

Ecophysiology and environmental distribution of organohalide-respiring bacteria

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Ecophysiology and environmental distribution of organohalide-respiring bacteria

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1

General introduction and outline

Organohalides

Organohalides are halogenated organic compounds containing one or more carbon-halogen bonds in their structure. With different halogens, i.e. fluorine, chlorine, bromine and iodine, substituting hydrogen atoms in aliphatic and aromatic hydrocarbons, diverse organohalides are formed and referred to as organofluorine, organochlorine, organobromine and organoiodine compounds.

The organohalides found in the environment are of natural as well as anthropogenic origin. Natural sources include geogenic processes (forest fires, meteorites, volcanoes and other geothermal activities) and biosynthesis of organohalides by a broad range of different (micro)organisms, plants and animals [1, 2]. Additionally, enormous quantities of organohalides have been produced via anthropogenic activities [3]. Due to their extensive structural, chemical and physical variety and desirable industrial properties, organohalides have been manufactured for a wide range of industrial and agricultural applications, including fire extinguishants, solvents used e.g. for dry cleaning and degreasing of metal surfaces, refrigerants, pesticides and pharmaceutical agents [3]. As a result of massive use and uncontrolled disposal on one hand and high persistence and bioaccumulation in ecosystems on the other hand, organohalides have become problematic environmental chemicals [4], and some of them are proven to be toxic and threaten the health of humans and other animals [5].

Reductive dehalogenation

To clean up organohalide pollutants in the environment, many efforts have been made [6]. However, organohalides are found recalcitrant to biodegradation, which is mainly due to a combined influence of site biogeochemical parameters and the lack of (the activity of) degrading microbes [7]. Especially highly halogenated compounds are hardly mineralized under oxic conditions, leading to their (bio)accumulation [8, 9]. For their biotransformation and/or detoxification under anoxic conditions, reductive dehalogenation, also termed as organohalide-respiration (OHR) [7], is the only documented microbial process [6] and has been observed in many oxygen-depleted environments such as subsurface soil, wastewater treatment reactors, groundwater, aquifers and marine sediments. During OHR, organohalides are utilized as terminal electron acceptors for anaerobic respiration. Because organohalide-respiring bacteria (OHRB) use organohalides in their energy metabolism, OHR is considered as a feasible process to produce less-halogenated metabolites that can ultimately lead to the complete detoxification of organohalides and/or render them susceptible to further

downstream (bio)transformations, and therefore contribute to alleviating the environmental problem of organohalide pollutants [10].

Organohalide-respiring bacteria (OHRB)

OHRB are able to use organohalides as terminal electron acceptors and couple OHR to energy conservation. Since the first organohalide-respiring isolate, *Desulfomonile tiedjei* DCB1 [11], that was characterized as a 3-chlorobenzoate respirer, a large number of OHRB have been isolated/described. OHRB isolates are reported to utilize various organohalides for growth, including (but not limited to) trichloromethane, trichloroethane, tetra- and tri-chloroethene, chlorinated/brominated phenols and benzenes, dioxins, polychlorinated biphenyls and polybrominated diphenyl ethers [12]. Therefore, these organisms have received considerable scientific/applied interests due to their only recently discovered mode of anaerobic respiration as well as their potential application in organohalide bioremediation [12, 13]. A good example is the acceleration of *in situ* reductive dehalogenation of chlorinated ethenes by adding electron donors and/or exogenous OHRB containing enrichment cultures via biostimulation and bioaugmentation, respectively [14]. However, the known OHRB are fastidious microbes, and their efficient application in organohalide bioremediation has been held back by lack of thorough understanding of metabolic capabilities, optimized growth conditions, dependency on other microbial guilds for their nutritional requirements as well as interactions with physical chemical parameters and their environmental distribution. To fill this knowledge gap, this dissertation focuses on enhancing our understanding of the growth and physiology of OHRB, improving our knowledge of dehalogenation activity and interactions of OHRB with non-dechlorinating microbes as well as the affecting physical chemical parameters at bench and field scales, and exploring their environmental distribution in contaminated and pristine sites. To this end, a combination of analytical chemistry, physiological approaches and molecular techniques including quantitative PCR, next generation sequencing, transcriptional analysis and shotgun proteomics are applied (Figure 1.1).

Molecular approaches applied to study OHRB and their reductive dehalogenase (RDase) genes

To investigate potential OHRB in the studies described in this thesis, culture-independent methods based on 16S ribosomal RNA (rRNA) gene amplicon sequencing and quantitative PCR (qPCR) that target phylogenetic markers of OHRB and their genes encoding RDases, the key enzymes catalyzing OHR, are applied. qPCR is a powerful diagnostic technique that has been applied as a fast and sensitive strategy for identifying and monitoring OHRB and RDase

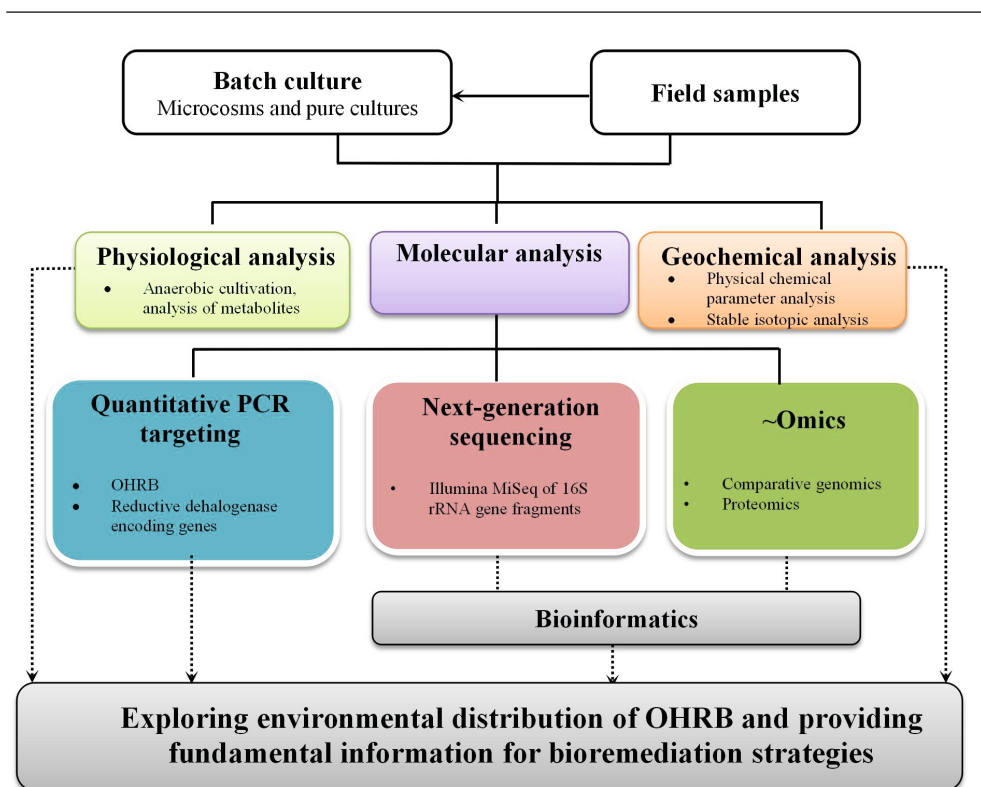


Figure 1.1 Approaches used in this thesis.

genes involved in the biotransformation of organohalides, and it has been widely used to guide bioremediation efforts [15]. In this study, a series of qPCR assays are applied to target 16S rRNA and RDase encoding genes of OHRB to quantify their absolute or relative abundance. To explore broader bacterial community composition based on 16S rRNA gene, high-throughput sequencing is conducted on the Illumina MiSeq platform. Compared to the low-resolution molecular methods that generate a fingerprint of main microbial community members, e.g. a combination of terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE) with cloning and Sanger sequencing, next generation technology sequencing platforms such as those developed by Illumina (MiSeq, HiSeq) can provide vast quantities of data and the cost per sequence is much lower [16, 17]. On the downside, these new sequencing technologies also have their limitations like generally shorter read length and higher error rates than Sanger sequencing [17-19].

To further explore the metabolism of OHRB isolates, ~omics techniques, including comparative genomics and proteomics analyses, are applied in this thesis. With the increasing

number of released genomes of OHRB, comparative genomics studies provide the necessary basis for comprehensive understanding of their physiology, metabolism and regulation mechanisms [20-22]. Furthermore, these approaches offer insights into the mechanisms of evolution and can reveal patterns/evidence of acquisition of e.g. RDase encoding genes via horizontal gene transfer [23]. In our study, comparative genomics is applied to investigate the organization of corrinoid operons in six available genomes of *Dehalobacter* spp. to better understand the corrinoid auxotrophy of *Dehalobacter restrictus*. Additionally, high-throughput proteomics has been applied in many studies of OHRB isolates [24-29], which aids in functional characterization of OHRB strains at the protein level, and can expand our knowledge of their potential substrate spectrum and regulation of RDases, the electron transport mechanism of OHR, and the metabolism and activity of OHRB. In our study, shotgun proteomics is applied to elucidate the response of *D. restrictus* under partial corrinoid starved conditions and to reveal its corrinoid metabolism and regulation mechanism at the protein level.

Stable isotopic analyses to study OHR(B)

Stable isotopic analysis has been widely used in the study of OHR(B) at both field and laboratory scales to i) distinguish abiotic and biotic transformation of organohalides [30-33], ii) identify the specific stable isotope fractionation caused by different OHRB and define reaction mechanisms [30, 31, 34, 35], iii) reveal the factors (e.g. growth substrates, transport across membranes, binding to the enzyme) that may control the stable isotope fractionation of different organohalides during OHR [31], iv) and explore the central metabolism [36], such as study the Wood-Ljungdahl pathway in *Dehalococcoides mccartyi* [37]. Stable isotopic analysis, and in particular the high resolution dual carbon-chlorine isotopic analysis, has been applied as a useful complementary tool to evaluate (bio)remediation of organohalide pollutants [33, 34, 38]. In our study, carbon isotopic signatures of *cis*-dichloroethene (DCE) and vinyl chloride are investigated during biostimulation of a *cis*-DCE-contaminated aquifer and applied as an informative line of evidence to build up a conceptual model of metabolic interactions within microbial communities.

