S rRNA composition and reductive dehalogenase (*rdh*) gene diversity. Percentages indicate the proportion of the variation in composition or diversity that could be explained by the different parameters.

	River Elbe	River Ebro	
	Dehalococcoides spp 16S rRNA composition	*Dehalococcoides* spp 16S rRNA composition	*rdh* gene diversity
Sampling location	1.4% ns	6.2% *	12% •
Sample depth	6.6% +	2.1% ns	6.8% ns
Sampling period	4.4% ns	16.9% •	4.9% ns
Dehalococcoides spp. 16S rRNA copy / g sample	14.1% **	2.5% ns	na
Twater	na	5.0% +	na
pHwater	na	4.8% +	na
TOC	1.6% ns	4.8% +	9.0% *
TKN	12.8% **	3.4% ns	10.2% *
TP	3.4% ns	2.8% ns	9.1% *
HCB	16.7% •	1.7% ns	4.4% ns
All	50.2% •	37.7% **	40.3% **

na: not applicable; ns: not significant; +: P≤0.10; *P≤0.05; **: P≤0.01; •: P≤0.001

The first two RDA axes could explain 48% of the total variation in the *Dehalococcoides spp.* composition in the river Elbe (Fig. 7B). The distribution of samples in the ordination diagram was strongly influenced by the HCB contamination ($p \leq 0.001$), which accounted for 16.7% of the variation in species composition (Table 4). Moreover, Monte-Carlo significance tests showed that variances can be significantly related to 16S rRNA copy numbers (p=0.006) and TKN (p=0.005). Grouping the samples in river sediment and flood plain soils showed that these two environments did not share the same species composition. Most of the species negatively correlated with increasing HCB concentrations and increasing sampling depth. In contrast to the results in the river Ebro, temporal gradients (sampling period) and TOC did not significantly affect the species composition.

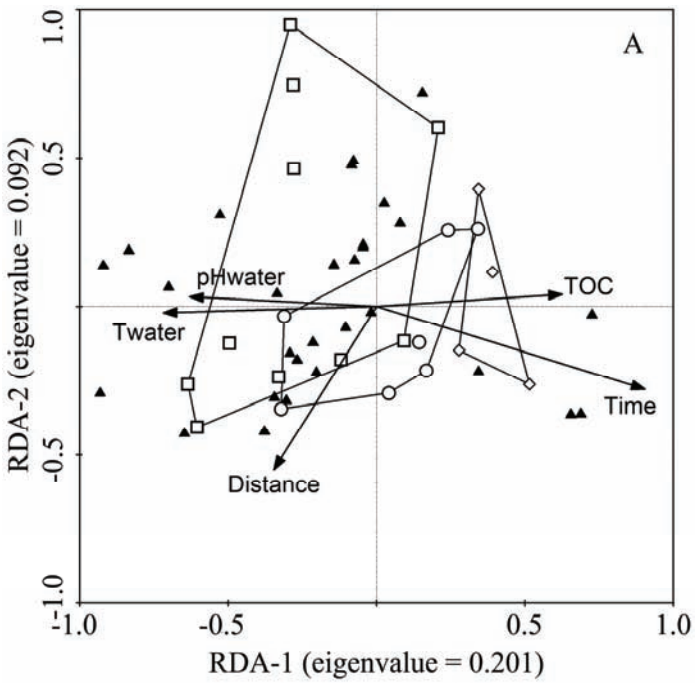

Figure 7. Ordination triplots for RDA analysis. Species (each *Dehalococcoides spp.* DGGE band) are displayed by triangles (▲). Samples are displayed by open circles, squares and diamonds as indicated below. Arrows represent environmental parameters (p<0.1). The length of each gradient (eigenvalue) is indicated on the corresponding redundancy axis. The plot can be interpreted qualitatively by following the direction of arrows for environmental parameters. The arrow length corresponds to variance that can be explained by the environmental variable. The direction of an arrow indicates an increasing magnitude of the environmental variable. The perpendicular distance between species and environmental variable axes in the plot reflect their correlations. The smaller the distance the stronger the correlation, whereas distances among species symbols are not explanatory. **(A)** DGGE band analysis for samples taken along the Ebro River. Samples are grouped according to sampling period (years 2004, □; 2005, ○; 2006, ◇).

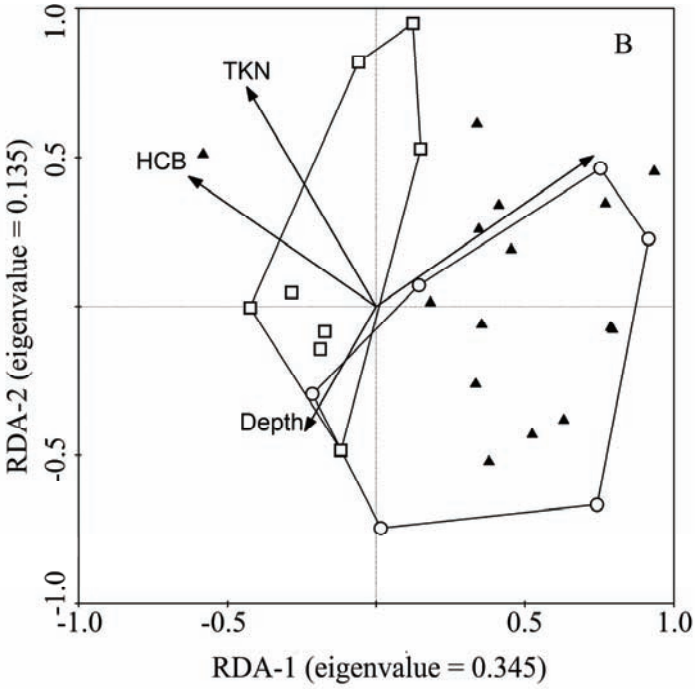

Figure 7. *cont*. (B) DGGE band analysis for Elbe River samples. Samples are grouped according to the sample type (soil, □; or sediment, ○).

GeoChip analysis of *rdh* gene diversity

Functional gene array (GeoChip) analysis was used to assess the variation of the reductive dehalogenase-encoding gene (*rdh* gene) diversity in upstream (Flix) and downstream (River Delta-Rice Fields) areas of the Ebro River basin. To assess the effect of environmental parameters on *rdh* gene profiles, RDA was conducted using normalized signal intensities. Redundancy axes ($p \leq 0.01$) were found to explain 40.3% of the overall variance within the *rdh* gene diversity. Monte-Carlo permutation tests showed that the *rdh* gene diversity changed significantly between different sampling locations ($p \leq 0.001$). As a result, a clear separation could be observed between the upstream and downstream samples (Fig. 8). Besides sampling location, TOC, TKN and TP were found to strongly correlate with variation in *rdh* gene diversity. Hierarchical cluster analysis of *rdh* genes showed that the upstream location was dominated by *rdh* genes of *Dehalococcoides* spp. CBDB1 (namely, cbdbA88, cbdbA1535, cbdbA1578, cbdbA1582, cbdbA1595, cbdbA1624, cbdbA1638) and *Dehalococcoides ethenogenes* strain *195* (namely, DET0088, DET0173, DET1522, DET1545) (Fig. 9, Appendix I, Table A3), which are the only cultivated anaerobic bacteria known to degrade HCB (3, 18). These genes were amongst the most abundant *rdh* genes and negatively

correlated with increasing TOC and TKN concentrations (Fig. 8). Downstream samples hybridized with probes specific for a variety of *rdh* genes from mainly *Desulfitobacterium* spp. and *Dehalococcoides* spp., including reductive dehalogenases of strains FL2 (RdhA7), VS (*vcrA*), CBDB1 (cbdbA1582, cbdbA1535, cbdbA1578, cbdbA1638) and *D. ethenogenes* strain *195* (DET0088, DET0173, DET1538, DET1528). The top layers of the sediment and agricultural soil samples were dominated by genes originating from *Dehalococcoides* spp., whereas a mixture of *Desulfitobacterium* spp., and *Dehalococcoides* spp. genes was detected in bottom layers (Fig. 9, Appendix I, Table A3). Abundance of *rdh* genes varied drastically during the sampling period. Sampling depth, however, as well as sampling period and HCB concentrations did not significantly affect the *rdh* gene diversity (Fig. 8).

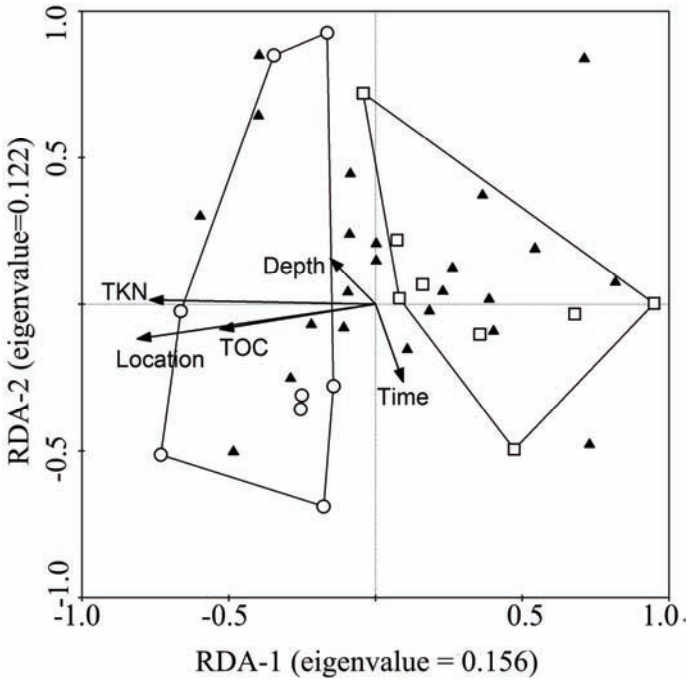

Figure 8. Ordination triplot for RDA analysis of *rdh* gene diversity in the Ebro River (based on GeoChip analysis). Each *rdh* gene is displayed by triangles (▲). Samples are grouped according to sample location (upstream, □; or downstream, ○). This grouping also represents sample type (agricultural soil or sediment). Arrows represent environmental parameters ($p<0.1$). See the Fig. 7 legend for further explanation.

Discussion

The aim of this study was to assess the composition and abundance of active *Dehalococcoides* spp. in river basins polluted with HCB, using a set of complementary cultivation-independent approaches. Previous biomolecular studies on *Dehalococcoides* spp. have shown their

presence in various environments and geographical locations (24, 26, 30, 34, 60). However, to the best of our knowledge, this study addressed for the first time the potential effects of temporal and spatial gradients on species composition and relative abundance in river basins.

Dehalococcoides spp. 16S rRNA relative abundance changed significantly between and within different sampling locations, depths and periods. In some locations, relative abundance could reach up to 1% (Fig. 2A and 2C). The only reported 16S rRNA relative abundance for *Dehalococcoides* spp. in the environment is two to six percent in a PCE and TCE contaminated groundwater aquifer (17). Additionally, HCB and PCE transforming batch scale enrichments from Ebro and Elbe River sediment samples had a higher relative abundance of *Dehalococcoides* spp. (two to six percent) than the corresponding environmental samples (**Chapter 5**). In enrichment cultures containing *D. ethenogenes* strain 195, 16S rRNA gene copy abundance was calculated at 7-62% during PCE degradation (47). When taken together, our results demonstrate that the relative abundance of *Dehalococcoides* spp. in the river basins studied here is lower than in contaminated aquifers or enrichment cultures.

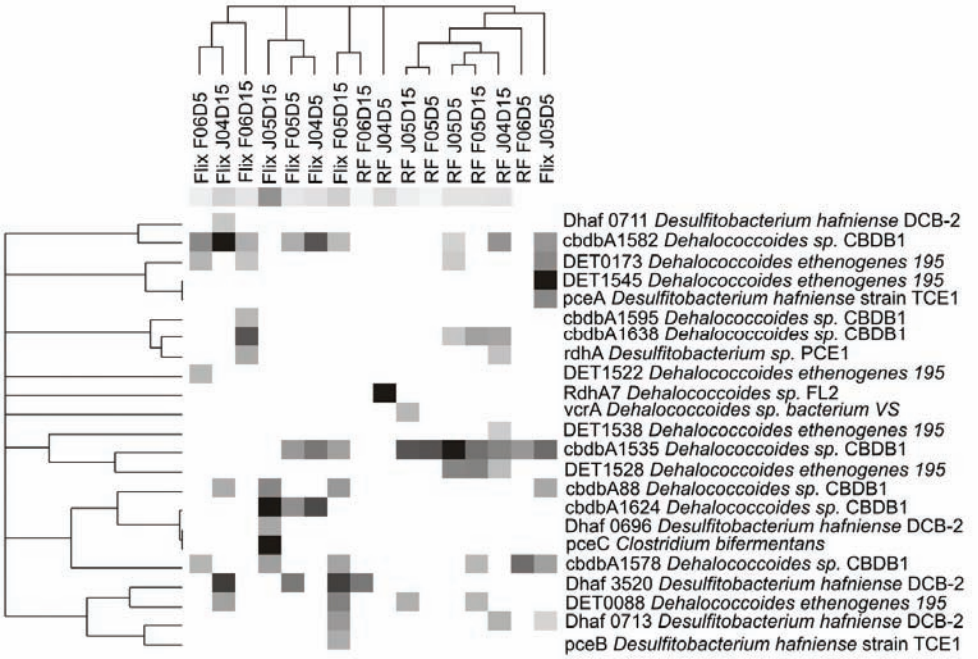

Figure 9. Hierarchical cluster analysis of *rdh* gene-profiles based on hybridization signals for samples from Flix and Rice Fields (RF) in the Ebro River. White represents no hybridization above background level and grey represents positive hybridization. The grey-scale intensity indicates differences in hybridization signal intensity, with black representing the strongest signals. Samples are represented according to sampling month, year and sampling depth (i.e. F06D5: February 2006 depth 0-5 cm; J04D15: June 2004 depth 10-15 cm). For accession numbers of *rdh* gene targets, see Appendix I,Table A3.

In geographically distant locations, as in the samples from the Ebro River, variance between the different sampling locations and periods could be so influential that the effects of other environmental parameters can be too small to explain the variations in the species composition. Eventhough water temperature, pH and TOC appeared to be relevant parameters in explaining the variation in the species composition, they were not highly significant. It can not be excluded that other factors, which could not be included in this study due to the lack of uninterrupted and reproducible measurements, might be of importance. Sediment transport in the river system and oxygen content in different depths of sediment (or soil), are two of these factors. The flow of the Ebro River is highly (57%) regulated by reservoirs that are used for irrigation and hydropower production. In the past years significant decreases were reported in the flood discharges (8). Reservoirs were reported to trap most of the sediment transported in the river stream, resulting in drop of the annual sediment contribution of the Ebro to its delta by up to 99% in the past century (13, 48, 55). During the sampling period dissolved oxygen content in Ebro River water varied between 6.2-16.7 mg O_2/L (data not shown). Molecular oxygen is typically being depleted between 0.1-mm up to 1-cm depending on the carbon content of the sediment (27), resulting in anoxic conditions in the deeper layers. However given the low flow rates of the river and variable sediment deposition, it is not possible to confidently estimate how much O_2 could be introduced to deeper layers of sediments and soils. Especially in the Ebro Delta, due to agricultural practices, presumably more O_2 could be introduced to the soil. In addition, varying salt concentrations (approx. 1-5 g/l from seashore to inland at one meter depth) caused by seawater intrusion (50) could also negatively influence the presence and activity of *Dehalococcoides* spp. in the river delta. Previous surveys conducted in the sampling area between 1999-2003 and more recent studies showed that besides HCB, DDT, PCE, and TCE, polycyclic aromatic hydrocarbons (PAH's), polybrominated diphenyl ethers (PBDEs) and brominated flame retardants could be detected in water, sediments and biota of this river (12, 16, 19). Therefore, the lack of significant correlation between HCB pollution and *Dehalococcoides* spp. composition and relative abundances may suggest that *Dehalococcoides* spp. in this river system does not depend only on HCB for their growth and possibly use alternative electron acceptors. These are not necessarily the compounds mentioned in Table 1, but the presence of this mixture of chlorinated compounds suggests that also other unidentified chlorinated compounds may be present that induce the activity of *Dehalococcoides* spp..

In accordance with the above, the distribution and diversity of *rdh* genes in the Ebro River could not be significantly related to the dominant contamination at the sampling locations. However, selective pressure of the contaminants could be demonstrated by differences in *rdh* gene distributions in the Ebro River up- and downstream locations. Upper layers of the sediment sampled at the HCB contamination hot-spot Flix were enriched with

rdh genes from *Dehalococcoides sp.* strain CBDB1 and *D. ethenogenes* strain 195, the only two cultured isolates currently known for their HCB-dechlorinating activity (3, 18). In contrast, samples taken at downstream locations within the Ebro Delta, which receives numerous halogenated compounds, were found to contain a variety of *rdh* genes, including those from various other species. From the detected *rdh* genes from *Dehalococcoides* spp. only one was previously characterized; *vcrA* of *Dehalococcoides sp*. bacterium VS. The *vcrA* gene product is involved in reductive dehalogenation of vinylchloride to ethene (39). Even though the rest of the detected *rdh* genes are uncharacterized, DET0088, DET0173 and DET1545 were reported to be up-regulated during PCE degradation in ANAS enrichments and mixed cultures containing *D. ethenogenes* strain 195 (46, 57). Furthermore, cbdbA1624 was found to be expressed during HCB degradation in batch scale enrichments from Flix sediment (**Chapter 5**).

Screening of the samples with 16S rRNA-targeted RT-qPCR and DGGE in the Elbe River demonstrated that *Dehalococcoides* spp. are more active and have a higher diversity in river sediments compared to flood plain soils (Fig. 7B). A major part of their activity was located in the upper layers (0-10 cm) of the river sediments that could provide the desired conditions for the growth and activity of *Dehalococcoides* spp. Unlike the situation in the Ebro River, HCB contamination was a significantly explanatory variable. A smaller number of *Dehalococcoides* spp. rRNA copies was found in flood plain soils, which had higher HCB contamination than river sediment. However, the floodplain soils of the Elbe River were shown to be contaminated with high concentrations of various heavy metals (31), which could be inhibitory for *Dehalococcoides* spp., resulting in the observed low diversity and activity.

Dehalococcoides spp. emerged as the most abundant dechlorinating bacteria in comparison to *Desulfitobacterium* spp. and *Dehalobacter* spp. in HCB contaminated river basins. Active *Dehalobacter* spp. could not be detected in either river basin during the two years of sampling. *Desulfitobacterium* spp., however, could be detected in most locations, albeit usually in lower numbers than *Dehalococcoides* spp. Unlike *Dehalococcoides* spp., relative abundances of *Desulfitobacterium* spp. were higher in flood plain soils of the Elbe River. However, a similar trend was not observed for the Ebro Delta. *Desulfitobacterium* spp. have not yet been reported to degrade chlorinated benzenes, and have only been associated with the degradation of chlorinated ethenes and ethanes, and chlorophenols. The functional gene array analysis of these samples confirmed the presence of potentially PCE and/or TCE dechlorinating *Desulfitobacterium* spp. both in river sediment and agricultural soil. Whereas at upstream locations *rdh* genes from *Desulfitobacterium hafniense* strains DCB-2 and TCE1 (*pceA* and *pceB*) were detected, the Ebro Delta was also shown to harbor a putative chloroethene reductive dehalogenase *rdhA* gene from *Desulfitobacterium sp.* PCE1. Moreover, *Desulfitobacterium* spp. are more flexible in their choice for electron acceptors than *Dehalococcoides* spp.. They can also use a wide variety of non-chlorinated compounds,

such as nitrate, sulfite, metals, and humic acids (56). Therefore it cannot be excluded that numbers obtained via 16S rRNA based detection of *Desulfitobacterium* spp. in the Elbe flood plains could also be originating from non-dechlorinating members of the species.

Conclusions

This study showed that high amounts of 16S rRNA *Dehalococcoides* spp. can be detected in river sediments exposed to HCB for a long period of time. However, spatial and temporal variations play a crucial role in affecting activity and diversity of abundant populations. Our findings indicate that the *Dehalococcoides* spp. activity is highly heterogeneous and varies significantly between different locations. In open environments like river basins, it will remain challenging to unequivocally link species composition and activity to changes in environmental conditions. From the data presented here, it can be concluded that river sediment emerges as a preferred environment for *Dehalococcoides* spp. as compared to agricultural or flood plain soils. As could be expected from current knowledge on the ecophysiology of halorespiring bacteria, *Dehalococcoides* spp. are more dominant in HCB polluted locations within river basins than *Desulfitobacterium* spp. and *Dehalobacter* spp. Hence, monitoring of *Dehalococcoides* spp. activity in HCB contaminated river basins provides valuable information about changes in the environmental conditions and contributes to our understanding of the life of these interesting bacteria in natural environments.

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Chapter 04

Magnifying Glass for Ecosystems: Functional Gene Array Analysis of Polluted River Sediments

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This study was conducted in two locations in the Ebro River (Spain), which were contaminated with chlorinated compounds. The aim of this study was to gain insight in functional gene diversity of microbial communities in the river and to identify microbial community members involved in the cycling of nutrients and degradation of pollutants. River sediment and agricultural soil samples were analyzed with the GeoChip, a functional gene array that has more than 10,000 genes targeting over 150 functional groups belonging to carbon, nitrogen and sulfur cycling, metal reduction and resistance, and organic contaminant degradation. The results indicated a high abundance and diversity of carbon, nitrogen and sulfur cycling genes. The majority of genes of eukaryotic and archaeal origin were involved in carbon cycling, while the most abundant contaminant degradation genes were from bacterial populations. With this study we could demonstrate that the GeoChip is a useful tool for comprehensive initial screening to identify functional aspects of microbial communities that can be used as biomarkers to design more specific and quantitative tools for their detection.

Manuscript in preparation

Introduction

Almost all European rivers are under pressure of agricultural and industrial activities (38). These activities have been shown to have adverse effects on the functioning of the river ecosystems (4). Microbial communities in river sediments take part in many environmental processes such as decomposition of organic material, recycling of nutrients and degradation of sediment-associated pollutants. Identifying the effects of changing environmental conditions and pollution on the river microbial communities is not only a valuable addition to the existing knowledge but also necessary to implement correct measures for sustainable river management.

Most freshwater sediments are heterogeneous ecosystems, which can create favorable conditions for the development of many different environmental niches (42). Microbial communities in the environment can be characterized via cultivation-dependent and independent methods. Recently, microarrays became important tools in the characterization of microbial communities in environmental samples (50, 58, 60). Most of the reported microarray applications use phylogenetic markers like 16S rRNA genes for detection (12, 26, 32). Phylogenetic markers are informative targets for assessing community structure and dynamics (16). However, they give little or no information about the functioning of microbial communities, including their potential functional diversity. In contrast, functional gene arrays (FGAs) target genes involved in microbial processes (functional genes) instead of phylogenetic markers (20, 61). Hence, FGAs provide information about the metabolic functions and the functional guilds present in the environment. A variety of different FGAs targeting single or multiple microbial processes have been applied in different environments (44, 46, 53-55). One of these arrays, the GeoChip, allows simultaneous detection of over 10,000 gene variants on a single array (He et al, 2007). The Geochip contains a variety of probes for the most important microbially-driven nutrient cycle processes and catabolic routes for degradation of pollutants. As a result, the GeoChip provides an ideal platform for comprehensive detection of different microbial processes in complex environments like river sediments, without the need for prior knowledge of predominant processes.

This study was conduced in the Ebro River (928 km), which is located in the northeast of Spain. The Ebro River discharges into the Mediterranean Sea where it forms a large delta of more than 30,000 ha (**Chapter 3**, Fig. 1A). The Ebro river basin (approximately 85,000 km^2) is the largest irrigated area in Spain. The river basin is populated by more than 2.7 million inhabitants and various industrial complexes. Past industrial activities and extensive agriculture lead to the discharge of various pollutants especially halogenated organic compounds, into the river. These compounds were reported to be the predominant pollution in the downstream section (200 km from Mediterranean Sea shore) of the river (23). In this part of the river, DDTs, polybrominated diphenyl ethers (PBDEs) and chlorobenzenes (CBz) are

the most widely detected pollutants not only in sediment but also in fish. Besides halogenated organic pollutants, pesticides –i.e. atrazine- were detected in high concentrations in the Ebro River basin (10). The delta of the Ebro is mainly used for rice cultivation and orchards (Terrado, 2007). As a result of the agricultural practices various pesticides are applied to the soils of the delta. The water flow of the delta is controlled by means of upstream barrages and water canalization for irrigation.

As previously discussed in **Chapter 3**, environmental conditions can influence the composition and the activity of *Dehalococcoides* spp. in the river environments. It has also been shown that *Dehalococcoides* spp. depend on the H_2 supply (43) from other microorganisms (i.e. fermentative bacteria) and co-factors like vitamin B_{12} (19) which might have a great influence in their *in situ* performance (37). Thus, it is necessary to have insight in factors affecting nutrient fluxes, namely carbon, nitrogen, sulfate cycling, to be able to understand the survival and functioning of *Dehalococcoides* spp. in the river basins. The main goal of this study, therefore, was to describe the functional diversity of microbial communities in the Ebro River, and to identify key functional guilds in the cycling of nutrients and degradation of pollutants. Functional community analyses with the GeoChip focused on samples taken from the river sediment and agricultural soils of the river delta. The major ecological questions of this study were: i) Which abiotic factors control the abundance and distribution of microorganisms in the river systems? ii) What are the differences between microbial communities and their metabolic activity profiles present in different compartments of the river ecosystem (e.g. various depths and locations)? iii) What are the effects of the selected pollutants on microbial functions in the river ecosystem? iv) To what extent are specific populations and catabolic routes responsible for biodegradation of pollutants affected by the presence of additional major microbial processes? To this end, we here provide a detailed analysis of functional gene distribution and effects of spatial and temporal gradients on functional gene distribution across the Ebro River basin.

Materials and Methods

Sampling sites

Samples from the Ebro River were collected at two locations: river sediment samples from Flix (N 41º13’43.6”, E 00º33’09.0”) and agricultural soil samples from rice fields in the river delta (N 40°38’22.7”, E 00°42’39.5”). Sediment/soil samples were collected once every six months between July 2004 and February 2006. Sterilized PVC tubes (25 cm, inner diameter 4 cm) were used for sampling. Tubes were inserted vertically into the sediment, retracted, and openings were sealed with rubber caps. Sediment/soil cores were immediately frozen in liquid nitrogen, transported on dry ice, and stored at -80°C. Under sterile laboratory conditions

frozen sediment/soil cores were divided into several pieces and homogenized by mixing with a spoon. Divided samples were stored at -80°C until further processing. In this study, samples representing 0-5 cm and 10-15 cm in sediment/soil depth were used for analysis. Samples were analyzed by AGROLAB (Al-West B.V., Deventer, The Netherlands) for geochemical parameters according to standardized methods. Pesticides and organic contaminant concentrations were measured according to ISO 10382. The compounds measured were hexachlorobenzene (HCB), DDT, DDE and DDD isomers (total DDT); Aldrin, Telodrin, Dieldrin and Endrin (total Drins) and hexachlorocyclohexane (HCH) isomers (total HCH).

Nucleic acid extractions and rolling circle amplification

High molecular weight DNA was extracted from 25 g sediment (wet weight). Prior to extraction, sediment samples were mixed with 2 g of sterile sand and immediately frozen with liquid nitrogen. The frozen mix was grinded in a mortar, and the freeze-grinding procedure was repeated three times. DNA extraction was performed by lysis in a CTAB buffer at 60°C using a phenol-chloroform purification protocol (59). DNA extracts were purified via minicolumn purification (Wizard DNA clean-up system; Promega, Madison, WI). Rolling circle amplification (RCA) was carried out by using the TempliPhi kit (Amersham, Piscataway, NJ), following manufacturer's instructions with modifications in the reaction mixture (54). RCA has been demonstrated to be well adapted for the amplification of DNA from low biomass microbial communities prior to microarray hybridization (54). 50-75 ng of genomic DNA was mixed thoroughly with 25 µl of reaction buffer containing 0.1 µM spermidine and a single-strand binding protein (final concentration 260 µM) to facilitate amplification. After incubation for 3 hours at 30°C, the enzyme was inactivated by incubation for 10 min at 65°C. The amplification products were purified with a QIAquick PCR purification column (Qiagen, Valencia, CA) and used for labeling.

Real-Time Quantitative PCR

Real-time quantitative PCR (qPCR) was performed using an iQ5 iCycler (BioRad, Veenendaal, Netherlands) with the thermocycling program as previously described elsewhere (49). The reductive dehalogenase-encoding genes of *Dehalococcoides* sp. CBDB1, cbdbA84 (AJ965256.1), cbdbA1624 (AJ965256.1), cbdbA1588 (AJ965256.1) (49), and *Dehalococcoides ethenogenes* strain 195, DET0318 (NC_002936.3) and DET0079 (NC_002936.3) (5), were detected via PCR reactions which were prepared in 25 µl total reaction volume containing 5 µl template DNA, 1× BioRad SYBR Green PCR master mix (BioRad, Veenendaal, Netherlands), 0.2 µM of each primer and 6.5 µl sterilized milli Q. Samples were analyzed in triplicate, no-template controls were included. qPCR standards were prepared by cloning PCR-amplified reductive dehalogenase (*rdh*) genes of dehalogenating bacteria into the pGEM-T Easy plasmid vector (Promega, Madison, WI).

DNA labeling and Microarray hybridization

DNA labelling with Cy-5 dye (Amersham) was performed as described elsewhere (55, 60). The GeoChip (20) was used to detect functional genes in sediment- and agricultural soil samples. The current version of the GeoChip does not include all the reductive dehalogenase (*rdh*) gene sequences deposited in public databases. Detection of *rdh* genes was important for the scope of this study since they are pivotal to the degradation of halogenated organic pollutants (41) which are predominantly found in the Ebro River. Therefore, approximately 138 new probes were designed and added to the microarray to have a better coverage of these genes, based on publicly available gene sequences as of January 2007. Oligonucleotide probe design, synthesis and fabrication was performed as described previously (20). Hybridizations were performed using a HS4800 Hybridization Station (TECAN US, Durham, NC). The hybridization protocol was as follows. The first wash was done at 50°C for 1 min with a pre-hybridization solution (5× SSC, 0.1% SDS and 0.1% BSA) followed by a 45 min prehybridization. The slides were then washed four times with water at 23°C for 5 min. Labelled DNA was dissolved in the hybridization solution and then injected into the chamber at 60°C. Hybridization was carried out at 42°C for 10 hours with high agitation. Slides were then washed four times at 50°C for 1 min using wash buffer I (1× SSC, 0.1%SDS), four times at 23°C for 1 min using wash buffer II (0.1× SSC, 0.1%SDS) and four times at 23°C for 1 min using wash buffer III (0.1× SSC). Slides were finally dried at 23°C for 3 min under a flow of nitrogen gas.

Scanning and image processing

The ProScanArray microarray analysis system (Perkin-Elmer, Wellesley, MA) was used to scan microarrays at 10 µm resolution for data collection (laser power of 95%, PMT gain of 80%). Scanned images were analyzed by quantifying the pixel density (intensity) of each spot using ImaGene 6.0 (BioDiscovery, El Segundo, CA). The GeoChip included one, two or three (and more for few of the cases) probes for each gene sequence or each group of homologous sequences. Due to the variation in the total hybridization signal between the different replicates, genes were scored as positive when more than 1/3 of the available probes for each gene gave a signal in all replicates. In addition the fluorescence intensity of the probe had to be at least 1.2 times higher than the background (signal-to-noise ratio, SNR). SNR was calculated as, $SNR = (X_p - X_b)/Y_b$ where X_p is the mean pixel intensity of all replicate probe spots, X_b is the mean pixel intensity of the local background area around all replicate probe spots and Y_b is the standard deviation of the pixel intensity of the local background area around all replicate probe spots. When the absolute signal intensity of a spot minus the mean signal intensity of all probes of a gene was greater than 2σ, the spot was considered to be an

Table 1. Sediment and soil characteristics for the Ebro River.

	DM	TKN	TOC	TP	C/N	Total DDT	Total Drins	Total HCH	HCB
	%	g kg^{-1} DM^{-1}	% DM	g kg^{-1} DM^{-1}		mg kg^{-1} DM^{-1}	mg kg^{-1} DM^{-1}	mg kg^{-1} DM^{-1}	mg kg^{-1} DM^{-1}
Flix									
June 04 D5	58.20	0.46	0.49	260	1.83	0.990	n.d.	n.d.	<0.040
June 04 D15	79.30	0.50	0.48	250	1.21	1.100	n.d.	0.012	<0.003
Feb 05 D5	63.60	0.97	1.30	370	2.11	0.250	n.d.	n.d.	0.009
Feb 05 D15	71.70	0.49	1.30	280	3.70	0.086	n.d.	n.d.	<0.004
June 05 D5	48.90	0.53	1.30	210	5.02	0.370	n.d.	n.d.	<0.060
June 05 D15	67.90	0.75	0.85	210	1.67	0.420	n.d.	n.d.	0.038
Feb 06 D5	62.40	0.90	0.88	300	1.57	0.097	n.d.	n.d.	0.120
Feb 06 D15	73.60	0.86	0.76	290	1.20	0.007	n.d.	n.d.	<0.001
Rice Fields									
June 04 D5	63.00	2.40	2.40	470	1.59	0.080	0.004	0.002	0.006
June 04 D15	63.80	2.70	2.70	470	1.57	0.054	n.d.	0.007	<0.001
Feb 05 D5	68.80	2.60	2.20	530	1.23	0.110	0.008	n.d.	<0.003
Feb 05 D15	68.80	2.50	2.40	490	1.40	0.059	n.d.	n.d.	<0.003
June 05 D5	28.90	3.00	2.60	890	3.00	0.074	n.d.	n.d.	0.015
June 05 D15	65.90	1.70	1.70	540	1.52	0.130	n.d.	0.003	0.005
Feb 06 D5	59.50	2.50	2.90	380	1.95	0.046	0.004	0.009	<0.001
Feb 06 D15	68.60	2.50	2.90	440	1.69	0.057	n.d.	0.006	<0.001

Abbreviations: DM: dry matter; TKN: total Kjeldahl nitrogen; TOC: total organic carbon; TP: total phosphorus; Total DDT: sum of DDT, DDE and DDD isomers; Total Drins: sum of Aldrin, Telodrin, Dieldrin and Endrin; Total HCH: sum of hexachlorocyclohexane (HCH) isomers; HCB: Hexachlorobenzene, (<) absolute concentration could not be measured, below detection limit. Samples are labeled according to sampling year and sampling depth (i.e. June 04 D5 being, sample taken in June 2004 at 0-5 cm depth or Feb 05 D15 being, sample taken in February 2005 at 10-15 cm depth)

outlier and removed (54). The abundance of each spot is defined as the averaged and normalized signal intensity of the spot in the up to six replicate probes. Relative abundance values were calculated by dividing the intensity of the spots by the sum of the intensity for all the spots. For some analyses, the relative intensity was summed per gene family.

Statistical analyses

Functional genes were divided into the following groups for statistical analysis: carbon cycle (carbon degradation and fixation, methane production and oxidation), nitrogen cycle (N_2-fixation, nitrification, denitrification), sulfate reduction, metal reduction and resistance, and contaminant degradation. Carbon cycle, nitrogen cycle and sulfate reduction genes are sometimes referred as C,N,S cycling genes. All ANOVAs and correlations analyses were carried out in R (2.7.1) software (Bell Laboratories, NJ, USA) with vegan, MASS and car packages. Association strengths between different parameters were tested for significant Pearson linear correlation (r) and also for non-parametric Spearman correlation (r_s). Correlations were considered highly significant at a $P<0.05$ baseline and significant at a $P<0.10$ baseline. Spearman correlation (r_s) was assumed to be high (strong) when $r_s> 0.80$.