Thesis outline

This dissertation has two sections, i.e. **Section one** with two review chapters (**Chapter 2-3**) and **Section two** with four research chapters (**Chapter 4-7**). In **Chapter 2**, phylogeny, genetic characteristics and ecophysiology of the different OHRB known to date are described. Further, important factors impacting the ecology and environmental distribution of OHRB are

discussed in this chapter. **Chapter 3** provides an overview of the primers targeting functional genes of OHRB. Primers that have been developed to target reductive dehalogenase-encoding genes are summarized and their application in the characterization and quantification of OHRB and reductive dehalogenase homologous genes are described in this chapter.

Chapter 4 aims at exploring the impact of corrinoid starvation on *Dehalobacter restrictus* strain PER-K23, which is restricted to OHR for energy conservation. *D. restrictus* is known for its corrinoid auxotrophy, and the strategy it develops to satisfy this key nutritional need is studied by combining physiological and molecular techniques including comparative genomics, transcriptional analysis and proteomics.

Chapter 5 reports dechlorination of three tetrachlorobenzene (TeCB) isomers by anoxic microcosms derived from contaminated harbor sludge. The thermodynamically preferred biotransformation pathways of TeCB isomers are investigated, and molecular tools are utilized to identify microorganisms responsible for TeCB dechlorination. The aim is to broaden current knowledge of the under-explored non-dechlorination community members involved in chlorobenzene dechlorination and their potential relationship with dechlorinators. Increased data availability on the key microbial players is a priority to boost the applicability of bioremediation.

Chapter 6 reports biostimulation with glycerol to enhance reductive dechlorination in a *cis*-DCE-polluted aquifer and monitoring of geochemical and microbial evolution to reveal subsurface responses to biostimulation. The results identify some common but also some previously overlooked microbial guilds that, intertwined with site geochemical and hydrological parameters, can promote/prohibit enhanced reductive dechlorination.

The research described in **Chapter 7** seeks OHR potential from extreme pristine environments, and more specifically in hypersaline lake sediments collected from Western Australia that have recently been reported as sources of biological chloromethane and chloroform emission [39]. The OHR potential is evaluated using microcosms prepared from hypersaline sediment, and microbial community analysis in combination with quantification of the known OHRB and reductive dehalogenase genes are applied. This extends OHR to extreme pristine environments and provides important implications for a local chlorine cycle between biotic chlorinators and dechlorinators.

Chapter 8 provides a general discussion of the results presented in this dissertation, and concludes with an outlook for future perspectives in the study of ecophysiology and environmental distribution of OHRB, the physical and chemical parameters affecting OHR(B), the interactions of OHRB with other microbial guilds in the community, and the application of this fundamental information for future bioremediation strategies.

Overview of known organohalide- respiring bacteria - phylogenetic diversity and environmental distribution

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#equal contribution

Abstract

To date, organohalide-respiration (OHR) has been restricted to the bacterial domain of life. Known organohalide-respiring bacteria (OHRB) are spread among several phyla comprising both Gram-positive and Gram-negative bacteria. As a unique trait, OHRB benefit from reductive dehalogenase enzymes enabling them to use different organohalides as terminal electron acceptors and occupy a wide range of terrestrial and aquatic environments. This chapter comprises three sections: Firstly, we give an overview of phylogeny of known OHRB and briefly discuss physiological and genetic characteristics of each group. Secondly, the environmental distribution of OHRB is presented. Owing to the application of molecular diagnostic approaches, OHRB are being increasingly detected not only from organohalide-contaminated groundwaters and sediments but also from pristine environments, including deep oceanic sediments and soils that are ample sources of naturally-occurring organohalides. Finally, we highlight important factors that impact the ecology of OHRB and their interaction with other microbial guilds.

Introduction

The vast functional diversity of microorganisms and their metabolic capabilities have made them successful mediators of electron liberation from the oxidation of inorganic and organic matter coupled to the reduction of a wide array of inorganic and organic electron acceptors including organohalides [7]. Although most well-known representatives of organohalides are considered to be man-made products of industrial origin, thousands of naturally occurring organohalides have been reported from geogenic and biogenic sources [2]. Numerous reports exist on natural production of organohalide compounds from biogenic sources such as a broad range of seaweeds, sponges, terrestrial plants, fungi, as well as through geogenic processes such as volcanic activity, forest fires, and other geothermal processes, some of which predate the industrialization era. Such ancient natural production of organohalides might have contributed to the development of biochemical strategies capable of unlocking the chemically stable carbon-halogen bond in organohalides. This is particularly important for non-oxygenolytic dehalogenation processes that probably have developed in the originally oxygen-free atmosphere on Earth. Taking advantage of organohalides as thermodynamically favourable electron acceptors under anoxic conditions, reductive dehalogenation is used as a terminal electron accepting process by organohalide-respiring bacteria (OHRB). These microbes have greatly contributed to global cycling of halogens by breathing (rather) toxic organohalides and preventing their accumulation in the environment. Exploiting reductive dehalogenases (RDase in case of functionally characterized enzymes, and RdhA for yet uncharacterized reductive dehalogenases predicted from genomes and molecular surveys) dedicated to organohalide respiration (OHR), OHRB occupy a wide range of niches/environments. Hence, understanding factors governing their evolution, distribution and ecology will help to unravel their role in the fate of organohalides. It should be noted that organohalide degradation processes have been described for a wide range of redox conditions (from highly oxidising to strictly reducing), and mediated by a wide variety of (micro)organisms in co-metabolic and/or energy-yielding modes, however, in this chapter we specifically discuss the phylogeny and environmental distribution of OHRB that are known to gain energy and grow using organohalides as electron acceptors.

Phylogeny of OHRB

Since the description of *Desulfomonile tiedjei* as the first isolated OHRB [11], numerous bacterial strains capable of OHR have been obtained in axenic culture (Figure 2.1), providing

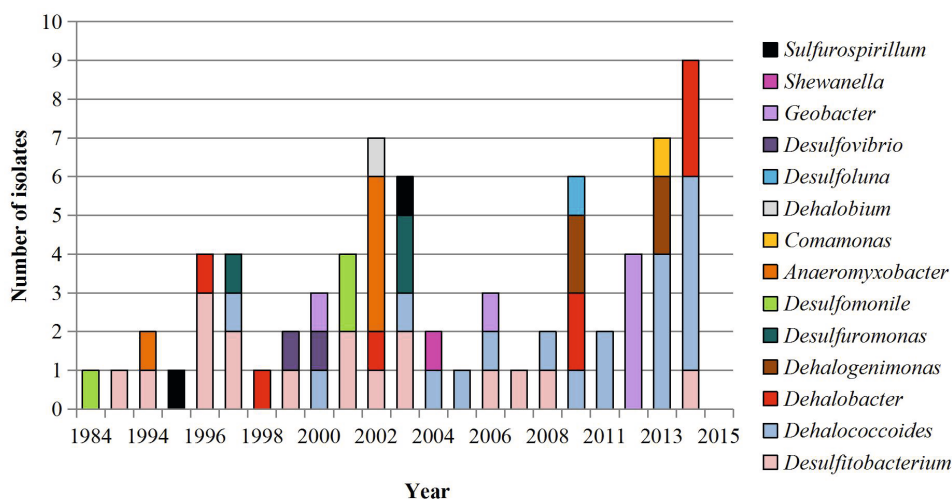


Figure 2.1 Number of OHRB available in axenic culture. The data is based on the number of isolates as of July 2015.

indispensable insights into their phylogenetic, physiological, and biochemical traits. Members of the genus *Dehalococcoides* comprise the biggest groups of isolates to date (19 isolates) followed by strains of *Desulfitobacterium* (17 isolates) (Figure 2.1). The known OHRB are spread among several distinct phyla comprising both Gram-positive and Gram-negative bacteria (Figure 2.2). The known isolates can by and large be divided into facultative and obligate groups based on whether OHR is their only energy-gaining metabolism [13]. The members of the facultative OHRB are characterized by a more versatile metabolism, in general have the ability to grow on a wide range of electron acceptors, and include proteobacterial OHRB such as *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter*, *Desulfomonile*, *Desulfovibrio*, *Desulfoluna*, *Sulfurospirillum*, *Comamonas*, *Shewanella* as well as *Desulfitobacterium* from the phylum *Firmicutes*. The fact that some facultative OHRB such as *Comamonas*, *Geobacter* and *Shewanella* belong to phylogenetic groups that mostly comprise non - OHRB points towards horizontal acquisition of reductive dehalogenase genes. The obligate OHRB on the other hand are restricted to OHR for energy conservation and growth and include *Dehalobacter* (phylum *Firmicutes*) and the OHRB belonging to the *Dehalococcoidia* class (phylum *Chloroflexi*) including strains of *Dehalococcoides mccartyi*, *Dehalogenimonas* spp. and the single isolate ‘*Dehalobium chlorocoercia*’ DF-1 [13, 40, 41]. However, recent studies showed fermentative growth of *Dehalobacter* spp. on chloromethane

[42, 43], suggesting that at least some of the isolates previously considered obligate OHRB might harbour additional modes of metabolism beyond the canonical OHR. It is also interesting to note that recent single cell genomic studies of marine *Dehalococcoidia* did not reveal any evidence for catabolic reductive dehalogenation, indicating that microorganisms closely related to known obligate OHRB do not rely on OHR for energy conservation, but rather utilize organic matter degradation pathways [44, 45].