Functional gene diversity was calculated using the Shannon-Weaver index (H'). Hierarchical clustering of samples was based on Bray-Curtis coefficients of similarity, which were calculated by the R package "vegan". Common measures of diversity (alpha, beta and gamma diversities) treat all species as being equal and do not take phylogenetic relationships into account (48). Taxonomic (or phylogenetic) diversity (TD) is calculated as the average taxonomic distance between any two species (functional genes in this case), chosen at random from the sample (8). The average taxonomic distinctness (AvTD) of a sample is the mean of this distance. AvTD is a measure of the taxonomic diversity which is not influenced by samples' species richness. Expected AvTD was calculated from the the complete data set for the hypothesis of "no taxonomic difference between the samples". Variation in taxonomic distinctness (VarTD) is the variance between every pair of samples in the dataset. These pairwise path lengths reflect the unevenness of the taxonomic (or phylogenetic) distances. In other words, VarTD represents a measure for the degree to which functional gene-based taxa are over- or under-represented in samples is reflected in.

In order to relate the changes in the functional gene variations to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands). Relative abundances of functional genes were used as predictors. The environmental variables tested were time of sampling, distance between sampling locations (absolute distance between sampling points in km are used to classify samples), sediment depth from which samples were taken, total organic carbon (TOC), total Kjeldahl nitrogen (TKN), total phosphorus (TP), C/N ratio and total pollutant contamination (as sum of HCB and total DDT, Drins and HCH) measurements

that were made on soil and sediment samples. A Monte-Carlo permutation test based on 999 random permutations was used to test the null hypothesis of "Variances in functional gene relative abundances are not related to environmental variables". Microarray data was normalized with Box-Cox transformation (6) prior to analysis. Community structure was visualized via ordination triplots with scaling focused on inter-sample differences. Hierarchical cluster analysis of genes was done using the pairwise complete-linkage clustering algorithm in CLUSTER software (14) and visualized with TREEVIEW (http://rana.stanford.edu).

Results

Sediment and soil geochemistry

Measurements done in the river sediment and in the rice field soils of the river delta showed clear differences between the two locations (Table 1). The river sediment was characterized by variable dry matter (DM) content, and lower total carbon (TOC), total Kjeldahl nitrogen (TKN) and total phosphorus (TP) content when compared to rice field soils. Furthermore, in most of the cases the total carbon (TOC) content of the selected locations (Flix and Rice Fields) was lower than the rest of the Ebro (**Chapter 3**, Table 1). Overall total DDT, HCH, Drins and hexachlorobenzene (HCB) contamination in river sediment was higher than in rice fields. The C/N ratio varied within and between the sampling locations and was noticeably lower than reported literature values for similar rivers (29). Even though rare, low C/N values were previously reported in the Ebro (17) and few other rivers (11). Approximately two to four fold increases (or decreases) in C/N content were observed within the time frame of sampling. These measurements might suggest high carbon mineralization and enrichment of inorganic nitrogen in these sediments and soils. The correlation between sediment (soil) geochemical parameters and sampling time was not significant (Table 2). The strong positive correlation between TOC, TKN and C/N with sampling location confirmed the higher nutrient content of the rice field soils. In addition, total Drins and total HCH concentrations were also found to be significantly higher in this location. On the other hand, total DDT and HCB concentrations were significantly higher in Flix river sediment. Only DM, total Drins and HCB showed significant correlations with sampling depth. While strong positive correlation of DM showed a higher dry matter content in the deeper sediments (soils), contamination of total Drins and HCB were higher in the upper sections of the sediments (soils).

Diversity of functional genes

In total 2334 different functional genes were detected (Table 3) which covered 18%-29% of the targeted genes for each function. The majority of the eukaryotic and archaeal genes were

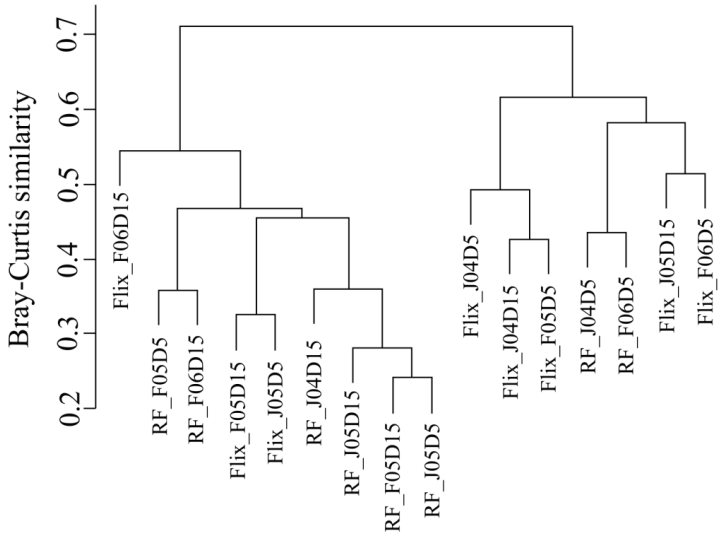
involved in the carbon cycle (70.3 and 43.7%, respectively), whereas the majority of bacterial genes was involved in contaminant degradation (41.1%). Analysis of the GeoChip data showed strong variations in functional gene profiles between different samples. Bray–Curtis

Table 2. Spearman's rank-order correlations between sediment (soil) parameters and sampling scheme.

	DM	TKN	TOC	TP	C/N	Total DDT	Total Drins	Total HCH	HCB
Sampling location	ns	0.78•	0.87 •	ns	0.81•	-0.54*	0.48+	0.42+	-0.48 +
Sampling time	ns	ns	ns	ns	ns	Ns	ns	ns	ns
Sampling depth	0.75 •	ns	ns	ns	ns	Ns	-0.48+	ns	-0.51*

Abbreviations: DM: dry matter; TKN: total Kjeldahl nitrogen; TOC: total organic carbon; TP: total phosphorus; HCB: Hexachlorobenzene; DDT: total DTT isomers; ns: not significant; +: P≤0.10; *P≤0.05; **: P≤0.01; •: P≤0.001

A



B

	Time	Location	Depth	DM	TKN	TOC	C/N	TP	Pollutant
r^2	0.07 ns	0.13 *	0.10 +	0.07 ns	0.07 ns	0.041 ns	0.08 +	0.07 ns	0.054ns

ns: not significant; +: P≤0.10; *P≤0.05; **: P≤0.01; •: P≤0.001

Figure 1. (A) Hierarchical clustering of samples based on Bray-Curtis similarity indices. **(B)** Results of permutational multivariate analysis of variance using in the Bray-Curtis indices.

Table 3. Summary of the functional gene array results and distribution of the functional genes into functional gene groups

	Total	**Nitrogen Cycling**	**Carbon Cycling**	**Sulfate Reduction**	**Contaminant Degradation**	**Metal Reduction and Resistance**
Number of targeted genes	10416	2743	1951	702	3224	1796
Number of detected genes	2334	507	353	137	930	407
Number of genes detected in each kingdom						
Eukaryota	119	5	83	0	28	3
Bacteria	2151	494	242	137	885	393
Archaea	64	8	28	0	16	12
Percentage distribution of genes detected in each kingdom*						
Eukaryota		4.2 %	70.3%	0,0 %	23.6 %	1.8 %
Bacteria		22.9 %	11.2 %	6.4 %	41.1 %	18.2 %
Archaea		12.5 %	43.7 %	0,0 %	25.0 %	18.7 %

*Percentages calculated as: [Total number of detected functional gene per kingdom/ Total number of all genes per kingdom]*100

similarity indices were calculated to construct a hierarchical clustering of the different samples (Fig.1), and a multivariate regression analysis was used to test association of the sample dissimilarities with environmental variables (56). The largest portion of variation between the samples could be explained by the distance between different sampling locations (r^2=0.13, p≤0.05). Sampling depth and C/N ratio of the samples contributed to almost equal parts to the overall variation. However, the remaining environmental variables did not significantly contribute to explaining the variance between different samples. As a result of the observed high variation between and within the sampling locations, and low correlation with environmental variables, when clustered, no obvious grouping could be observed in the samples (Fig.1).

Table 4. (A) Diversity indices for each sample calculated based on all detected genes. Significance of VarTD values observed for different samples was assessed by comparison to the VarTD normal distribution. **(B)** Spearman's rank-order correlations between taxonomic diversity indices (TD and AvTD) and environmental variables.

A

Location, Time, Depth	Shannon (H')	Evenness	Richness	TD	AvTD	VarTD
Flix						
June 04 D5	6.87	0.994	1002	75.6	75.6	345.3 ns
June 04 D15	6.64	0.938	1189	76.4	74.9	348.2 ns
Feb 05 D5	6.50	0.934	1060	76.6	75.5	355.4 ns
Feb 05 D15	7.15	0.954	1796	75.7	74.9	353.0*
June 05 D5	6.97	0.947	1566	75. 3	74.9	345.6*
June 05 D15	6.44	0.932	998	75.7	74.8	354.3 ns
Feb 06 D5	6.07	0.928	688	75.5	74.8	348.3 ns
Feb 06 D15	6.98	0.951	1521	76.0	75.2	351.6 ns
Rice Fields						
June 04 D5	5.78	0.926	516	75.7	76.8	360.8*
June 04 D15	6.86	0.948	1383	74.9	74.3	352.7•
Feb 05 D5	6.72	0.944	1234	74.3	74.7	358.9*
Feb 05 D15	7.28	0.950	2138	74.8	74.8	353.6•
June 05 D5	7.34	0.959	2117	75.2	74.8	352.6•
June 05 D15	7.19	0.955	1856	74.6	74.6	355.3•
Feb 06 D5	5.87	0.934	536	73.1	74.2	344.0+
Feb 06 D15	6.59	0.937	1133	74.2	74.8	359.2 ns

B

	Location	Time	Depth	DM	TKN	TOC	TP	C/N	Pollutant
TD	-0.79•	ns	ns	ns	-0.65**	-0.73•	-0.53*	ns	0.66**
AvTD	-0.73•	ns	ns	ns	-0.61*	-0.67**	-0.47+	ns	0.60+

ns: not significant; +: P≤0.10; *P≤0.05; **: P≤0.01; •: P≤0.001

Shannon (H') diversity, richness, evenness and taxonomic (TD, AvTD and VarTD) diversity indices based on all detected genes of each sample are given in Table 4A. Shannon

indices varied within and between different sampling locations and sampling periods. No significant correlations between Shannon indices and environmental or spatial parameters (data not shown) were detected. The number of functional genes detected (richness) changed considerably among the samples and also showed no correlation to any of the environmental parameters (data not shown). Additionally the evenness was comparable among all sediment sampling times, locations and depths. Furthermore, Shannon diversity (H') indices were calculated for functional subsets, focusing on carbon, nitrogen and sulfate cycling genes (Appendix IV, Table A1). PCA analysis of Shannon diversity indices showed no significant correlation with the sampling location but could be significantly related to sampling depth, C/N ratio and DM content (Fig.2). When the complete data set was considered, in both locations, the top layers of the sediments (0-5 cm) were found to be less diverse than bottom sediments (10-15 cm) ($p \leq 0.05$). This difference was on average 10-15%, and was more pronounced in rice fields (19-29%) (data not shown). The maximum diversity was observed in cellulase, chitinase, *nifH* and *dsrA* like sequences. Between the two locations rice field

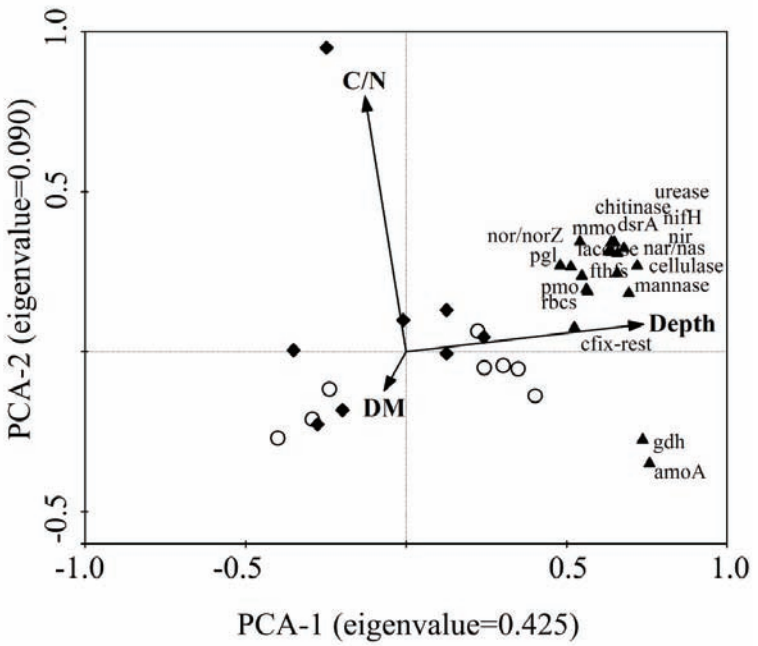


Figure 2. Ordination triplot for principal component analysis of Shannon (H') diversities of functional gene groups. Each functional gene group is displayed by a triangle (▲). Samples are grouped according to sample location (Flix river sediment, ♦; or rice field soil, ○). Arrows represent environmental parameters ($p<0.1$). The length of each gradient (eigenvalue) is indicated on the corresponding axis.

samples were more diverse and rich in these genes. Lowest gene diversity and richness was observed in nitrification (*amoA*) genes.

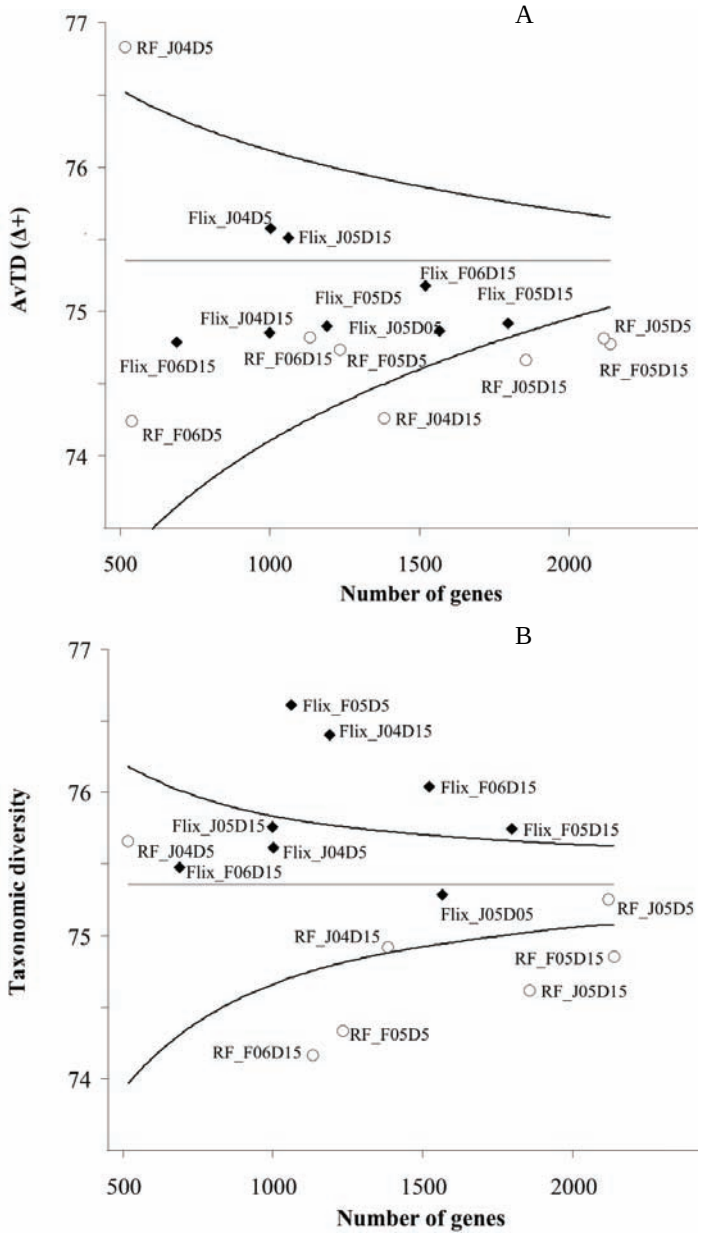


Figure 3. **(A)** Average taxonomic distinctness (AvTD) **(B)** Taxonomic diversity of 16 samples from Flix river sediment (♦) and rice field soils (○) plotted against the number of genes detected by GeoChip for each sample. Also shown are **(A)** AvTD expected and **(B)** the expected taxonomic diversity for all the samples (thin line) and the 95% confidence limits.

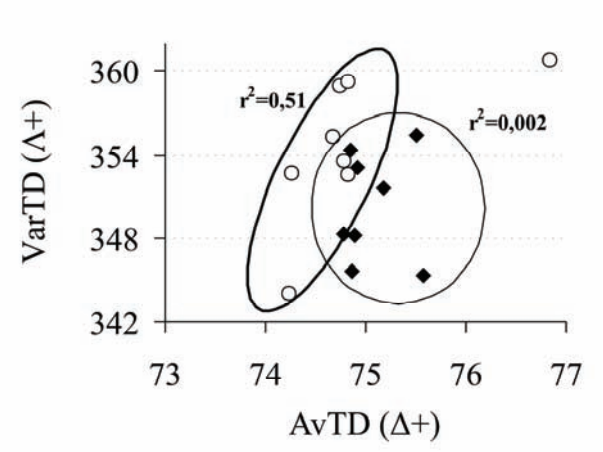


Figure 4. Scatter plot of VarTD against AvTD values from rice field soils and river sediment. Samples are grouped according to sample location (Flix river sediment, ♦; or rice field soil, ○).

Comparison of the average taxonomic distinctness (AvTD) with the expected range of values showed that most of the samples were within 95% confidence limits (Fig. 3A). AvTD and taxonomic diversity (TD) of river sediment samples did not increase significantly with increasing functional gene richness. However, for some of these samples, observed taxonomic diversity was higher than expected (Fig. 3B). On the other hand there were significant differences ($p \leq 0.05$) in AvTD of samples from rice field soils. Especially in samples from 10-15 cm depth, calculated AvTD and TD values were lower than expected, indicating a loss in the overall taxonomic diversity despite the high functional gene richness. TD and AvTD decreased significantly with increasing TOC, TKN and TP concentrations and increased with increasing pollutant concentrations (Table 4B). Overall, a narrow range for AvTD (74.2–76.8) and VarTD (344.0-360.8) values were observed. The highest taxonomic variation (VarTD) was observed in rice field soils. Correlation between in AvTD and VarTD was weak ($r^2=0.51$, $p \leq 0.001$), in rice fields, whereas in Flix no correlation could be observed (Fig. 4).

Distribution of functional gene groups

The variations in the functional gene diversity were explored by partitioning redundancy analysis with environmental and spatial variables (Table 5). Due to lack of measurements in sulphate and metal concentrations functional gene groups “sulfate reduction” and “metal reduction and resistance” were only tested for physical parameters. 69 different groups of contaminant degradation genes were detected by GeoChip (data not shown). From these groups, 20 genes were selected that either had the highest relative abundance or relate to the degradation of pollutants that were found in samples analyzed in this study.

When all the functional gene groups were analyzed together, sampling depth accounted for 10.3% of the overall gene variation. In total, sampling location, TKN and C/N could explain 16.6% of changes in the functional gene diversity. The model built by forward selection of the significant environmental parameters could explain 26.5% of the overall functional gene variation ($p \leq 0.1$). The distribution of the samples in the ordination space showed that for most of the samples, the functional gene content was correlated with either sampling depth or sampling location (Fig. 5). Most of the Flix river sediment samples were negatively correlated with high TKN concentrations. For the entire functional gene groups DM and sample depth explained the majority of the variation in diversity (Table 5). The two

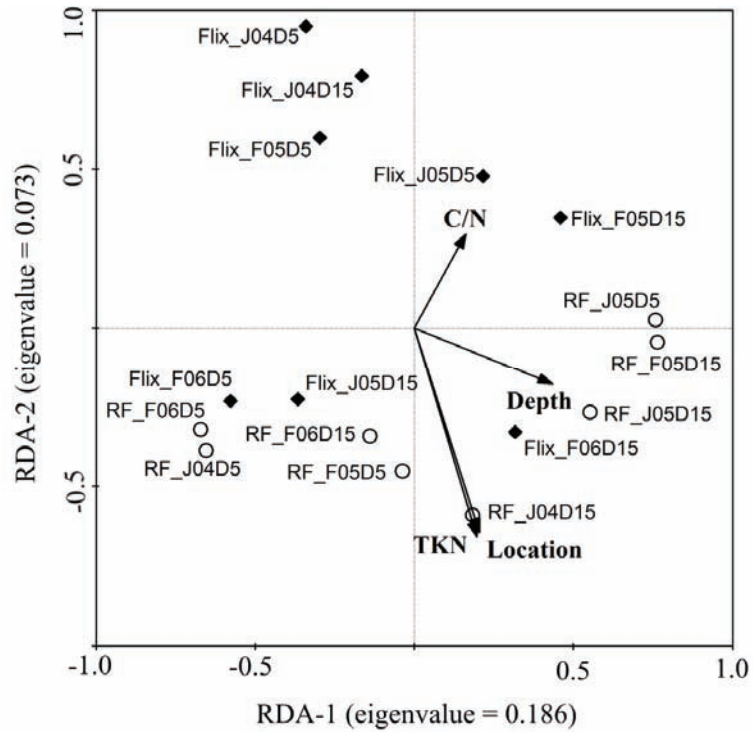


Figure 5. Ordination biplot for redundancy analysis of functional gene diversity. Samples are grouped according to sampling location (Flix river sediment, ♦; or rice field soil, ○). Arrows represent environmental parameters ($p<0.1$). The length of each gradient (eigenvalue) is indicated on the corresponding axis. Arrow length corresponds to variance that can be explained by the environmental variable. The direction of an arrow indicates an increasing magnitude of the environmental variable. Samples are labeled according to sampling location, sampling year and sampling depth (i.e. Flix_F06D5 being, Flix river sediment, sampled in February 2006 at 0-5 cm depth or RF_J04D15 being, rice field soils, sampled in June 2004 at 10-15 cm depth)

Table 5. Summary of the results obtained for RDA test for the significance of environmental variables in explaining the variance in each functional gene group. Percentages indicate the proportion of the variation in functional gene diversity that could be explained by the environmental variables.

	All genes	**Carbon Cycling**	**Nitrogen Cycling**	**Sulfate Reduction**	**Metal Reduction and Resistance**	**Contaminant Degradation**
Sampling location	5.0%*	8.9% *	8.1% +	9.8% ns	8.4% ns	12.4% •
Sample depth	10.3% +	7.9% +	9.4% +	11.4% +	8.9% +	8.1% +
Sampling time	6.4% ns	6.5% ns	5.0% ns	4.5% ns	6.5% ns	9.6% **
DM	9.9% ns	12.7% **	14.1% **	10.1% *	13.0% **	4.6% ns
TOC	10.1% ns	7.1% ns	6.9% ns	nt	nt	5.4% ns
TKN	4.3%+	8.9% *	8.2% +	nt	nt	4.5% ns
C/N	7.3% *	9.5% *	10.0% *	nt	nt	5.0% ns
TP	10.1% ns	5.2% ns	5.0% ns	nt	nt	5.8% ns
Pollutant	6.6% ns	4.8% ns	4.2% ns	nt	nt	6.7% ns
First RDA axis	18.6%**	27.2% *	30.5% **	19.2% +	18.5% *	15.0% *
Total	26.5%+	54.4%*	49.8%*	21.5%+	21.9%+	30.1% **

nt: not tested; ns: not significant; +: P≤0.10; *P≤0.05; **: P≤0.01; •: P≤0.001

sampling locations significantly explained the changes in carbon and nitrogen cycle genes and were insignificant for sulfate reduction genes and metal reduction and resistance genes. TKN and C/N were found to contribute to the explanation of the variation in carbon and nitrogen cycle genes. Explained variation in the contaminant degradation gene diversity mostly originated from sampling time, location and depth. Geochemical parameters and measured pollutant contamination had no significant effect on the diversity of these genes (Table 5).

Relative abundance of different gene families

With respect to the relative abundance of different gene groups, no significant correlation was found with any of the environmental and geochemical parameters measured in this study (data not shown). Therefore, to pinpoint those environmental parameters that affect the relative gene abundances, the Spearman's rank correlations for individual gene families (e.g. *amoA*) instead of functional gene groups (e.g. nitrogen cycling) were calculated and only the ones with significant correlation is reported (Table 6). The relative abundance of the carbon degradation genes encoding cellulase, chitinase and laccase decreased significantly with increasing TOC and was elevated in samples with high pollutant concentrations. Cellulase and laccase encoding genes were more abundant in Flix sediment whereas mannanase genes were more abundant ($p \leq 0.10$) in rice field soils (Table 6). Mannanase-encoding genes were also the only carbon cycle genes that correlated with sampling time. CO dehydrogenase-encoding genes showed a weak correlation with DM content of the samples. No significant correlations could be detected between environmental parameters and genes that code for 6-phosphogluconolactonase (*pgl*), methyl coenzyme M reductase (*mtr*), methane monooxygenase (*mmo*) and particulate methane monooxygenase (*pmo)*. Most of these genes originated from uncultured microorganisms. Methane generation genes from *Methanothermobacter thermautotrophicus* had the highest abundance and could be found in both sampling locations. Besides the genes from uncultured species, methane oxidation genes from *Methylocystis* sp. M, *Methylomonas* sp. KSPIII, *Methylococcus capsulatus* str. Bath and *Methylosinus trichosporium* were abundant in the sampling locations. While functional genes coding for protocatechuate 3,4 dioxygenase (*pca*) (EC 1.13.11.3) and *D*-cysteine desulfhydrase (*dcd*) (EC 4.4.1.15) were found to be the most abundant functional genes in rice fields, polygalacturonase genes (*pg*) (EC:3.2.1.15) were most abundant in river sediment (Table 6). *Pg* gene abundance was high at high pollutant concentrations ($r_s = 0.66$, $p<0.01$). *Pca* and *dcd* genes showed a significant positive correlation with TOC.

With respect to nitrogen cycle genes, no significant correlations were observed between relative abundances of nitric oxide reductase (*nor*) genes with environmental variables. Nitrite reductase (*nir*), nitrate oxide reductase (*nosZ*), nitrate reductase (*nar*/*nas*) and N_2-fixation (*nifH*) genes were positively correlated with sampling location indicating their higher abundance in rice field soils than Flix river sediment (Table 6). Relative abundance of

Table 6. Spearman's rank-order correlation of relative abundance of different functional gene families with environmental parameters. Functional gene families that did not show any significant correlations are not presented in this table.

	laccase	**mannanase**	***cox***	***nifH***	***amoA***	***gdh***	***nar/nas***	***nir***	***nosZ***
Time	ns	-0.49*	ns	ns	-0.49*	ns	0.44+	ns	ns
Depth	ns	ns	ns	ns	ns	ns	ns	ns	ns
Location	-0.81•	0.46+	ns	0.43+	ns	ns	0.43+	0.84•	0.67**
DM	ns	ns	0.46+	ns	ns	ns	ns	ns	ns
TKN	-0.60*	ns	ns	0.44+	-0.43+	0.51*	ns	0.71**	0.57*
TOC	-0.78•	ns	ns	ns	ns	ns	ns	0.75•	0.52**
C/N	ns	ns	ns	ns	ns	ns	ns	Ns	-0.48*
TP	-0.51*	ns	ns	ns	ns	ns	ns	0.63**	0.57*
Pest	0.66**	ns	ns	-0.46+	0.47+	ns	ns	-0.73•	-0.55*

	alkylS	***arylesterase***	***dcd***	***dma***	***hca***	***hoa***	***pca***	***pg***	***rdh***	**γ-HCH**
Time	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Depth	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.59*
Location	-0.66**	0.51*	0.46+	ns	0.79**	0.59*	0.43+	-0.84•	-0.43+	ns
DM	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.45+
TKN	-0.49+	0.67**	ns	0.55*	0.61*	0.54*	ns	ns	-0.47+	ns
TOC	-0.48+	0.67**	0.52*	0.66+	0.73**	0.63+	0.44+	-0.69**	ns	ns
C/N	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
TP	-0.59*	ns	ns	ns	0.62*	ns	0.45+	-0.43+	-0.61*	ns
Pest	0.63**	-0.64**	ns	-0.64**	-0.73**	-0.45+	ns	0.66**	0.56**	ns

Abbreviations: *cox*: CO dehydrogenase gene; *alkylS*: alkyl sulfatase gene; *arylesterase*: arylesterase gene; *dcd*: D-cysteine desulfhydrase gene; *dma*: dimethyl sulfoxide reductase gene; *hca*: phenylpropionate dioxygenase gene; *hoa*: 4-hydroxy-2-keto-pentanoic acid aldolase gene; *pca*: protocatechuate 3,4-dioxygenase gene; pg: polygalacturonase gene ; *rdh*: reductive dehalogenase gene; ns: not significant; +: P≤0.10; *P≤0.05; **: P≤0.01; •: P≤0.001

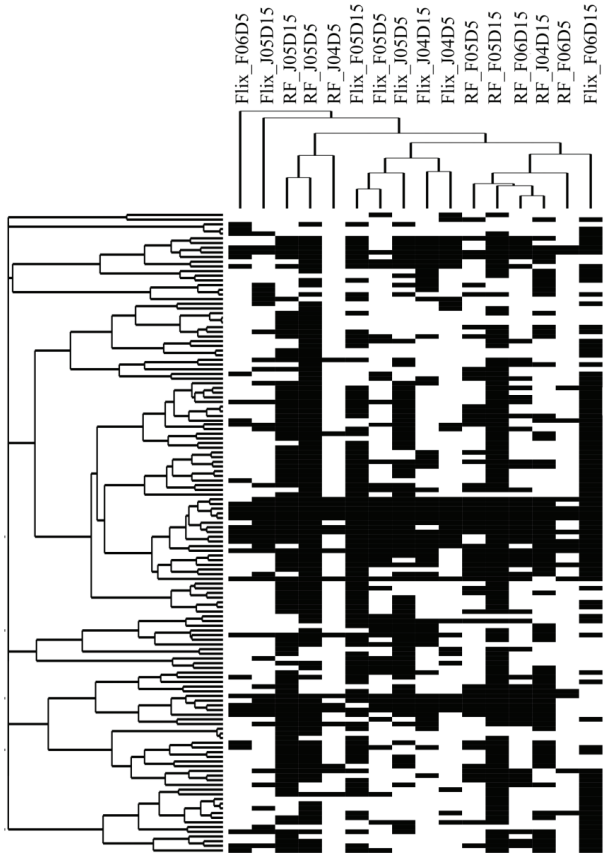


Figure 6. Hierarchical cluster analysis of *dsrA* gene-profiles based on hybridization signals for samples from Flix and Rice Fields (RF) in the Ebro River. White represents no hybridization above background level and grey represents positive hybridization. The grey-scale intensity indicates differences in hybridization signal intensity, with black representing the strongest signals. Samples are labeled according to sampling location, sampling year and sampling depth (i.e. Flix_F06D5 being, Flix river sediment, sampled in February 2006 at 0-5 cm depth or RF_J04D15 being, rice field soils, sampled in June 2004 at 10-15 cm depth)

a*moA* genes showed a significant decrease with sampling time, and there was a weak negative correlation with TKN concentrations. In contrast, there was an increase in relative abundance of nitrate reductase genes *nosZ* and *nir* with high TKN and TOC, where *nosZ* also had weak a negative correlation with C/N. Concentrations of these genes decreased with high pollutant concentrations. None of *nor* genes of cultured species was abundant in any of the samples (data not shown). Overall, relative abundance of nitrogen cycle genes was similar between the

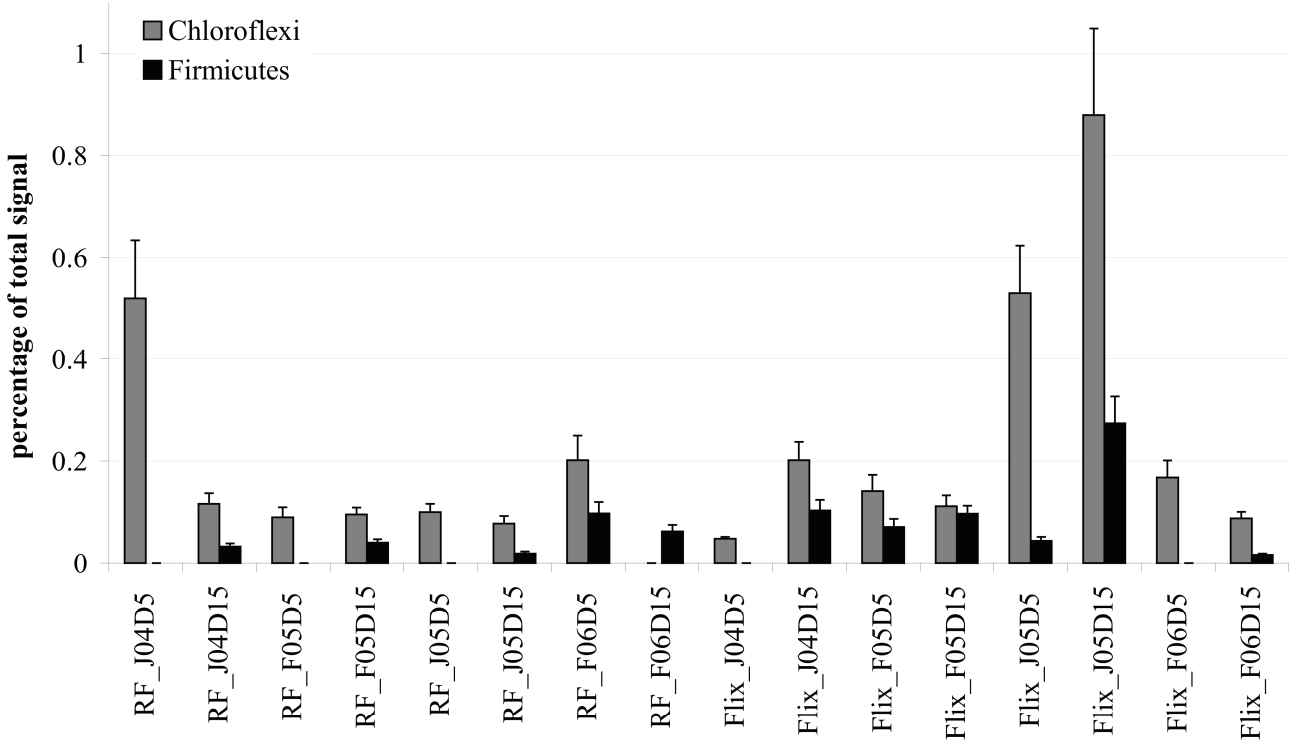


Figure 7. Relative abundance of *rdh* degradation genes. The value presented is total of the signal obtained for (all genes of) the respective phylum per sample. Error bars represented the standard error between the replicates. For sample labeling please refer to Figure 5.

two sampling locations. The only noteworthy difference was the higher abundance of denitrification genes in the rice field soils.

No significant correlations were observed between the detected sulfate reduction genes'(*dsrA*) relative abundance and environmental parameters (data not shown). Although most of the detected genes were from environmental clones or uncultured organisms (57% of all detected *dsrAB* genes), genes from *Desulfosporosinus orientis*, *Desulfovibrio desulfuricans* subsp. *desulfuricans* and *Pelotomaculum* sp. MGP were most abundant in both locations during the sampling period. Clustering of the *dsrA* genes did not result in any distinct pattern other than clustering by sampling locations (Fig. 6). The *dsrA* genes had a higher relative abundance (3.7-6.1% of the total signal) and lower diversity in Flix river sediment than rice field soils (2.2-5.6% of the total signal) (Appendix IV, Table A2).