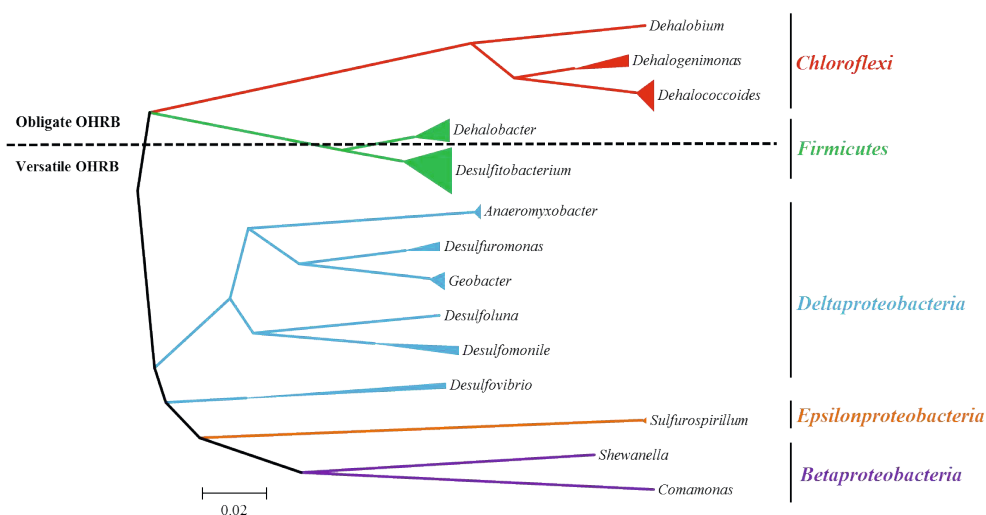


Figure 2.2 Phylogenetic tree of known OHRB based on bacterial 16S rRNA gene sequences. Alignment and phylogenetic analysis were performed with MEGA and the tree was constructed using the neighbour-joining (NJ) method. The reference bar at the bottom indicates the branch length that represents 2% sequence divergence. Colour Key: *Chloroflexi* (red), *Firmicutes* (green), *Deltaproteobacteria* (blue), *Betaproteobacteria* (violet), *Epsilonproteobacteria* (brown).

With few exceptions, OHRB from different phyla, i.e. members of the *Firmicutes*, *Chloroflexi* and different classes of *Proteobacteria*, benefit from similar enzymes/pathways for OHR indicating that these genes could be acquired from common ancestors via transposon mediated dissemination. However, to date little to no correlation could be found between the type of organohalides used as electron acceptor and the phylogenetic affiliation of OHRB, and haloaliphatic and haloaromatic compounds can be dehalogenated by isolates of taxonomically different genera [46]. Furthermore, isolates with similar phylogeny and physiology have been obtained from very different environments. For example, *Desulfuromonas michiganensis* strain BB1 was isolated from pristine river sediment while the closely related strain BRS1 was obtained from chloroethene-contaminated aquifer material [47]. Similarly, the closely

related uncultured *Chloroflexi* Lahn and Tidal Flat Clusters were both capable of dechlorination of perchloroethene (PCE) to *trans*-DCE while being enriched from sediments from river and marine environments, respectively [48, 49]. Another example is the case of *Dehalococcoides mccartyi* strains KS and RC. Both bacteria grow with 1,2-dichloropropane (1,2-D) as an electron acceptor in enrichment cultures while being derived from hydrocarbon-contaminated and pristine river sediments, respectively [50, 51].

In the following, we will separately discuss OHRB from different phyla.

Firmicutes

Dehalobacter

Dehalobacter has been first described as a genus in 1998, belonging to the *Firmicutes* (Phylum), *Clostridia* (Class), *Clostridiales* (Order) and *Peptococcaceae* (Family) [52]. *Dehalobacter restrictus* PER-K23^T, the first isolate and the type species of this genus, was obtained from an anaerobic PCE-dechlorinating packed-bed column, and was found to be able to couple the reductive dehalogenation of PCE to growth and energy conversation [52, 53]. Subsequently, additional pure cultures were isolated and characterized from this genus (Figure 2.1) [54-58]. *Dehalobacter* spp. are known as obligate OHRB with relatively small genomes (2.60 Mb - 3.09 Mb, with exception of *Dehalobacter* sp. FTH1 with a genome size of 6.33 Mb), and are restricted to organohalide respiration with H₂ as sole electron donor except for two reports on fermentative growth of *Dehalobacter* spp. with chloromethane [42, 43]. The presence of up to 39 copies of putative RDH catalytic subunit encoding *rdhA* genes in currently available genomes indicates their potentially broader substrate spectrum. Cultivation and environmental studies also revealed an increasing number of halogenated compounds that can be used by *Dehalobacter* spp. as electron acceptors including PCE and trichloroethene (TCE) [53, 54, 58], chlorinated ethanes [56, 59], 2,4,6-trichlorophenol (2,4,6-TCP) [58], 2,4,6-tribromophenol (2,4,6-TBP) [60], chloroform [61], chlorinated benzenes [57, 62], β -hexachlorocyclohexane (β -HCH, known as lindane) [63], and phthalide (4,5,6,7-tetrachlorophthalide) [55]. One RDase (PceA) was functionally characterized from *Dehalobacter* spp. [64], which displayed a corrinoid as essential cofactor. Recent studies showed that most *Dehalobacter* spp. genomes have a complete anaerobic cobalamin biosynthesis pathway [65], however, no *Dehalobacter* spp. have been confirmed to biosynthesize cobalamin *de novo* [52, 55, 56, 63]. Salvaging cobinamide from the environment, e.g., from cobalamin-producing non-dehalogenating community members such

as *Sedimentibacter* spp. [63, 66] was suggested as the strategy *Dehalobacter* spp. employ to ensure corrinoids supply for growth and reductive dechlorination [65, 67].

Desulfitobacterium

Desulfitobacterium is a genus belonging to the *Firmicutes* (Phylum), *Clostridia* (Class), *Clostridiales* (Order) and *Peptococcaceae* (Family). *Desulfitobacterium* spp. isolates are among the best characterized facultative OHRB that are known for their versatile metabolism with the capability to use a wide variety of electron donors and acceptors [68]. The first OHRB isolated in this genus was originally related to the genus *Clostridium* [69]. Strain DCB-2, isolated from municipal sludge, is a Gram-positive, strictly anaerobic, *ortho*- and *meta*-chlorophenol-dechlorinating bacterium [69]. Two years later, with the isolation of the monotypic strain *Desulfitobacterium dehalogenans* JW/IU-DC1^T, the *Desulfitobacterium* genus was proposed as a new taxon [70]. This genus was designated *Desulfitobacterium* because strain JW/IU-DC1^T grows with sulfite, but not with sulfate, as a terminal electron acceptor [70]. This was followed by re-classification of strain DCB-2 as the type strain of *Desulfitobacterium hafniense* [71]. The species *Desulfitobacterium frappieri* was proposed with the isolation of strain PCP-1, an anaerobic pentachlorophenol-dechlorinating bacterium isolated from a methanogenic consortium [72]. Based on DNA-DNA hybridization and comparative physiological studies *D. hafniense*, *D. frappieri*, and the non-OHRB strain GBFH were later united into the species *D. hafniense* [73]. Other OHRB belonging to the genus *Desulfitobacterium* isolated in the following years are indicted in Table 2.1.

Completed [20, 74, 75] and ongoing (Kruse et al., in preparation) genome sequencing projects have identified 1-7 copies of RDH encoding genes in the genomes of *Desulfitobacterium* spp., far less than the closely related dehalogenating clade of *Dehalobacter* spp. that contain up to 39 copies [23]. The loss of the OHR trait upon growth on alternative electron acceptors further confirmed the physiological opportunism of organohalide-respiring strains of *Desulfitobacterium* spp., and it was proposed that the RDase genes were acquired by horizontal gene transfer from closely related obligate organohalide-respiring strains of *Dehalobacter* spp. [76]. Nevertheless, *Desulfitobacterium* spp. strains are of high interest in the OHR field due to their high growth rate and respiration with a diverse range of organohalides, though none of the isolates were able to dechlorinate PCE past *cis*-DCE. Diverse RDases were purified and characterized from *Desulfitobacterium* spp. including chlorophenol RDase [77, 78], PCE RDase [79-82] and dichloroethane (DCA) RDase [83].

Additionally, a complete *de novo* corrinoid synthesis pathway was identified from the genome of *D. hafniense* Y51 [74].

Chloroflexi

Dehalococcoides

Dehalococcoides bacteria belong to *Chloroflexi* (Phylum), *Dehalococcoidia* (Class), *Dehalococcoidales* (Order) and *Dehalococcoidaceae* (Family) [40], which are well known for their metabolic restriction to OHR. The genus was first established in 1997 with the isolation of *D. ethenogenes* 195^T capable of complete dechlorination of PCE to ethene [84]. In following years, additional isolates of this genus were obtained in axenic culture including CBDB1, BAV1, VS, FL2 and GT strains. These five strains and the type strain *D. ethenogenes* 195 were re-classified as the new genus and species, *Dehalococcoides mccartyi* [40]. Recently, more strains from this species were isolated including strain JNA [85, 86], CG1 [87, 88], CG4 [87, 88], CG5 [87, 88], SG1 [87, 88], AD14-1 [89] and AD14-2 [89]. Based on an earlier 16S rRNA gene-based classification, Hendrickson, Payne [90] divided isolates and clones available at the time into three phylogenetic subgroups, a classification which has been widely referred to ever since. Accordingly, *D. ethenogenes* 195^T belongs to the ‘Cornell’ subgroup, strain VS belongs to the ‘Victoria’ subgroup, and subgroup ‘Pinellas’ contains most of the cultured strains such as CBDB1, BAV1, FL2 and GT and the majority of environmental sequences. Common traits of *D. mccartyi* strains are streamlined genomes, small and non-motile coccoid cells, resistant to penicillin-type antibiotics, obligately anaerobic, and restricted to OHR as the only mode of metabolism using H₂ as the only electron donor [40]. Similar to the situation described above for *Dehalobacter* spp., genomic studies of known isolates showed the lack of *de novo* vitamin B12 synthesis pathways [91, 92] although they are capable of corrinoid salvaging and remodelling from their growth medium [93-95].