Most abundant contaminant degradation gene groups did not show any significant correlation with sampling time. Moreover, there were no correlations between individual gene groups and corresponding individual pollutants (e.g. no correlations were found between hexachlorobenzene (HCB) concentrations and reductive dehalogenase-encoding gene abundances). The total signal obtained from the contaminant degradation genes was variable within and between the two locations (Appendix IV, Table A2). The most abundant contaminant degradation genes originated from *Actinobacteria* and *Proteobacteria* (Appendix IV, Table A3). Phenylpropionate dioxygenase (*hca*), arylesterase protein, dimethyl sulfoxide reductase (*dma*) and 4-hydroxy-2-keto-pentanoic acid aldolase (*hoa*) genes were negatively correlated with high pollutant concentrations (Table 3). From the four reported dehalogenases, which might be involved in aldrin and endrin degradation (21), only one (gi 26986508) was detected in rice field soils of 10-15 cm depth. Seven dehydrogenases which are involved in γ-HCH degradation were detected. These genes are originating from *Methanosarcina acetivorans* C2A, *Amycolatopsis mediterranei*, *Caulobacter crescentus* CB15, *Sphingobium japonicum*, *Sphingomonas paucimobilis* and *Xylella fastidiosa* 9a5c (gi 20089308, 7839576, 16124349, 4521186, 4587228, 24935279 and 15838327). These genes were more abundant in deeper levels of the sediment and soils. In addition, relative abundance of γ-HCH degradation genes was higher when DM content was high. 23 different reductive dehalogenase (*rdh*) genes were detected belonging to *Desulfitobacterium* spp., *Dehalococcoides* spp. and *Clostridium bifermentas* (Chapter 3). These genes showed a negative correlation with sampling location, indicating their higher abundance in Flix river sediments compared to rice field soils. These genes also positively correlated with pollutants, which in this part of the river originates predominantly from chlorinated organic compounds (23). Genes from *Dehalococcoides* spp. (*Chloroflexi*) were more abundant in the river system than those from *Desulfitobacterium* spp. and *Clostridium bifermentas* (*Firmicutes*) (Fig. 7). Relative gene abundances for reductive dehalogenase *cbdb1624* were significantly correlated to the number of gene copies estimated by qPCR per ng of DNA (r_s = 0.78, $p<0.001$). Several other

Table 7. Results of qPCR measurements based on reductive dehalogenase (*rdh*) genes of *Dehalococcoides* sp. CBDB1 (*cbrA*, *cbdbA1588* and *cbdbA1624*) and *Dehalococcoides ethenogenes* strain*195* (DET0079 and DET0318) and relative abundance of *cbdbA1624* gene as detected by GeoChip. qPCR is represented as *rdh* gene copies /ng DNA with standard deviations. Detection limit for *rdh* gene qPCR was 10 gene copies/ng DNA.

	qPCR					**GeoChip**
	cbrA (cbdbA84)	*cbdbA1588*[1]	*cbdbA1624*	*DET0079*	*DET0318*	*cbdbA1624*
Flix						
June 04 D5	nd	nd	3000 ± 240	nd	nd	0.019 ± 0.007
June 04 D15	nd	nd	nd	nd	nd	nd
Feb 05 D5	nd	nd	35000 ± 630	nd	nd	0.057 ± 0.034
Feb 05 D15	50±9	nd	nd	nd	nd	nd
June 05 D5	nd	nd	nd	nd	nd	nd
June 05 D15	nd	nd	110000 ± 87000	nd	nd	0.780 ± 0.064
Feb 06 D5	310 ± 73	nd	nd	nd	nd	nd
Feb 06 D15	350 ± 11	nd	nd	990 ± 120	310 ± 90	nd
Rice Fields						
June 04 D5	60 ± 38	nd	nd	210 ± 50	nd	nd
June 04 D15	62 ± 28	nd	nd	100 ± 27	nd	nd
Feb 05 D5	63 ± 24	nd	nd	nd	nd	nd
Feb 05 D15	120 ± 16	25 ± 2	nd	490 ± 120	500 ± 180	nd
June 05 D5	1100 ± 140	nd	nd	nd	560 ± 220	nd
June 05 D15	82 ± 22	nd	nd	22 ± 4	nd	nd
Feb 06 D5	160 ± 47	nd	nd	460 ± 92	390 ± 140	nd
Feb 06 D15	99± 9	11 ± 3	130 ± 10	100 ± 20	nd	nd

Abbreviations: nd: not detected

reductive dehalogenase genes from *Dehalococcoides* spp. which were not detected with the GeoChip (Table 7) could be amplified using qPCR. This indicates a detection threshold of the GeoChip for these genes of approximately 10^3 copies/ng DNA.

Discussion

River systems play an important role in the functioning of terrestrial ecosystems. Extending our understanding on the functioning of microbial processes in the river systems will help us to assess possible impacts of human activities. The functional gene microarray (GeoChip) is a powerful tool to gather information about all currently known microbially mediated C, N, S cycling and contaminant degradation processes (20). In this study we present the first in depth functional analysis of microbial communities in river sediments using the GeoChip.

Various tests of significance (Table 5 and 6) showed that functional gene diversity is restrained by several different environmental and spatial parameters. Distribution of functional genes between different microbial kingdoms demonstrated that in the Ebro River basin archaeal and eukaryotic communities are mainly involved in carbon cycling, whereas the bacterial communities are largely involved in contaminant degradation and nitrogen cycling. In general, statistical analyses showed an absence of correlation between diversity indices, species richness and relative abundance of genes with sampling time. However, this could be a result of the relatively short sampling period (two years), rather than a lack of temporal variability. The spatial parameters sampling depth and location correlated or significantly explained the changes in functional gene diversity and abundance, suggesting that different sediment depths and locations within the same river system emerge as distinct microenvironments. Both Shannon (H') diversity and taxonomic diversity indices varied more strongly and significantly in the rice field soils than in river sediments. Thus, it can be speculated that microbial species in Ebro River delta are under bigger stresses (i.e. deposition of upstream pollution, irrigation management, sea water intrusion and agricultural practices) than species in river sediments.

Carbon cycling

All of the detected carbon degradation genes - cellulase, laccase and mannanase- are involved in degradation of plant tissue. Significant correlation of cellulase and laccase encoding genes with river sediment suggests that decomposition of available vegetation is an important source of carbon input to this relatively carbon poor river sediment. Polygalacturonases, on the other hand, are enzymes secreted by fungal pathogens, and their degradation of pectin in the plant cell wall is believed to play a major role in tissue invasion and maceration (18). Additionally, laccase genes are not only involved in lignin degradation but are also associated with

degradation of phenolic compounds (9, 39). This might indicate their involvement in contaminant degradation in these locations as well. In the rice field soils, mannanase encoding genes were detected besides those coding for cellulases, chitinases and laccases. As a part of a predominantly photosynthetic process, carbon fixation genes were low in abundance and diversity in light-scarce systems like river sediment and deep layers of soil. Rice field soils are important contributors to the of global methane production (24). However, our results showed a low abundance and diversity of methane oxidation and production genes both in rice field soils and river sediments. Given the fact that most of these genes were originating from uncultured species, it is highly likely that the Ebro River system harbors unknown species of methane oxidizing and producing microorganisms.

Nitrogen cycling and sulfate reduction

The genes involved in N_2-fixation (*nifH*), denitrification (e.g. nitrite reductase genes *nir*), and sulfate reduction (e.g. *dsrA/B*) have previously been shown to be diverse in natural environments (62). Our results also support this proposition, since the majority of the genes detected in these gene groups were originating from various phyla and uncultured species of bacteria (Table 1B). Interestingly N_2-fixation (*nifH*) genes were positively correlated with high TKN concentrations. There are several other reported cases of high *nifH* gene abundance and diversity in estuary and river sediments in non N-limiting conditions (2, 7, 57). Strong positive correlation between nitrite reductase (*nir*) and nitrate oxide reductases (*nosZ*) and sampling location, TOC and TKN concentrations underlines the importance of denitrification in the nitrogen supply to the rice field soils. *AmoA* genes were found in low numbers and abundance at both locations. The decrease in the *amoA* abundance with high TKN concentrations and sampling time suggests a disturbance in conditions favourable for nitrification. However, this finding might also be the result of an experimental underestimation of the diversity of the nitrification genes. It recently has been shown that, in sediments, ammonia oxidizing archaea (AOA) are numerically more abundant and transcriptionally more active than ammonia oxidizing bacteria (AOB) (Leininger et al., 2006). Since recently characterized archaeal *amoA* genes were not included in the GeoChip probes, the observed richness of this particular gene family is likely to be lower than the actual value.

In anoxic environments, such as river sediments, sulfate-reducing bacteria are major contributors to carbon and sulfur cycles (28). Our results demonstrated the high abundance and diversity of *dsrA* genes in these samples, yet clustering analysis showed no clear separation between river sediment and rice field soils. On large distances (on a kilometer scale) sulfate-reducing communities have been shown to cluster with those from geographically widely separated sites and not necessarily with the site with the closest proximity (33). In our case clustering analysis not only supported this previous finding but also showed a homogeneous distribution and continuous diversification of the *dsrA* genes

during the sampling period. None of the environmental parameters correlated with *dsrA* gene relative abundance, and partitioning RDA analysis could only explain a small portion of the variance in *dsrA* gene diversity. It could therefore be speculated that these results are somehow biased. Given the fact that more than half of the *dsrAB* sequences were originating from environmental clones it is quite likely that high abundance and diversity of this group is a result of overrepresentation of these environmental sequences in comparison to the rest of the array.

Contaminant degradation

Microbial communities are largely involved in degradation of organic contaminants. Organisms degrading a wide range of these pollutants under aerobic and/or anaerobic conditions have been described (22). Many microorganisms developed specialized biodegradation pathways to degrade organic contaminants as a sole carbon or energy source (51). Contamination degradation genes, which were most abundant in the rice field soils, encode for enzymes involved in intermediate steps of aromatic compound degradation. While protocatechuate 3,4-dioxygenase catalyses a critical ring-opening step in the biodegradation of aromatic compounds (30), detected phenylpropionate dioxygenases were reported to be involved in pyrene degradation pathway (40). In addition, several other genes concerning aromatic compound degradation were detected in lower abundances (data not shown). Benzyl-CoA is produced in reductive benzoate degradation, which is the central pathway for the anaerobic biodegradation of aromatic pollutants (13). Anaerobic dimethyl sulfoxide reductases constitute cytoplasmic enzyme complexes that have a broad substrate specificity, ranging from S and N oxides (i.e. DMSO, trimethylamine N-oxide, adenosine N-oxide) to sodium chlorate and hydroxylamine (52). Naturally the presence of any gene in the environment can not be directly linked to the process catalysed by the encoded protein. However, the high abundance of these genes in the river sediment and rice field soils presents the potential for degradation of contaminants with aerobic and anaerobic processes.

Biological degradation of DDT to DDE and DDD, and of aldrin into dieldrin, by some bacteria and fungi was previously described (3, 31). Several pathways for aerobic, anaerobic and cometabolic degradation of these compounds were proposed (27, 35) yet none has been fully verified. Also, byproducts of these degradations, namely DDE and dieldrin, are believed to be more persistent in the environment than the mother compounds. In addition, it should be noted that these pesticides are not only toxic for macroorganims but also affect the functioning of the microorganism. For example aldrin has been shown to cause a decrease in denitrification rates and the number of denitrifying bacteria in water samples (25). Here, a weak negative correlation was found between the N_2-fixation (*nifH*), nitrite reductase (*nir*) and nitrate oxide reductase (*nosZ*) genes, which all showed a weak but significant negative correlation with pollutant concentrations (Table 6). Up to date, complete degradation of DDT or aldrin by a microorganism has not been reported (47). In the case of DDT, estimation of

microbial degradation in the environment becomes complicated by the fact that DDT can also be transformed to DDE via photochemical reactions (34). Given the fact that very little is known about the genes involved in the microbial transformation of these pollutants, it is challenging to demonstrate correlations with the detected functional genes. Changes in the diversity of the contaminant degradation genes were not significantly related to pollutant contamination. Nevertheless, though the observed effect was weak, TD and AvTD of microbial communities showed an increase with high pollutant concentrations. Furthermore, relative abundance of the most abundant contaminant degradation genes was in general negatively correlated to high pollutant levels. Both clustering and other statistical analyses consistently pointed towards the sampling location as the most important environmental parameter affecting the diversity and abundance of contaminant degradation genes. As a result it could be postulated that, unlike C,N,S cycling genes, the functional diversity and abundance of contaminant degradation genes will most strongly be controlled by pollutants rather than other environmental parameters.

Anaerobic reductive dehalogenation by *Dehalococcoides* spp. is the only known HCB biodegradation pathway, and results in formation of less chlorinated benzenes (1). The GeoChip results showed that the *rdh* genes of *Dehalococcoides* spp. were the most abundant in the overall river system. The presence and activity of HCB-degrading *Dehalococcoides* spp. in HCB contaminated river sediments was shown via 16S rRNA-targeted DGGE and qPCR analyses (**Chapters 3** and **5**). The GeoChip findings also suggested the presence of several other *rdh* genes besides those of *Dehalococcoides* spp. Quantitative PCR analysis of detected and several undetected *rdh* genes showed that GeoChip's detection limit for this group of genes was 10^3 *rdh* copies/ng DNA (Table 7). Previous applications of the GeoChip concluded that roughly 10^7 cells are needed to accomplish strong hybridization (36). Whole community genome amplification prior to GeoChip hybridization enhanced the sensitivity to such an extent that low-abundant genes could also be detected (54). Hence, the targeted *rdh* genes appear to be low in abundance in the river environments investigated in the present study. Since neither the targeted *rdh* genes nor any of the other dedicated biodegradation genes (like genes involved in atrazine, dioxin or benzene degradation) were found to be abundant it can be speculated that the Ebro river system might harbor novel genes for degradation of these compounds.

Conclusions and perspectives

Microarrays are powerful tools to evaluate the functional gene diversity in the environment. However, it remains very challenging to relate environmental changes to changes in microbial diversity in open and dynamic systems. Our results showed that even though the functional gene diversity and abundance changes, C, N and S cycling genes can always be detected in the river systems. However the direct influence of these genes on *Dehalococcoides* spp.

remains unclear. Understanding the underlying mechanisms behind the preservation of ecological functions will not only increase our knowledge on the ecological processes but could also result in better elucidation of functional gene data. The fact that microarrays can only detect known sequences causes an underestimation of functional gene diversity and abundance in different environments. Application of newly developed metagenomic methods (45) and/or GeoChip applications with environmental mRNA (15) can provide some indication towards this goal. In addition our results show that targeting dedicated microorganisms and enzymes could be more efficient for assessing the *in situ* conditions than targeting the entire ecosystem. Development and application of specialized microarrays, which can focus on organic contamination genes and their quantification, can be an ideal tool for decision making in the management of river basins.

Acknowledgements

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Chapter 05

Hexachlorobenzene Degradation Potential and Activity of Dehalococcoides spp. in European Rivers

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River basins are important sinks for chlorinated pollutants. The fate and persistence of these compounds is often a concern, because in many cases the prevailing environmental conditions don't support their degradation. *Dehalococcoides* spp. are important contributors to the degradation of chlorinated pollutants in the environment. For compounds like hexachlorobenzene (HCB), *Dehalococcoides* spp. are the only known microorganisms capable of anaerobic transformation. Therefore it is important to understand the effect of environmental parameters on the functioning of *Dehalococcoides* spp. and the dechlorination of HCB. *Dehalococcoides* spp. in the river sediments under study were exposed to different concentrations of nutrients and the effect of incubation temperature and medium salinity was assessed. HCB dechlorination could be linked to *Dehalococcoides* spp. activity by 16S rRNA based methods. Analysis of the transcription of reductive dehalogenase (*rdh*) genes showed the dominance of *cbrA* (encoding a chlorobenzene reductive dehalogenase), whereas *cbdbA*1624 (predicted to code for a 1,2,4-trichlorobenzene reductase) was transcribed at lower levels. Even though the observed growth yields were lower then previously reported for *Dehalococcoides* spp., this study demonstrates that *Dehalococcoides* spp. have the capability to dechlorinate HCB to lower chlorinated chlorobenzenes under a variety of environmental conditions.

Manuscript in preparation

Introduction

Hexachlorobenzene (HCB) has accumulated in the environment due to excessive usage in industry and agriculture and poor disposal standards in the early 1980's (5). HCB-containing pesticides and fungicides are no longer allowed in most of the world due to the toxic and carcinogenic nature of HCB. However, production and emission of the compound still occurs as an intermediate of chemical processes and from natural sources such as volcanoes (4, 15). Accepted as one of the twelve most persistent organic pollutants by the United Nations Environment Program, HCB poses a great risk for human health and wildlife (31). In aquatic environments HCB is mainly deposited in the sediment as a result of its hydrophobicity. Bacterial reductive dechlorination plays an important role in the degradation of chlorinated aromatic contaminants like HCB in anaerobic environments such as aquifers and river sediments (13, 19). Under anaerobic conditions bacteria can use HCB dechlorination in their energy metabolism by coupling reductive dehalogenation to electron transport phosphorylation. This is the only known pathway for the microbial degradation of HCB. Reductive dechlorination of HCB and its lower chlorinated derivatives was previously reported in river sediments at different rates, quantities and locations (7, 9, 18, 19). Nevertheless, in none of these studies, the microorganisms responsible for the process were identified.

Until now three strains of bacteria able to degrade HCB via reductive dechlorination could be isolated, namely *Dehalococcoides sp.* strain CBDB1, *Dehalococcoides ethenogenes* strain 195 and "Dehalobium chlorocoercia" DF-1. Both *Dehalococcoides sp.* strain CBDB1 and *D. ethenogenes* strain 195 dechlorinate HCB to 1,3-DCB (dichlorobenzene), 1,4-DCB and 1,3,5-TCB (trichlorobenzene) (3, 12). Moreover, production of 1,2-DCB was also observed with *D. ethenogenes* strain 195. A distant relative of *Dehalococcoides spp.*, "Dehalobium chlorocoercia" DF-1, dechlorinates HCB only to 1,3,5-TCB (34). Members of the genus *Dehalococcoides* are phylogenetically distant from other dechlorinating bacteria and grow on a variety of chlorinated compounds (29). Besides HCB, *Dehalococcoides sp.* strain CBDB1 can also degrade dibenzodioxins and polychlorinated ethenes, phenols and benzenes (1, 3, 8), whereas *D. ethenogenes* strain 195 can also use chlorinated ethenes and ethanes as electron acceptors (26). Several enzymes catalyzing the respiratory reductive dechlorination of chloroaromatics have been isolated and characterized (29), but none of them is involved in the initial dechlorination of HCB. Recently, however, a reductive dehalogenase-encoding gene from *Dehalococcoides sp.* strain CBDB1, *cbrA* (*cbdbA*84, GenBank accession number CAI82345) was characterized as a 1,2,3,4-TeCB (tetrachlorobenzene) and 1,2,3-TCB reductase (2).

The goal of this study was to assess the effect of different environmental conditions on the HCB dechlorination potential of *Dehalococcoides* spp. in contaminated river sediments

using batch microcosm incubations. River sediments were collected from three different locations in the Ebro River (Spain) and the Elbe River (Germany). Changes in the *Dehalococcoides* spp. activity and diversity were detected via 16S ribosomal RNA (rRNA)-targeted real-time quantitative PCR (qPCR) and denaturing gradient gel electrophoresis (DGGE), respectively. Terminal restriction fragment length polymorphism (TRFLP) was used to detect *rdh* gene diversity. Changes in the transcription levels of two genes from *Dehalococcoides sp.* strain CBDB1, *cbdbA*84 and *cbdbA*1624, a potential 1,2,4-TCB reductase encoding gene (33), were also explored. For comparison, the activity of two other dechlorinating genera (*Desulfitobacterium* and *Dehalobacter*) was also followed. Given the importance of river sediments as sinks for compounds like HCB, it is important to be aware of the effect of changing environmental conditions on the activity of *Dehalococcoides* spp. With the evidence presented in **Chapters 2, 3 and 4,** it is likely that HCB transformation end-products, *Dehalococcoides* spp. activity and *rdh* gene content are variable between different geographical locations. Furthermore, temperature was identified as one of the significant parameters that affect *Dehalococcoides* spp. composition and activity (**Chapter 3**), which suggests that phylogenetically different *Dehalococcoides* spp. may be responsible for HCB transformation at different temperatures. In addition, the effect of synthetic (basal) growth medium (20) and river water on the activity and composition of the genus was investigated. Finally, Ebro delta sediment was exposed to a range of sea salt concentrations to determine the impact of sea salt on the dechlorinating activity of sediments. To this end, this study aims to increase the knowledge with respect to the potential of these dedicated degraders for the biotransformation of HCB in these environments.

Materials and Methods

Chemicals

All chemicals were at least of analytical grade. HCB, 1,2,4,5-TeCB (tetrachlorobenzene), 1,2,3,5-TeCB, 1,3,5-TCB (trichlorobenzene) and 1,2-DCB (dichlorobenzene) were obtained from Aldrich Chemicals Co.Ltd. (Dorset, England). QCB (pentachlorobenzene), 1,2,3,4-TeCB, 1,2,4-TCB, 1,3-DCB and 1,4-DCB were purchased from Merck (Darmstadt, Germany). 1,2,3-TCB was from Janssen Chimica (Beerse, Belgium). All chemicals (purity >99%) were used as received without further purification. Sea salt (Sigma-Aldrich, St. Louis, MO) stock was prepared from an artificial salt mixture resembling the salt composition of ocean water.

Origin of the river water and sediments

Samples were collected from two European rivers, the Ebro in Spain and the Elbe in Germany, within the frame work of the European framework 6 project AquaTerra (6). Both rivers have a history of HCB contamination, but they are also polluted with other chlorinated compounds. Sediment samples from the Ebro River were collected in February 2005 in Flix (N 41º13'43.6'', E 00º33'09.0'') and in February 2006 both in Flix and Ebro River delta estuary (N 40°38'22.7'', E 00°42'39.5''). The sediment sample from the Elbe River was taken in October 2005 in Schönberg-Deich (N 52°90'73.2", E 11°87'21.9"). A river water sample was taken from Flix in February 2006. River water and sediment samples were taken approximately 1.5 m away from the river shore. A closed 0.5-1 liter sterilized air-tight jar was submerged and opened just above the water-sediment interface. The jar was completely filled with top layer sediment (approximately from 5 cm in depth) or with river water, closed before it was surfaced, and transported to the laboratory in a cooler. Sediments and water were stored at 4°C until further use.

Batch Experiments

The transformation of chlorinated benzenes was tested with 5 or 10 g (wet weight) of sediment in 120 or 250 ml glass bottles containing 50 or 100 ml basal medium (0.1g sediment/ml basal medium) as described earlier, but without the addition of the fermented yeast extract (20). The pH in the batch bottles was 7.0-7.3. The gas phase consisted of N_2 (80%) and CO_2 (20%). The bottles were sealed with Viton stoppers (Maag Technic AG, Dubendorf, Switzerland). Fifty mM lactate was added as electron donor, and 30 or 50 μM HCB were added from a stock solution prepared in acetone. Experiments were started up in an anaerobic glove box. The oxygen concentration in the anaerobic glove box was kept low with a palladium catalyst. The batches were incubated statically at 30°C in the dark, unless stated otherwise. All experiments were carried out in triplicate. Sterile control batches did not contain any sediment. Sterile sediment controls were prepared by autoclaving the sediments twice for 20 min at 120°C prior to the start of the experiment. Batch experiments were sampled in intervals of 7 to 10 days to monitor dechlorination. Liquid-phase extractions for chlorinated benzene measurements were performed on the sampling day. Around 1.5 ml of sediment-liquid mixture per sampling point were stored at -80°C for nucleic acid extraction. Four different sets of batch scale experiments were conducted between 2005 and 2007: (1) To test degradation of HCB in two river sediments, sediment samples taken in 2005 from the Elbe and the Ebro rivers were used. (2) To assess the effect of basal medium compared to river water, samples that were collected in February 2006 from the Ebro River were used. The batch experiments were started within 2 weeks of sampling. In this experiment two sets (n=3) of batch scale bottles with river water as a growth medium were prepared with and without addition of an external electron (lactate) donor. River water controls (without sediment

addition) were prepared to check if dechlorination occurs in the river water. Experiments that addressed the effect of temperature (3) and sea salt (4) were started in October 2006 with sediments collected in February 2006 from the Ebro River.

Analytical Methods

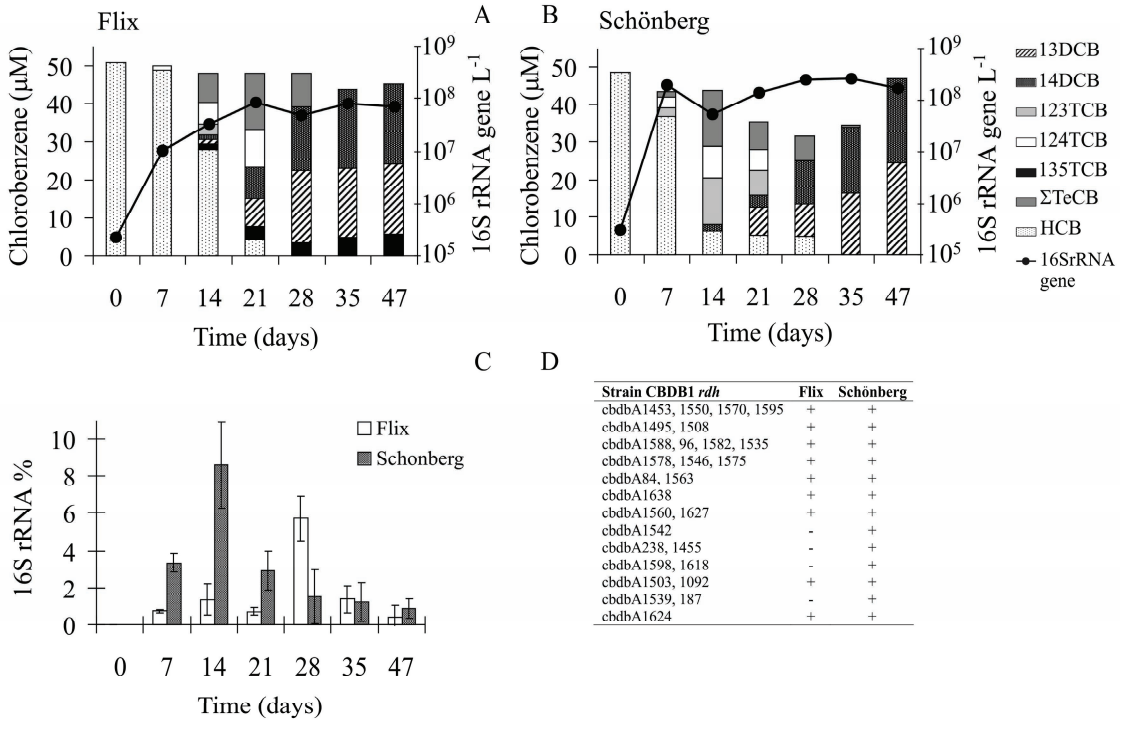
Total masses of the chlorinated benzenes were determined by GC-mass spectrometry analysis of liquid phase samples. One ml sediment culture fluid was extracted to 1ml hexane-acetone (4:1, vol/vol) solution via sonication for 20 min followed by overnight mixing. One µl of this solution was injected into a TRACE DSQ System (TRACE DSQ MS Detector and TRACE GC ULTRA; Milan, Italy) equipped with an injection splitter (split ratio, 2:1) and a FID detector connected to a capillary column (30 m by 0.25 µm [inner diameter], Rtx® 5MS [0.25 mm thick], Restek, PA). The carrier gas was helium, and the inlet pressure was 3 kPa. The operating temperatures of the injector and detector were 220 and 250°C, respectively. The column was operated with the following temperature program: initial column temperature, 60°C (1 min); increase of 5°C/min to a final temperature of 180°C (3 min). Output data were analyzed with TRACE Xcalibur Data System 1.3 (Milan, Italy). The chlorobenzenes were quantified using 1,2-DCB and HCB as the internal standards. For each bottle duplicate measurements were made. Graphs were drawn as an average of triplicate bottles.

16S rRNA targeted reverse transcription, Real-Time PCR and DGGE

Extraction of nucleic acids, cDNA synthesis, PCR, denaturing gradient gel electrophoresis (DGGE), cloning and sequence analysis were performed as described previously (**Chapter 3**). Real-time quantitative PCR (qPCR) was performed using an iQ5 iCycler (BioRad, Veenendaal, Netherlands) with the primers and thermocycling program as previously described (30) for 16S rRNA genes of dehalogenating bacteria (*Dehalococcoides*, *Desulfitobacterium*, *Dehalobacter*) and Universal Bacteria using SYBR Green Dye. From these data, the relative abundance of 16S rRNA copies of dechlorinating bacteria compared to the number of total bacterial 16S rRNA copies was calculated.

Reductive dehalogenase gene diversity and expression

Terminal restriction fragment length polymorphism (TRFLP) was used to fingerprint reductive dehalogenase gene diversity with methods described elsewhere (33). For the analysis of gene expression, cDNA was synthesized from 0.1 µg of total RNA using SuperScriptTM III First-Strand Synthesis System (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. The expression of the reductive dehalogenase-encoding genes of *Dehalococcoides* spp. CBDB1 *cbrA* (AJ965256.1) and cbdbA1624 (AJ965256.1) were detected via qPCR using an iQ5 iCycler (BioRad, Veenendaal, Netherlands) with the primers and thermocycling program as previously described (33).



Strain CBDB1 *rdh*	Flix	Schönberg
cbdbA1453, 1550, 1570, 1595	+	+
cbdbA1495, 1508	+	+
cbdbA1588, 96, 1582, 1535	+	+
cbdbA1578, 1546, 1575	+	+
cbdbA84, 1563	+	+
cbdbA1638	+	+
cbdbA1560, 1627	+	+
cbdbA1542	-	+
cbdbA238, 1455	-	+
cbdbA1598, 1618	-	+
cbdbA1503, 1092	+	+
cbdbA1539, 187	-	+
cbdbA1624	+	+

Figure 1. (A-B) Degradation of 50µM HCB in river sediment microcosms from Flix (Ebro, Spain), and Schönberg (Elbe, Germany). Bars represent concentration of HCB and daughter products. Lines represent *Dehalococcoides* spp. 16S rRNA gene copies/L. Error bars represent the standard deviation of duplicate measurements (standard deviations are not displayed if they are smaller than the symbol). **(C)** Changes in the relative abundance (%) of *Dehalococcoides* spp. 16S rRNA copies as compared to total bacterial 16S rRNA molecules. Error bars represent the standard deviation of duplicate measurements. **(D)** TRFLP fingerprinting of *Dehalococcoides* sp. strain CBDB1 reductive dehalogenase (*rdh*) genes in from Flix and Schönberg sediment microcosms. (+) and (-) represent presence or absence of the *rdh* gene, respectively. Genes that did not give any results in both cases are not presented.

Substrate consumption rate and observed growth yield

Substrate consumption rate was calculated in the point of total HCB depletion. Kinetic constants used in the growth yield calculation are included in Appendix V.

Table 1. Comparison of observed growth yields of *Dehalococcoides* spp. in sediment microcosms

Microcosm	Sampling Year	e^- donor	Observed Growth Yields (16S rRNA gene copies /μmol Cl^-)	Growth Temperature	Growth Medium
Flix (1)	2005	+	$(4.6\pm0.3) \times 10^5$	30°C	dechlorinating medium
Schönberg	2005	+	$(1.4\pm0.2) \times 10^6$	30°C	dechlorinating medium
Flix (2)	2006	+	$(6.3\pm0.9) \times 10^5$	30°C	river water
Flix (3)	2006	-	$(7.5\pm0.1) \times 10^5$	30°C	river water
Flix (4)	2006	+	$(3.5\pm0.1) \times 10^6$	30°C	dechlorinating medium
Flix (5)	2006	+	$(2.7\pm0.3) \times 10^6$	15°C	dechlorinating medium
Estuary (1)	2006	+	$(6.3\pm0.8) \times 10^5$	30°C	dechlorinating medium[a]
Estuary (2)	2006	-	$(6.0\pm0.4) \times 10^6$	30°C	dechlorinating medium[a]
Estuary (3)	2006	+	$(3.2\pm0.4) \times 10^6$	30°C	dechlorinating medium[b]

(+),with lactate as e^- donor; (-),without lactate; [a]0.0M and [b]0.2M sea salt added to growth medium

Results

Dechlorination of HCB in river sediment from different geographical locations

The primary objective of this experiment was to test the dechlorination potential of HCB by *Dehalococcoides* spp. in the river sediments under a variety of different environmental conditions, including changes in nutrient concentration, temperature and salinity. All of the sediments used in this study were collected from river basins, which have been exposed to the compound during the past decades (14, 24). Fifty μM HCB was completely dechlorinated in all the batch microcosms from HCB contaminated river sediments of Flix (Ebro River, Spain) and Schönberg (Elbe River, Germany) (Fig.1A and 1B). No dechlorination was observed in the sterile controls in any of the tested conditions (data not shown). Dechlorination of HCB started somewhat earlier in the Schönberg sediment batches than in the Flix batches. In the Schönberg sediment batches 87% of HCB was dechlorinated within two weeks, after which the apparent concentration of HCB did not change for another two weeks. However, a continued increase in 1,4-DCB and 1,3-DCB concentrations was observed. After 47 days, HCB was quantitatively converted to 1,4- and 1,3-DCB. In the Flix sediment batches dechlorination started in the second week and HCB was completely depleted within four weeks. In both cases (Flix and Schönberg) 1,4-DCB and 1,3-DCB were produced in approximately equal quantities. The only substantial difference between the dechlorination pattern of HCB in the two locations was the absence of 1,3,5-TCB as an end-product in the

Schönberg sediment batches. Throughout the experiment (91 days) monochlorobenzene (MCB) production was never detected. As the dechlorination of HCB started, 16SrRNA gene copy numbers of *Dehalococcoides* spp. increased rapidly to approximately 10^8 copies/L. However, dechlorination proceeded without further increase in 16S rRNA gene copy numbers (Fig. 1), this was also observed in *Dehalococcoides*-containing enrichment cultures and isolated strains (11, 21, 22, 26). The highest abundance (Fig. 1C) of *Dehalococcoides* spp. 16S rRNA copies could be detected after most of the HCB was dechlorinated (compare with Fig. 1A-B). There was no clear change in the relative abundance of *Desulfitobacterium* spp. or *Dehalobacter* spp. 16S rRNA copies during HCB dechlorination (data not shown). The observed growth yield for *Dehalococcoides* spp. was three times higher in Schönberg batches when compared to Flix batches (Table 1). *Dehalococcoides* spp. 16S rRNA targeted DGGE analysis showed that there were no changes in the composition of the *Dehalococcoides* spp. population during dechlorination (data not shown). The 16S rRNA fragments corresponding to the dominant DGGE band were sequenced. The DGGE band corresponded to a single *Dehalococcoides* strain (EU700498) which had 98.1% (~1200bp) similarity to *Dehalococcoides* sp. strain CBDB1 and 96.8% similarity to *Dehalococcoides ethenogenes* strain 195 in the Flix sediment batches. Likewise, the active *Dehalococcoides* strain in the Schönberg batches (EU700501) had a sequence identity of 97.9% to *Dehalococcoides* sp. strain CBDB1 and 96.6% to *Dehalococcoides ethenogenes* strain 195. These two strains were found to be 98.2% similar to each other. Furthermore, we have used TRFLP to assess whether any of the *Dehalococcoides* spp. in the river sediments possessed *rdh* genes corresponding to those identified in the genome of *Dehalococcoides* sp. strain CBDB1. Samples taken in the third week of dechlorination showed that the Schönberg sediment batches had 28 of the 32 *rdh* genes from *Dehalococcoides* sp. strain CBDB1 (Fig.1D). The number of *rdh* genes in the Flix sediment batches was found to be less than Schönberg sediment batches. The recently characterized chlorobenzene reductase-encoding gene, *cbrA*, could be detected in both of the sediment batches.