In spite of sharing more than 98% identity with respect to their 16S rRNA gene sequences, different strains respire on different ranges of organohalides. For example, vinyl chloride (VC) respiration is limited to strains BAV1, GT and VS while none of them can respire on PCE. This might be due to large diversity of *rdhA* genes that are present in multiple non-identical copies on individual genomes. However, in spite of multiple homologous RDH genes the number of which currently ranges from 11 copies in strain BAV1 up to 36 copies in strain VS [96], only a limited number of RDases was purified and characterized from *D. mccartyi*

strains including PCE RDase [97], TCE RDase [98], *trans*-DCE producing RDase [99], vinyl chloride RDase [100-102], chlorobenzene RDase [103, 104], dichloropropane RDase [104] and polychlorinated biphenyls (PCBs) RDase [88].

Dehalogenimonas

The genus *Dehalogenimonas* belongs to the *Chloroflexi* (Phylum), *Dehalococcoidia* (Class), *Dehalococcoidales* (Order) and *Dehalococcoidaceae* (Family) [40]. Up to now, two species were described that were isolated from groundwater contaminated with chlorinated alkanes and alkenes. *Dehalogenimonas lykanthroporepellens* was proposed as a new species in 2009 [105], with the isolation of two strains BL-DC-9^T and BL-DC-8, both Gram-negative, non-motile, non-spore forming, polychlorinated aliphatic alkanes-dechlorinating bacteria [106]. The genome of *D. lykanthroporepellens* BL-DC-9 has been released recently [107], and multiple *rdhA* genes were found simultaneously transcribed during OHR with different organohalides as the electron acceptors [108]. *D. lykanthroporepellens* strains show 10–11% 16S rRNA gene sequence divergence compared to *Dehalococcoides* [108], perform dihaloelimination and have a higher tolerance to oxygen exposure [106]. The second species, *D. alkenigignens*, was proposed in 2013 [109] with the isolation of two strictly anaerobic strains IP3-3^T and SBP-1. Both species (*D. alkenigignens* and *D. lykanthroporepellens*) have similar morphological features and substrate spectra with 96.2% pairwise identity of their 16S rRNA gene sequence [109]. 1,2-DCA, 1,2-DCP, 1,2,3-DCP, 1,1,2-TCA and 1,1,2,2-TeCA can serve as electron acceptor with H₂ as sole electron donor [105, 106, 109].

Dehalobium

Next to *Dehalogenimonas* and *Dehalococcoides*, the class *Dehalococcoidia* also contains the genus *Dehalobium* [40] represented by a single isolate informally named ‘*Dehalobium chlorocoercia*’ strain DF-1. This bacterium was highly enriched in a PCB-dechlorinating co-culture with a sulfate-reducing *Desulfovibrio* sp. from estuarine sediment of Charleston Harbor, South Carolina [110, 111]. *Dehalobium chlorocoercia* DF-1 selectively dechlorinates PCB congeners with double-flanked chlorines with formate or H₂-CO₂ as the electron donor and carbon source [110, 111]. Strain DF-1 is the only reported representative of the genus *Dehalobium* and the classification as ‘*Dehalobium chlorocoercia*’ was first proposed in 2008 [48], however, the current genus and species names have yet to be validated. *D. chlorocoercia* DF-1 shares 89% 16S rRNA sequence similarity to the *ortho*-PCB-dechlorinating bacterium *o*-17 [112] while having ~87.5% similarity with *Dehalococcoides* [40]. Unlike *o*-17 and *D. mccartyi* strains, strain DF-1 uses formate as the electron donor, however, it requires

Desulfovibrio spp. in co-culture or its cell extract for growth with hydrogen and PCB in mineral medium [41]. Other phylotypes phylogenetically related to the *D. chlorocoercia* DF-1/ *Bacterium o-17* clade within the *Chloroflexi* were reported to be able to dechlorinate PCB congeners [113, 114]. In addition to PCB, *D. chlorocoercia* DF-1 dechlorinates highly chlorinated benzenes [115] and PCE and TCE producing higher amounts of *trans*-DCE than *cis*-DCE compared with canonical PCE/TCE dechlorinators [116]. Based on its unique dechlorinating activity, DF-1 has shown merits in PCBs bioaugmentation in contaminated soil [117] and sediment [118].

Deltaproteobacteria

Anaeromyxobacter

Anaeromyxobacter dehalogenans belongs to the *Proteobacteria* (Phylum), *Deltaproteobacteria* (Class), *Myxococcales* (Order) and *Myxococcaceae* (Family). *Anaeromyxobacter* was established as a genus with the monotypic species *Anaeromyxobacter dehalogenans* 2CP-1^T to describe a myxobacterium that can grow without air [119]. This bacterium is a Gram-negative, motile by gliding, spore-forming, bright red pigments-producing slime rod that is capable of facultative anaerobic growth, and was isolated from stream sediment near Lansing, MI, USA [120]. Subsequently, another four isolates were obtained from different sources including pond sediment, Cameroon rain forest soil and yard compost with acetate and 2-chlorophenol (2-CP) as electron donor and acceptor, respectively [119]. Halophenols such as 2-CP, 2,6-dichlorophenol (2,6-DCP), 2,5-dichlorophenol (2,5-DCP), 2-bromophenol (2-BP) as well as nitrate, fumarate and oxygen can serve as electron acceptors with acetate, H₂, succinate, pyruvate, formate, and lactate as electron donors [119, 120]. *A. dehalogenans* is able to grow microaerophilically [119, 120], and the genome analysis of *A. dehalogenans* 2CP-C showed that it harboured genotypic characteristics of obligately aerobic myxobacteria as well as anaerobic *Deltaproteobacteria* [121].

Desulfuromonas

Desulfuromonas, a genus the name of which refers to an elemental sulfur-reducing bacterium, belongs to the *Proteobacteria* (Phylum), *Deltaproteobacteria* (Class), *Desulfuromonadales* (Order) and *Desulfuromonadaceae* (Family). To date, two species of *Desulfuromonas* capable of chloroethenes respiration have been reported [47, 122]. *Desulfuromonas chloroethenica* strain TT4B was isolated from chloroethene-contaminated freshwater sediments in eastern Massachusetts, USA [122, 123]. Strain TT4B uses PCE, TCE as well as fumarate, polysulfide

and Fe(III) nitriloacetate as electron acceptor with acetate or pyruvate as electron donors [122]. The second species, *Desulfuromonas michiganensis*, currently comprises two strains, BB1 and BRS1, which were isolated from pristine river sediment and contaminated aquifer materials, respectively, both collected from the state of Michigan, USA, as indicated in the species name [47]. Compared to *D. chloroethenica*, strains BB1 and BRS1 use a wider range of electron donors and can grow by fermentation on fumarate or malate with succinate as major end product [47]. Besides, they showed higher growth yield and PCE tolerance [47].

Geobacter

Geobacter is a genus belonging to the *Proteobacteria* (Phylum), *Deltaproteobacteria* (Class), *Desulfuromonadales* (Order) and *Geobacteraceae* (Family), which is well-studied for its capabilities to oxidize organic compounds coupled to the reduction of insoluble metal oxides [124]. This genus was established with isolation of the non-OHRB *Geobacter metallireducens* [125]. The first OHRB characterized in this genus was *Geobacter thiogenes* strain K1 (formerly described as *Trichlorobacter thiogenes*), an anaerobic trichloroacetate-dechlorinating, sulfur-producing, fast-growing (doubling time=6 h) bacterium isolated from contaminated soil [126]. Acetate and acetoin were used as electron donor for reductive dechlorination of trichloroacetate, and fumarate served as alternative electron acceptor [126]. Subsequently, *Geobacter lovleyi* was described as a novel species with strain SZ as type strain, isolated from non-contaminated freshwater sediment collected from Su-Zi Creek near Seoul, South Korea [127]. This bacterium couples acetate oxidation to PCE dechlorination with *cis*-DCE as the end product for growth. Strain SZ can use pyruvate and H₂ as electron donors with a wide range of electron acceptors including PCE, TCE, Fe(III), Mn(IV), U(VI), malate, nitrate, fumarate and elemental sulfur, and no fermentation metabolism was found [127]. Additionally, 16S rRNA gene amplicons derived from *G. lovleyi* strain SZ-like organisms were found in uranium- and chlorinated compounds-polluted environmental samples by using strain SZ-specific primers. Recent genomic analysis of strain SZ identified the presence of a 77 kbp plasmid, pSZ77, encoding 15 out of the 24 genes required for *de novo* cobalamin biosynthesis [128]. Another OHRB belonging to the *Geobacter* genus is *G. lovleyi* strain KB-1, a PCE-to-*cis*-DCE-dechlorinating bacterium identified in the PCE to ethene dechlorinating consortium KB-1 [129]. More recently, four *G. lovleyi* isolates Geo7.1, Geo7.2, Geo7.3, and Geo7.4 were obtained from PCE to *cis*-DCE dechlorinating microcosms established with soil from Fort Lewis, WA, that share 99–100% nucleotide identity with strain SZ based on 16S rRNA gene sequences [128].

Desulfoluna

The genus *Desulfoluna* belongs to the *Proteobacteria* (Phylum), *Deltaproteobacteria* (Class), *Desulfobacterales* (Order) and *Desulfobacteraceae* (Family) [130]. *Desulfoluna spongiiphila* strain AA1^T, an anaerobic Gram-negative bromophenol- dehalogenating bacterium, was isolated from the marine sponge *Aplysina aerophoba* collected from the Mediterranean Sea [131], which is so far the only isolate of the genus *Desulfoluna* with confirmed ability to grow via OHR and the only pure OHRB culture isolated from sponge to date (Figure 2.4). To enrich and isolate strain AA1^T, lactate was used as electron donor and carbon source, and 2-BP served as sole electron acceptor [131, 132]. Diverse halogenated compounds can be completely dehalogenated by strain AA1^T including 2-, 3- and 4-BP, 1,4- and 2,6- 2,6-DBP, 2,4,6-tribromophenol (2,4,5,TBP), 2- and 3-iodophenol, 2-bromo-4-fluorophenol and 3,5-dibromo-4-hydroxybenzoate, but not monochlorinated or fluorinated phenols. NaCl was found essential for growth and no fermentation was observed with lactate, pyruvate, fumarate or malate as carbon source [131].