Dechlorination of HCB in in-situ conditions

To mimic the *in situ* conditions, the Flix sediment was subjected to river water instead of the defined dechlorinating medium. Batch scale anaerobic bottles were kept at 26-28°C, which corresponds to the higher end of the summer water temperatures (10). In all of the river sediment batches, methane production could be observed 20 days after the start of the experiment (data not shown), indicating the development of fully methanogenic conditions. HCB dechlorination in bottles with lactate started after 10 days while bottles without an external electron donor had a lag phase of 30 days before dechlorination could be observed. Full dechlorination of HCB was achieved in both cases. End products were similar to those observed in the previous experiment –with dechlorinating medium-, namely 1,3,5-TCB, 1,3-

DCB and 1,4-DCB. However, more 1,3,5-TCB was produced (Compare Fig 1A and 1B and Fig. 2A and 2B). Molecular screening showed that the activity of *Dehalococcoides* spp. (based on 16S rRNA) increased during dechlorination, covering 0.5-0.9% of the bacterial 16S rRNA pool, both in the absence and presence of the external electron donor, respectively (Fig. 2D). Bottles with the external electron donor showed a pattern of activity similar to that observed in the bottles with a defined medium. On the other hand incubations without an external electron donor showed a more gradual increase and decrease in the 16S rRNA copy numbers, as well as a two-fold lower maximal relative abundance. Comparison of DGGE fingerprints showed that active species in the sediment batches had the same fingerprints as *Dehalococcoides* sp. CBDB1. On the other hand, these fingerprints were different than those from the predominant endogenous populations observed in the original sediment samples from the sampling location (Fig. 2C).

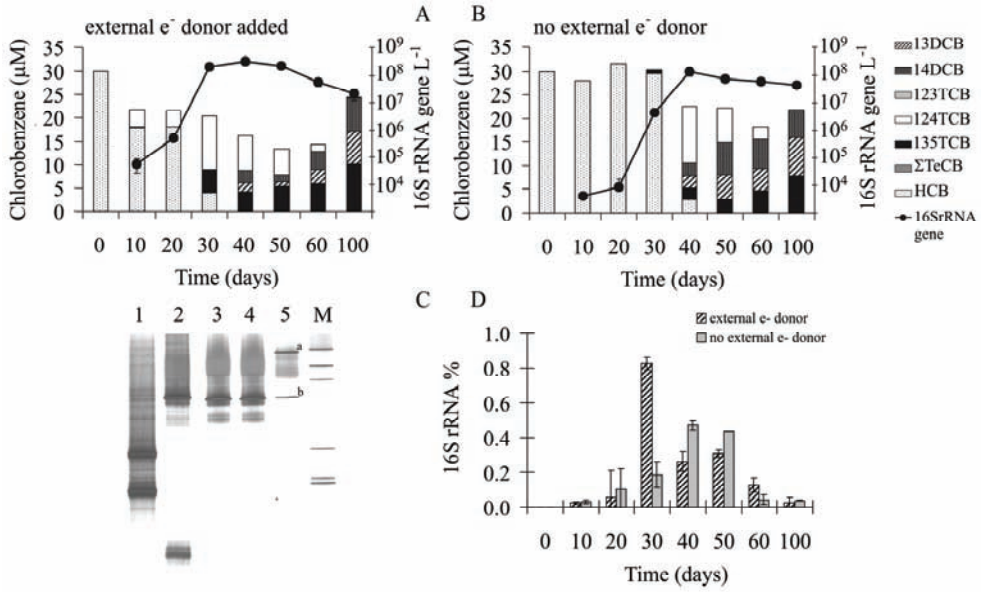


Figure 2. (A-B) Degradation of 30µM HCB in river sediment microcosms from Flix (Ebro, Spain). Bars represent concentration of HCB and the end products of degradation with and without electron donor added. Line represents *Dehalococcoides* spp. 16S rRNA gene copies/L. Error bars represent the standard deviation of duplicate measurements (standard deviations are not displayed if they are smaller than the symbol). Lacking data points indicate that concentrations were below detection limit **(C)** DGGE fingerprints of environmental samples and river sediment microcosms (Lane 1,2) Flix sediment from 2005 and 2006; (Lane 3) River water enrichment; (Lane 4) Dechlorinating medium enrichment; (Lane 5) a: *Dehalococcoides* sp. Strain 195 b: *Dehalococcoides* sp. CBDB1; (Lane M) Marker **(D)** Changes in the relative abundance (%) of *Dehalococcoides* spp. 16S rRNA copies as compared to total bacterial 16S rRNA molecules during HCB dechlorination in river water. Error bars represent the standard deviation of duplicate measurements.

Dechlorination of HCB at different temperatures

The effect of different temperatures on the reductive dechlorination in Flix river sediment was measured by subjecting the sediment to temperatures ranging from 4 to 37°C. Degradation of 30μM HCB was observed at all tested temperatures, except at 4°C (Fig. 3). Even though 30°C seemed to be the optimal temperature; the activity of the sediment at 25°C was still high enough to complete the dechlorination process in a similar time frame. However, at temperatures below 20°C or above 30°C the speed of dechlorination dropped considerably (Fig. 3). The results indicated that it was still possible to have reductive dechlorination at low

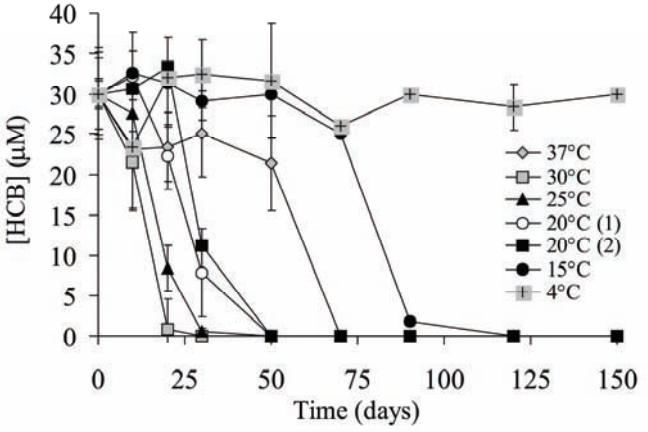


Figure 3. Transformation of HCB at various temperatures in Flix river sediment with dechlorinating medium as a growth medium. Data points are averages of duplicate measurements of triplicate bottles. Error bars represent the standard deviation between the triplicates. "20°C (1)" represents the batch microcosm at 20°C with lactate as an e- donor. "20°C (2)" represents the batch microcosm at 20°C without any additional e^- donor.

temperatures, yet the time needed for complete degradation almost doubled (from 50 days at 20°C to more than 100 days at 15°C). There were no significant differences between the sediment batches incubated at 15°C and 30°C in the observed growth yields (Table 1), nor with respect to the concentration or nature of the end products of the process (Fig. 4A and 4B). A rapid increase in 16S rRNA gene copies of *Dehalococcoides* spp. parallel to HCB depletion was observed at 30°C. Likewise, the number of 16S rRNA gene copies increased gradually at 15°C, reaching its highest level at the point of total depletion of HCB. The relative abundance of *Dehalococcoides* spp. 16S rRNA reached its highest level (5.2±1.0% at 30°C and 5.5±1.2% at 15°C; Fig. 4D) during HCB dechlorination. DGGE fingerprinting showed that the active species in the batches had the same fingerprints (data not shown). This experiment showed that similar to the laboratory isolates (3, 17, 26), naturally occurring

Dehalococcoides spp. are mesophilic with an optimum temperature of 25 to 30˚C. However, it was still possible to achieve complete dechlorination at suboptimal temperature.

Dechlorination of HCB in estuary sediment

The Ebro delta estuary is exposed to a wide range of pollutants carried by the river (24) and has a dynamic profile of sea salt concentrations due to tidal movements and irrigation practices (28). Previous studies indicated that the salt concentrations at the sampling location ranged from 0 to 0.5M (28). The influence of several factors (changing sea salt concentrations and external electron donor addition) on the reductive dechlorination of HCB in the estuary sediment was studied for a period of 70 days. The HCB concentration decreased gradually during the first 20 days in the sediments amended with 0.0-0.2M sea salt, whereas no dechlorination was observed in sediment with 0.5M sea salt during the experimental period (Fig. 5).

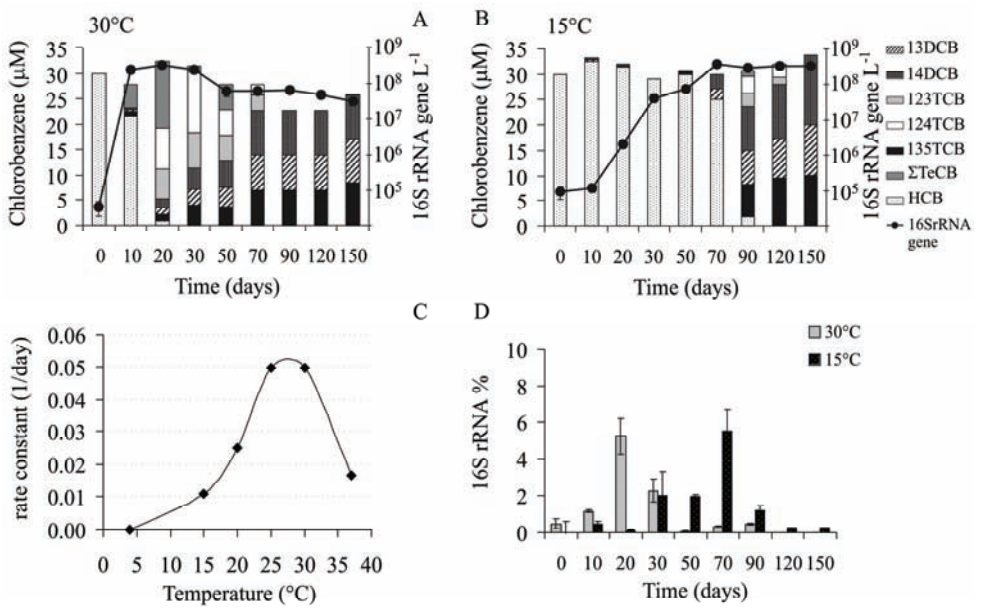


Figure 4. (A-B) Degradation of 30μM HCB in river sediment microcosms from Flix (Ebro, Spain), at 30°C and 15°C. Bars represent concentration of HCB and end products. Line represents *Dehalococcoides* spp. 16S rRNA gene copies/L. Error bars represents the standard deviation of duplicate measurements. **(C)** Substrate consumption rate (1/day) of *Dehalococcoides* spp. during HCB dechlorination at different temperatures. **(D)** Changes in the relative abundance (%) of *Dehalococcoides* spp. 16S rRNA copies as compared to total bacterial 16S rRNA molecules during HCB dechlorination. Error bars represent the standard deviation of duplicate measurement.

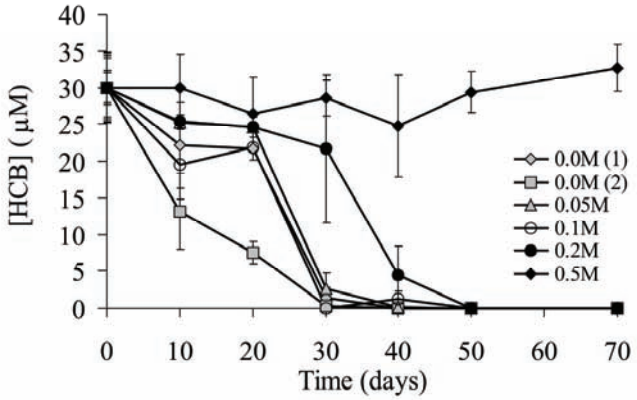


Figure 5. Degradation of HCB in various sea salt concentrations in Ebro delta estuary, Spain, with dechlorinating medium as a growth medium. Data points are averages of duplicate measurements of triplicate bottles. Error bars represent the standard deviation between the triplicates. "0.0M (1)" represents the batch microcosm without addition of sea salt but with an e-donor. "0.0M (2)" represents the batch microcosm without addition of lactate and sea salt.

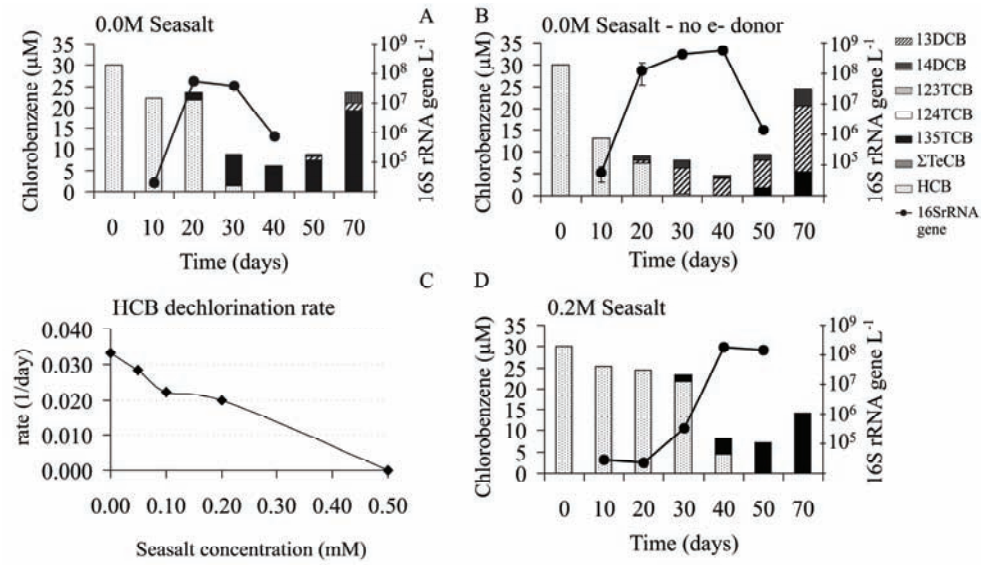


Figure 6. (A-B-D) Degradation of 30µM HCB in river sediment microcosms from Ebro delta estuary, Spain, in different sea salt concentrations. Bars represent concentration of HCB and end products. Line represents *Dehalococcoides* spp. 16S rRNA gene copies/L. Error bars represent the standard deviation of duplicate measurements. Standard deviations smaller than figure bullet are not shown. Lacking data points indicate that concentrations were below the detection limit. **(C)** Substrate consumption rate (1/day) of *Dehalococcoides* spp. during HCB dechlorination at different sea salt concentrations.

Interestingly, sediments incubated without the addition of an external electron donor showed increased rates of HCB dechlorination compared to sediments with addition of lactate. Moreover, the distribution and the quantity of the end products were different. Lactate-amended sediments dechlorinated HCB primarily to 1,3,5-TCB whereas sediments without an external e^- donor dechlorinated HCB primarily to 1,3-DCB (Fig. 6 A,B,D). High sea salt concentrations clearly delayed the dechlorination. We only detected 1,3,5-TCB as an end product in the presence of 0.2M sea salts. Overall, HCB degradation rates (day^{-1}) observed in the estuary sediment were lower than those found in river sediments and further decreased with increasing sea salt concentration (Fig. 6C). At the start of the experiments 16S rRNA gene copy numbers of *Dehalococcoides* spp. were below the detection limit, but increased as HCB dechlorination proceeded. Unlike with the river sediments, *Dehalococcoides* spp. 16S rRNA gene copies eventually decreased below the detection limit after HCB was depleted (Fig. 6A,B,D). Moreover, *Dehalococcoides* spp. 16S rRNA copies were only 1.1-3.1% of total bacterial 16S rRNA molecules (Fig. 7). Another noteworthy difference with the results obtained with Flix and Schönberg sediments was the increased relative abundance (up to 2.1%) of *Desulfitobacterium* spp. in the early and later phases of the experiment. Again, active *Dehalobacter* spp. could not be detected.

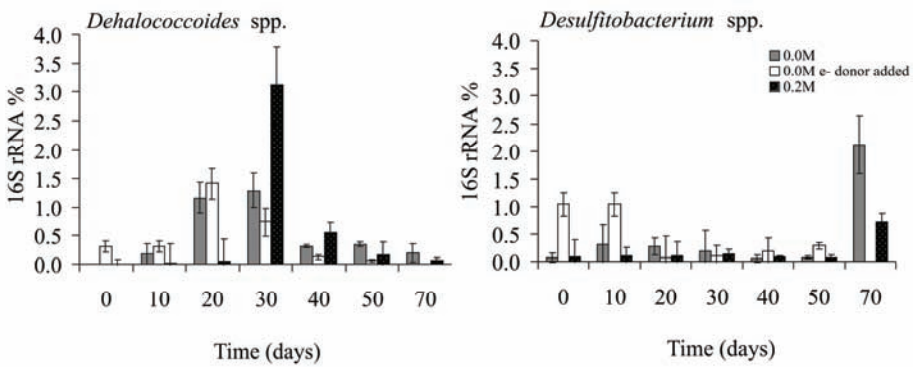


Figure 7. Changes in the relative abundance (%) of *Dehalococcoides* spp. 16S rRNA copies as compared to total bacterial 16S rRNA molecules during HCB dechlorination in microcosms from Ebro delta estuary, Spain, in different sea salt concentrations. Error bars represent the standard deviation of duplicate measurements. Data points not shown were below the detection limit of 10 copies/gr sample.

rdh gene transcript levels in sediments under different environmental conditions

Transcript levels of *cbrA* and *cbdbA*1624 were normalized to 16S rRNA and examined during the transformation of HCB at two different temperatures in Flix sediments and two different sea salt concentrations in Estuary sediments (Fig. 8). In most of the batches, transcription of

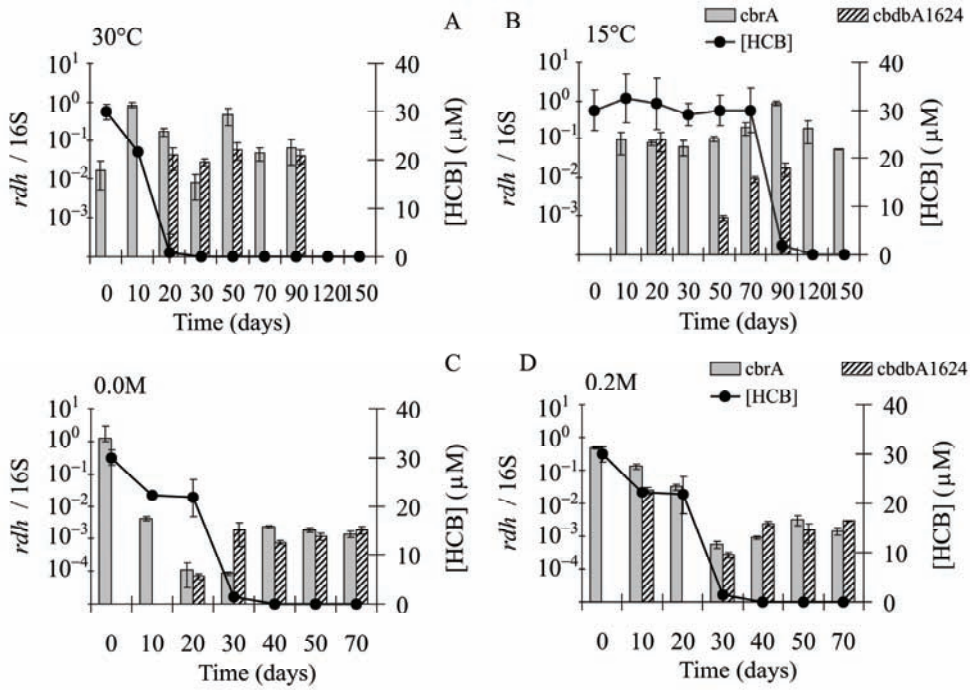


Figure 8. Normalized expression of *cbrA* and *cbdbA1624* genes at two different temperatures **(A-B)** and sea salt concentrations **(C-D)**. Normalization was based on 16S rRNA copies. Bars represents the ratio of each *rdh* transcript to 16S rRNA. Error bars represents the standard deviation of duplicate measurements. When not detected, the gene expression in samples is not displayed. Line represents changes in HCB concentration; error bars represent average measurements of triplicates.

cbdbA84 was already detected at the start of the experiment whereas transcription of *cbdbA*1624 could only be observed during the later phases of dechlorination. During the degradation of HCB, transcription levels of both genes were higher in the river sediment compared to the estuary sediment. In the river sediments at 30°C, the relative transcript levels of *cbrA* increased an order of magnitude compared to the beginning of the experiment (Fig. 8A). Transcript levels were 3 to 50-fold higher than start-up transcript levels during the dechlorination of HCB to TCB and DCBs. When detected, transcription of *cbdbA*1624 was less than of *cbrA*. At 15°C transcription of the two genes was not detected at the start-up of the experiment. Once HCB dechlorination started the *cbrA* transcription level increased by one order of magnitude (Fig. 8B). On the other hand, *cbdbA*1624 did not follow a particular pattern and its transcription level remained clearly lower than *cbrA* transcription, except for the sample taken at day 20 of incubation. In the estuary sediments, similar to the river sediment, transcription of *cbrA* was observed at the start of the experiment in all of the batches (Fig. 8C and 8D). During and after dechlorination of HCB, the normalized amount of

cbrA transcripts was 2-4 orders of magnitude lower than the starting levels. Transcription of *cbdbA*1624 was first detected when *cbrA* transcription was minimal, which corresponds to 20-30 days of incubation. Batches with 0.0M-0.2M sea salt had a similar transcription pattern whereas batches without external electron donor (0.0M sea salt) were different from both (data not shown). The number of *Dehalococcoides* 16S rRNA copies present in batch cultures was compared to the sum of *rdh* gene transcript copies (*cbdbA*84 and *cbdbA*1624). This comparison was made to determine whether or not a positive linear correlation existed between 16S rRNA and *rdh* gene transcription (Fig. 9A and B). The only significant linear correlation was found in the Flix sediment batches at 15°C showing that, as the *Dehalococcoides* spp. become active, these genes were also expressed. On the other hand, the correlation for the Flix sediment batches at 30°C and the Ebro delta estuary was not as strong.

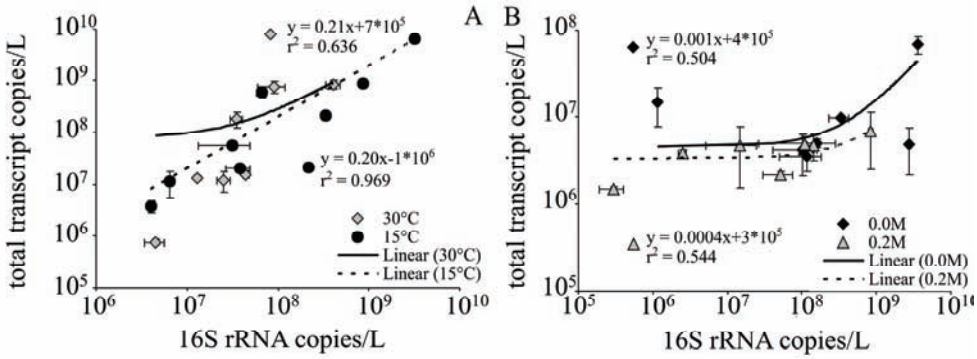


Figure 9. Sum of *rdh* concentrations compared to Dehalococcoides spp. 16S rRNA **(A)** in Flix sediment microcosms at two different temperatures **(B)** in Ebro delta estuary sediment microcosms in two different sea salt concentrations. Error bars represent the standard deviation calculated from duplicate measurements. Transcript numbers are given in logarithmic scale. Each error bar represents the standard deviation for the quantitative PCR method (measured in duplicates). Dashed and straight lines represent fitted linear regression between two variables.

Discussion

In this study we show the effect of several environmental factors on the biodegradation of HCB and link the effects to changes in the activity and growth of *Dehalococcoides* spp.

Differences between *Dehalococcoides* spp. of HCB-contaminated river sediments

Significant increases in the 16S rRNA relative abundance and 16S rRNA gene copy numbers of *Dehalococcoides* spp. were observed in all the sediment batches in which HCB was degraded. Results suggest that the *Dehalococcoides* spp. in these sediments are responsible for the observed reductive dechlorination of HCB to mainly 1,3-DCB, 1-4,DCB and 1,3,5-TCB. The end products of HCB dechlorination and their quantities, however, as expected

were different at different conditions and for different sources of inoculum (Fig. 1, Fig. 4). Likewise, growth yields of *Dehalococcoides* spp. from different locations or sampling periods were notably different (Table 1).

Sequence analysis of 16S rRNA gene fragments and TRFLP fingerprinting of *rdh* genes confirmed the differences between *Dehalococcoides* spp. from different sediments. The *Dehalococcoides* spp. 16S rRNA gene is highly conserved among currently known species. However, there are considerable differences in reductive dehalogenase genes (25). 16S rRNA gene sequence analysis confirmed the high sequence identity of *Dehalococcoides* spp. from the sediment batches. For the analysis of reductive dehalogenase-encoding gene diversity, we have focused on the *rdh* genes from *Dehalococcoides* sp. strain CBDB1 in the sediment enrichments of Flix and Schönberg, because we previously detected genes similar to *rdh* genes of this strain by PCR amplification with degenerate primers and subsequent cloning and sequencing of amplicons, at all locations except the Ebro delta estuary (data not shown). Using a series of PCR assays targeting all 32 *rdh* genes predicted from the genome sequence of *Dehalococcoides* sp. strain CBDB1, Schönberg sediment batches showed a higher number of CBDB1-like *rdh* genes compared to Flix sediments. Nevertheless, chemical analysis showed that Flix sediment batches had an end product profile most similar to that reported for strain CBDB1. This finding suggests that similarities in *rdh* gene content or 16S rRNA based identity are not conclusive parameters for the prediction of the degradation pathways. However, they could be informative for dechlorination capability. In the chlorinated ethene degrading *Dehalococcoides* strains 10-17 copies of *rdh* genes were detected (16, 17, 27). In river sediment samples studied here, we were able to detect a high number of *rdh* genes (21 *rdh*'s in Flix and 28 *rdh*'s in Schönberg) from *Dehalococcoides* sp. strain CBDB1, which has 32 predicted *rdh* genes (23). The future efforts focusing on gene-trait matching and elucidation of structure-function relations for members of the *rdh* gene family could further improve the predictive power of these molecular analyses.

Effects of environmental conditions on HCB dechlorination by *Dehalococcoides* spp.

Our results clearly showed that environmental conditions - in this case growth medium, temperature and sea salt concentration - significantly affect the dechlorination of HCB and activity of *Dehalococcoides* spp. in the river sediment. We observed 1.8-2.7 times higher growth yields in river water batches compared to batches with dechlorinating medium, indicating that river sediment and water can together support the growth of *Dehalococcoides* spp (Table 1). The lag-phase of the degradation was longer in river water batches; however, this is likely to be a result of the more slowly developing methanogenic conditions. The dechlorinating medium is adjusted to methanogenic conditions from the start, which creates favorable growth conditions for *Dehalococcoides* spp.. Additionally, the dechlorinating medium contains various vitamins and trace elements which might not be readily available in

the river water. These may also be the main reasons for the 7.5-fold difference in relative abundances of *Dehalococcoides* spp. in the dechlorinating medium compared to the river water batches (Fig. 1, Fig. 2). There was no significant difference in the absolute copy numbers of *Dehalococcoides* spp. 16S rRNA between the river water and the dechlorinating medium (data not shown), but the universal bacterial 16S rRNA copy numbers were higher in the batches with river water. It is likely that various facultative bacteria had an opportunity to grow before full methanogenic conditions developed; thus increasing the copy numbers for universal bacterial counts. This experiment showed that river sediment and water can supply the resources and conditions to nutrient and carbon transfer within the sediments are sufficient to achieve reductive dechlorination. Not only the addition of an external electron donor speeds up HCB degradation, but also results in a higher the growth yields than without an external electron donor (Table 1). Additionally, observed growth yields in batches with river water were higher than the batches with dechlorinating medium.

Decreasing the temperature caused a significant delay in the dechlorination of HCB and growth of *Dehalococcoides* spp.. Our results also showed that *Dehalococcoides* spp. in the Flix sediment are not adapted to temperatures below 15°C. Since there was no difference in the diversity of the genus, observed growth yields and relative abundance between 30°C and 15°C, we can conclude that below the optimum growth temperature (30°C-25°C for this study) *Dehalococcoides* spp. dechlorination of HCB is possible but at lower rates. A significant decrease in HCB degradation rates (Fig. 3C) also at 37°C further reinforces the notion that the HCB transformation rates of *Dehalococcoides* spp. strongly correlate to temperature.

In the batches inoculated with Ebro delta estuary sediment, exposure to high sea salt concentrations resulted in a longer acclimation period for HCB transformation, a shift in end products and a significant decrease in dechlorination rate (Fig. 4). The Ebro delta estuary sediment ecosystem is largely different from the river sediments. Ebro delta estuary sediment batches showed, in the presence or absence of lactate as additional the electron donor, significant differences with respect to the quantity of end products and growth yields, which were 9.5-times higher in batches without lactate. No significant differences were found for Flix sediment batches (Table 1). Remarkably, sediments without addition of lactate as e^- donor dechlorinated HCB faster than cultures with lactate. This may indicate that there is a well established e^- transfer network amongst the (micro)organisms that occurs naturally in the sediment. After HCB was dechlorinated, and even though there was a production of TCB and DCB's, *Dehalococcoides* spp. 16S rRNA gene copies decreased below the detection limit. Besides, significant increases in *Desulfitobacterium* spp. relative abundance were detected. *Desulfitobacterium* spp. were never reported to be involved in chlorinated benzene degradation. Hence, it is tempting to speculate that this observation broadens the spectrum of dehalogenation activities of this versatile genus of low GC Gram positive halorespirers (32),

or alternatively, suggests the presence of another group of yet unknown dechlorinating bacteria, which were not targeted in this study.

The transcription of two potential chlorinated benzene reductase-encoding genes (*cbrA* and *cbdbA*1624) was detected both in river and estuary sediment. In general, the transcription of *cbrA* and *cbdbA*1624 was not affected by tested environmental conditions. As *Dehalococcoides* spp. dechlorinate HCB, these genes are being transcribed. The 16S rRNA concentrations and *cbrA* transcription increased concurrently during HCB dechlorination supporting previous reports on the metabolic involvement of this gene in one of the HCB degradation pathways (2). The transcription of *cbrA* in the early phases of the degradation could also be an indication of the involvement of this gene in dechlorination of highly chlorinated benzenes. However, *cbrA* was consistently transcribed in most of the microcosms even after dechlorination was completed. As a result, substrate specificity of this gene in our case is uncertain. *cbdbA*1624 transcription was only observed when HCB dechlorination had already started. Lack of expression in early HCB dechlorination suggests that the corresponding gene product is also involved in dechlorination of lower chlorinated benzenes. Moreover, it can be speculated that expression of *cbdbA*1624 is induced by lower chlorinated benzenes, rather than by HCB. Once dechlorination stops, transcription of *cbdbA*1624 was not observed confirming the gene's specificity for the particular process. There were no significant differences between the transcript levels of both genes at different temperatures or sea salt concentrations. The main distinction was observed between river and estuary sediments. The measured transcription of the *rdh* genes and 16S rRNA during the HCB degradation in estuary sediment verified that *Dehalococcoides* spp. were physiologically active even though no increase in *rdh* gene transcript concentration was observed. Given the fact that the dechlorination end products from the estuary sediment were also different than the end products of the river sediments it could be concluded that these two *rdh* genes are not involved in the degradation pathway leading to 1,3,5-TCB.

Conclusions

In this study HCB the dechlorination potential of *Dehalococcoides* spp. under several different environmental settings was tested. As strictly anaerobic and mesophilic bacteria *Dehalococcoides* spp. were shown to be resistant and adaptable to the different temperatures. However, high sea salt concentrations were shown to be inhibitory to sediment-associated *Dehalococcoides* spp. The broad HCB transformation capability of *Dehalococcoides* spp. under a variety of different environmental conditions relevant to the river systems studied here is a promising indicator for their *in situ* competency with changing environmental conditions.

Acknowledgements

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Chapter 06

Concurrent Hexachlorobenzene and Chloroethene Degradation by Endogenous Dechlorinating Microorganisms in the Ebro River Sediment

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The diffuse pollution of chlorinated organic compounds in the river basins is an issue of great concern, due to their potential adverse effects on human health and the environment. The chloroaromatics, like hexachlorobenzene (HCB) and the chloraliphatics, like solvents tetrachloroethene (PCE) and trichloroethene (TCE) are common soil and groundwater pollutants. These compounds are recalcitrant to aerobic microbial degradation, but shown to be dechlorinated under anaerobic conditions. This anaerobic process, called reductive dechlorination, possesses a great potential for the transformation of chlorinated organics in anoxic environments. Up to date only members of one bacterial genus, *Dehalococcoides* spp., are shown to be able to reductively dechlorinate HCB, PCE and TCE. Our knowledge, however, of dechlorination by *Dehalococcoides* spp. under different conditions is limited. Most of the research so far has focused on polluted groundwater or soils while river systems remain poorly studied. The main goal of this study was to investigate the role of *Dehalococcoides* spp. in the transformation of chlorinated benzenes and chlorinated ethenes in river sediments. The study area was a HCB contaminated location in the Ebro River basin, Spain. Lab-scale batch microcosms containing the river sediment were used to follow the growth and the abundance of *Dehalococcoides* spp. during dechlorination of HCB, TCE and PCE. Molecular analysis targeting 16S ribosomal RNA (rRNA) and functional genes, i.e. genes of *Dehalococcoides* spp. that are involved in reductive dechlorination, was used to delineate the dynamics of the reductive dechlorination in the river sediments. Our results showed that the Ebro River sediment is able to dechlorinate chlorinated ethenes alongside with chlorinated benzenes. Growth of *Dehalococcoides* spp. and the expression of several reductive dehalogenase (*rdh*) genes during dechlorination process could be demonstrated. Our findings underline the remarkable potential of this rivers' sediment to tackle these compounds.

Submitted manuscript

Introduction

Chlorinated compounds in the environment originate from anthropogenic, biogenic and geogenic sources (16, 39). Two important groups are the chloroaromatics, like hexachlorobenzene (HCB) and the chlorinated solvents, e.g. tetrachloroethene (PCE) and trichloroethene (TCE), which are found worldwide as soil and groundwater contaminants(19). Hexachlorobenzene (HCB) is a pesticide component with high toxic and carcinogenic potential. Such polychlorinated compounds are in general highly resistant to bacterial aerobic biodegradation, but under anaerobic conditions chlorinated compounds can be used by bacteria as terminal electron acceptors in their energy metabolism. Reductive dehalogenation of the chlorinated compounds by this process, also termed halorespiration, or dehalorespiration or chloridogenesis, presents a great potential in bioremediation applications in anoxic environments. Until now only three strains of bacteria could be isolated, which are able to degrade HCB to lower chlorinated benzenes via reductive dechlorination, *Dehalococcoides sp.* strain CBDB1 (4), *Dehalococcoides ethenogenes* strain *195* (13) and "Dehalobium chlorocoercia" strain DF-1 (42). No bacterium, however, has so far been shown to completely dechlorinate HCB to benzene. Microorganisms from various genera are able to dechlorinate PCE and TCE to predominantly dichloroethene (DCE). The only genus known to fully dechlorinate PCE and TCE to non-toxic ethene, however, groups around the members of the *Dehalococcoides* spp. (29). Dechlorination of chloroethenes has been demonstrated in batch scale enrichments (12, 21, 22), fed-batch reactors (23) and soil columns (5). Despite the fact that reductive dechlorination is an energy yielding process for *Dehalococcoides* spp., laboratory scale microcosm studies showed that their growth (at least in pure culture) is relatively slow (9, 20). The reductive dechlorination of chlorinated compounds is achieved by reductive dehalogenase (*Rdh*) enzymes. A large number of putative *rdh* genes has been identified in currently available genome sequences (15, 27, 37). Nevertheless, only a few have been characterized for their function, and in case of *Dehalococcoides* spp., 4 chloroethene- and chlorobenzene reductive dehalogenases have been studied in detail (3, 25, 32, 34).