Desulfomonile

The genus *Desulfomonile* belongs to the *Proteobacteria* (Phylum), *Deltaproteobacteria* (Class), *Syntrophobacterales* (Order) and *Syntrophaceae* (Family) [11]. Two species of *Desulfomonile* were characterized as strictly anaerobic, 3-chlorobenzoate dechlorinating, sulfidogenic bacteria with special ‘collar’ girdling cells [11, 133]. *Desulfomonile tiedjei* was proposed as a novel and type species in 1990 [11] with strain DCB-1^T, a Gram-negative, non-motile, non-spore-forming, anaerobic rod-shaped bacterium, which was isolated from a municipal sewage sludge collected from Adrian, MI, USA [134]. Strain DCB-1 is able to reductively dehalogenate *meta*-halobenzoates [134, 135], and sulfate, sulfite and thiosulfate can also be use as electron acceptors, however, the presence of sulfoxo anions inhibited dechlorination as well as high concentrations of formate and H₂ [135]. Fermentation was observed with pyruvate supplied as sole carbon source [11]. Thiamine, nicotinamide and 1,4-naphthoquinone are required in the defined media, and MoO₄²⁻, SeO₄²⁻, tetracycline, chloramphenicol, kanamycin and streptomycin are potential inhibitors [11]. Additionally, cytochrome c₃ and desulfovirdin were detected in the growth medium of *Desulfomonile tiedjei* [11].

Desulfomonile limimaris is another organohalide-respiring species with two strains DCB-M^T and DCB-F that were isolated from marine sediments [56]. These two strains share 99% similarity based on 16S rRNA gene sequences and are both capable to dechlorinate 3-

chlorobenzoate (CB) to benzoate with formate and H_2 as electron donor. A broad range of electron donors can be used such as benzoate, H_2 , formate, butyrate, lactate, propionate and pyruvate. Besides 3-CB, sulfite, sulfate, thiosulfate, nitrate, and fumarate can be used as electron acceptors for growth. Compared to *Desulfomonile tiedjei* DCB-1 [11], these two isolates of *Desulfomonile limimaris* are not able to dechlorinate *meta*-halobenzoates using acetate or ferment pyruvate, and NaCl (>0.32%, w/w) is required for their growth [133]. Sulfite and thiosulfate and not sulfate inhibit OHR of *Desulfomonile tiedjei* DCB-1^T [11, 136] and *Desulfomonile limimaris* DCB-M^T, which was explained by the competition for electron flow.

Desulfovibrio

Desulfovibrio is a genus of Gram-negative sulfate-reducing bacteria (SRB) that belongs to the *Proteobacteria* (Phylum), *Deltaproteobacteria* (Class), *Desulfovibrionales* (Order) and *Desulfovibrionaceae* (Family). Up to now, two isolates of *Desulfovibrio* spp. were published with OHR capability, and both were isolated from marine sediments with halophenols as electron acceptors. *Desulfovibrio* sp. TBP-1 was isolated from estuarine sediments of the Arthur Kill in the New York/New Jersey harbor with 2,4,6-TBP as electron acceptor and supplied with hydrogen and acetate [137]. Only *ortho*-bromophenols and/or bromophenols without adjacent bromines at *para*- position were dehalogenated with lactate, pyruvate, formate or hydrogen as electron donors. Strain TBP-1 accumulated acetate when lactate and sulfate were provided, which indicated that this microorganism is an incomplete oxidizer and NaCl is required for its growth [137]. The other isolate is strain SF3^T, the type strain of species *Desulfovibrio dechloracetivorans*, which is able to couple acetate oxidation to OHR for growth [138]. Strain SF3 was isolated from San Francisco Bay sediment with acetate as electron donor and 2-CP as electron acceptor [138]. Several electron donors can be used to couple to OHR for growth, such as acetate, pyruvate, fumarate, propionate, lactate, ethanol and alanine but not with sulfate, and only *ortho*-chlorophenols like 2-CP and 2,6-DCP were dechlorinated [138].

Besides, *Desulfovibrio desulfuricans*, a sulfate-reducing bacterium, pre-coated with Pd⁰ was used as a biomass-supported chemical (bioinorganic) catalyst for dehalogenation of 2-CP and polychlorinated biphenyls with formate or H_2 as an electron donor [139]. Enhanced dechlorination was observed using the bioinorganic catalyst while negligible chloride release occurred from organohalides using biomass alone, chemically reduced or commercially available Pd⁰ [139]. The same concept was used to dechlorinate tris(chloroisopropyl)-

phosphate (TCPP, a potential flame retardant) in a groundwater sample [94].

Epsilonproteobacteria

Sulfurospirillum

Sulfurospirillum, a genus of Gram-negative, elemental sulfur-reducing spirilla, belongs to the *Proteobacteria* (Phylum), *Epsilonproteobacteria* (Class), *Campylobacterales* (Order) and *Campylobacteraceae* (Family) [140]. The first OHRB characterized in this genus was *Dehalospirillum multivorans* strain K, a strictly anaerobic PCE-dechlorinating bacterium isolated from activated sludge of a wastewater treatment plant with pyruvate and PCE [141], which was later renamed as *Sulfurospirillum multivorans* [142]. Additionally, strain PCE-M2^T, an anaerobic bacterium that is also able to reductively dechlorinate PCE to *cis*-DCE via TCE with lactate as electron donor, was isolated from a site polluted with chlorinated aliphatics in the Netherlands, and was assigned as a novel species *Sulfurospirillum halospirans* to the genus *Sulfurospirillum* [142]. AsO₄³⁻, SeO₄²⁻, PCE, nitrate and fumarate can serve as electron acceptors for strains K and PCE-M2^T [142, 143]. Strain PCE-M2^T is able to reduce nitrite while strain K is not, and the end product of nitrate reduction for strain PCE-M2^T and strain K is ammonium and nitrite, respectively [142]. Pyruvate, lactate, H₂ and formate can be used as electron donors to support PCE dechlorination, and fermentative growth was detected on pyruvate and fumarate [142]. Unlike all described RDases [46], PCE RDase of *S. multivorans* needs a special corrinoid cofactor (norpseudo-B12) [144] synthesized *de novo* by the strain itself [145].

Gammaproteobacteria

Shewanella

Shewanella belongs to the *Proteobacteria* (Phylum), *Gammaproteobacteria* (Class), *Alteromonadales* (Order) and *Shewanellaceae* (Family). *Shewanella sediminis* strain HAW-EB3^T is a psychrophilic rod-shaped marine bacterium isolated from Halifax Harbour sediment, Canada [146, 147]. This strain was originally noted for its ability to degrade hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) [146, 147], and its OHR capacity was later shown for PCE dechlorination to TCE [148]. This strain grows on several carbon sources such as N-acetyl-D-glucosamine, Tween 40, Tween 80, acetate, succinate, butyrate and serine [147]. Bacteria belonging to the *Shewanella* genus are also known for their versatile electron-accepting capabilities using a complex electron transfer network composed mainly of c-type cytochromes as well as iron-sulfur proteins [149]. To cope with low temperatures of marine

environments, psychrophilic strains of *Shewanella* seem to have adapted through a decreased genome G+C content and an increased proportion of alanine, proline and arginine in proteins to increase protein structural flexibility [150]. Genomic analysis of strain HAW-EB3^T revealed the presence of genes encoding five putative RDHs similar to characterized RDases, of which Ssed_3769 was responsible for PCE transformation [148]. The OHR potential of this bacterium was proposed to play a significant role in halogen and carbon cycles as it can uniquely access organic carbon resources locked as organohalogenes in sediments that may not be available to non-dehalogenating heterotrophic microbes [148].

Betaproteobacteria

Comamonas

Comamonas was defined as a genus belonging to the *Proteobacteria* (Phylum), *Betaproteobacteria* (Class), *Burkholderiales* (Order) and *Comamonadaceae* (Family). *Comamonas* sp. 7D-2 is the only isolate capable of OHR. It is a strictly aerobic bacterium that can completely degrade bromoxynil (a widely used brominated aromatic herbicide). This bacterium was recently isolated from a bromoxynil octanoate-contaminated soil [151]. The RDase (BhbA) of strain 7D-2, which catalysed the reductive debromination of 3,5-dibromo-4-hydroxybenzoate (DBHB) to 4-hydroxybenzoate (HB), was characterized as the first RDase isolated from strict aerobes, with exclusive substrate specificity for haloaromatic compounds with halogen substitutions at the *ortho* position of a hydroxyl group [151]. Similar to RDase genes from known OHRB, *bhbA* encodes two Fe-S cluster-binding motifs and is associated with a potential anchor protein encoding gene, however, unlike canonical RDases, it lacks a twin arginine signal peptide and B12-binding motif [151]. Widespread presence of putative RDHs homologous to BhbA in marine *Proteobacteria* and the fact that the *bhbA* gene was located on a large plasmid (pBHB) and surrounded by transposase genes suggested horizontal acquisition of *bhbA* from aerobic marine *Proteobacteria* [151]. Furthermore, the crystal structure of a similar aerobic RDase was recently published [152]. These elegant reports point to further catabolic plasticity of OHRB and indicates that the available OHRB represent the tip of the iceberg while a huge diversity awaits discovery.

Table 2.1 OHRB isolates.