Our knowledge of dechlorination by *Dehalococcoides* spp. under different conditions remains limited. For example, the impact of other halorespiring species, i.e. competition for chlorinated compounds or electron donors, or simultaneous exposure to different aromatic and/or aliphatic chlorinated compounds, has never been addressed. Moreover, most of the research up to date has focused on polluted groundwater or soil systems while other environments remain poorly studied. Our previous research on the river sediments indicated that only one strain of *Dehalococcoides* spp. was dominantly present in HCB transforming microcosms (**Chapter 5**). Nevertheless, *Dehalococcoides* spp. have been reported to be functionally diverse and our field survey indicated the presence of more than one active *Dehalococcoides* spp. strains at this location (**Chapter 3**). In case of concurrent exposure to

chlorinated ethenes and benzenes we hypothesized that more than one *Dehalococcoides* strain may be responsible from the transformation of the compounds. The main goal of this study was to investigate the role of *Dehalococcoides* spp. in the transformation of chlorinated benzenes and chlorinated ethenes in river sediments. To achieve this goal several lab-scale microcosms containing the river sediment were prepared. The abundance and growth of *Dehalococcoides* spp. during dechlorination of HCB, TCE and PCE in these microcosms were demonstrated via 16S ribosomal RNA (rRNA)-targeted molecular analysis. PCE and TCE were tested separately since some of the *Dehalococcoides* spp. isolates are shown dechlorinate TCE but not PCE, when supplied as the only electron acceptor (18). Additionally, 16S rRNA gene cloning and real-time quantitative PCR (qPCR) were used to delineate the composition of other putative dechlorinating and non-dechlorinating populations. Furthermore, the expression of several reductive dehalogenase (*rdh*) genes, potentially involved in different stages of the dechlorination process, could be demonstrated.

Materials and Methods

Chemicals

Hexachlorobenzene (HCB), 1,2,4,5-TeCB (tetrachlorobenzene), 1,2,3,5-TeCB, 1,3,5-TCB (trichlorobenzene) and 1,2-DCB (dichlorobenzene) were obtained from Sigma-Aldrich Chemicals Co. Ltd. (Dorset, England). QCB (pentachlorobenzene), 1,2,3,4-TeCB, 1,2,4-TCB, 1,3-DCB and 1,4-DCB were purchased from Merck (Darmstadt, Germany). 1,2,3-TCB was from Janssen Chimica (Beerse, Belgium). PCE, TCE, and 1,1-dichloroethene (1,1DCE) were obtained from E. Merck (Amsterdam, The Netherlands). *Cis*-1,2-dichloroethene (*cis*DCE) was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), and *trans*-1,2-dichloroethene (*trans*DCE) from Janssen Chimica. All chemicals were at least of analytical grade (purity >99%) and were used as received without further purification.

Origin of the river sediment and experimental procedures

The sediment sample used in this study was collected from the Ebro River in Spain in the framework of the European FP6 project Aquaterra (6). The sampling took place nearby the town of Flix (N 41º13’43.6’’, E 00º33’09.0’’) in February 2006. In this location hexachlorobenzene (HCB) and hexachlorocyclohexane (HCH) were found to be the dominant chlorinated contaminants over the past ten years (28). River sediment up to a depth of five cm was sampled anaerobically with an air-tight glass jar, approximately 1.5 meters from the river shore. Sample was transported to the laboratory in a cooler and stored in the dark at 4°C. The sediment was analyzed by AGROLAB (Al-West B.V., Deventer, the Netherlands) for geochemical parameters according to standardized methods. HCB concentrations were measured according to the ISO 10382 protocol. Analysis showed that the sediment had 67.5%

dry matter (DM) content, 0.90 g kg^{-1} DM^{-1} total Kjeldahl nitrogen (TKN), 0.88 % DM total organic carbon (TOC), 300 g kg^{-1} DM^{-1} total phosphorus (TP) and 0.12 mg kg^{-1} DM^{-1} HCB. HCH was not detected.

The transformation of chlorinated compounds was tested with 10 g (wet weight) of sediment in 250 ml glass bottles (0.1 g sediment/ml basal medium) as described earlier, but without the addition of fermented yeast extract (22). The pH in the batch bottles was 7.0-7.3. The bottles were sealed with Viton stoppers (Maag Technic AG, Dubendorf, Switzerland). 50 mM acetate was added as carbon source. HCB, PCE and TCE were added from a stock solution prepared in acetone. Experiments were started up in an anaerobic glove box. The oxygen concentration in the anaerobic glove box was kept low with a palladium catalyst and H_2. The gas phase in the bottles was composed of 0.8 bars of H_2 (80%) and CO_2 (20%). In the first week of the experiments, additional H_2/CO_2 was added to the bottles under sterile conditions when a drop in headspace pressure occured. All experiments were carried out in triplicate. The batches were incubated statically at 30±2°C in the dark. Sterile control batches did not contain any sediment. Sterile sediment controls were prepared by autoclaving the sediments twice for 20 min at 120°C at the start of the experiment. Liquid-phase extractions for chlorinated benzene measurements were performed on the sampling day. 1.5 ml sediment-liquid mixture per sampling point was stored at -80°C for nucleic acid extraction.

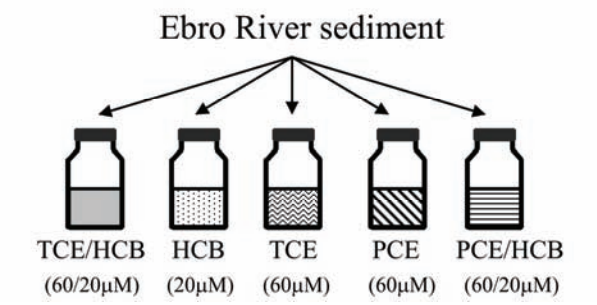


Figure 1. Experimental set-up of Ebro River sediment microcosms. Reductive dechlorination of HCB (20μM), TCE (60μM) and PCE (60μM) was tested as single chlorinated compound (single electron acceptors). The Ebro River sediment was also exposed to combinations of HCB with TCE (TCE/HCB) and PCE (PCE/HCB) to test the capability of these sediments to degrade a mixture of two chlorinated compounds. Microcosms with individual pollutants HCB, TCE and PCE were used for construction of 16S rRNA gene libraries.

Analytical Methods

Total masses of the chlorinated benzenes were determined by GC-mass spectrometry analysis of liquid phase samples. 1 ml sediment culture slurry was extracted to 1 ml hexane-acetone (4:1, vol/vol) solution via sonication for 20 min followed by overnight mixing. 1μl of this solution was injected into a TRACE DSQ System (TRACE DSQ MS Detector and TRACE

GC ULTRA; Milan, Italy) equipped with an injection splitter (split ratio, 20:1) and a FID detector connected to a capillary column (30 m by 0.25 μm [inner diameter], Rtx® 5MS [0.25 mm thick], Restek, PA). The carrier gas was helium, and the inlet pressure was 3 kPa. The operating temperatures of the injector and detector were 220 and 250°C, respectively. The column was operated with the following temperature program: initial column temperature, 60°C (1 min); increase of 5°C/min to a final temperature of 180°C (3 min). Output data were analyzed with the TRACE Xcalibur Data System 1.3 (Milan, Italy). The chlorobenzenes were quantified using 1,2-DCB and HCB as internal standards. For each bottle duplicate measurements were done. Graphs were drawn from averages of values measured in triplicate bottles with corresponding standard deviations. The total masses of chlorinated ethenes were determined via headspace analysis. PCE, TCE and DCEs were analyzed by injecting 0.2 ml headspace to a Gas Chromatograph (Chrompack, Bergen op Zoom, The Netherlands) equipped with a Flame Ionization Detector (GC-FID) connected to a Sil 5CB capillary column (25m x 0.32 mm by 1.2 μm film) and a split injection (ratio 1:50). N_2 with an inlet pressure of 50 kPa was used as the carrier gas. The operating temperatures of the injector and detector were 250ºC and 300ºC respectively. The operation temperature of the oven was 50ºC (4 min) which was increased by 10ºC/min to 110ºC and held at 110ºC for 1 min. The retention times at 50ºC (oven temperature) were (in minutes) 6.8, 3.2, 1.8, 1.5, 0.75, 0.72 for PCE, TCE, cDCE, tDCE/1,1DCE, methane and ethene, respectively. Due to technical difficulties undisrupted VC measurements could not be done. The substrate consumption rate is calculated in the point of total HCB dechlorination. Calculation of observed growth yields was done as previously described (35).

16SrRNA targeted Real-Time PCR and DGGE

Extraction of nucleic acids, cDNA synthesis from 16S rRNA, PCR, denaturing gradient gel electrophoresis (DGGE), and cloning and sequence analysis were performed as described previously (**Chapter 3**) . The list of primers used in this study is given in Appendix I. Real-time quantitative PCR (qPCR) was performed using an iQ5 iCycler (BioRad, Veenendaal, Netherlands) with the primers and thermocycling program as previously described (40) for 16S rRNA genes of dehalogenating bacteria (*Dehalococcoides*, *Desulfitobacterium*, *Dehalobacter*) and all Bacteria using SYBR Green Dye. The expression of the reductive dehalogenase-encoding genes of *Dehalococcoides* sp. CBDB1 *cbrA* (*cbdbA*84) and *cbdbA*1624 (41) and *Dehalococcoides ethenogenes 195 pceA* (DET0318) and *tceA* (DET0079) were detected via reverse transcriptase qPCR (RT-qPCR) with the primers and the thermocycling program as previously described (14). cDNA for this detection was synthesized from 0.1 μg of total RNA using SuperScriptTM III First-Strand Synthesis System (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. qPCR reactions were prepared in 25 μl total reaction volume containing 5 μl template cDNA or DNA, 1× BioRad

SYBR Green PCR master mix (BioRad, Veenendaal, Netherlands), 0.2 μM of each primer and 6.5 μl sterilized milli Q. Samples were analyzed in triplicate, and no-template controls were included. Standard curves were generated from triplicate dilution series. qPCR standards were prepared by cloning PCR-amplified 16S rRNA or reductive dehalogenase genes of targeted dehalogenating bacteria into the pGEM-T Easy plasmid vector (Promega, Madison, WI).

Table 1. End products of HCB degradation in HCB, TCE/HCB and PCE/HCB microcosms. Data points are given in μM as the average of duplicate measurements of triplicate bottles with their respective standard deviation.

Time (days)		**0**	**7**	**14**	**21**	**28**	**35**
HCB	1,3-DCB	0	1.1±0.5	1.5±0.4	2.6±0.3	4.2±2.7	5.2±2.1
	1,4-DCB	0	1.3±0.7	2.1±0.5	3.4±0.3	5.2±2.6	5.9±2.2
	1,3,5-TCB	0	1.2±0.2	2.6±0.4	2.9±0.2	6.8±3.4	6.8±3.1
TCE/HCB	1,3-DCB	0	2.8±0.7	4.0±1.8	6.7±2.3	6.6±2.1	6.8±2.1
	1,4-DCB	0	3.5±1.0	5.3±1.9	8.8±2.5	8.9±2.4	8.6±2.4
	1,3,5-TCB	0	1.5±0.6	2.4±1.1	3.3±1.8	3.2±1.7	3.7±1.4
PCE/HCB	1,3-DCB	0	0.3±0.1	1.0±0.8	1.3±0.7	3.6±1.8	3.7±1.7
	1,4-DCB	0	0.4±0.1	1.2±0.7	1.2±0.6	3.0±1.7	2.6±1.9
	1,3,5-TCB	0	1.2±0.7	1.5±1.2	4.8±2.4	15.0±5.3	14.9±5.3

Results

PCE, TCE and HCB were used in 5 different combinations to set-up the microcosms (Fig. 1). When chlorinated compounds were provided as the sole electron acceptor, PCE and TCE microcosms performed similarly by fully dechlorinating the compounds to ethene. HCB was degraded to 1,3,5-TCB, 1,4-DCB and 1,3-DCB (Fig. 2, Fig. 3, Table 1). Methane production was observed in all bottles immediately after the start-up (Fig.2D and Fig.3E), however, methane accumulation in PCE and PCE/HCB amended bottles occurred faster than in TCE and TCE/HCB microcosms. No dechlorination was observed in the sterile controls or in the sediment controls in any of the tested conditions (data not shown). In the TCE and TCE/HCB microcosms complete dechlorination of TCE was achieved within three weeks. *cis*DCE was the only detected intermediate, which was consecutively dechlorinated to ethene (Fig.2). There were no noteworthy differences between HCB and TCE, and TCE/HCB microcosms in the degradation of either chlorinated compound. The major difference was in the end products of HCB degradation. TCE/HCB microcosms dechlorinated HCB predominantly to 1,3-DCB and 1,4-DCB, whereas in HCB microcosms 1,3-DCB, 1,4-DCB and 1,3,5-TCB could be detected in similar concentrations (Table 1). Absolute 16S rRNA gene copy numbers and rRNA relative abundance (*Dehalococcoides* spp. 16S rRNA copies / total Bacteria 16S rRNA

copies) were used to relate growth of the species to the degradation of the compounds. When the dechlorination of HCB started, 16S rRNA gene copy numbers of *Dehalococcoides* spp. increased to a level of 10^8 copies / L and stayed at this level during the degradation (Fig.4). In microcosms with TCE and TCE/HCB substantial increases in the *Dehalococcoides* spp. rRNA gene copy numbers were observed concurrent to *cis*DCE dechlorination (Fig.2A, Fig.4B).

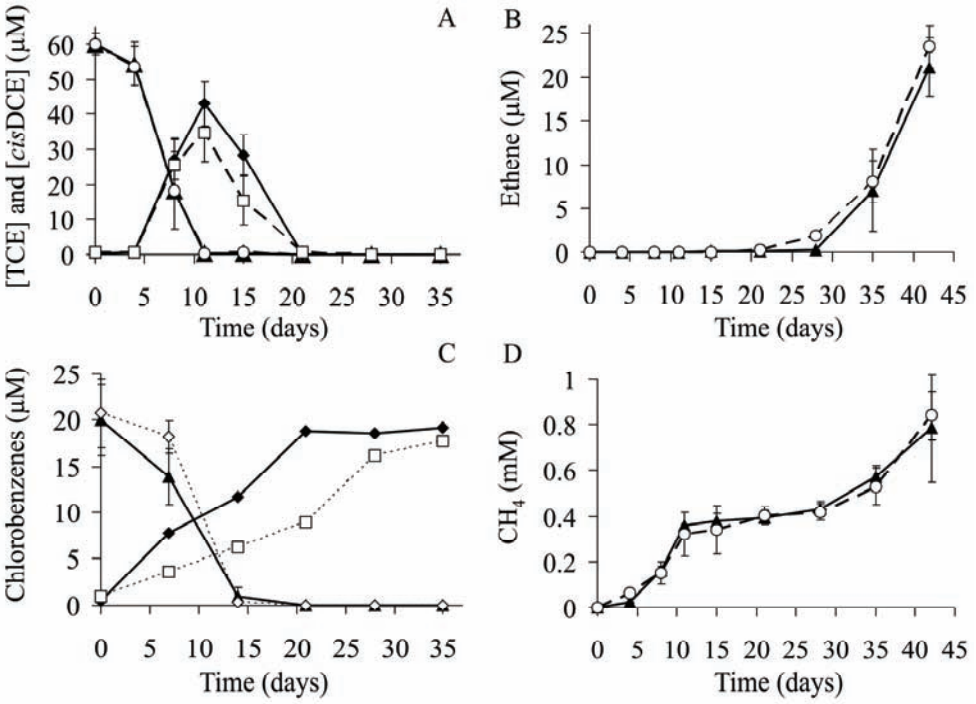


Figure 2. Degradation of 60μM TCE and 20μM HCB in river sediment microcosms. Error bars represent the standard deviation of triplicate measurements. When the standard deviation is smaller than the symbol, it is not presented. **(A)** Dechlorination of TCE in TCE amended microcosms (○, long dashed line) and TCE/HCB amended microcosms (▲, continuous line) was completed in two weeks. Production of *cis*DCE in TCE (□, long dashed line) and TCE/HCB (◆, continuous line) microcosms was observed between the first and the second week of the experiment. Accumulated *cis*DCE was consumed by day 21. **(B)** Ethene production in TCE (○, long dashed line) and TCE/HCB (▲, continuous line) microcosms. **(C)** Dechlorination of HCB in HCB amended microcosms (◇, dotted line) and TCE/HCB amended microcosms (▲, continuous line) was also completed in two weeks. Accumulation of 1,3-DCB, 1,4-DCB and 1,3,5-TCB was presented as sum of end products in HCB (□, dotted line) and TCE/HCB (◆, continuous line) microcosms. Concentrations of the individual products and standard deviations are given in Table 1. **(D)** Methane production in TCE (○, long dashed line) and TCE/HCB (▲, continuous line) microcosms.

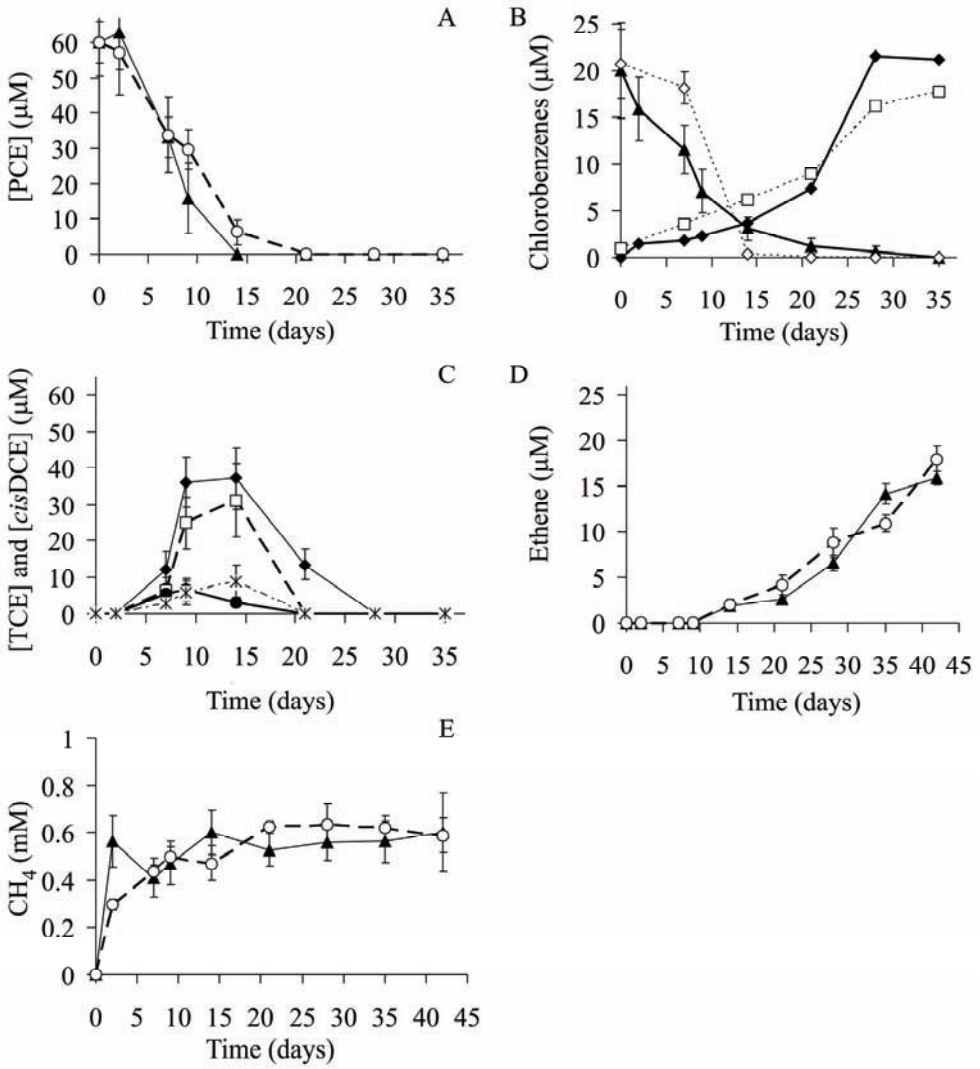


Figure 3. Degradation of 60µM PCE and 20µM HCB in river sediment microcosms. Error bars represent the standard deviation of triplicate measurements. When the standard deviation is smaller than the symbol, it is not presented. **(A)** PCE in PCE amended microcosms (○, long dashed line) and PCE/HCB amended microcosms (▲, continuous line) was completely dechlorinated. **(B)** Dechlorination of HCB in HCB amended microcosms (◇, dotted line) and PCE/HCB amended microcosms (▲, continuous line). Accumulation of 1,3-DCB, 1,4-DCB and 1,3,5-TCB was presented as sum of end products in HCB (□, dotted line) and TCE/HCB (◆, continuous line) microcosms. For concentration of individual products and standard deviations refer to Table 1. **(C)** TCE has accumulated in PCE (∗, long dash dot line) and PCE/HCB (●, continuous line) microcosms prior to its degradation. However, *cis*DCE accumulation in both PCE (□, long dashed line) and PCE/HCB (◆, continuous line) microcosms was higher than TCE accumulation. **(D)** Ethene and **(E)** Methane production in PCE (○, long dashed line) and PCE/HCB (▲, continuous line) microcosms.

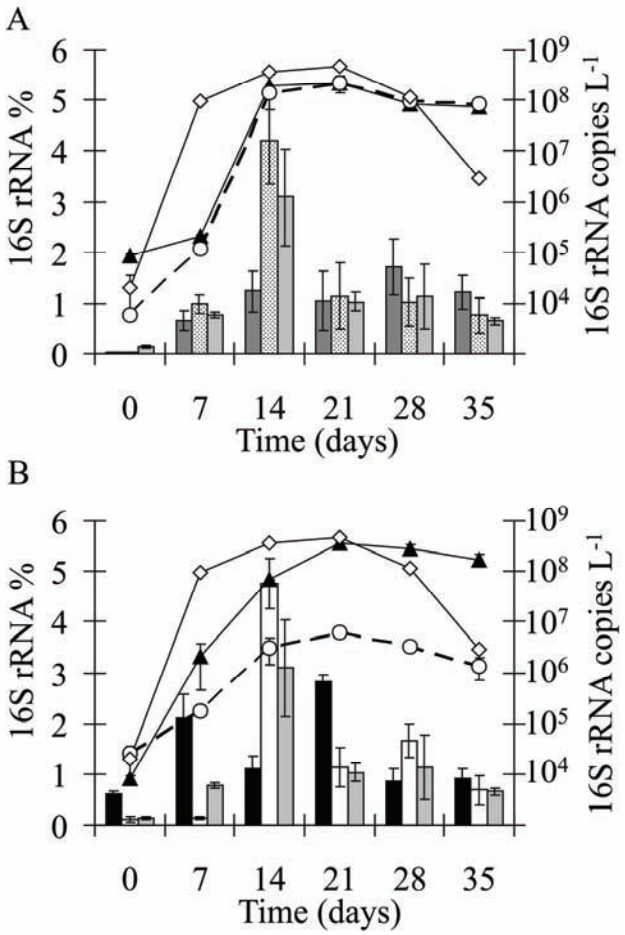


Figure 4. Changes in the relative abundance of 16S rRNA and 16S rRNA gene copies of *Dehalococcoides* spp. during dechlorination of the chlorinated compounds. Bars represent the relative abundance (%) of *Dehalococcoides* spp. 16S rRNA copies as compared to total bacterial 16S rRNA. Lines represent *Dehalococcoides* spp. 16S rRNA gene copies L^{-1}. Error bars represent the standard deviation of triplicate measurements (n=3). When the standard deviation is smaller than the symbol, it is not presented. **(A)** *Dehalococcoides* spp. 16S rRNA gene copies in TCE (○, long dashed line), HCB (◇, continuous line) and TCE/HCB (▲, continuous line) microcosms. Relative 16S rRNA abundance of *Dehalococcoides* spp. was higher in TCE microcosms (dot filled bars) than in TCE/HCB (dark grey) and HCB (light grey) microcosms. **(B)** *Dehalococcoides* spp. 16S rRNA gene copies in PCE/HCB amended microcosms (▲, continuous line), HCB amended microcosms (◇, continuous line) and PCE amended microcosms (○, long dashed line). Relative 16S rRNA abundance of *Dehalococcoides* spp. was higher in PCE (white bars) and HCB (light grey bars) microcosms than in PCE/HCB (black bars) microcosm.

The relative abundance of *Dehalococcoides* spp. rRNA in HCB degrading microcosms was at its highest at 3.1% after 14 days. Likewise the relative abundance of *Dehalococcoides* spp. rRNA in TCE degrading microcosms increased to a maximum of 4.2% at day 14. On the other hand in the TCE/HCB microcosms the relative abundance of *Dehalococcoides* spp. rRNA did not increase as much and stayed between 0.7-1.8%. *Dehalococcoides* spp. 16S rRNA targeted DGGE analysis showed that there were no changes in the composition of the *Dehalococcoides* spp. population during dechlorination (data not shown). The 16S rRNA fragments corresponding to the predominant DGGE bands at the 14th day of the experiment were sequenced. The DGGE band in the TCE/HCB microcosms was related to two *Dehalococcoides* strains (HCBTCE_T226 and HCBTCE_T211), the 16S rRNA of which had 99.1% and 98.9% (*E.coli* positions 102 to 1234) similarity to that of *Dehalococcoides* sp. strain CBDB1 (Fig.5). In the HCB and TCE degrading microcosms the DGGE band corresponded to a single strain with 98.7% (HCB_T233) and 99.5% (TCE_T230) 16S rRNA sequence similarity to *Dehalococcoides* sp. strain CBDB1, respectively.

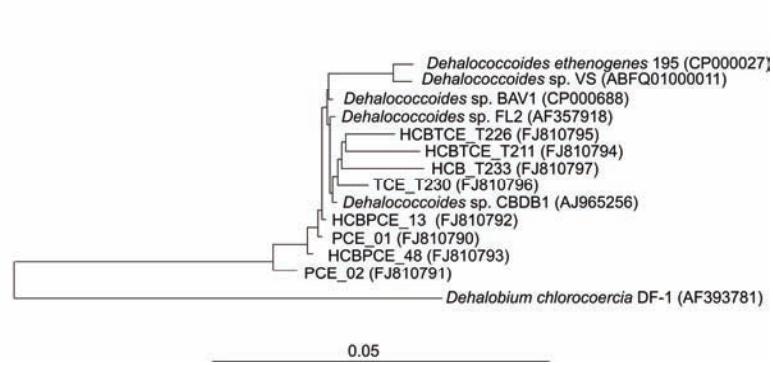


Figure 5. Neighbor-joining tree of *Dehalococcoides* spp. 16S rRNA sequences from different microcosms and appropriate reference sequences within the *Dehalococcoidetes*. Sequences HCBTCE_T226 and HCBTCE_T211 originate from TCE/HCB amended microcosms. Sequences HCB_T233 andTCE_T230 are from HCB and TCE microcosms respectively. Sequences PCE_01 and PCE_02 originate from PCE microcosms while HCBPCE_13 and HCBPCE_48 are from PCE/HCB microcosms. The tree was constructed based on partial 16S rRNA sequences (E. coli positions 103 – 1234). Scale bar represents 5% dissimilarity. Accession numbers are given in parentheses.

In the PCE and PCE/HCB microcosms, PCE was dechlorinated to ethene via TCE and *cis*DCE within four weeks (Fig. 3A and C). In both cases most of the PCE was transformed to *cis*DCE, which accumulated in the system as an intermediate prior to its degradation. HCB was dechlorinated more slowly in the presence of PCE, and end product of dechlorination was mainly 1,3,5-TCB (Table 1). As previously observed in HCB and TCE/HCB microcosms, 16S rRNA gene copy numbers of *Dehalococcoides* spp. increased in PCE/HCB microcosms with HCB dechlorination. In PCE microcosms, the amount of 16S rRNA gene copies of

Dehalococcoides spp. was lower than in any other microcosm. Conversely, the relative abundance of *Dehalococcoides* spp. 16S rRNA copies in these microcosms was as high as 4.8% on day 14 (Fig.4). In PCE/HCB microcosms the relative abundance of *Dehalococcoides* spp. rRNA was at its highest (2.8%, day 21) in the later phases of the experiment when the accumulated *cis*DCE was degraded. Similar to HCB and TCE/HCB microcosms there was no change in the DGGE fingerprints of *Dehalococcoides* spp. (data not shown). Sequencing of

Table 2. Comparison of observed growth yields of *Dehalococcoides* spp. in sediment microcosms

Chlorinated compound	e^- donor	Yield[a]	T[b]	Source	End Product	Reference
HCB	Lactate	$(3.5\pm0.1) \times 10^6$	30°C	Ebro river sediment	1,3,5-TCB, 1,3-DCB, 1,4-DCB	Chapter 5
HCB	Acetate/H_2	$(8.5\pm0.2) \times 10^6$	30°C	Ebro river sediment	1,3,5-TCB, 1,3-DCB, 1,4-DCB	This study
TCE	Acetate/H_2	$(3.2\pm1.1) \times 10^6$	30°C	Ebro river sediment	Ethene	This study
PCE	Acetate/H_2	$(7.4\pm0.7) \times 10^4$	30°C	Ebro river sediment	Ethene	This study
TCE/HCB	Acetate/H_2	$(1.6\pm0.2) \times 10^6$	30°C	Ebro river sediment	1,3,5-TCB, 1,3-DCB, 1,4-DCB, Ethene	This study
PCE/HCB	Acetate/H_2	$(3.6\pm0.3) \times 10^6$	30°C	Ebro river sediment	1,3,5-TCB, 1,3-DCB, 1,4-DCB, Ethene	This study
1,2-DCA	Methanol	$(1.6\pm0.8) \times 10^8$	30°C	KB-1/TCE enrichment	Ethene	(11)
VC	Methanol	$(1.5\pm0.3) \times 10^8$	30°C	KB-1/TCE enrichment	Ethene	(11)
cisDCE	Methanol	$(0.9\pm0.3) \times 10^8$	30°C	KB-1/TCE enrichment	Ethene	(11)
VC	Lactate	$(1.3\pm0.3) \times 10^7$	25°C	ANAS enrichment	Ethene	(23)
cisDCE	Lactate	$(1.1\pm0.1) \times 10^7$	25°C	ANAS enrichment	Ethene	(23)
TCE	Lactate	$(1.4\pm0.4) \times 10^7$	25°C	ANAS enrichment	Ethene	(23)
2,3-DCP[c]	Methanol	6.3×10^7	28°C	strain CBDB1	3-MCP[d]	(2)
2,3-DCP[c]	Methanol	8.3×10^7	35°C	strain 195	3-MCP[d]	(2)

Abbrv: [a]Yield, Observed growth yield as 16S rRNA gene copies /µmol Cl^-); [b]T,Growth temperature; [c]DCP, dichlorophenol; [d]MCP, monochlorophenol

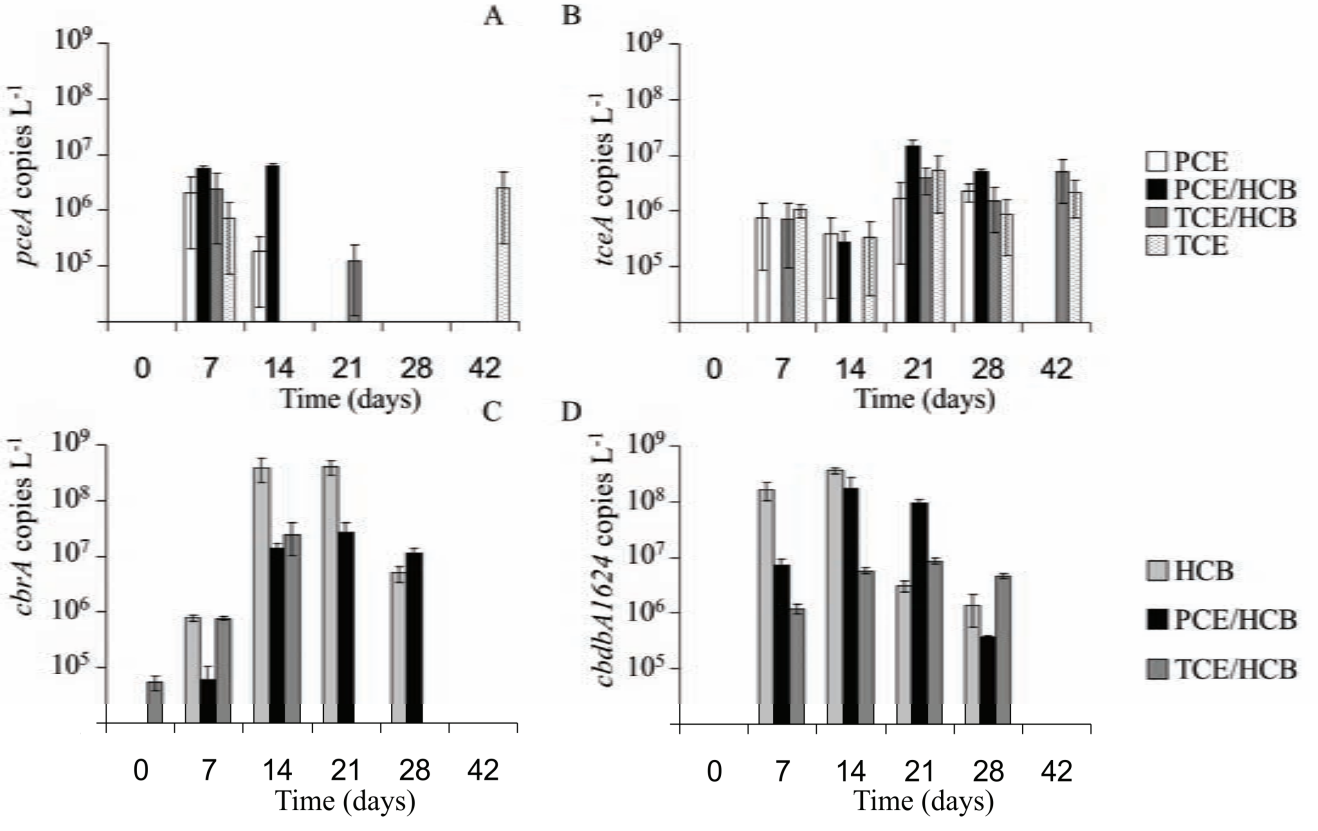


Figure 6. Expression of reductive dehalogenase (*rdh*) genes in the microcosms. Detected *rdh* gene transcript copies per litre for *pceA* **(A)**, *tceA* **(B)**, *cbrA* **(C)** and *cbdbA1624* **(D)** presented in the y-axis. Bars represent average values of replicate microcosms. Error bars represent the standard deviation of triplicate measurements.

the corresponding 16S rRNA fragments in PCE microcosms showed that the band corresponded to two strains of *Dehalococcoides* with respectively 99.8% (PCE_01) and 99.6% (PCE_02) similarity to *Dehalococcoides* sp. strain CBDB1. Likewise the DGGE profile from the PCE/HCB microcosms originates from two other strains of *Dehalococcoides* with 99.8% (HCBPCE_13) and 99.6% (HCBPCE_48) similarity to *Dehalococcoides* sp. strain CBDB1. In all cases there was no considerable change in the relative abundance of *Desulfitobacterium* spp. rRNA during the dechlorination process (0.05-0.1% during experimental period, data not shown). *Dehalobacter* spp. 16S rRNA was not detected at any time (data not shown).

The growth yields of *Dehalococcoides* spp. in the presence of chlorinated ethenes and chlorinated benzenes were calculated and indicated that in these sediments growth yields were lower than those previously reported for enrichment cultures and isolates (Table 2). The *Dehalococcoides* yield on HCB was higher than with chlorinated ethenes or any of the combinations of HCB with chlorinated ethenes. Not only the type of the chlorinated compound but also the electron donor supplied to the microcosms had an impact on the growth yields. In addition, *Dehalococcoides* yield on HCB with Acetate/H_2 was 2.8 times higher than previously observed growth yields with lactate.

The transcripts of the *rdh* genes *cbrA*, *cbdbA1624*, *pceA* and *tceA* were detected in sediment microcosms (Fig. 6). The *pceA* gene could in general only be detected in the beginning of the PCE degradation whereas *tceA* transcripts could be detected throughout PCE and TCE degradation. However, the high standard variation between measurements and the inconsistencies observed in the melting curve analysis of these genes (such as one to two degrees differences between the melting curves of the standards and samples, data not shown) suggest that quantitative interpretation of the data should be done cautiously. The *cbrA* transcription was detected in HCB amended microcosms during HCB degradation, but not in TCE and PCE microcosms (data not shown). The *cbrA* transcription was at its highest in HCB microcosms. On the other hand, *cbrA* transcript levels were not only 1.5 times lower in TCE/HCB microcosms as compared to HCB microcosms, but also no *cbrA* transcripts could be detected after day 14 of the experiment. In HCB and PCE/HCB microcosms, 10^6-10^8 copies of *cbrA* transcripts could be detected during degradation of HCB and production of TCB and DCBs. A higher amount of *cbdbA1624* transcripts was detected in HCB and PCE/HCB microcosms than in TCE/HCB microcosms. An increase in *cbdbA1624* transcription during the degradation of HCB was observed in HCB and PCE/HCB microcosms between day 7 and 21 (Table 1 and Fig. 6). In TCE/HCB microcosms transcription of this gene could also be found day 7 and 28 of the experiment.

Table 3. Class-level distribution of clones obtained by cloning and sequence analysis of 16S rRNA genes amplified by PCR from HCB, TCE and PCE microcosms. Values in parentheses show the fraction of each class in the clone library. Others include classes of *Verrucomicrobiae*, *Erysipelotrichi*, *Anaerolineae*, *Lentisphaerae*, and *Sphingobacteria*

	PCE	**TCE**	**HCB**
Total number of clones	29	44	49
Clostridia	16 (0.55)	18 (0.41)	24 (0.49)
Betaproteobacteria	2 (0.07)	4 (0.09)	4 (0.08)
Alphaproteobacteria	0 (0.00)	1 (0.02)	1 (0.02)
Epsilonproteobacteria	3 (0.10)	4 (0.09)	3 (0.06)
Bacteroidetes	1 (0.03)	6 (0.14)	5 (0.10)
Deltaproteobacteria	0 (0.00)	7 (0.16)	8 (0.16)
Bacilli	0 (0.00)	1 (0.02)	1 (0.02)
Flavobacteria	1 (0.03)	1 (0.02)	1 (0.02)
Gammaproteo	1 (0.03)	1 (0.02)	0 (0.00)
Others	5 (0.17)	1 (0.02)	2 (0.04)

Microbial community composition in the sediment microcosms

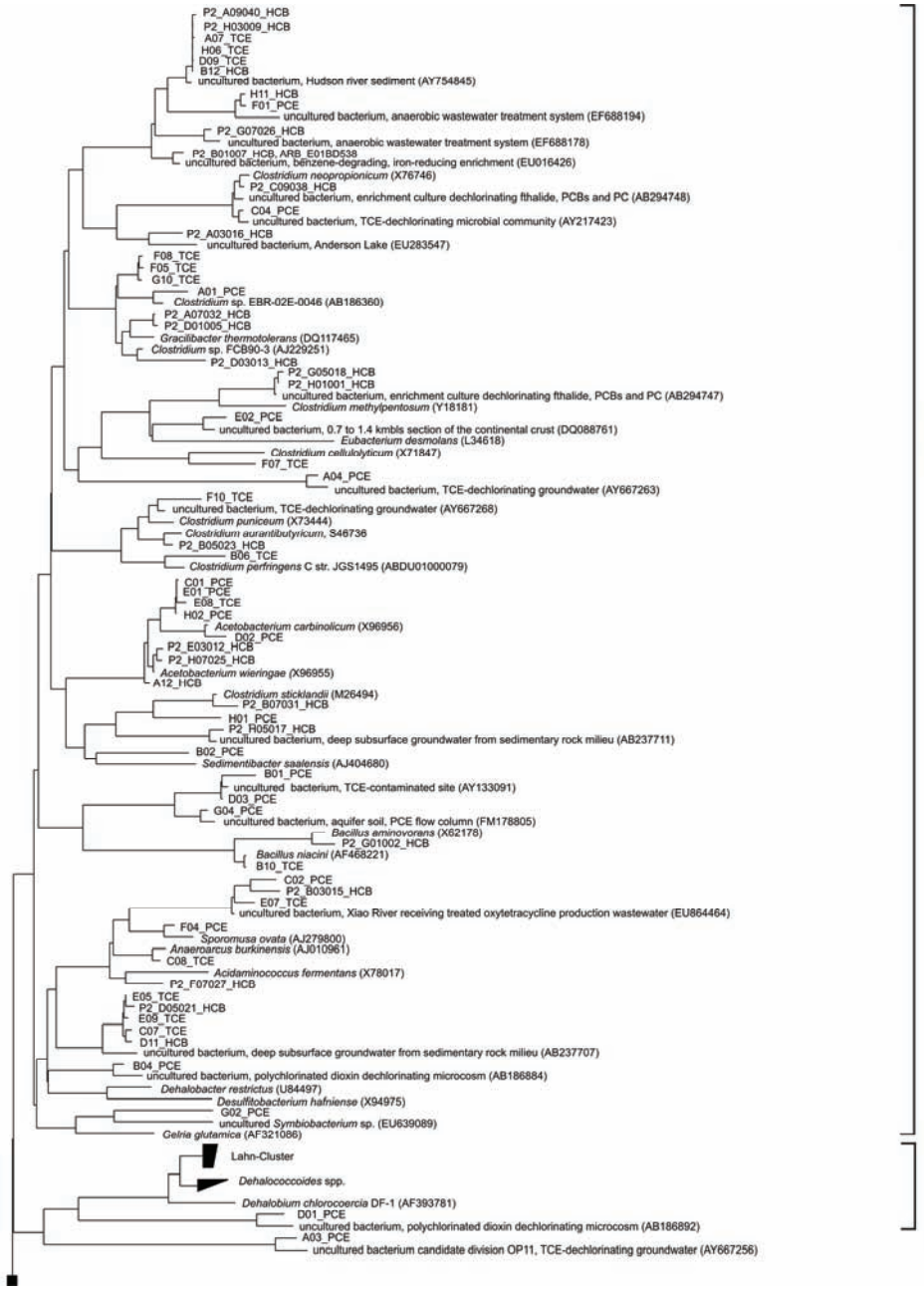
The bacterial community structure in PCE, TCE and HCB-degrading microcosms was investigated via cloning and sequence analysis of 16S rRNA gene fragments amplified with universal bacterial primers from microcosm samples taken at day 14. Only bacterial rRNA genes were targeted here due to the fact that no archaeon has ever been shown to respire with chlorinated compounds. 192 colonies were selected for each microcosm. Restriction fragment length polymorphisms (RFLP) analysis showed that the PCE degrading microcosm harbored 29 unique operational taxonomic units (OTUs), while TCE and HCB microcosms had 44 and 49 unique OTUs, respectively. In general, the composition of OTUs found in the TCE and PCE clone libraries was similar to those detected in locations which are polluted with these contaminants (31). Likewise, the HCB clone library showed similarities with PCB-degrading enrichment cultures or dioxin degrading microcosms (43, 44). In all of the clone libraries *Clostridia* was the dominant class-level phylum (40-55% of all sequences, Table 3). Based on phylogenetic analysis, the compositions of TCE- and HCB-degrading microcosms were more similar to each other than PCE-degrading microcosms (Fig. 7). Only a fraction of the OTUs in the clone libraries could be identified to the genus level because of the low sequence identity to known isolates (Table 3 and Fig.7). *Acetobacterium* was the most frequently found genus (9 clone sequences) in all of the clone libraries while *Bacteroidetes*-like bacteria were common in TCE and HCB libraries. Even though most of the sequences clustered with uncultured bacteria, close relatives of *Sulfuricurvum kujiense* and *Desulfovibrio ferrireducens* could be identified in all of the libraries (Fig. 7). OTUs from dechlorinating bacteria closely related to *Desulfitobacterium* spp. or *Dehalobacter* spp. were not found in the clone libraries,

confirming the RT-qPCR findings. On the other hand, several clones were found to be most closely related to the PCE-dehalogenating *Sulfurospirillum multivorans*, one clone from PCE-degrading microcosm showed closest phylogenetic affiliation with *Sporomusa ovata*, and one OTU from the TCE library was closely related to *Desulfuromonas chloroethenica*. No *Dehalococcoides*-like sequences were detected in the clone libraries, however, this could be a result of overall low abundance of *Dehalococcoides* spp. in the sediment samples.

Discussion

In this study we used molecular tools and chemical analysis to investigate the diversity of the bacterial communities, and especially the role of *Dehalococcoides* spp., during the transformation of chlorinated benzenes and chlorinated ethenes in HCB contaminated river sediments. Our previous work in the Ebro River showed the HCB degradation capacity of this river's sediment under different environmental conditions (**Chapter 5**). In the present study we could demonstrate that this sediment-associated microbiota is also capable of fully dechlorinating chlorinated ethenes. PCE and TCE contamination in the Ebro's water and sediment has not been reported. However, our previous work at the sampling location has already shown the presence of several different strains of *Dehalococcoides* spp. and close phylogenetic relatives in various depths of the river sediment (**Chapter 3**). *Dehalococcoides* spp. have been shown to be able to halorespire using a variety of different chlorinated compounds (39). *D. ethenogenes* strain *195* can use chlorinated ethenes, benzenes and ethanes as electron acceptors (33). Likewise, *Dehalococcoides* sp. CBDB1 utilizes dibenzodioxins, chlorinated phenols and ethenes besides chlorinated benzenes (2, 4, 8). With the methods used in the present study, it was not possible to unequivocally distinguish, which OTU was responsible for the degradation of chlorinated benzenes or chlorinated ethenes in TCE/HCB and PCE/HCB amended microcosms. However, since the *Dehalococcoides* sp. 16S rRNA sequences detected from HCB-, TCE- and PCE-degrading microcosms were different from each other, it can be assumed that several different, but closely related strains were involved in the degradation of each compound, supporting our hypothesis on the issue (Fig. 5).

The growth and yield of *Dehalococcoides* spp. was strongly related to the degradation of HCB (Table 2 and Fig. 4). Given the fact that up to date no other microorganism has ever been reported to degrade HCB in anaerobic conditions, it can be assumed that *Dehalococcoides* spp. were the only bacteria in the sediments that were responsible for HCB degradation. In contrast, similarly strong correlations could not be found with chlorinated ethene degradation. Overall growth yields of *Dehalococcoides* spp. were considerably lower when PCE was present. One noticeable difference between TCE and PCE amended bottles was high methane production in the early phases of dechlorination (Fig.2 and Fig.3). From the analysis performed it is not clear what the cause of this limited growth of *Dehalococcoides*


P2_A09040_HCB
P2_H03009_HCB
A07_TCE
H06_TCE
D09_TCE
B12_HCB
uncultured bacterium, Hudson river sediment (AY754845)
H11_HCB
F01_PCE
uncultured bacterium, anaerobic wastewater treatment system (EF688194)
P2_G07026_HCB
uncultured bacterium, anaerobic wastewater treatment system (EF688178)
P2_B01007_HCB, ARB_E01BD538
uncultured bacterium, benzene-degrading, iron-reducing enrichment (EU016426)
Clostridium neopropionicum (X76746)
P2_C09038_HCB
uncultured bacterium, enrichment culture dechlorinating fthalide, PCBs and PC (AB294748)
C04_PCE
uncultured bacterium, TCE-dechlorinating microbial community (AY217423)
P2_A03016_HCB
uncultured bacterium, Anderson Lake (EU283547)
F08_TCE
F05_TCE
G10_TCE
A01_PCE
Clostridium sp. EBR-02E-0046 (AB186360)
P2_A07032_HCB
P2_D01005_HCB
Gracilibacter thermotolerans (DQ117465)
Clostridium sp. FCB90-3 (AJ229251)
P2_D03013_HCB
P2_G05018_HCB
P2_H01001_HCB
uncultured bacterium, enrichment culture dechlorinating fthalide, PCBs and PC (AB294747)
Clostridium methylpentosum (Y18181)
E02_PCE
uncultured bacterium, 0.7 to 1.4 kmbls section of the continental crust (DQ088761)
Eubacterium desmolans (L34618)
Clostridium cellulolyticum (X71847)
F07_TCE
A04_PCE
uncultured bacterium, TCE-dechlorinating groundwater (AY667263)
F10_TCE
uncultured bacterium, TCE-dechlorinating groundwater (AY667268)
Clostridium puniceum (X73444)
Clostridium aurantibutyricum, S46736
P2_B05023_HCB
B06_TCE
Clostridium perfringens C str. JGS1495 (ABDU01000079)
C01_PCE
E01_PCE
E08_TCE
H02_PCE
Acetobacterium carbinolicum (X96956)
D02_PCE
P2_E03012_HCB
P2_H07025_HCB
Acetobacterium wieringae (X96955)
A12_HCB
Clostridium sticklandii (M26494)
P2_B07031_HCB
H01_PCE
P2_H05017_HCB
uncultured bacterium, deep subsurface groundwater from sedimentary rock milieu (AB237711)
B02_PCE
Sedimentibacter saalensis (AJ404680)
B01_PCE
uncultured bacterium, TCE-contaminated site (AY133091)
D03_PCE
G04_PCE
uncultured bacterium, aquifer soil, PCE flow column (FM178805)
Bacillus aminovorans (X62178)
P2_G01002_HCB
Bacillus niacini (AF468221)
B10_TCE
C02_PCE
P2_B03015_HCB
E07_TCE
uncultured bacterium, Xiao River receiving treated oxytetracycline production wastewater (EU864464)
F04_PCE
Sporomusa ovata (AJ279800)
Anaeroarcus burkinensis (AJ010961)
C08_TCE
Acidaminococcus fermentans (X78017)
P2_F07027_HCB
E05_TCE
P2_D05021_HCB
E09_TCE
C07_TCE
D11_HCB
uncultured bacterium, deep subsurface groundwater from sedimentary rock milieu (AB237707)
B04_PCE
uncultured bacterium, polychlorinated dioxin dechlorinating microcosm (AB186884)
Dehalobacter restrictus (U84497)
Desulfitobacterium hafniense (X94975)
G02_PCE
uncultured Symbiobacterium sp. (EU639089)
Geiria glutamica (AF321086)
Lahn-Cluster
Dehalococcoides spp.
Dehalobium chlorocoercia DF-1 (AF393781)
D01_PCE
uncultured bacterium, polychlorinated dioxin dechlorinating microcosm (AB186892)
A03_PCE
uncultured bacterium candidate division OP11, TCE-dechlorinating groundwater (AY667256)
Firmicutes
Dehalococcoidetes

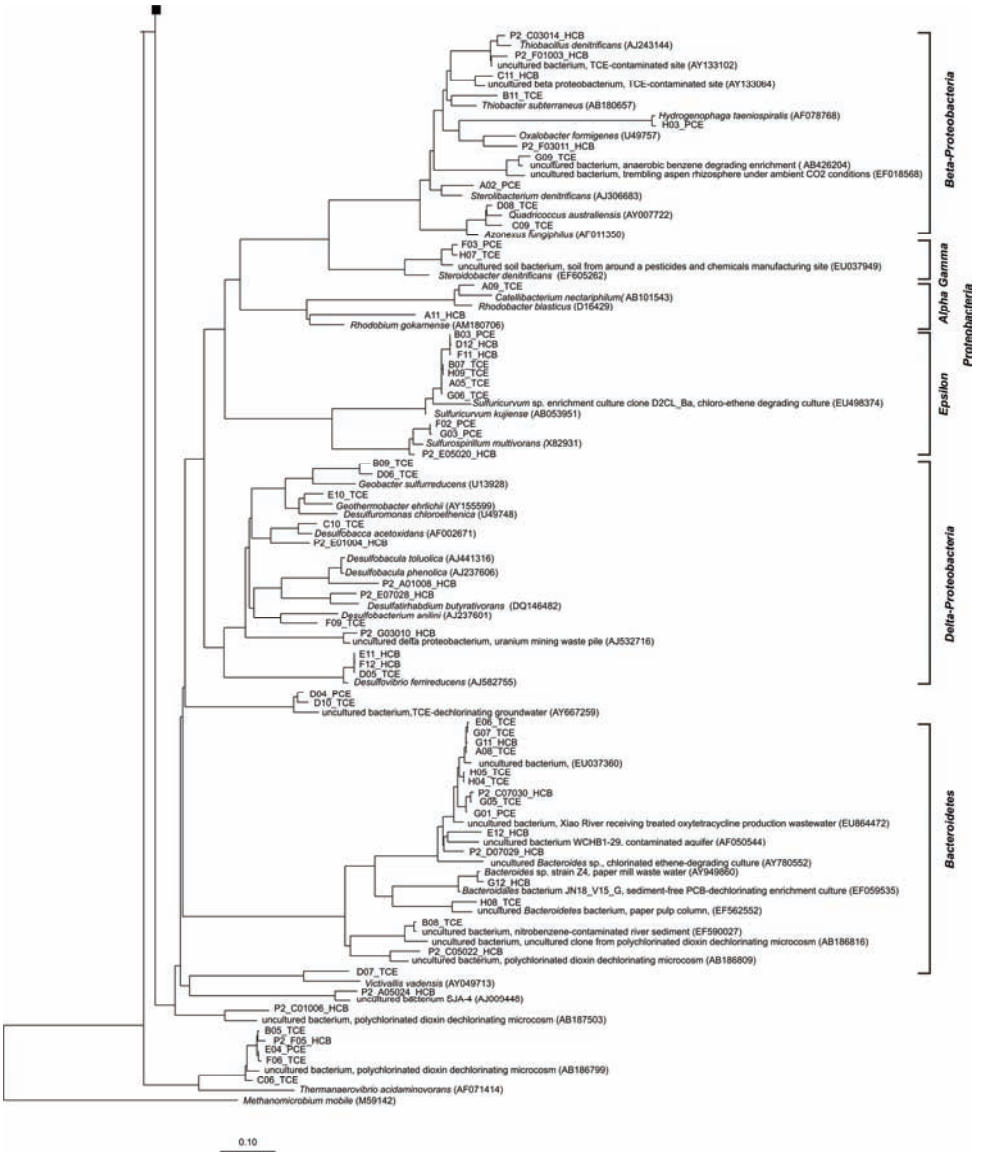


Figure 7. Neighbor-joining tree of 16S rRNA gene sequences from TCE, PCE and HCB microcosms The tree was constructed based on partial sequences (*E. coli* positions 27 – 800) of the 16S rRNA-encoding gene. Scale bar represents 10% dissimilarity. Accession numbers of reference sequences are given in parentheses.

spp. in PCE amended microcosms may be. However, methanogenesis may have caused shortage of H_2 (30) or other growth supporting elements for *Dehalococcoides* spp. thus limiting their growth. Increases in *Dehalococcoides* spp. 16S rRNA gene copies and rRNA relative abundance, taken as an indicator of growth, were not observed during TCE or PCE degradation, but during degradation of the intermediate dechlorination product *cis*DCE. From these data it is tempting to speculate that complete dechlorination of TCE or PCE in these sediments occurs sequentially, while bacteria not belonging to the genus *Dehalococcoides* are responsible for initial dechlorination of PCE and TCE, and native *Dehalococcoides* spp. dechlorinate *cis*DCE. *Dehalococcoides* sp. strains BAV1 and VS have been shown to grow on *cis*DCE and VC, but not on PCE or TCE (10, 17). Cloning and sequence analysis of bacterial 16S rRNA genes from PCE- and TCE-degrading microcosms indicated some likely candidates for this commensal relationship. One phylotype in the PCE-degrading microcosm was most similar to *Sporomusa ovata* which has been shown to dechlorinate PCE to TCE with homoacetogenesis from methanol and carbon dioxide (7). Similarly three phylotypes in the PCE- and TCE-degrading microcoms were affiliated with *Desulfuromonas chloroethenica* and *Sulfurosprillium multivorans,* which have been shown to degrade PCE and TCE to *cis*DCE (26, 36).

Full-genome sequence analyses and amplification of *rdh* genes with degenerate primers revealed that there are seven to 32 different *rdh* genes in various *Dehalococcoides* strains (24, 25, 27, 37). Even though the function of most of these genes is unknown, the majority of those presented in this manuscript (*cbrA*, *pceA* and *tceA*) have been linked to a specific activity (3, 14). *CbrA* gene expression was observed in *Dehalococcoides* sp. CBDB1 during its growth with 1,2,3,4-TeCB and 1,2,3-TCB (3). The function of *cbdbA1624* is unknown, however, its homologues were frequently found in clone libraries from HCB degrading microcosms of Ebro's sediment (**Chapter 5**). Therefore, the transcription of *cbdbA1624* was followed in parallel to the *cbrA* transcription during HCB degradation. In Ebro's sediment, transcription of *cbrA* was detected from the beginning of HCB dechlorination, while transcription of *cbdbA1624* was also high during the degradation of HCB (Fig.6). One of the end products of 1,2,3-TCB dechlorination by *Dehalococcoides* sp. CBDB1, 1,2-DCB (1), was not detected in any of the microcosms (data not shown). The other end product, 1,3-DCB was detected in all microcosms, with higher concentrations in HCB and TCE/HCB microcosms than in PCE/HCB microcosms (Table 1). The major end products of degradation, however, were 1,4-DCB and 1,3,5-TCB, which are produced from 1,2,4-TCB and 1,2,3,5-TeCB (1), respectively. As a result, even though transcription of *cbrA* and *cbdbA1624* was observed, it can not be excluded that other *rdh* genes not targeted in this study were expressed during HCB degradation. It should also be noted that pure culture studies previously suggested that expression levels of *rdh* genes in batch systems are highly dependent on the sampling time. Therefore the expression does not necessarily correlate with

detected dechlorination of the chlorinated compound (36). Still, the genes involved in the various steps of HCB dechlorination are left to be discovered. From the chemical and molecular data presented earlier it was assumed that *Dehalococcoides* spp. in this sediment were only capable of dechlorinating *cis*DCE (Fig.4). Our results show that it will be challenging to use *rdh* gene expression to follow degradation of complex compounds like HCB and PCE. Not only are several different *rdh* genes likely to be involved in their degradation but also several different organisms are likely to have a role in the degradation of these compounds. Such complicated networks can be studied more accurately with specialized microarrays that allow for more comprehensive and simultaneous detection of presence and activity of a large number of genes (45).

This study shows the ability of Ebro River sediment to dechlorinate chlorinated ethenes alongside with chlorinated benzenes. Even though there is no record of chlorinated ethene contamination in this location, *Dehalococcoides* spp. and other dechlorinating bacteria were able to degrade all of the chlorinated compounds. Our findings underline the potential of this rivers' sediment to tackle these compounds once the favorable environmental conditions are achieved. Although it is encouraging to find a potential for bioremediation, it is still challenging to assess, which organisms are responsible for degradation of these compounds. *Dehalococcoides* spp. are believed to be an important part of bioremediation of PCE and TCE polluted groundwater (5, 38) and their capacity and function in the river systems remains to be discovered.

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Chapter 07

General Discussion and Concluding Remarks

Neslihan Taş

To be submitted with parts from **Chapter 01**

The little bacteria that can

In 1997, Maymó-Gatell and co-authors isolated the first anaerobic bacterium, *Dehalococcoides ethenogenes* strain 195 (35), that can transform toxic tetrachloroethene (PCE) to non-toxic ethene. Since then, *Dehalococcoides* spp. were found to be dechlorinating a variety of hazardous chlorinated pollutants like chlorophenols, polychlorinated dibenzo-*p*-dioxins (16), polychlorinated biphenyl (PCB) congeners (10) and chlorinated benzenes (2, 16). In **Chapter 1** of this thesis a general introduction was provided about the traits of this interesting group of microorganisms. *Dehalococcoides* is a species of many irregularities. Even though several representatives of this genus have one of the smallest genomes of free-living bacteria (29, 45), they also contain the highest number of reductive dehalogenase (*rdh*) genes within all known dechlorinating genera (**Chapter 1**, Table 4). Regardless of their general specialization to reductive dechlorination, every strain isolated so far has its own choice of favorite chlorinated compound(s). The unusual dependence of *Dehalococcoides* spp. on chlorinated organic compounds for their growth made it very interesting to study their application in bioremediation. Yet our knowledge about presence, activity and capabilities of this genus in the environment is rather limited, including its response to changes in environmental conditions.

It can be envisaged that global climate changes are and will keep on shaping the aquatic biota in the river basins. The anticipated river basin management will, even more than before, need to deal with frequent flooding of flood planes. This causes an introduction of pollutants and altered geochemical conditions. This is an important issue to tackle, since the degradation of environmental pollutants is strongly determined by the prevailing conditions. In the summer of 2004, The EU 6th Framework Integrated Project Aqua Terra was initiated to address these issues and to investigate the fate and impact of persistent organic pollutants and heavy metals in several European river basins (8). To detect the effect of environmental changes on the microbial ecology it was crucial to know the fate and functioning of microbial communities involved in biodegradation of pollutants. Therefore, the aim of this research was to study the microbial ecology of *Dehalococcoides* spp. and to learn more about their capacity for the biodegradation of chlorinated organic pollutants – specifically hexachlorobenzene (HCB) - in the sediments and flood plain soils of European river basins.

Biogeography of *Dehalococcoides* spp.

As the ecologists' quest prevails to delve Becking and Beijerinck's long running argument: "Everything is everywhere, but, the environment selects." (9, 11), with the development of novel molecular tools, it is now possible to study the full extent of microbial diversity and describe the biogeographical patterns exhibited by microorganisms at large spatial scales (17, 34). Sequencing of 16S rRNA genes from environmental samples has not only changed the

way that microbial diversity of ecosystems is studied but also our understanding of microbial systematics and diversity in general (43). Additionally, ribosomal RNA copy numbers have the potential to be indicative of ecosystem functioning and could be important for predicting the response of ecosystems to environmental change (21).

With the growing interest on *Dehalococcoides*' presence and functioning in the environment, several studies were conducted using *Dehalococcoides*-specific 16S rRNA gene-based approaches in uncontaminated and chlorinated ethene contaminated sediments, soils and groundwater aquifers (23, 28, 31). Additionally, lab-scale microcosm experiments reported the enrichment of *Dehalococcoides* spp. in actively dechlorinating batches, using sequencing and/or quantitative detection of the 16S rRNA gene (4, 10, 13, 15, 18, 24). In summary, all of these studies showed the presence of *Dehalococcoides* spp. in different locations and environments in the Northern Hemisphere (mainly concentrated in North America, Europe, and Japan). In **Chapters 2** and **3** we have presented a large scale survey of presence, activity and dechlorination potential of *Dehalococcoides* spp. focusing on river sediments and floodplain soils from different locations in Europe (Fig. 1). HCB deposition due to industrial and agricultural activities was evident for the selected locations prior to this study (8, 30). In highly heterogeneous environments like river basins, however, it is challenging to establish clear links between microbial species composition or activity and changes in environmental conditions and pollutants concentrations. Almost all of the tested sediment and soils samples showed the capacity to dechlorinate HCB irrespective of the *in situ* HCB levels (**Chapter 2,** Table 1). The HCB transformation rates observed in the laboratory scale microcosms and the number of 16S rRNA gene copies of *Dehalococcoides* spp. in the environmental samples did not show a strong correlation. Nevertheless, it was striking to observe a relatively wide spread endogenous HCB transformation capacity in the sediments and soils of European river basins. Even though dechlorination capacity and *Dehalococcoides* spp. were present in these samples, HCB contamination is still prevalent in the environment. This is not entirely unexpected since the transformation experiments described were carried out under ideal conditions for the dechlorinating bacteria, e.g. the medium contains every nutrient the microorganisms may require. This discrepancy with the real-life situation of HCB being still present in the sediment raises questions about *Dehalococcoides*'s usefulness in the bioremediation or natural attenuation of HCB in contaminated sediments. However, when compared to chemical (e.g. chemical reaction with hydroxyl (OH) radicals, with a half-life of 156 days to 4.2 years (6)) or physical (e.g. atmospheric photolysis, with a suggested half-life of about 80 days (41)) processes, biological reductive dechlorination of HCB, with the half-lives observed in this study ranging from 2 to 62 days (**Chapter 2**, Table 1), is a relatively fast process provided that the conditions are suitable for dechlorination. Moreover, the results presented in this thesis support the findings of recent multimedia fate models, which predict that on a global scale the greatest losses of

		●	◘	✦	⅄
Ebro	1-Reinosa	-	na	-	-
	2-Conchas de Haro	++	na	+++	-
	3-Zaragoza	+	na	+++	++
	4-Lerida	++	+	+++	++
	5-Flix	+++	+	+++	+++
	6-Tortosa	+++	+	+++	++
	7-Delta	+++	+	+++	++
Elbe	8-Roßlau	+	na	-	-
	9-Schönberg [a]	+	+++	+/-	++
	10-Schönberg [b]	+++	+	+++	++
Danube	11-Budapest	+	na	++	na
Others	12-PCE contaminated soil	+++	na	+	+++
	13-Rhine River	+	na	+++	++
	14-Baltic Sea & Waddenzee	+	na	-	++

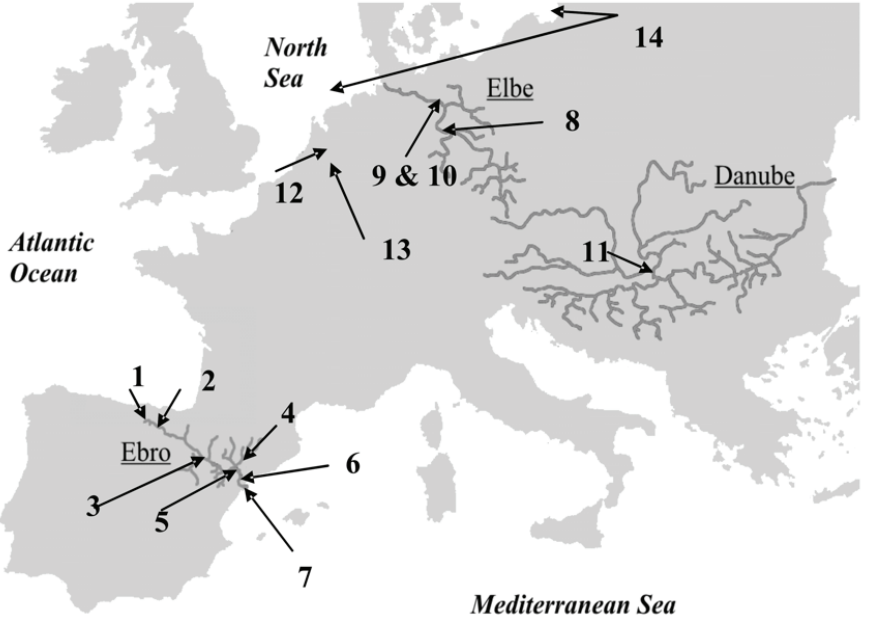

Figure 1. Summarized results from the locations studied in this thesis with culture dependent and independent (molecular) methods. ●: *Dehalococcoides* spp. detection with 16S rRNA and/or 16S rRNA gene targeted methods; ◘: *Desulfitobacterium* spp. detection with 16S rRNA targeted methods; ✦: HCB transformation; ⅄: chlorinated ethene transformation; (-) no detection or no transformation, (+/++/+++) low to high rRNA copies or long to short lag phases in HCB and chlorinated ethene transformation; na: not available; (a) soil and (b) river sediment sample from Schönberg. Map is re-drawn from Barth *et al.(7)*

HCB in the environment may occur from sediment and soils (5, 6). As a result it became even more essential to identify the parameters that affect the activity and diversity of *Dehalococcoides* spp. in field conditions in these key compartments.

The results of detailed monitoring of *Dehalococcoides* spp. activity and diversity over a period of two years in two selected European rivers, the Ebro and the Elbe, are presented in **Chapter 3**. In the overall framework of this thesis the (relative) abundance of *Dehalococcoides* spp. 16S rRNA copy numbers was used as a proxy for metabolic activity of these dedicated degraders. Even though details for the case of *Dehalococcoides* spp. are not known, a strong positive correlation between growth and rRNA content has been shown for a variety of other microorganisms (26, 27). Active species of *Dehalococcoides* could be detected in the majority of the samples. *Dehalococcoides* spp. 16S rRNA relative abundance (i.e. *Dehalococcoides* spp. 16S rRNA copies / total Bacteria 16S rRNA copies) changed significantly along temporal and spatial gradients, reaching 1% in some locations (**Chapter 3,** Fig. 2). In most of the cases statistical analysis did not find any significant and/or strong correlations between sampling time and *Dehalococcoides* spp. activity. Species composition, however, strongly changed during the monitoring period, with soils being less diverse than river sediments (**Chapter 3,** Fig. 4 and Fig. 5). In the Ebro these changes were significantly correlated with sampling time. In the Elbe, on the other hand, changes were significantly correlated with HCB concentrations and number of *Dehalococcoides* spp. 16S rRNA copies (**Chapter 3**, Table 4, Fig. 7). However, at this point it should also be mentioned that lack of measurements for parameters such as the dissolved oxygen concentration or levels of metal contamination (in the Elbe) did not allow us to provide a complete assessment of the environmental conditions. Moreover, it has been shown in pure culture studies that the activity and dechlorination rate of *Dehalococcoides* spp. also depend on e.g. the presence of co-factors such as vitamin B_{12} (22). The effect of these parameters on the composition and activity of *Dehalococcoides* spp. in river sediments remains to be discovered. It is necessary to note that the relative abundance of representatives of a certain group of microorganisms is not necessarily linked to the importance of that group in the functioning of the community. Highly abundant groups may not readily play an important role in the functioning of the system, and organisms that only cover 0.1% of the community (e.g. nitrogen fixers) (14) can be of critical importance. The data presented in **Chapter 3** suggest that biogeography of *Dehalococcoides* spp. is strongly influenced by environmental factors. As non-fermentative microorganisms *Dehalococcoides* spp. depend on the H_2 supply from other microorganisms for their growth (48). Recently, it has also been suggested that the activity of *Dehalococcoides* spp. in *in situ* conditions is linked to the performance of fermentative communities (44). Therefore, it is crucial to have insight in factors affecting nutrient fluxes and microbial communities involved in carbon, nitrogen, sulfate (C, N, S) cycling in the river basins to be able to understand the survival and functioning of *Dehalococcoides* spp. in wide spread geographical locations.

The introduction of functional gene arrays in microbial ecology

Functional gene arrays (FGAs) are very useful tools for high throughput analysis of the genomic potential and activities of microbial populations (55). The GeoChip (35) that can detect 10,000 catabolic genes involved in the biogeochemical processes, was applied in the Ebro river basin (**Chapter 3** and **4**) to learn more about the functional diversity of microbial communities and to identify important guilds involved in C, N, S cycles, as well as contaminant degradation. The results of this analysis showed a great abundance and diversity in genes involved in C, N, S cycles. Genes from all microbial kingdoms could be detected with the majority of the eukaryotic and archaeal genes being involved in the C cycle. The abundance of bacterial genes, on the other hand, could be ranked as N > C > S (**Chapter 4**, Table 1). It was found that the river basin has the genomic potential for performing complete C, N, S cycles. Specialized aerobic or anaerobic metabolic pathways to degrade organic contaminants, as a sole carbon or energy source, are common in many microorganisms (53). The investigation of the contaminant degradation genes in the Ebro river basin showed that the most significant environmental parameter affecting the diversity and abundance of this gene group was the sampling location (**Chapter 4**, Table 2). This could most strikingly be demonstrated for *rdh* genes of dechlorinating bacteria (**Chapter 3**, Fig. 8 and 9). GeoChip analysis, targeting 153 *rdh* genes, showed that *rdh* gene diversity changed significantly between different sampling locations ($p \leq 0.001$). More specifically, samples from the location Flix, which is especially characterized by high HCB pollution (30), were dominated by *rdh* genes of *Dehalococcoides* spp. strain CBDB1 and *Dehalococcoides ethenogenes* strain 195. In contrast, in samples taken at downstream locations (rice fields in the estuary), which are characterized by more diffuse pollution with a broader range of contaminants, a wide spectrum of *rdh* genes was detected including those from various other halorespiring microorganisms (**Chapter 3**, Fig. 9). Thus, it could be suggested that, since the diversity and abundance of the contaminant degradation genes will be mainly controlled by pollutants rather than other environmental parameters, these genes possess a great potential to be used as biomarkers of degradation capacity. However, to serve this purpose, developers of PCR or array based techniques will need to address technical problems like sensitivity (e.g. **Chapter 4**, Table 7) or sequence variability and availability. Here, it should also be noted that microarrays can only detect known sequences, which can cause an underestimation of functional gene diversity and abundance in environments with limited sequence information. Application of FGAs in combination with newly developed techniques such as high throughput non-gel based proteomics (33) and sequencing of the metatranscriptome (20, 50) offer a remarkable promise. During the contaminant biodegradation, gene transcripts can be studied using transcriptomic techniques with FGAs (19), in combination with proteomics methods to identify the proteins with significant functional impact (47).