Strain	Source	Year of isolation	Reference
<i>Anaeromyxobacter dehalogenans</i> 2CP-1	A small stream sediment	1994	[119, 120]
<i>Anaeromyxobacter dehalogenans</i> 2CP-2	Michigan pond sediment	2002	[119]
<i>Anaeromyxobacter dehalogenans</i> 2CP-3	Michigan yard compost	2002	[119]
<i>Anaeromyxobacter dehalogenans</i> 2CP-5	Michigan yard compost	2002	[119]
<i>Anaeromyxobacter dehalogenans</i> 2CP-C	Cameron rain forest soil	2002	[119]
<i>Comamonas</i> sp. 7D-2	Bromoxynil octanoate contaminated soil	2013	[151]
<i>Dehalobacter restrictus</i> PER-K23	Anaerobic Rhine River sediment and ground anaerobic granular sludge	1998	[52, 53]
<i>Dehalobacter</i> sp. 12DCB1	Sediment from water-saturated drainage ditch	2014	[57, 62]
<i>Dehalobacter</i> sp. 13DCB1	Sediment from water-saturated drainage ditch	2014	[57, 62]
<i>Dehalobacter</i> sp. FTH1	Ploughed layer of a paddy field	2009	[55]
<i>Dehalobacter</i> sp. FTH2	Ploughed layer of a paddy field	2009	[55]
<i>Dehalobacter</i> sp. TCA1	Upper Hudson River sediment	2002	[56]
<i>Dehalobacter</i> sp. TCP1	Digester sludge from an industrial wastewater treatment plant	2014	[58]
<i>Dehalobacter</i> sp. TEA	Industrially polluted, anaerobic groundwater in a fixed-bed reactor	1996	[54]
<i>Dehalobium chlorocoercia</i> DF-1	Estuarine sediment	2002	[110, 111]
<i>Dehalococcoides mccarryi</i> ANAS1	Contaminated soil	2011	[153, 154]
<i>Dehalococcoides mccarryi</i> ANAS2	Contaminated soil	2011	[153, 154]
<i>Dehalococcoides mccarryi</i> 11a	Sediments from Wuhan industrial districts	2013	[155, 156]
<i>Dehalococcoides mccarryi</i> 11a5	Sediments from Wuhan industrial districts	2013	[155, 156]
<i>Dehalococcoides mccarryi</i> BAV1	Aquifer material from the chloroethene-contaminated site	2003	[157]
<i>Dehalococcoides mccarryi</i> CBDB1	Saale River sediment	2000	[158, 159]
<i>Dehalococcoides mccarryi</i> DCMB5	Creek Spittelwasser sediment	2008	[160]
<i>Dehalococcoides mccarryi</i> FL2	Non-contaminated Red Cedar River sediment	2005	[161]
<i>Dehalococcoides mccarryi</i> GT	Aquifer material from a chloroethene-contaminated site	2006	[162]

(continued)

<i>Dehalococcoides mccarthyi</i> INA	Housatonic River sediment with Aroclor 1260-contamination	2014	[85, 86]
<i>Dehalococcoides mccarthyi</i> VS	Aquifer material from a PCE-contaminated site	2004	[101, 163]
<i>Dehalococcoides mccarthyi</i> 195	Anaerobic sewage digester sludge	1997	[84]
<i>Dehalococcoides mccarthyi</i> CG1	Sand and silt near Liangjiang River	2014	[87, 88]
<i>Dehalococcoides mccarthyi</i> CG4	Sand and silt of ditch sediment near electronic waste dump site	2014	[87, 88]
<i>Dehalococcoides mccarthyi</i> CG5	Sand and silt of ditch sediment near electronic waste dump site	2014	[87, 88]
<i>Dehalococcoides mccarthyi</i> SG1	Digester sludge of an industrial wastewater treatment plant	2014	[87, 88]
<i>Dehalococcoides mccarthyi</i> AD14-1	An anaerobic digester in a wastewater treatment plant	2013	[89]
<i>Dehalococcoides mccarthyi</i> AD14-2	An anaerobic digester in a wastewater treatment plant	2013	[89]
<i>Dehalococcoides mccarthyi</i> MB	Sediment in the San Francisco Bay area	2009	[164]
<i>Dehalogenimonas alkenigenens</i> IP3-3	Contaminated groundwater	2013	[109]
<i>Dehalogenimonas alkenigenens</i> SBP-1	Contaminated groundwater	2013	[109]
<i>Dehalogenimonas lykanthroporepellens</i> BL-DC-8	Contaminated groundwater	2009	[105, 106]
<i>Dehalogenimonas lykanthroporepellens</i> BL-DC-9	Contaminated groundwater	2009	[105, 106]
<i>Desulfitobacterium chlororespirans</i>	2,3-CP dehalogenating compost soil	1996	[165]
<i>Desulfitobacterium dehalogenans</i> JW/IU-DC1	Freshwater sediments-pond	1994	[70]
<i>Desulfitobacterium dichloroelminans</i> LMG P-21439	Soil matrix of an anoxic Water-saturated layer (1 m in depth)	2003	[166]
<i>Desulfitobacterium hafniense</i> DCB-2	Municipal sludge	1992	[69, 71]
<i>Desulfitobacterium hafniense</i> G2	An active Dry Branch Kaolin Company mine	2003	[167]
<i>Desulfitobacterium hafniense</i> JH1	Ditch sludge (mixed with sewage)	2008	[168, 169]
<i>Desulfitobacterium hafniense</i> PCP-1	A mixture of PCP-contaminated soil and anaerobic sewage sludge	1996	[72]
<i>Desulfitobacterium hafniense</i> TCE-1	Chloroethene-contaminated soil	1999	[170]
<i>Desulfitobacterium hafniense</i> TCP-A	River sediments	2001	[171]
<i>Desulfitobacterium metallireducens</i>	River sediments	2002	[172]
<i>Desulfitobacterium</i> sp. B31e3	Unsaturated subsurface soils	2007	[173]
<i>Desulfitobacterium</i> sp. KBC1	Crop field soil	2006	[80]

(continued)

<i>Desulfotobacterium</i> sp. PCE1	Various freshwater sediments	1996	[174]
<i>Desulfotobacterium</i> sp. PCE-S	PCE-contaminated soil	1997	[175]
<i>Desulfotobacterium</i> sp. PR	Chloroethenes and chloroethanes-dechlorinating bioreactor	2014	[176]
<i>Desulfotobacterium</i> sp. Viet-1	Parfume River sediment	1997	[177]
<i>Desulfotobacterium</i> sp. Y51	PCE-polluted soil	2001	[79]
<i>Desulfoluna spongiiphila</i> AA1	Marine sponge <i>Aplysina aerophoba</i>	2009	[131]
<i>Desulfomonile liminaris</i> DCB-F	Marine sediments	2001	[133]
<i>Desulfomonile liminaris</i> DCB-M	Marine sediments	2001	[133]
<i>Desulfomonile tiedjei</i>	Sewage sludge	1984	[11, 134]
<i>Desulfovibrio dechloracetivorans</i>	Marine sediment	2000	[138]
<i>Desulfovibrio</i> sp. TBP-1	Estuarine sediments	1999	[137]
<i>Desulfuromonas chloroethenica</i> TT4B	Chlorinated ethylenes contaminated freshwater sediments	1997	[122]
<i>Desulfuromonas michiganensis</i> BB1	Pristine river sediment	2003	[47]
<i>Desulfuromonas michiganensis</i> BRS1	Chloroethene-contaminated aquifer material	2003	[47]
<i>Geobacter lovleyi</i> Geo7.1	Soil	2012	[128]
<i>Geobacter lovleyi</i> Geo7.2	Soil	2012	[128]
<i>Geobacter lovleyi</i> Geo7.3	Soil	2012	[128]
<i>Geobacter lovleyi</i> Geo7.4	Soil	2012	[128]
<i>Geobacter lovleyi</i> SZ	Freshwater sediment	2006	[127]
<i>Geobacter thiogenes</i> K1	Contaminated soil	2000	[126]
<i>Shewanella sediminis</i> HAW-EB3T	Halifax Harbour sediment	2004	[146, 147]
<i>Sulfurospirillum halorespirans</i> PCE-M2	Anaerobic soil polluted with chlorinated aliphatic compounds	2003	[142]
<i>Sulfurospirillum multivorans</i> K	Activated sludge	1995	[141]

Environmental distribution of OHRB

Our current understanding of the environmental distribution of OHRB has to some extent been obtained by domestication of microbial communities capable of OHR in the form of laboratory culturing. Furthermore, monitoring of the presence of OHRB at a particular site was mediated by application of a suit of molecular techniques targeting phylogenetic and/or functional gene markers such as 16S rRNA and *rdhA* genes. Combined with field data and compound specific isotope analysis (CSIA) as a tool for *in situ* monitoring of OHR, the allied application of these tools has created a complementary toolbox for examining the dehalogenation potential mediated by OHRB. Based on the worldwide detection of OHR potential, a cosmopolitan distribution of OHRB is likely, however, an overview of the geographical distribution of isolated OHRB indicates that nearly all OHRB were isolated from a restricted number of discrete parts of the world (Figure 2.3) though from different source environments (Figure 2.4). Most probably, this is not due to the restricted geographical distribution of OHRB, but rather due to the initiatives taken by a limited number of laboratories to isolate OHRB from these locations. Hence, a great diversity of OHRB awaits discovery that have not been studied from remote/ill-defined geographical locations. This fact is further illuminated in repeated isolation of different OHRB [42, 47, 161] and identification of OHR potential [48, 50, 178, 179] from pristine environments where naturally-occurring organohalides are believed to feed OHRB.

Groundwater and aquifer

The infamous popularity of OHR studies centred around groundwater and aquifer is due to anthropogenic contamination of these environments with organohalides as a serious threat to aquatic as well as terrestrial ecosystems [180-185].

In an early attempt to detect OHRB from aquifer samples, of the six different chloroethene-contaminated aquifer samples, only one tested positive for the presence of *Dehalococcoides*, and another tested positive for *Desulfuromonas* [186]. A similar study on a TCE-contaminated aquifer at Cape Canaveral Air Station, Florida, indicated heterogeneous distribution of dechlorinating activity and a specific dechlorinating organism, *Dehalococcoides*, at the site where the aquifer in question showed a non-uniform pattern of daughter product concentrations [187]. In a more comprehensive study, distribution of *Dehalococcoides* species was investigated in 10 groundwater samples collected from very diverse chloroethene-contaminated sites representing different geographical locations and a variety of climatic zones in North America, England, and The Netherlands [90].