Reductive dechlorination of HCB in river systems

Chapter 5 described the effect of several environmental factors on abundance and diversity of the *Dehalococcoides* spp. in the environment, focusing on parameters relevant to the river ecosystems studied here. To this end, sediments from the Ebro river basin were exposed to varying temperature and sea salt concentrations to test the fitness of the sediment-associated *Dehalococcoides* spp. to an environmental stress. Tetrachloroethene (PCE) and polychlorinated biphenyls (PCBs) can be transformed in low temperatures (4-12°C) (12, 39) and estuary sediments were shown to degrade dioxins in high salinity medium (46). However involvement of *Dehalococcoides* spp. in these transformations is not known. In most of the cases HCB dechlorination was achieved even in submesophilic and psychrophilic conditions (15-20°C) or at high salinities (up to 0.2M). Lag phases of various lengths (several days to tens of days) and dominance of the thermodynamically most favorable pathways (i.e. under most of the extreme environmental conditions dechlorinating bacteria favored 1,3,5-TCB production) were observed. We could show that *Dehalococcoides* spp. are able to adapt to changes in the environment in a relatively fast manner but in the case of extreme alterations (e.g. exposure to low <15°C or high >30°C temperatures or very high salinity levels, 0.5M) adaptation might not be possible. Here it should be noted that temperature was already identified as an important parameter explaining the variances in *Dehalococcoides* spp. composition in the river basins studied (**Chapter 3**, Table 4). Additionally simultaneous exposure to different aromatic and/or aliphatic chlorinated compounds were also studied in Flix sediment (**Chapter 6**). PCE and trichloroethene (TCE) amended microcosms performed similarly by fully dechlorinating the compounds to ethene, when chlorinated compounds were provided as the sole electron acceptors. HCB was degraded to 1,3,5-TCB, 1,4-DCB and 1,3-DCB. Mixes of compounds were also degraded, either at the same time or sequentially. Unlike previous tests (**Chapter 5**), sequencing analysis show the presence of more than one *Dehalococcoides* spp. in each microcosm. Another remarkable finding was that even though there is no record of chlorinated ethene contamination in this location, *Dehalococcoides* spp. and other dechlorinating bacteria were able to degrade all of the chlorinated compounds. These results together underline again the potential of endogenous microorganisms present in European rivers' sediment to tackle these compounds.

Future perspectives: Reductive dechlorination, systems microbiology and microbial networks

The broad aim of systems microbiology is to define and understand the relationships between the individual components that build a cellular organism, a community and an ecological niche (51). As a result, in the past, the focus of systems microbiology was on microbial isolates or enrichments (36). To date, the majority of the research conducted in the field of reductive dechlorination has been predominantly focused on the identification of genes and

proteins involved in the dechlorination process (1, 13, 24, 37, 38, 52, 54). These experimental studies, so far, allowed the analysis and characterization of only a few genes. However, it is becoming evident that in order to understand microbial functions or the functioning of microbial communities one must study the entire system (51). The research presented in this thesis also supports this idea and suggests that with biomolecular assays targeting ribosomal and process-specific functional genes such as those encoding reductive dehalogenases, it will remain difficult to understand the full extent of the process, since the dechlorination process is a part of a complex web of metabolic and regulatory interactions (42, 52, 54) The application of novel, more comprehensive methods like whole genome shotgun (WGS) sequencing of environmental DNA and mRNA (functional metagenomics) (25, 49) as well as the development of new computational resources for comparative genomic analyses (3, 32, 40) enable us to develop and analyze datasets (and microbial networks) which are so far the closest to the actual environmental situations. Thus, today, it can be proposed to leave reductionist methodologies and to study reductive dechlorination and the function of *Dehalococcoides* spp. in larger communities and in the environments in which they belong. As the functional properties of such communities are elucidated, the true role and importance of *Dehalococcoides* spp. in the environment can be assessed.

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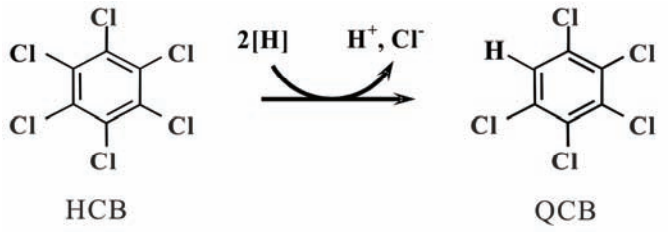
Nederlandse samenvatting

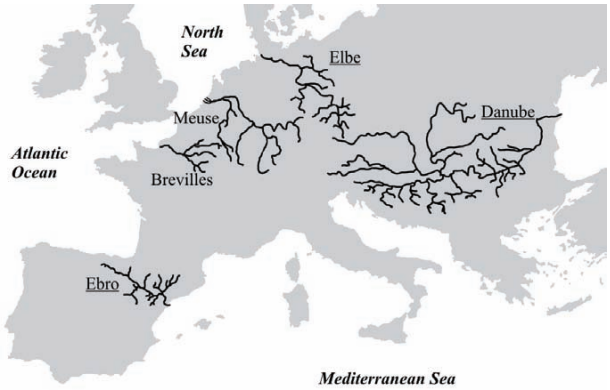
In 1997 isoleerden Maymó-Gatell en collegas *Dehalococcoides ethenogenes* stam 195. Dit is een anaërobe bacterie, dat wil zeggen een bacterie die onder volledig zuurstofloze omstandigheden leeft en in staat is het giftige tetra-chlooretheen (PCE) om te zetten in het niet-giftige etheen. Sindsdien is gebleken dat leden van het geslacht *Dehalococcoides* (*Dehalococcoides* spp.) een breed scala aan gechloreerde verbindingen kunnen afbreken. In dit proefschrift staan *Dehaloccoides* spp. centraal vanwege hun vermogen één van deze gechloreerde verbindingen, hexachloorbenzeen (HCB), af te breken tot een verbinding met minder chlooratomen. HCB werd tot voor kort voornamelijk toegepast als fungicide en pesticide en kan bijvoorbeeld vrijkomen bij de productie van synthetisch rubber. Daarnaast wordt het gevormd als bijproduct tijdens de productie van oplosmiddelen en pesticiden. HCB is kankerverwekkend, giftig en hoopt zich op in ecosystemen. Tegenwoordig is het gebruik ervan binnen de E.U. dan ook verboden, maar omdat de stof erg moeilijk afbreekbaar is zal deze nog gedurende lange tijd worden teruggevonden in het milieu.



Figuur 1. Dechlorering van HCB

Hoofstuk 1 van dit proefschrift bevat een algemene inleiding over *Dehalococcoides* spp. en de eigenschappen die deze familie van dechlorerende micro-organismen kenmerken. Ze zijn, zelfs naar bacteriemaatstaven, bijzonder klein. Zoals gezegd zijn het anaërobe bacteriën; ze overleven alleen in zuurstofloze omgevingen, zoals bodems en sedimenten, waar zij hun energie halen uit de omzetting van gechloreerde verbindingen door deze van hun chlooratomen te ontdoen. Dit proces wordt dechlorering genoemd (Figuur 1). *Dehalococcoides* spp.is een geslacht met unieke eigenschappen. Het genoom (de totale hoeveelheid erfelijk materiaal van een organisme) van *Dehalococcoides* spp. behoort tot het kleinste in het bacterierijk, maar van alle tot nu toe bekende bacteriën bevat het wel de meeste genen, die (mogelijk) coderen voor dechlorerende enzymen. Elke tot nog toe geïsoleerde *Dehalococcoides* stam heeft een uitgesproken voorkeur voor slechts enkele gechloreerde verbindingen. Deze ongebruikelijke specialisatie maakt deze bacteriën tot perfecte kandidaten voor het reinigen van bodem en riviersedimenten die zijn verontreinigd met deze verbindingen. Desalniettemin is onze kennis over de aanwezigheid, activiteit en vaardigheden van de *Dehalococcoides* spp. in hun natuurlijke leefomgeving vrij beperkt. Ook is grotendeels onbekend hoe *Dehalococcoides* reageert op veranderingen in het milieu; bijvoorbeeld als

gevolg van klimaatverandering. Het is niet ondenkbaar dat mondiale klimaatveranderingen een effect hebben en zullen blijven houden op ecosystemen in de stroomgebieden van rivieren. Er moet, meer dan voorheen, rekening worden gehouden met de mogelijkheid dat rivieren buiten hun oevers kunnen treden. Het riviersediment dat hierbij wordt afgezet verontreinigt en verandert de samenstelling van de bodem. Dit zal ook gevolgen hebben voor de aanwezigheid en activiteit van de micro-organismen die ter plekke aanwezig zijn.



Figuur 2. Stroomgebieden van de riviersystemen die in het Aqua Terra project zijn bestudeerd.

Het in dit proefschrift beschreven onderzoek maakte deel uit van het internationaal project AquaTerra". Dit project, gefinancierd door het 6^{e} kaderprogramma van de Europese Unie, richt zich op het integraal beheer van water, sediment en bodem in de stroomgebieden van Europese rivieren. Dit project had als doel problemen in kaart te brengen die als gevolg van klimaatverandering in de toekomst zouden kunnen optreden en de gevolgen van vervuiling met zware metalen en moeilijk afbreekbare organische verbindingen op de stroomgebieden te onderzoeken. Voordat de effecten van een veranderend milieu op micro-organismen in een ecosysteem kunnen worden ingeschat moet eerst inzicht verkregen worden in het functioneren van de microbiële gemeenschappen die verantwoordelijk zijn voor de biologische afbraak van verontreinigingen. Het doel van het hier beschreven onderzoek was, om in het kader van het AquaTerra project inzicht te krijgen in *Dehalococcoides* spp. en hun plaats in het ecosysteem, en zo te bepalen in welke mate *Dehalococcoides* spp. organische gechloreerde verbindingen (en dan met name HCB) kan afbreken in bodems en sediment.

Terwijl ecologen nog steeds de stelling van Baas Becking en Beijerinck: '*Alles is overal*: maar *het milieu selecteert*" onder de loep nemen is het nu met moderne moleculaire technieken mogelijk de volle omvang van de diversiteit van micro-organismen te bestuderen. Met behulp van deze technieken was het onder andere mogelijk de volgorde van de nucleotiden in16S rRNA genen te bepalen (zie kader). Dit proces, "16S-rRNA sequencing", heeft niet alleen geleid tot een verandering in de manier waarop we de microbiële diversiteit

van ecosystemen bestuderen, maar heeft ook bestaande inzichten in microbiële taxonomie en diversiteit veranderd. Tevens kan het aantal 16S rRNA genen dienen als een indicator voor het functioneren van een ecosysteem en kan het helpen te voorspellen hoe ecosystemen op veranderingen in het milieu zullen reageren.

Bijna alles wat je moet weten over DNA en het 16S-rRNA gen.
DNA is het erfelijk materiaal van al het leven op aarde. Een DNA-molecuul bestaat uit twee lange spiraalvormige ketens die zijn opgebouwd uit vier verschillende nucleotiden (bouwstenen met de namen adenine (A), guanine (G), thymine (T) en cytosine (C)). De zo gevormde lange ketens van A's, G's, T's en C's kunnen worden opgevat als een soort alfabet dat een instructiecode vormt voor erfelijke eigenschappen. De in het DNA opgeslagen informatie voor een bepaalde erfelijke eigenschap wordt een gen genoemd.
"Gene sequencing" is een moleculaire techniek waarmee de volgorde van de nucleotiden in een gen kan wordt bepaald, zodat de instructiecode kan worden gelezen. Voor dit onderzoek is veelvuldig van deze techniek gebruik gemaakt om, onder andere, het 16S-rRNA gen te sequencen.
Het 16S-rRNA gen is aanwezig in alle bacteriën. Bepaalde stukken van dit gen zijn hetzelfde voor elke bacterie binnen een soort. Hierdoor kan het gen worden gebruikt om soorten bacteriën te onderscheiden en te identificeren. De manier waarop dit gebeurt kan een beetje worden vergeleken met een identiteitskaart. Een identiteitskaart bevat kenmerken die in iedere kaart hetzelfde zijn: De vorm, de kleur de tekst maken de kaart herkenbaar als een identiteitskaart. Op een zelfde manier kan een 16S-rRNA gen worden herkend als een 16S-rRNA gen. De foto en de persoonsgegevens op een identiteitskaart zijn echter voor ieder individu verschillend. Aan de hand van deze gegevens kan je een persoon identificeren. Zo zijn er ook binnen een 16S-rRNA gen gebieden die van soort tot soort verschillen. Aan de hand van deze gebieden kan men dus via "16S-rRNA gene sequencing" een bacteriesoort identificeren.

In de **hoofdstukken 2** en **3** wordt een grootschalig onderzoek gepresenteerd naar de aanwezigheid, activiteit en het dechlorinerende vermogen van *Dehalococcoides* spp. in riviersedimenten en de bodems in uiterwaarden van verschillende Europese rivieren (zie figuur 2). Voorafgaand aan dit onderzoek was bekend dat de voor dit onderzoek geselecteerde locaties vervuild waren met hexachloorbenzeen (HCB) als gevolg van industriële en agrarische activiteiten. In een steeds veranderende omgeving als een stroomgebied van een rivier is het een grote uitdaging om duidelijke verbanden te leggen tussen enerzijds de activiteit en samenstelling van het microbiële leven en anderzijds het veranderende milieu en wisselende concentraties gechloreerde verbindingen. Verrassend genoeg werd HCB in bijna alle onderzochte sediment- en bodemmonsters afgebroken, onafhankelijk van de HCB

concentraties ter plekke (zie **hoofdstuk 2**, tabel 1). Onder laboratoriumomstandigheden werd geen sterke correlatie gevonden tussen de omzettingssnelheid van HCB en de hoeveelheid 16S rRNA genen van *Dehalococcoides* spp. in de sediment- en grondmonsters. Om deze reden is het uitermate belangrijk te begrijpen wat de factoren zijn die de activiteit en diversiteit van *Dehalococcoides* spp. in bodems en sedimenten bepalen. Hoofdstuk 3 bevat de resultaten van een twee jaar durend onderzoek naar de activiteit en diversiteit van *Dehalococcoides* in twee riviersystemen, de Ebro in Spanje en de Elbe in Duitsland, waarvan het sediment en de bodem in de uiterwaarden in beide gevallen op een aantal plaatsen verontreinigd is met HCB. De samenstelling van de microbiële gemeenschap is in de loop van het onderzoek aan veel veranderingen onderhevig geweest. De microbiële diversiteit was lager in de bodems dan in de sedimenten. (zie **hoofdstuk 3**, figuur 4 en 5). In de Ebro waren deze veranderingen sterke gecorreleerd aan het tijdstip van het nemen van de monsters, en in de Elbe aan de HCB concentraties en het aantal *Dehalococcoides* spp. (zie **hoofdstuk 3**, tabel 4, figuur 7). De resultaten in dit hoofdstuk suggereren dat de distributie van *Dehalococcoides* spp.in het milieu sterk wordt beinvloed door milieufactoren. Deze factoren worden niet altijd bepaald door het tijdstip en de locatie. Zo zijn *Dehalococcoides* spp. voor bijvoorbeeld de voorziening van waterstof en vitamine B_{12}, beiden essentieel voor de groei, afhankelijk van andere micro-organismen. Recentelijk is (door anderen) geoppperd dat er een verband bestaat tussen de activiteit van *Dehalococcoides* en de activiteiten van micro-organismen, die door fermentatie waterstof produceren. Het is dan ook cruciaal inzicht te verkrijgen in de factoren die bepalend zijn voor het transport van deze en andere voedingsstoffen en de betrokkenheid van microbiële gemeenschappen in koolstof-(C), stikstof- (N-) en sulfaat-(S) cycli in stroomgebieden om zo het functioneren van *Dehalococcoides* beter te begrijpen.

Nader bekeken: Microarrays in de microbiële ecologie

Het merendeel van de hedendaagse moleculaire technieken is er op gericht informatie te vergaren over één specifiek aspect van een bacterie, zoals bijvoorbeeld het 16S rRNA gen of een gen dat codeert voor het afbreken van bijvoorbeeld koolstofverbindingen. Microarrays daarentegen, maken deel uit van een nieuwe techniek die een microbioloog in staat stelt duizenden verschillende eigenschapppen tegelijkertijd te detecteren. De hier gebruikte Geochip is een voorbeeld van zo'n microarray. De Geochip is een soort catalogus van alle genen in het milieu die verantwoordelijk zijn voor C,N,S cycli en de afbraak van verontreinigingen. De Geochip is gemaakt door de DNA sequenties van geselecteerde genen te printen op glazen plaatjes. Als vervolgens DNA, dat eerst uit de sediment- en bodemmonsters moet worden gehaald, op zo'n plaatje wordt uitgesmeerd kan door middel van een laser worden uitgelezen welke genen er in dit DNA aanwezig zijn. Deze kennis helpt ons te begrijpen welk micro-organisme welk proces in het milieu uitvoert. Deze nieuwe

technieken leveren enorme hoeveelheden data op. Een goede statistische analyse is dan ook essentieel voor de verwerking van de gegevens.

De **hoofstukken 3** en **4** gaan over de experimenten die met de Geochip zijn verricht. Met behulp van deze microarray werd bepaald welke genen er in het stroomgebied van de Ebro aanwezig zijn. Na statistische analyse kon worden aangetoond dat zowel in het riviersediment als in de bodem een grote diversiteit aan micro-organismen aanwezig is die gezamenlijk bijna alle bekende ecologische functies (relevant voor de C, N, S cycli) kunnen vervullen en daarnaast ook verontreinigingen zoals HCB kunnen omzetten. De meeste genen die verantwoordelijk zijn voor de afbraak van HCB werden gevonden op locaties met de hoogste niveaus van HCB verontreining. Dit laat zien dat, in dit geval, het milieu een potentieel vermogen heeft om zichzelf, zij het erg langzaam, van HCB te ontdoen. Ook hebben we met dit experiment aangetoond dat genen die betrokken zijn bij de afbraak van veronreinigingen niet zozeer beinvloed worden door omgevingsfactoren, maar meer door mate en het type van de vervuiling. Micro-organismen (zoals *Dehalococcoides* spp.) hebben niet alleen de potentie vervuiling op te ruimen (dit proces wordt bioremediatie genoemd), maar de aanwezigheid van deze micro-organismen (of hun genen) kan ook dienen als indicator voor de conditie waarin een ecosysyteem verkeert.

Hoofstuk 5 behandelt het onderzoek naar het effect van verschillende omgevingsfactoren op de diversiteit en aanwezigheid van *Dehalococcoides* spp., waarbij rekening werd gehouden met de verschillende lokale kenmerken van elke rivier. Alle metingen in dit hoofstuk werden verricht in “microcosms”. Dit zijn glazen flessen met sediment en water waarin de zuurstofloze leefomstandigheden van *Dehalococcoides* werden nagebootst (zie afbeelding). Zo werden Ebro sedimenten blootgesteld aan verschillende temperaturen en (zee)zoutconcentraties om de effecten van deze stressfactoren op de in deze sedimenten aanwezige *Dehalococcoides* spp. te onderzoeken. In de meeste gevallen werd er zelfs HCB gedechloreerd onder omstandigheden die ver buiten de “comfort zone” van *Dehalococcoides* spp. lagen (Te warm, te koud, te zout etc.). De tijd tussen het begin van het experiment en de eerste waarneembare vermenigvuldiging van de bacteriën varieerde wel sterk onder wisselende stressniveaus.

We hebben aangetoond dat *Dehalococcoides* spp. in staat zijn zich relatief snel aan te passen aan veranderende omstandigheden, hoewel in extreme gevallen (b.v. blootstelling aan zeer lage of zeer hoge temperaturen of aan zeer hoge zoutconcentraties) aanpassing niet mogelijk lijkt te zijn. Daarnaast is in **hoofdstuk 3** (tabel 4) temperatuur reeds als belangrijke parameter geïndentificeerd om de variatie in de samenstelling van *Dehalocccoides* spp. gemeenschappen te kunnen verklaren.

Om het dechlorerend vermogen van de *Dehalococcoides* populatie in de sedimenten verder te testen zijn verschillende gechloreerde verbindingen in verschillende concentraties toegevoegd aan “microcosms” met sediment uit Flix (een locatie aan de Ebro, zie **hoofdstuk 6**). De *Dehalococcoides* in het sediment waren in staat zowel PCE als trichlooretheen volledig om te zetten naar etheen als deze verbindingen als enige gechloreerde verbinding werden aangeboden. HCB werd omgezet tot 1,3,5-trichloorbenzeen of 1,4- en 1,3-dichloorbenzeen. Cocktails van gechloreerde verbindingen werden ook gedechloreerd, ofwel tegelijkertijd ofwel na elkaar. In tegenstelling tot voorgaande experimenten, bleek uit “16S-rRNA sequencing” dat elke fles meer dan één soort *Dehalococcoides* bevatte. En ondanks het feit dat gechloreerde ethenen nooit zijn aangetroffen in Flix waren de aanwezige *Dehalococcoides* spp. uit deze locatie deze verbindingen toch in staat deze verbindingen zonder problemen om te zetten tot onschadelijke eindproducten. Uit deze resultaten blijkt opnieuw het potentiële vermogen van deze micro-organismen om verontreinigde bodems en sedimenten te zuiveren.

Wat heeft de toekomst voor ons in petto?

In de afgelopen jaren is door de komst van nieuwe technologiën de wijze waarop microbiologen en moleculair ecologen hun werk doen sterk veranderd. We beseffen meer en meer dat het noodzakelijk is micro-organismen niet meer voor per individuele soort te bestuderen, zoals in het verleden gebruikelijk was. Als we de rol van een micro-organisme in het milieu werkelijk willen begrijpen moeten we de hele leefgemeenschap bestuderen; vele micro-organismen zijn op de één of ander manier afhankelijk van elkaar en/of van dezelfde hulpbronnen.

In het verleden was het om technische reden niet mogelijk inzicht te krijgen in deze interacties tussen micro-organismen. Vandaag de dag zijn we door nieuwe sequencing technieken wel in staat meer- en meer gedetailleerde data te genereren over een micro-organisme en zijn omgeving. Daarom wordt in dit proefschrift dan ook voorgesteld een bacterie niet meer als een enkele entiteit, maar als onderdeel van een groter geheel te bestuderen.

Appendixes

Appendix I

Table A1. Sediment/Soil sample locations used in this thesis

Location	River	Sample type	Latitude	Longitude
Schönberg	Elbe	RS	N 52°90'73.2"	E 11°87'21.9"
Schönberg – Flood plain soil 1	Elbe	FPS	N 52º90'55.8"	E 11º87'09.2"
Schönberg – Flood plain soil 2	Elbe	FPS	N 52 º90'60.5"	E 11º87'09.4"
Lleida	Ebro	RS	N 41º32'10.8"	E 00º30'44.6"
Flix	Ebro	RS	N 41º13'43.6"	E 00º33'09.0"
Tortosa	Ebro	RS	N 40º44'58.3"	E 00º34'03.6"
Delta-Rice Fields	Ebro	AS	N 40°40'54.6"	E 00°37'49.2"
Delta-Estuaria	Ebro	RS	N 40°38'22.7"	E 00°42'39.5"

Abbreviation: RS, river sediment, AS, agricultural soil, FPS, floodplain soil

Table A2. Primers used in this thesis

Primer	Sequence	Positions (16SrRNA gene)	Reference
DeR	ACTTCGTCCCAATTACC	1406–1422, *D. ethenogenes* strain 195[c]	(1)
DeF	GCAATTAAGATAGTGGC	49–65, *D. ethenogenes* strain 195 [c]	(1)
DHC1	GATGAACGCTAGCGGCG	1-17, *D. ethenogenes* strain 195 [c]	(4)
DHC1350R	CACCTTGCTGATATGCGG	1350-1357 *Dehalococcoides spp.* [a]	(3)
27-F	GTTTGATCCTGGCTCAG	27-43 All Bacteria[a]	(5)
1492-R	GG(AT)TACCTTGTTACGACTT	1492-1510 All Bacteria[a]	(5)
968F*	AACGCGAAGAACCTTAC	968-984 All Bacteria[a]	(7)
341F[#]	CCTACGGGAGGCAGCAG	341-357 All Bacteria[a]	(6)
534R[#]	ATTACCGCGGCTGCTGGC	534-517 All Bacteria[a]	(6)
Dre441F[#]	GTTAGGGAAGAACGGCATCTGT	441-461 *Dehalobacter spp.* [a]	(8)
Dre645R[#]	CCTCTCCTGTCCTCAAGCCATA	645-666 *Dehalobacter spp.* [b]	(8)
Dsb406F[#]	GTACGACGAAGGCCTTCGGGT	406-426 *Desulfitobacterium spp.* [a]	(8)
Dsb619R[#]	CCCAGGGTTGAGCCCTAGGT	610b-619 *Desulfitobacterium spp.* [a]	(8)
Dco728F[#]	AAGGCGGTTTTCTAGGTTGTCAC	728-750 *Dehalococcoides spp.* [a]	(8)
Dco944R[#]	CTTCATGCATGTCAAAT	928-944 *Dehalococcoides spp.* [a]	(8)
T7[+]	TAATACGACCACTATAGGG	-	Promega
SP6[+]	TAAATCCACTGTGATATCTT	-	Promega
cbdbA84f[#]	CTTATATCCTCAAAGCCTGA	118-138 strain CBDB1 cbrA[d]	(9)
cbdbA84r[#]	TGTTGTTGGCAACTGCTTC	301-320 strain CBDB1 cbrA[d]	(9)
cbdbA1624f[#]	CTGCTAAAGGATTAGCCAAAA	500-521 strain CBDB1 cbdbA1624[d]	(9)
cbdbA1624r[#]	CAGTCACTCGAAGCTACCA	654-673 strain CBDB1 cbdbA1624[d]	(9)
tceA-500f[#]	TAATATATGCCGCCACGAATGG	500-522 strain 195 tceA[d]	(2)
tceA-795r[#]	AATCGTATACCAAGGCCCGAGG	795-817 strain 195 tceA[d]	(2)
DET0311-373f[#]	GCTGTCCGGCGTAACATGGATTAT	373-397 strain 195 pceA[d]	(2)
DET0311-552r[#]	AAAGCTGCATTCTGTTCGGGTGTG	552-576 strain 195 pceA[d]	(2)

(*) Primer with GC-clamp of 5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3'

([#]) Real time quantitative PCR primers; ([+]) Clone selection and sequencing

[a] Corresponding to the relative position in the *E. coli* 16S rRNA gene; [b] Corresponding to the position in the *Desulfitobacterium hafniense* strain TCE1 16S rRNA gene; [c] Corresponding to the position in the *D. ethenogenes* strain 195 16S rRNA gene; [d] Corresponding to the position in the *Dehalococcoides spp. rdh*A operon

Table A3. Accession numbers and genome annotations of *rdh* genes detected by the Geochip

Accession Number	Genome Annotation	Organism
CAC37919	*pceC*	*Clostridium bifermentans*
CAD28792	*pceA*	*Desulfitobacterium hafniense* strain TCE1
CAD28793	*pceB*	*Desulfitobacterium hafniense* strain TCE1
CAD62438	*rdhA*	*Desulfitobacterium sp.* PCE1
AAQ94119	*vcrA*	*Dehalococcoides sp.* bacterium VS
AAR24337	RdhA7	*Dehalococcoides sp.* FL2
YP_180839	DET0088	*Dehalococcoides ethenogenes 195*
YP_180921	DET0173	*Dehalococcoides ethenogenes 195*
YP_182243	DET1545	*Dehalococcoides ethenogenes 195*
YP_182236	DET1538	*Dehalococcoides ethenogenes 195*
YP_182226	DET1528	*Dehalococcoides ethenogenes 195*
YP_182220	DET1522	*Dehalococcoides ethenogenes 195*
YP_307266	cbdbA88	*Dehalococcoides sp.* CBDB1
YP_308474	cbdbA1535	*Dehalococcoides sp.* CBDB1
YP_308518	cbdbA1578	*Dehalococcoides sp.* CBDB1
YP_308522	cbdbA1582	*Dehalococcoides sp.* CBDB1
YP_308534	cbdbA1595	*Dehalococcoides sp.* CBDB1
YP_308560	cbdbA1624	*Dehalococcoides sp.* CBDB1
YP_308569	cbdbA1638	*Dehalococcoides sp.* CBDB1
EAT49845	Dhaf_0696	*Desulfitobacterium hafniense* DCB-2
EAT49860	Dhaf_0711	*Desulfitobacterium hafniense* DCB-2
EAT49862	Dhaf_0713	*Desulfitobacterium hafniense* DCB-2
ZP_01373225	Dhaf_3520	*Desulfitobacterium hafniense* DCB-2

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Appendix II

Table A1. List of probes added to GeoChip

No	Prot GI	Organism	Annotation
1	27228284	bacterium PM-VC1	trichloroethene reductive dehalogenase
2	27228287	bacterium RC-VC2	trichloroethene reductive dehalogenase
3	27228281	bacterium YK-TCE1	trichloroethene reductive dehalogenase
4	115251004	*Clostridium difficile 630*	putative reductive dehalogenase
5	13751043	*Dehalobacter restrictus*	putative CprA
6	31337508	*Dehalobacter restrictus*	chloroethene reductive dehalogenase RdhA1
7	49613984	*Dehalobacter restrictus*	tetrachloroethene reductive dehalogenase
8	31338100	*Dehalobacter restrictus*	chloroethene reductive dehalogenase RdhA2
9	57233548	*Dehalococcoides ethenogenes 195*	reductive dehalogenase domain protein
10	57233710	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
11	57233724	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
12	57233735	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
13	57233751	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
14	57233757	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
15	57234100	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
16	57234338	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
17	57234851	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
18	57234856	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
19	57234868	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
20	57234963	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
21	57235084	*Dehalococcoides ethenogenes 195*	reductive dehalogenase, putative
22	48995922	*Dehalococcoides sp. BAV1*	putative reductive dehalogenase
23	48995925	*Dehalococcoides sp. BAV1*	putative reductive dehalogenase
24	48995928	*Dehalococcoides sp. BAV1*	putative reductive dehalogenase
25	49339402	*Dehalococcoides sp. BAV1*	putative vinyl chloride reductive dehalogenase bvcA
26	88914601	*Dehalococcoides sp. BAV1*	reductive dehalogenase, putative
27	88932748	*Dehalococcoides sp. BAV1*	reductive dehalogenase, putative
28	73659733	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
29	73659738	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
30	73659743	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
31	73659751	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
32	73659867	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
33	73659872	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
34	73660584	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
35	73660906	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
36	73660912	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
37	73660919	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
38	73660956	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
39	73660959	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
40	73660967	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
41	73660979	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
42	73660987	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
43	73660999	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
44	73661011	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
45	73661037	*Dehalococcoides sp. CBDB1*	putative reductive dehalogenase
46	38569297	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA1

47	38569300	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA2
48	38569303	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA3
49	38569306	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA4
50	38569309	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA5
51	38569312	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA6
52	38569315	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA7
53	38569318	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA8
54	38569321	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA9
55	38569324	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA10
56	38569327	*Dehalococcoides sp. FL2*	reductive dehalogenase homologous protein RdhA11
57	73913556	*Dehalococcoides sp. FL2*	putative reductive dehalogenase RdhA12
58	73913559	*Dehalococcoides sp. FL2*	putative reductive dehalogenase RdhA13
59	27228278	*Dehalococcoides sp. FL2*	trichloroethene reductive dehalogenase
60	77176878	*Dehalococcoides sp. KB0*	reductive dehalogenase protein
61	77176881	*Dehalococcoides sp. KB1*	reductive dehalogenase protein
62	77176875	*Dehalococcoides sp. KB1*	reductive dehalogenase protein
63	77176884	*Dehalococcoides sp. KB2*	reductive dehalogenase protein
64	77176872	*Dehalococcoides sp. KB2*	reductive dehalogenase protein
65	77176887	*Dehalococcoides sp. KB3*	reductive dehalogenase protein
66	77176866	*Dehalococcoides sp. KB3*	reductive dehalogenase protein
67	77176869	*Dehalococcoides sp. KB4*	reductive dehalogenase protein
68	77176863	*Dehalococcoides sp. KB4*	reductive dehalogenase protein
69	77176860	*Dehalococcoides sp. KB5*	reductive dehalogenase protein
70	77176857	*Dehalococcoides sp. KB6*	reductive dehalogenase protein
71	77176854	*Dehalococcoides sp. KB7*	reductive dehalogenase protein
72	77176851	*Dehalococcoides sp. KB8*	reductive dehalogenase protein
73	77176848	*Dehalococcoides sp. KB9*	reductive dehalogenase protein
74	3002555	*Dehalospirillum multivorans*	tetrachloroethene reductive dehalogenase catalytically active subunit
75	19073919	*Desulfitobacterium chlororespirans*	o-chlorophenol reductive dehalogenase
76	12003192	*Desulfitobacterium chlororespirans*	putative o-chlorophenol reductive dehalogenase
77	5531941	*Desulfitobacterium dehalogenans*	ortho-chlorophenol reductive dehalogenase catalytically active subunit precursor
78	17220533	*Desulfitobacterium dehalogenans*	putative reductive dehalogenase RdhA
79	5531941	*Desulfitobacterium dehalogenans*	ortho-chlorophenol reductive dehalogenase catalytically active subunit precursor
80	115430532	*Desulfitobacterium dichloroeliminans*	dichloroethane reductive dehalogenase
81	115430534	*Desulfitobacterium dichloroeliminans*	putative membrane bound regulatory protein
82	14625484	*Desulfitobacterium frappieri*	cprA-like protein
83	15809257	*Desulfitobacterium frappieri*	putative reductive dehalogenase RdfA
84	15809259	*Desulfitobacterium frappieri*	putative reductive dehalogenase RdfA
85	27461024	*Desulfitobacterium frappieri*	chlorophenol reductase precursor
86	27461024	*Desulfitobacterium frappieri*	chlorophenol reductase precursor
87	15809262	*Desulfitobacterium hafniense*	putative ortho-chlorophenol reductive dehalogenase catalytically active subunit precursor CprA
88	23304942	*Desulfitobacterium hafniense*	tetrachloroethene reductive dehalogenase
89	19421873	*Desulfitobacterium hafniense*	o-chlorophenol reductive dehalogenase
90	15809262	*Desulfitobacterium hafniense DCB-2*	putative ortho-chlorophenol reductive dehalogenase catalytically active subunit precursor CprA
91	15809264	*Desulfitobacterium hafniense DCB-2*	putative reductive dehalogenase RdfA

92	19421860	*Desulfitobacterium hafniense DCB-2*	putative reductive dehalogenase
93	19421873	*Desulfitobacterium hafniense DCB-2*	o-chlorophenol reductive dehalogenase
94	19421883	*Desulfitobacterium hafniense DCB-2*	putative reductive dehalogenase
95	19421900	*Desulfitobacterium hafniense DCB-2*	reductive dehalogenase
96	19421902	*Desulfitobacterium hafniense DCB-2*	putative reductive dehalogenase
97	23304942	*Desulfitobacterium hafniense DCB-2*	tetrachloroethene reductive dehalogenase
98	45738230	*Desulfitobacterium hafniense DCB-2*	3,5-dichlorophenol reductive dehalogenase
99	109639513	*Desulfitobacterium hafniense DCB-2*	reductive dehalogenase
100	109640265	*Desulfitobacterium hafniense DCB-2*	reductive dehalogenase
101	109640287	*Desulfitobacterium hafniense DCB-2*	reductive dehalogenase
102	109640289	*Desulfitobacterium hafniense DCB-2*	reductive dehalogenase
103	109640304	*Desulfitobacterium hafniense DCB-2*	reductive dehalogenase
104	109640306	*Desulfitobacterium hafniense DCB-2*	reductive dehalogenase
105	109640310	*Desulfitobacterium hafniense DCB-2*	reductive dehalogenase
106	21623558	*Desulfitobacterium hafniense Y51*	PCE dehalogenase
107	58615701	*Desulfitobacterium hafniense Y51*	PCE dehalogenase
108	89333349	*Desulfitobacterium hafniense Y51*	probable chlorophenol reductive dehalogenase
109	89335033	*Desulfitobacterium hafniense Y51*	tetrachloroethene dehalogenase
110	89893901	*Desulfitobacterium hafniense Y51*	probable chlorophenol reductive dehalogenase
111	89895585	*Desulfitobacterium hafniense Y51*	tetrachloroethene dehalogenase
112	76573856	*Desulfitobacterium sp. KBC1*	putative ortho-chlorophenol reductive dehalogenase
113	76573858	*Desulfitobacterium sp. KBC1*	tetrachloroethene reductive dehalogenase
114	12240020	*Desulfitobacterium sp. PCE1*	o-chlorophenol reductive dehalogenase
115	15809253	*Desulfitobacterium sp. PCE1*	putative ortho-chlorophenol reductive dehalogenase catalytically active subunit precursor CprA
116	31337506	*Desulfitobacterium sp. PCE1*	chloroethene reductive dehalogenase RdhA
117	15809255	*Desulfitobacterium sp. PCE1*	putative reductive dehalogenase RddA
118	28932812	*Desulfitobacterium sp. PCE-S*	tetrachloroethene reductive dehalogenase precursor
119	12240023	*Desulfitobacterium sp. Viet-1*	o-chlorophenol reductive dehalogenase
120	118680181	*Geobacter lovleyi SZ*	reductive dehalogenase
121	118680182	*Geobacter lovleyi SZ*	reductive dehalogenase
122	118746906	*Geobacter lovleyi SZ*	reductive dehalogenase
123	118746907	*Geobacter lovleyi SZ*	reductive dehalogenase
124	88864008	*Jannaschia sp. CCS1*	Reductive dehalogenase
125	88866014	*Jannaschia sp. CCS1*	Reductive dehalogenase
126	89054459	*Jannaschia sp. CCS1*	Reductive dehalogenase
127	89056465	*Jannaschia sp. CCS1*	Reductive dehalogenase
128	90329402	*Photobacterium profundum 3TCK*	putative reductive dehalogenase
129	90410049	*Photobacterium profundum 3TCK*	putative reductive dehalogenase
130	99035689	*Silicibacter sp. TM1040*	Reductive dehalogenase
131	99078551	*Silicibacter sp. TM1040*	Reductive dehalogenase
132	15809266	*Sulfurospirillum halorespirans*	putative tetrachloroethene reductive dehalogenase catalytically active subunit PceA
133	31337504	*Sulfurospirillum multivorans*	chloroethene reductive dehalogenase RdhA
134	115430538	uncultured bacterium	dichloroethane reductive dehalogenase
135	116061992	uncultured bacterium	reductive dehalogenase homologue
136	116061994	uncultured bacterium	reductive dehalogenase homologue
137	116061996	uncultured bacterium	reductive dehalogenase homologue
138	116061998	uncultured bacterium	reductive dehalogenase homologue

Appendix III

Results of RT-qPCR measurements based on 16SrRNA of dechlorinating and all bacteria (16SrRNA copies /gr sediment) in (A) 1. River Ebro absolute copy numbers 2. Standard deviations (B) 1. River Elbe absolute copy numbers 2. Standard deviations

A-1

Depth		**2004**			**2005**		
		All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.	All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.
0-5cm	Lleida	9.51E+09	1.69E+06	2.50E+06	1.52E+10	2.01E+06	1.70E+06
	Flix	2.45E+07	2.47E+04	1.97E+03	5.79E+07	3.47E+04	6.26E+02
	Tortosa	2.21E+07	7.60E+04	6.54E+02	1.55E+08	1.94E+03	1.47E+03
	RF	3.62E+08	0.00E+00	0.00E+00	1.09E+07	6.84E+02	0.00E+00
	DS	4.21E+08	0.00E+00	0.00E+00	4.31E+07	3.86E+02	4.68E+02
5-10cm	Lleida	6.06E+08	2.03E+06	1.26E+05	1.93E+10	0.00E+00	3.42E+06
	Flix	1.03E+08	4.75E+04	2.70E+03	3.92E+07	1.36E+05	0.00E+00
	Tortosa	2.85E+08	3.16E+03	3.82E+04	1.92E+08	4.36E+03	6.32E+03
	RF	1.53E+08	0.00E+00	0.00E+00	1.36E+07	0.00E+00	0.00E+00
	DS	1.40E+08	0.00E+00	0.00E+00	1.26E+07	2.11E+04	3.58E+02
10-15cm	Lleida	NA	NA	NA	1.06E+10	3.94E+05	9.80E+04
	Flix	1.03E+06	0.00E+00	7.32E+02	2.96E+07	9.14E+03	0.00E+00
	Tortosa	3.03E+06	7.12E+03	0.00E+00	5.40E+07	3.52E+03	9.30E+02
	RF	6.78E+06	5.30E+04	0.00E+00	1.30E+07	3.90E+03	0.00E+00
	DS	1.68E+06	6.58E+03	0.00E+00	2.84E+07	4.70E+03	1.16E+03
15-20cm	Lleida	NA	NA	NA	NA	NA	NA
	Flix	3.23E+06	5.82E+03	0.00E+00	5.99E+06	7.08E+03	0.00E+00
	Tortosa	1.13E+08	1.28E+04	7.46E+03	2.26E+08	6.74E+03	7.08E+02
	RF	8.68E+06	4.24E+04	0.00E+00	2.40E+07	0.00E+00	0.00E+00
	DS	2.85E+06	0.00E+00	0.00E+00	NA	NA	NA

Abbreviations: RF:Rice Field, DS: Delta Estruary

A-1 (*cont.*)

Depth		2006		
		All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.
0-5cm	Lleida	2.08E+11	4.32E+05	3.33E+04
	Flix	4.87E+08	6.54E+06	8.30E+03
	Tortosa	2.58E+10	0.00E+00	1.09E+06
	RF	3.90E+08	0.00E+00	1.43E+04
	DS	2.88E+08	0.00E+00	2.98E+04
5-10cm	Lleida	9.46E+10	1.07E+06	7.09E+04
	Flix	1.90E+08	8.94E+05	0.00E+00
	Tortosa	1.85E+10	8.52E+05	6.88E+05
	RF	3.85E+07	0.00E+00	0.00E+00
	DS	1.33E+08	0.00E+00	1.47E+04
10-15cm	Lleida	6.50E+10	1.05E+06	0.00E+00
	Flix	5.50E+06	7.40E+04	0.00E+00
	Tortosa	NA	NA	NA
	RF	5.57E+07	0.00E+00	0.00E+00
	DS	NA	NA	NA
15-20cm	Lleida	NA	NA	NA
	Flix	8.50E+06	1.21E+04	0.00E+00
	Tortosa	NA	NA	NA
	RF	1.63E+08	0.00E+00	0.00E+00
	DS	5.51E+07	0.00E+00	0.00E+00

Abbreviations: RF:Rice Field, DS: Delta Estruary

A-2

Depth		2004			2005		
		All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.	All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.
0-5cm	Lleida	5.63E+07	1.91E+05	4.84E+03	3.58E+07	1.88E+04	7.89E+03
	Flix	3.34E+05	9.54E+02	7.53E+00	7.53E+04	7.02E+03	4.79E-01
	Tortosa	1.35E+06	9.22E+03	2.41E+00	5.14E+06	1.40E+00	2.44E+00
	RF	9.25E+05	0.00E+00	0.00E+00	6.39E+05	3.04E+00	0.00E+00
	DS	5.74E+06	0.00E+00	0.00E+00	4.45E+04	4.54E+01	5.81E-01
					1.77E+09	0.00E+00	1.59E+04
5-10cm	Lleida	7.75E+06	2.29E+05	2.44E+02			
	Flix	3.35E+05	1.84E+03	1.03E+01	2.08E+06	2.75E+04	0.00E+00
	Tortosa	3.88E+06	3.83E+02	1.41E+02	6.26E+06	3.13E+00	1.05E+01
	RF	2.28E+06	0.00E+00	0.00E+00	5.92E+05	0.00E+00	0.00E+00
	DS	4.16E+06	0.00E+00	0.00E+00	2.42E+04	2.48E+03	4.44E-01
					5.00E+08	3.68E+03	4.55E+02
10-15cm	Lleida	NA	NA	NA			
	Flix	9.09E+03	0.00E+00	2.80E+00	1.10E+05	1.85E+03	0.00E+00
	Tortosa	1.19E+04	8.63E+02	0.00E+00	2.54E+04	2.53E+00	1.54E+00
	RF	1.11E+05	2.19E+03	0.00E+00	5.58E+03	1.73E+01	0.00E+00
	DS	1.23E+04	4.94E+02	0.00E+00	7.35E+05	5.53E+02	1.44E+00
15-20cm	Lleida	NA	NA	NA	NA	NA	NA
	Flix	1.98E+05	2.25E+02	0.00E+00	2.04E+03	1.43E+03	0.00E+00
	Tortosa	2.89E+05	1.56E+03	2.75E+01	1.62E+07	4.85E+00	1.17E+00
	RF	1.18E+05	1.75E+03	0.00E+00	9.94E+05	0.00E+00	0.00E+00
	DS	3.65E+04	0.00E+00	0.00E+00	3.58E+07	NA	NA

Abbreviations: RF:Rice Field, DS: Delta Estruary

A-2 (*cont.*)

Depth		2006		
		All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.
0-5cm	Lleida	8.42E+09	2.83E+02	1.39E+00
	Flix	8.71E+05	2.02E+05	2.57E+01
	Tortosa	3.02E+09	0.00E+00	4.04E+02
	RF	5.45E+06	0.00E+00	3.71E+01
	DS	8.22E+06	0.00E+00	4.41E+01
		1.23E+09	7.02E+02	2.96E+00
5-10cm	Lleida			
	Flix	8.30E+06	2.76E+05	0.00E+00
	Tortosa	1.61E+09	2.77E+03	2.55E+02
	RF	1.11E+06	0.00E+00	0.00E+00
	DS	3.45E+06	0.00E+00	2.17E+01
		7.41E+08	6.89E+02	0.00E+00
10-15cm	Lleida			
	Flix	5.38E+04	2.29E+04	0.00E+00
	Tortosa	NA	NA	NA
	RF	6.97E+06	0.00E+00	0.00E+00
	DS	NA	NA	NA
15-20cm	Lleida	NA	NA	NA
	Flix	1.03E+05	7.95E+00	0.00E+00
	Tortosa	NA	NA	NA
	RF	4.78E+06	0.00E+00	0.00E+00
	DS	8.86E+05	0.00E+00	0.00E+00

Abbreviations: RF:Rice Field, DS: Delta Estruary

B-1

Depth		Autumn-2004			Spring-2005		
		All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.	All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.
0-5cm	FPS1	4.48E+10	0.00E+00	1.75E+06	6.40E+09	4.23E+07	1.35E+06
	FPS2	6.52E+10	0.00E+00	4.34E+07	6.76E+08	6.19E+05	5.88E+05
	RS	7.98E+10	8.94E+06	5.93E+06	3.36E+09	1.49E+07	3.98E+05
5-10cm	FPS1	6.24E+10	0.00E+00	1.56E+06	6.97E+07	5.50E+04	4.56E+04
	FPS2	4.92E+10	0.00E+00	2.68E+07	4.81E+08	4.21E+05	6.10E+05
	RS	5.47E+10	1.70E+07	1.89E+06	1.20E+09	0.00E+00	0.00E+00
10-15cm	FPS1	6.82E+10	0.00E+00	7.62E+05	2.82E+08	0.00E+00	3.82E+04
	FPS2	4.92E+10	0.00E+00	2.68E+07	1.62E+07	0.00E+00	0.00E+00
	RS	3.85E+10	0.00E+00	0.00E+00	1.10E+09	2.91E+05	0.00E+00
15-20cm	FPS1	NA	NA	NA	3.22E+08	1.70E+04	6.55E+04
	FPS2	NA	NA	NA	1.73E+07	0.00E+00	0.00E+00
	RS	4.23E+10	0.00E+00	0.00E+00	1.28E+09	3.31E+04	0.00E+00

Abbreviations: FPS: flood plain soil, RS: river sediment

B-1 (*cont.*)

Depth		Autumn-2005		
		All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.
0-5cm	FPS1	9.40E+08	0.00E+00	3.00E+06
	FPS2	3.26E+09	2.24E+06	2.28E+07
	RS	3.95E+09	4.15E+06	8.33E+06
5-10cm	FPS1	1.60E+08	3.38E+03	6.98E+05
	FPS2	1.59E+07	3.08E+03	9.81E+04
	RS	2.01E+09	7.89E+05	5.21E+06
10-15cm	FPS1	1.90E+07	1.20E+01	2.80E+05
	FPS2	1.30E+07	0.00E+00	1.23E+05
	RS	9.92E+08	2.07E+06	0.00E+00
15-20cm	FPS1	NA	NA	NA
	FPS2	NA	NA	NA
	RS	6.19E+08	6.24E+03	0.00E+00

Abbreviations: FPS: flood plain soil, RS: river sediment

B-2

Depth		Autumn-2004			Spring-2005		
		All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.	All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.
0-5cm	FPS1	4.67E+07	0.00E+00	6.88E+04	4.73E+07	8.61E+06	3.47E+05
	FPS2	2.21E+07	0.00E+00	1.29E+06	1.51E+05	1.10E+05	1.59E+04
	RS	2.23E+07	2.30E+05	1.31E+05	3.72E+04	2.73E+06	3.52E+04
5-10cm	FPS1	1.89E+06	0.00E+00	8.62E+05	8.15E+04	1.75E+04	1.07E+05
	FPS2	1.33E+06	0.00E+00	2.36E+05	1.11E+06	2.83E+05	4.96E+04
	RS	7.25E+08	1.42E+06	5.61E+05	1.23E+06	0.00E+00	0.00E+00
10-15cm	FPS1	1.45E+07	0.00E+00	4.12E+05	0.00E+00	0.00E+00	8.62E+03
	FPS2	1.23E+07	0.00E+00	1.58E+05	0.00E+00	0.00E+00	0.00E+00
	RS	4.43E+07	0.00E+00	0.00E+00	1.58E+07	1.83E+04	0.00E+00
15-20cm	FPS1	NA	NA	NA	0.00E+00	9.69E+02	2.45E+03
	FPS2	NA	NA	NA	2.11E+05	0.00E+00	0.00E+00
	RS	1.72E+06	0.00E+00	0.00E+00	3.41E+04	2.14E+03	0.00E+00

Abbreviations: FPS: flood plain soil, RS: river sediment

B-2

Depth		Autumn-2005		
		All Bacteria	*Dehalococcoides* spp.	*Desulfitobacterium* spp.
0-5cm	FPS1	2.09E+07	0.00E+00	3.10E+05
	FPS2	8.43E+07	1.54E+03	4.18E+06
	RS	2.33E+05	1.12E+05	2.34E+05
5-10cm	FPS1	2.50E+06	1.69E+03	2.74E+04
	FPS2	5.84E+05	1.76E+02	6.74E+03
	RS	1.02E+05	2.29E+04	2.56E+04
10-15cm	FPS1	6.94E+06	1.44E+01	1.25E+04
	FPS2	7.80E+06	0.00E+00	3.52E+04
	RS	3.14E+07	3.77E+02	0.00E+00
15-20cm	FPS1	NA	NA	NA
	FPS2	NA	NA	NA
	RS	8.43E+07	1.54E+02	0.00E+00

Abbreviations: FPS: flood plain soil, RS: river sediment

Table A1. Shannon (H') indices of mineral cycling genes for each sample.

	Carbon Degradation				Carbon Fixation		Methane Generation	Methane Oxidation	
	cellulase	*chitinase*	*laccase*	*mannase*	*fthfs*	*rbcs*	*mtr*	*pmo*	*mmo*
Flix									
June 04 D5	4.0	3.3	3.2	1.1	1.4	3.1	1.7	2.6	2.3
June 04 D15	4.1	3.8	3.3	1.8	1.8	3.3	1.9	2.8	2.8
Feb 05 D5	3.9	3.5	3.3	1.6	1.8	3.3	1.9	2.2	2.6
Feb 05 D15	4.6	4.3	3.8	1.8	2.8	3.9	2.0	3.2	3.0
June 05 D5	4.3	4.0	3.6	1.8	2.2	3.5	2.4	2.6	2.8
June 05 D15	3.9	3.6	3.1	1.4	1.4	2.9	1.8	2.1	2.6
Feb 06 D5	3.4	2.7	2.6	1.4	1.4	2.8	1.4	1.9	1.8
Feb 06 D15	4.6	4.0	3.7	1.8	2.2	3.7	2.3	3.1	3.0
Rice Fields									
June 04 D5	3.2	2.5	2.6	1.1	1.1	2.6	1.7	2.3	1.6
June 04 D15	4.3	4.0	3.3	2.4	2.1	3.4	2.2	2.6	2.8
Feb 05 D5	4.0	3.7	3.4	1.4	1.9	3.4	2.1	2.1	2.6
Feb 05 D15	5.0	4.6	4.0	2.5	2.9	4.3	2.6	3.1	3.1
June 05 D5	5.0	4.5	4.1	2.6	2.9	4.2	2.6	3.3	3.2
June 05 D15	4.5	4.1	3.7	2.1	2.2	3.7	2.5	2.7	2.9
Feb 06 D5	3.1	2.6	2.3	1.4	1.4	2.4	1.0	1.9	2.2
Feb 06 D15	3.9	3.4	2.9	1.4	1.6	3.2	2.0	2.1	2.7

Appendix IV

Table A1. Shannon (H') indices of mineral cycling genes for each sample. (*cont*)

	Nitrification		**Denitrification**			**Nitrogen fixation**	**Sulfate reduction**
	amoA	*gdh*	*nor/norZ*	*nir*	*nar/nas*	*nifH*	*dsrA*
Flix							
June 04 D5	0.7	1.4	0.0	2.3	2.6	3.4	3.6
June 04 D15	0.7	1.6	1.6	2.5	2.5	3.9	4.2
Feb 05 D5	0.7	0.0	1.6	2.4	2.2	3.7	4.1
Feb 05 D15	0.7	0.7	1.9	3.1	3.0	4.7	4.9
June 05 D5	0.0	0.0	2.1	3.0	2.8	4.4	4.7
June 05 D15	1.8	1.6	1.1	2.3	2.6	3.9	3.9
Feb 06 D5	1.6	0.7	0.7	2.1	2.2	3.3	3.6
Feb 06 D15	1.6	1.6	2.5	3.0	3.1	4.5	4.9
Rice Fields							
June 04 D5	0.7	0.0	0.0	1.6	1.9	3.0	3.0
June 04 D15	2.1	2.2	2.3	2.8	2.8	4.3	4.5
Feb 05 D5	0.0	1.6	1.4	2.6	2.5	4.0	4.0
Feb 05 D15	2.8	2.9	3.1	3.5	3.3	5.0	5.3
June 05 D5	2.6	2.4	2.7	3.5	3.1	4.9	5.2
June 05 D15	2.1	1.9	1.9	3.0	3.0	4.4	4.8
Feb 06 D5	1.1	0.0	0.0	1.9	1.8	3.2	2.9
Feb 06 D15	1.1	1.8	0.7	2.6	2.7	3.9	4.1

Table A2. Relative abundance of functional gene groups (presented as percentage) in each sample. Relative abundance is calculated by dividing the total signal intensity from the spots of each functional gene group by the sum of the intensity for all the spots in the replicate arrays.

	C-degradation	C-fixation	CH_4 oxidation	CH_4 generation	Contaminant degradation
Flix					
June 04 D5	11.2	2.9	2.4	0.6	27.9
June 04 D15	11.7	2.6	2.6	0.6	20.4
Feb 05 D5	11.4	3.5	1.9	0.6	23.2
Feb 05 D15	11.0	2.6	2.4	0.5	13.9
June 05 D5	10.2	2.8	2.1	0.8	15.9
June 05 D15	11.4	2.1	1.7	0.9	24.4
Feb 06 D5	9.1	3.5	2.1	0.6	29.5
Feb 06 D15	11.5	2.2	2.2	0.6	17.8
Rice Fields					
June 04 D5	7.7	2.6	2.7	0.7	29.9
June 04 D15	8.4	1.8	2.2	0.8	20.5
Feb 05 D5	9.8	2.5	2.4	0.6	18.5
Feb 05 D15	9.4	3.1	2.5	0.7	13.8
June 05 D5	9.7	3.0	1.9	0.7	11.2
June 05 D15	8.7	3.2	2.2	0.7	13.9
Feb 06 D5	7.6	2.6	3.4	0.3	30.8
Feb 06 D15	8.1	3.0	2.6	0.5	20.9

Table A2. Relative abundance of functional gene groups (presented as percentage) in each sample. (*cont*)

	N_2-fixation	Nitrification	Denitrification	SO_4 reduction	Metal reduction and resistance
Flix					
June 04 D5	3.0	7.1	9.5	3.7	9.2
June 04 D15	3.4	7.9	8.2	4.6	10.9
Feb 05 D5	2.4	7.7	9.3	4.3	11.7
Feb 05 D15	3.3	7.3	10.9	5.0	11.3
June 05 D5	3.5	8.5	10.6	5.2	10.9
June 05 D15	3.6	7.8	11.8	4.5	7.9
Feb 06 D5	4.3	6.0	11.6	4.3	9.9
Feb 06 D15	3.2	7.7	9.8	6.1	10.6
Rice Fields					
June 04 D5	2.9	7.7	12.6	2.2	16.0
June 04 D15	3.7	7.6	11.4	4.8	11.7
Feb 05 D5	3.6	8.2	13.9	3.8	9.9
Feb 05 D15	3.9	7.1	12.5	4.7	9.9
June 05 D5	3.5	7.1	12.1	5.6	10.5
June 05 D15	3.8	7.1	12.1	4.8	11.4
Feb 06 D5	4.5	8.0	13.9	2.8	11.4
Feb 06 D15	3.4	8.0	14.6	4.0	11.0

Table A3. List of contamination degradation genes with highest relative abundance in river sediment and rice field soil samples

NCBI gi	Definition	Kingdom	Phylum	Species
33333869	phenylpropionate dioxygenase	Bacteria	*Actinobacteria*	*Mycobacterium* sp. S65
2190579	benzoyl-CoA reductase	Bacteria	*Proteobacteria*	*Rhodopseudomonas palustris* CGA009
5360705	p-cumate 2,3-dioxygenase	Bacteria	*Proteobacteria*	*Rhodopseudomonas palustris*
15965201	arylesterase protein	Bacteria	*Proteobacteria*	*Sinorhizobium meliloti* 1021
23005049	alkyl sulfatase and related hydrolases	Bacteria	*Proteobacteria*	*Magnetospirillum magnetotacticum*
23107737	phenylpropionate dioxygenase	Bacteria	*Proteobacteria*	*Novosphingobium aromaticivorans*
27380842	cyanate hydratase	Bacteria	*Proteobacteria*	*Bradyrhizobium japonicum* USDA 110
38490070	protocatechuate 3,4-dioxygenase	Bacteria	*Proteobacteria*	*marine alpha proteobacterium* SE45
22975204	protocatechuate 3,4-dioxygenase	Bacteria	*Proteobacteria*	*Ralstonia metallidurans*
22987905	allophanate hydrolase	Bacteria	*Proteobacteria*	*Burkholderia fungorum*
26665349	hydroxylamine oxidoreductase	Bacteria	*Proteobacteria*	*Nitrosospira multiformis* ATCC 25196
425214	4-hydroxy-2-oxovalerate aldolase	Bacteria	*Proteobacteria*	*Pseudomonas* sp.
2507064	4-hydroxy-2-keto-pentanoic acid aldolase	Bacteria	*Proteobacteria*	*Escherichia coli* K12
23062663	protocatechuate 3,4-dioxygenase beta subunit	Bacteria	*Proteobacteria*	*Pseudomonas fluorescens* PfO-1
27228503	dienelactone hydrolase	Bacteria	*Proteobacteria*	*Pseudomonas resinovorans*
29144474	putative dimethylsulfoxide reductase	Bacteria	*Proteobacteria*	*Salmonella enterica* subsp. Typhi s
40890127	nitrilase	unclassified prokaryote		

Appendix V

Calculation of Observed Growth Yields

This section provides information about methods used and assumptions made for the growth yield calculations. Since HCB can be degraded to various end products via different pathways, it is necessary to relate formation of new biomass to products formed. To do so, thermodynamic calculations were made according to Chapter 2 of Rittman and McCarty (2). The growth yield as such can have various units depending on how it is calculated. However to be able to compare different experiments observed growth yields of this study were based on μmol Cl^- released. In this section list of kinetic constants and half reactions are provided. From available genome data, it is assumed that *Dehalococcoides* spp. have only one genomic copy of the 16S rRNA gene. Thus copy numbers measured via qPCR can be used as cell number equivalents. Moreover the possibility of dechlorination of HCB and chlorinated ethenes by other bacteria is neglected. Since H_2 was not measured, 10^{-5} atm was chosen as a representative value regarding its partial pressure for thermodynamic calculations.

Table A1. Dechlorination half-reactions and their Gibb's standard free energy per electron equivalent at standard conditions. Standard free energies of formation for the compounds are adapted from Duhamel et al. (1) and Rittman and McCarty (2)

e^- acceptor	**Product**	**Acceptor Half Reactions**	**number of Cl^- released**	**eeq /mole of product**	**ΔG^0 kJ/eeq**
HCB	1,4-DCB	$\frac{1}{8}C_6Cl_6 + \frac{1}{2}H^+ + e^- \rightarrow \frac{1}{8}C_6H_4Cl_2 + \frac{1}{2}Cl^-$	4	8	-48.8
HCB	1,4-DCB	$\frac{1}{8}C_6Cl_6 + \frac{1}{2}H^+ + e^- \rightarrow \frac{1}{8}C_6H_4Cl_2 + \frac{1}{2}Cl^-$	4	8	-48.2
HCB	1,3,5-TCB	$\frac{1}{6}C_6Cl_6 + \frac{1}{2}H^+ + e^- \rightarrow \frac{1}{6}C_6H_3Cl_3 + \frac{1}{2}Cl^-$	3	6	-47.3
PCE	Ethene	$\frac{1}{8}C_2Cl_4 + \frac{1}{2}H^+ + e^- \rightarrow \frac{1}{8}C_2H_4 + \frac{1}{2}Cl^-$	4	8	-55.4
TCE	Ethene	$\frac{1}{6}C_2HCl_3 + \frac{1}{2}H^+ + e^- \rightarrow \frac{1}{6}C_2H_4 + \frac{1}{2}Cl^-$	3	6	-36.6

Table A2. Relevant half-reactions and their Gibb's standard free energy per electron equivalent at standard conditions. Standard free energies of formation for the compounds are adapted from Rittman and McCarty (2)

e⁻ donor	**Donor Half Reactions**	**eeq/mole compound**	**ΔG^0 kJ/eeq**
Lactate	$\frac{1}{12}CH_3CHOHCOO^- + \frac{1}{3}H_2O \leftrightarrow \frac{1}{6}CO_2 + \frac{1}{12}HCO_3^- + H^+ + e^-$	12	-32.3
Acetate	$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \leftrightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^-$	8	-27.4
H_2	$H^+ + e^- \leftrightarrow \frac{1}{2}H_2$	2	39.9
Cell synthesis	$\frac{1}{5}CO_2 + \frac{1}{20}NH_4^+ + \frac{1}{20}HCO_3^- + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{9}{20}H_2O$	20	na

na: not applicable

Table A3. Theoretical cell yields of the chlorinated compounds of this study

e⁻ acceptor	**Product**	**A[a]**	**Cell Synthesis [b]**	**Energy Production [c]**	**Theoretical Yield [d]**
HCB	1,3-DCB[e]	1.39	0.42	0.58	4.06
	1,3-DCB[f]	1.71	0.37	0.63	3.31
	1,4-DCB[e]	1.39	0.42	0.58	4.04
	1,4-DCB[f]	1.70	0.37	0.63	3.32
	1,3,5-TCB[e]	1.41	0.42	0.58	4.00
	1,3,5-TCB[f]	1.68	0.37	0.63	3.36
PCE	Ethene	1.01	0.49	0.51	5.57
TCE	Ethene	0.96	0.51	0.49	5.88

[a] (eeq used for energy/eeq for cell synthesis)

[b] $f_s = 1/(A+1)$ (eeq cells/eeq substrates)

[c] $f_e = 1\text{-}f_s$ (eeq electron acceptor reduced/eeq substrates)

[d] (g cells/eeq acceptor), for ammonium as N-source, 20 electron equivalents are required per mole of cells, and assuming that one mole of cells has a mass of 113 g; 113/20 = 5.65 g dry biomass/electron equivalent

$$Y = \frac{f_s}{f_e} \times 5.65 \frac{gr\ dry\ cells}{eeq\ acceptor}$$

[e] acetate as e⁻ donor, [f] lactate as e⁻ donor

An example for overall growth reactions: Dechlorination of HCB to 1,3,5-TCB with lactate

Acceptor half-reaction → $R_a : \frac{1}{6}C_6Cl_6 + \frac{1}{2}H^+ + e^- \rightarrow \frac{1}{6}C_6H_3Cl_3 + \frac{1}{2}Cl^-$

Donor half-reaction→ $R_d : H^+ + e^- \rightarrow \frac{1}{2}H_2$

Energy reaction →

$$R_{cells} : \frac{1}{5}CO_2 + \frac{1}{20}NH_4^+ + \frac{1}{20}HCO_3^- + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{9}{20}H_2O$$

Fermentation of carbon source →

$$R_{CS} : \frac{1}{12}CH_3CHOCOO^- + \frac{1}{2}H_2O \rightarrow \frac{1}{6}CO_2 + \frac{1}{12}HCO_3^- + H^+ + e^-$$

$f_e * (R_a - R_d)$:

$$0.111C_6Cl_6 + 0.333H_2 \rightarrow 0.111C_6H_3Cl_3 + 0.333Cl^- + 0.333H^+$$

$f_s * (R_{cell} - R_{CS})$:

$$0.13CO_2 + 0.02NH_4^+ + 0.033CH_3CHOCOO^- \rightarrow 0.02C_5H_7O_2N + 0.046H_2O + 0.13HCO_3^-$$

Overall :

$$0.111C_6Cl_6 + 0.333H_2 + 0.13CO_2 + 0.02NH_4^+ + 0.033CH_3CHOCOO^-$$
$$\rightarrow 0.111C_6H_3Cl_3 + 0.333Cl^- + 0.333H^+ + 0.02C_5H_7O_2N + 0.046H_2O + 0.13HCO_3^-$$

1. **Duhamel, M., and E. A. Edwards.** 2007. Growth and yields of dechlorinators, acetogens, and methanogens during reductive dechlorination of chlorinated ethenes and dihaloelimination of 1,2-dichloroethane. Environ Sci Tech **41:**2303-2310.
2. **Rittmann, B. E., and P. L. McCarty.** 2001. Environmental biotechnology : principles and applications. McGraw-Hill.

Supplements

List of publications

Calli B, Mertoglu B, **Taş N**, Inanç B, Yenigün O and Öztürk I (2003) Investigation of Variations in Microbial Diversity in Anaerobic Reactors Treating Landfill Leachate. *Water Science & Technology* Vol. 48, No.4, pp 105–112

Calli B, **Taş N**, Mertoglu B, Inanc B and Öztürk I (2003) Molecular Analysis Of Microbial Communities In Nitrification And Denitrification Reactors Treating High Ammonia Leachate. *Journal Of Environmental Science And Health, Part A—Toxic/Hazardous Substances & Environmental Engineering* Vol. A38, No. 10, pp. 1997–2007

Taş N, van Eekert MHA, Schraa G, Zhou J, de Vos WM and Smidt H (2009) Tracking Functional Guilds: *Dehalococcoides* spp. in European River Basins Contaminated with Hexachlorobenzene *Applied and Environmental Microbiology:* doi:10.1128/AEM.02829-08

Taş N, van Eekert MHA, Wagner A, Schraa G, de Vos WM and Smidt H (2009) Hexachlorobenzene Degradation Potential and Activity of *Dehalococcoides* spp. in European Rivers *In Preparation for publication*

Taş N, van Eekert MHA, van Nostrand J, Deng J, Wu L, He Z, de Vos WM, Zhou J and Smidt H (2009) Magnifying Glass for Ecosystems: Functional Gene Array Analysis of Polluted River Sediments *In Preparation for publication*

Taş N, Heilig H.G.H.J., van Eekert MHA, Schraa G, de Vos WM and Smidt H (2009) Concurrent Hexachlorobenzene and Chloroethene Transformation by Endogenous Dechlorinating Microorganisms in the Ebro River Sediment *Submitted*

Taş N, van Eekert MHA, de Vos WM and Smidt H (2009) The Little Bacteria that Can – Ecophysiology of *Dehalococcoides* spp. in River Sediments *In Preparation for publication*

van Eekert MHA, **Taş N**, Langenhoff A.A.M., Smidt H and Schraa G (2009) Anaerobic Transformation of Hexachlorobenzene in European River Basins *In Preparation for publication*

About the author

Neslihan Taş was born on January 3, 1980, in Istanbul, Turkey. From 1996 to 2001 she studied in the Marmara University, Faculty of Engineering, Environmental Engineering Department. She moved to the Netherlands in 2002 to obtain her MSc degree in the Wageningen University. Her MSc thesis, titled "Design of Aerobic Landfills: Modelling of Carbon Degradation", was defended in the Wageningen University, Agrotechnology and Food Sciences, Sub-Department of Environmental Technology. In 2004 she had an internship in the Laboratory of Microbiology at Wageningen University on the detection of syntrophic interactions between propionate and butyrate degrading anaerobic bacteria with molecular tools. In the same year she continued practicing science as PhD student under the supervision of Prof. Willem M. de Vos and Dr. Hauke Smidt in the Laboratory of Microbiology at Wageningen University and Research Center. This research was part of EU 6th Framework project Aqua Terra "Integrated modeling of the river-sediment-soil-groundwater system; advanced tools for the management of catchment areas and river basins in the context of global change". The result of the project is presented in this thesis. Since August 2008 she has been appointed as a post-doctoral researcher at Department of Molecular Cell Physiology and at the Centre for Integrative Bioinformatics, Vrije Universiteit in Amsterdam.

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Sevgili HiTNet tayfası, bu son yıllarda çok görü$emesekte pe$imi bırakmadığınız için sağolun. Hande Teyze, iç açıcı mailler ve tüm sanal çiçekler için te$ekkürler. Sururicigim, sanırım hayatın yollarımızı çakı$tırdığı tesadüflerin en güzeli Hollanda'ya ta$ınmandı. Dilediğim kadar sık görü$emesek de, yine de yakınlarda olduğunu bilmek hep iyi geldi.

Sonunda “abi”lik görevini yerine getirebiliyor olmadan da cabası. Her$ey icin, ama her$ey için, te$ekkurler. Bak karde$in büyüdü de doktor bilem oldu. $imdi hepimiz için gelsin “haftaya bulu$alım haftaya”…

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

CERTIFICATE

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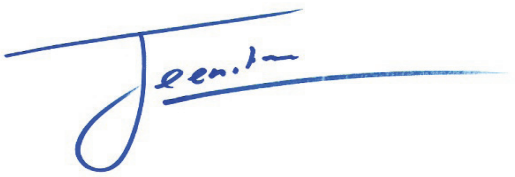
- **Structure and function of microbial communities: *Dehalococcoides* spp. in European river basins, Soil & Water Conference, 16 June 2006, Zeist, The Netherlands**
- **Tracing functional guilds: *Dehalococcoides* spp. In European river basins, ISEB 2006 Conference, Leipzig, Germany**
- **Ecology of dechlorinating bacteria in European river basins, NVvM Microbial Ecology 2007 Fall Symposium, 23 November 2007, Amsterdam, The Netherlands**



Mr. J. Feenstra
SENSE Coordinator PhD Education and Research

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