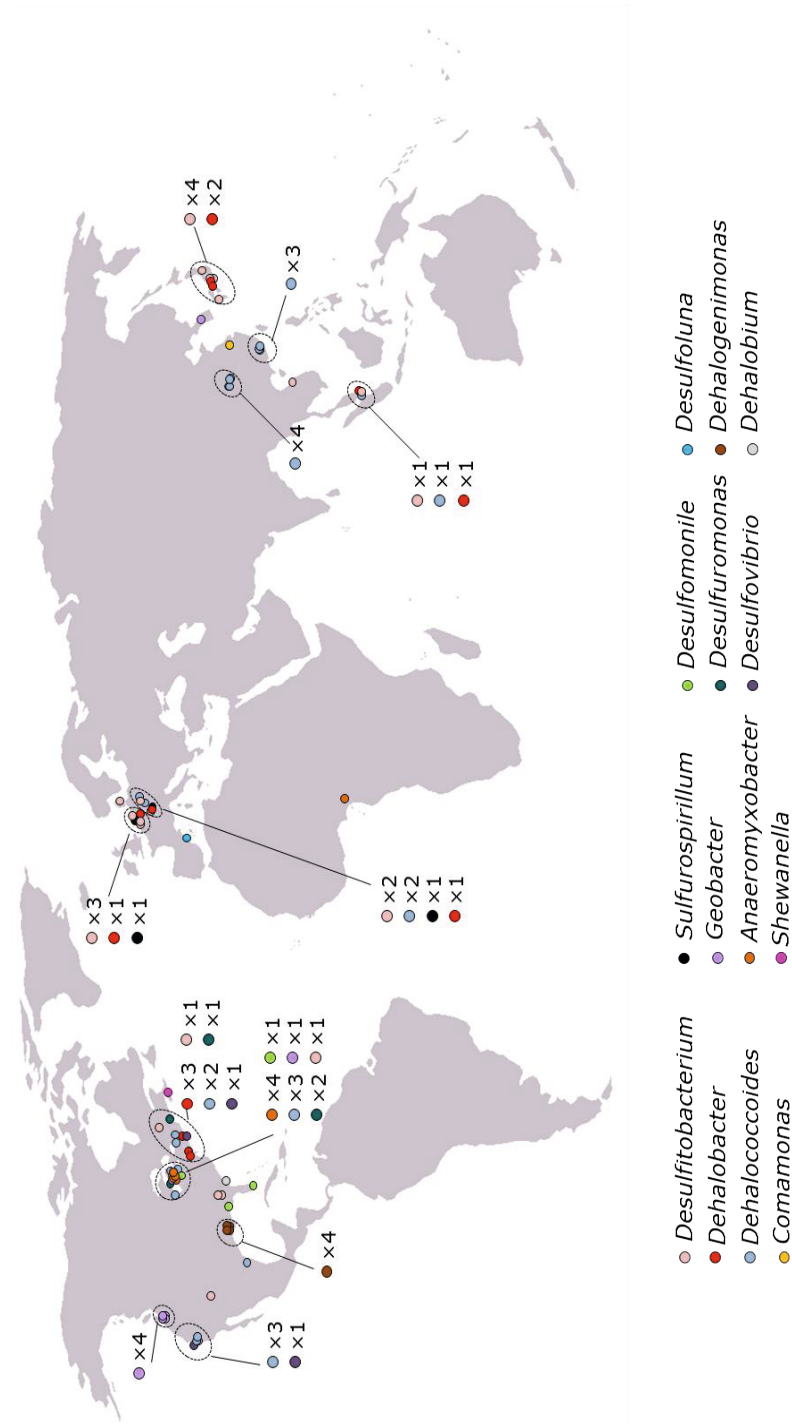


Figure 2.3 Global distribution of OHRB isolates. The data is based on the number of isolates until July 2015. The source locations of OHRB isolates are indicated, except *Sulfurospirillum multivorans*, *Desulfotobacterium dichloroeliminans*, *Desulfotobacterium hafniense* strain DCB-2 and PCP-1, *Desulfotobacterium* sp. strain B31e3, PCE1, PCE-S and PR. For those strains without defined inoculum source, the locations of the laboratory where the strains have been isolated are shown in the global map ([http://upload.wikimedia.org/wikipedia/commons/2/22/Blank_map_of_world_no_country_borders.PNG?use\[lang=en\]](http://upload.wikimedia.org/wikipedia/commons/2/22/Blank_map_of_world_no_country_borders.PNG?use[lang=en])).

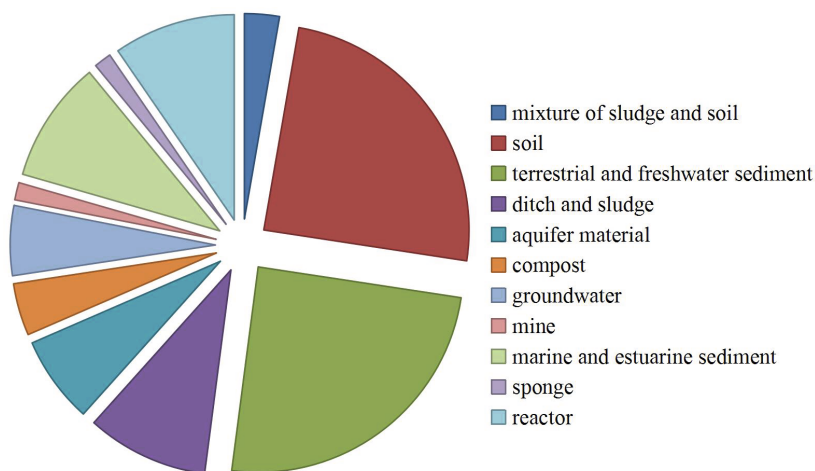


Figure 2.4 Sources from which OHRB were isolated.

Dehalococcoides sequences were not detected in samples from three sites at which partial dechlorination of chloroethenes occurred [90]. While 16S rRNA gene-based PCR characterization failed to detect *Dehalococcoides* populations in aquifer samples from the Kelly AFB site (Texas, USA) before bioaugmentation with the KB-1 culture [188], 16S rRNA genes-based qPCR of *Dehalococcoides* and *Desulfuromonas* spp. were successfully used to monitor the distribution and abundance of the OHRB from groundwater and aquifer solids in the Bachman Road Residential Wells Site, USA [189]. In a study to track natural attenuation of a chlorinated ethene contaminated aquifer in the Bitterfeld/Wolfen area in Germany, various potential OHRB including *Dehalococcoides*, *Desulfuromonas*, *Desulfitobacterium* and *Dehalobacter* were detected providing an extra line of evidence for occurrence of natural attenuation [190]. In a wider survey of 34 environmental samples from 18 different sites (including 19 aquifer samples and eight groundwater samples) in different European countries, *D. mccartyi* and *Desulfitobacterium* spp. were detected concomitantly in the majority of the samples suggesting either in parallel or stepwise catalysis of complete dehalogenation of PCE by OHR [191].

The environmental distribution of OHRB in samples retrieved from aquifers contaminated with chlorinated ethenes was also shown by tracking the abundance and distribution of RDase genes with assigned functions, i.e. TCE-to-VC RDase gene (*tceA*) [192] and VC-to-ethene RDase gene (*vcrA*) [101]. The recovered *rdhA* sequences from geographically distinct locations in the US exhibited between 95% and 98% identity with known *tceA* and *vcrA* genes, respectively [101, 192, 193]. A similar approach was recently used for the RDase gene *dcpA*

mediating 1,2-dichloropropane dichloroelimination to propene [104]. The *dcpA* gene was detected in all aquifer samples that yielded 1,2-D-dechlorinating microcosms [104], and *dcpA* sequences retrieved from geographically distinct samples shared >98% sequence identity with *dcpA* sequences from *D. mccartyi* strains RC and KS [50, 51] and *D. lykanthroporepellens* BL-DC-9 [105].

Studying the abundance and environmental distribution of OHRB was propelled by application of DNA/RNA-based quantitative PCR methods [194-196]. Using this method, the 16S rRNA and *tceA*, *bvcA* and *vcrA* genes of *D. mccartyi* were detected in groundwater samples collected from a contaminated site near Montague, MI, USA and a large number of unidentified *D. mccartyi rdhA* genes were discovered [195]. Analysis of 45 groundwater samples taken from Rugårdsvej 234, Odense, Denmark for *D. mccartyi* 16S rRNA and *vcrA* genes indicated the presence of indigenous biomarkers at below detection limit of qPCR [197]. Increases in ethene concentrations after lactate addition were accompanied by a 3-4 order of magnitude increase in concentrations of *vcrA* gene copies throughout the groundwater monitoring network [197]. The oligotrophic nature of aquifers as one of the main obstacles of subsurface OHR was noted in similar studies [198, 199]. Quantification of transcripts of the same biomarkers in groundwater samples from the Fort Lewis military base in Tacoma, WA, USA also verified an indigenous community of *D. mccartyi* cells carrying the *tceA*, *vcrA*, and *bvcA* genes [194]. A less active physiological state of the *D. mccartyi* cells carrying *tceA* was noted as opposed to the cells that harbour *vcrA* and *bvcA* [194] which was similar to a DNA based survey of an alluvial plain with complex geochemical and hydraulic conditions in France [200]. In an attempt to relate molecular data to geochemical conditions, 150 monitoring wells spread over 11 chlorinated ethene polluted European locations were analysed [201]. The *vcrA* gene copy numbers correlated most significantly to VC and chlorinated ethene concentrations while *bvcA* and especially *tceA* were more correlated with oxidizing conditions. The results showed geochemical conditions as the driver of geographical distribution of the RDase genes [201]. More recently, *Dehalobacter* was found to be more abundant than *D. mccartyi* populations in the clay till matrix collected from chlorinated ethenes and ethanes contaminated site in Vadsby, Denmark which corresponded with OHR being stalled at *cis*-DCE [202].

Cultivation independent approaches extended to subsurface aquifers further enhanced our understanding of metabolic plasticity of *Dehalococcoidia*-like bacteria. A recent metagenomic analysis from terrestrial aquifer sediment retrieved two *Dehalococcoidia* related

pan-genomes ('RBG-2' and 'RBG-1351') [203]. Rather than OHR mediating *rdhA* genes, versatile heterotrophic life styles were found [203]. These findings indicate that OHR should not be considered as sole mode of metabolism of *Dehalococcoidia*-like bacteria.

Various OHRB capable of respiration on diverse organohalides were isolated from groundwater and aquifer material as listed in Table 2.1.

Marine and estuarine environments

Marine and estuarine environments are repositories of numerous chlorinated, brominated and iodinated organic compounds that have been produced and released both from natural and anthropogenic sources. The study of King was the first report on OHR of 2,4-dibromophenol in coastal sediments [204]. Since then, numerous studies reported OHR as an essential step in the marine and estuarine biodegradation of halophenols [132], halogenated ethenes [148, 164], halobenzoates [133], lindane [205] and polychlorinated biphenyls [41, 206, 207].

Of all known OHRB, the organohalide-respiring *Chloroflexi* seem to play the most prominent role in OHR in marine, estuarine and tidal flat sediments. The *ortho*-PCB-dechlorinating bacterium *o*-17 was isolated from a PCB dechlorinating culture enriched from estuarine sediments of Baltimore Harbour (USA) [112]. Similarly, The PCB-dechlorinating *Dehalobium chlorocoercia* DF-1 is indigenous to estuarine sediment and was isolated from Charleston harbour in South Carolina [111]. An active role of these isolates and other *Chloroflexi* members that are distinct from *D. mccartyi* were shown in PCB dechlorination in sediment microcosms from Baltimore Harbour, USA [208]. Similar *Chloroflexi* phylotypes were reported for PCB dechlorination in enrichment from contaminated marine sediment of the Venice lagoon, Italy [209]. The Tidal Flat Cluster, a deep clade in the phylum *Chloroflexi*, was characterized from North Sea tidal flat sediment of the German Wadden Sea and was found to be involved in the dechlorination of PCE to *trans*-DCE as the predominant end product [49]. Molecular analysis of the bacterial community in enrichment cultures obtained from estuarine sediments of the San Diego Bay, USA, indicated involvement of *Chloroflexi*-like microorganisms related to *Dehalococcoides* in OHR of the model dioxin, 1,2,3,4-tetrachlorodibenzo-p-dioxin (TeCDD) [210].

In addition to former studies where detection of organohalide-respiring *Chloroflexi* was coupled to physiological/biochemical demonstration of OHR potential, there are other reports on abundant presence of bacteria affiliated with the *Dehalococcoidia* in marine sediments [211-213]. Wasmund and colleagues performed a comprehensive study on distribution and

diversity of *Dehalococcoidia*-affiliated bacteria along vertical profiles of various marine sediment cores obtained from Baffin Bay (Greenland), Aarhus Bay (Denmark), and tidal flat sediments of the Wadden Sea (Germany) [214]. qPCR analysis showed that *Dehalococcoidia*-affiliated bacteria constituted only low proportions of the total bacterial in shallow sediment and generally became more prevalent in deeper sediments. Pyrosequencing of 16S rRNA genes of different clades revealed diverse and divergent co-occurring *Dehalococcoidia* populations within single biogeochemical zones. The varying local biogeochemistry was proposed as the driving force for such abrupt shifts. Hence different subgroups of *Dehalococcoidia*-affiliated bacteria with different metabolic properties likely occupy a wide range of ecological niches over sediment depth [214].

A number of reports exist on distribution and diversity of *rdhA* genes in marine subsurface sediments. Using an array of degenerate primer sets, 32 putative *rdhA* phylotypes were detected from marine subsurface sediments collected from the southeast Pacific off the Peruvian coast, the eastern equatorial Pacific, the Juan de Fuca Ridge flank off the coast of Oregon, and the northwest Pacific off Japan down to a depth of 358 m below the seafloor [178]. OHR was noted using 2,4,6-tribromophenol and trichloroethene in sediment slurry collected from the Nankai Trough [178]. During another study on sediments of the northwest Pacific off the Kii Peninsula of Japan, OHR was restricted to 4.7 metres below the seafloor, despite detection of *rdhA* genes in deeper sediments [215]. A recent metagenomic survey on deep sub-seafloor further broadened the diversity of currently known *rdhA* genes [216]. These results are in contrast to single cell genomic studies of marine *Dehalococcoidia* where no evidence for OHR were found [44, 45]. These studies indicated that microorganisms closely related to known obligate OHRB do not rely on OHR for energy conservation, but rather utilize organic matter degradation pathways which is in line with a similar study from aquifer materials [203]

Marine and estuarine environment has been an important source for the isolation of OHRB given their likely exposure to abundant organohalides and remarkable distribution of *rdhA* genes. Various OHRB isolates with diverse OHR capabilities were obtained from marine sediment as outlined in section 5.2, and a comprehensive compilation is given in Table 2.1.

Riverbed and lake sediments

Due to the high diversity of organisms, the high load of organic nutrients and strong reducing power, sediment geochemical conditions strongly differ from those observed in aquifers, and

thus can support a wide diversity of OHRB. The first study on OHR potential from a lake sediment and sewage sludge was conducted by one of the pioneering labs of the OHR field, Tiedje and co-workers [217]. The observed reductive dehalogenation in the obtained enrichment culture was the first report of OHR as a novel anaerobic respiration pathway that has profoundly altered our perception of biodegradability of organohalides especially under anoxic conditions [217]. The methanogenic consortium was capable of dehalogenating and often mineralizing a variety of halobenzoates to CH₄ and CO₂ [217, 218]. Numerous studies reported anaerobic dechlorination of chlorinated ethenes in the groundwater surface water interaction zone from various geographical locations i.e. freshwater tidal wetlands of West Branch Canal Creek, Maryland, USA [219], Lake Michigan, USA [220], as well as streambed sediments in Angus, Ontario, Canada [221] and in Vilvoorde, Brussels, Belgium [222] without identification of responsible microorganisms. With growing interest on environmental distribution of OHRB, several 16S RNA gene-based methods were applied to track their presence in organohalide impacted or pristine sediments [48, 90, 186, 223]. In early attempts to detect OHRB from sediment environments, sediment samples of three pristine Michigan rivers yielded amplification products with *Desulfuromonas*- and *Dehalococcoides*-targeted primer sets independent of the sampling season [186]. RNA-based stable isotope probing was applied for detection of novel PCE to *cis*-DCE respiring OHRB from pristine river sediment of the river Lahn, Marburg, Germany [48]. These novel OHRB within the phylum *Chloroflexi* were only distantly related to cultivated *D. mccartyi* strains (92–94% sequence identity) [48]. In a chlorinated ethene impacted streambed in Toronto, Canada, *D. mccartyi* guilds were detected where soil organic contents were above 2% w/w and where transformation proceeded to ethene [223].

OHR in the riverbed sediments of the Zenne river, Brussels, Belgium impacted with *cis*-DCE, VC and 1,1-dichloroethane (1,1-DCA) coincided with the presence of *Dehalobacter* and *D. mccartyi* guilds [224]. Similar presence of *Dehalobacter* [225] and *D. mccartyi* [225, 226] was noticed in a number of follow up studies using microcosms prepared with material from the same site, whereas no *Desulfitobacterium* was detected. In both microcosm studies, VC RDase encoding *vcrA*, and *bvcA* genes were the most abundant *rdhA* genes [225, 226]. However, in spite of repeated detection of biomarkers associated with OHRB in the field, microcosm studies with Zenne riverbed sediments [224–226] and high OHR performance in microcosm studies with material from different locations at the Zenne river site [222, 226], high spatial variability of OHR potential was observed in field measurements from the

riverbed [224]. Hence, rather than the microbial community structure of the Zenne River sediments, the CAH discharge rate was hypothesized as the limiting factor for OHR in the Zenne sediments [227]. The high discharge rate and hence reduced residence time of discharging contaminated groundwater seems to be an important prerequisite to drive OHR within riverbed sediments [223, 227].

The composition and abundance of active OHRB was investigated in riverbed sediments collected from the Ebro river in Spain and the Elbe river in Germany [228]. *D. mccartyi* and *Desulfitobacterium* 16S rRNA were detected at the sampling locations whereas *Dehalobacter* rRNA could not be detected. *D. mccartyi* populations were more active and had a higher diversity in river sediments than in floodplain soils in the Elbe River and in general were more dominant in HCB-polluted locations within river basins than *Desulfitobacterium* spp. and *Dehalobacter* spp., albeit with significant temporal and spatial variation [228]. In a recent study 18 sediment samples from three different locations in the Yangtze River, China, were tested for OHR potential [229]. PCE to *cis*- and *trans*-DCE dechlorination was concomitant with increasing numbers of *Dehalobacter* spp., *Desulfomonile* spp., *Desulfitobacterium* spp., or *D. mccartyi* [229]. Krzmarzick and co-workers detected putative organohalide-respiring *Chloroflexi* in 67 of 68 samples collected from lake sediments across a geographical sulfur gradient. Higher quantities of these bacteria were noted where lower sulfate concentration was recorded, indicating an antagonistic relationship between both functional guilds [229].

Soil

Ubiquitous distribution and active involvement in halogen cycles was also shown for organohalide-respiring *Chloroflexi* in soil. An interesting study by Krzmarzick [179] documented widespread distribution of organohalide-respiring *Chloroflexi* in 116 pristine soil samples collected from different locations in USA where their number of 16S rRNA genes positively correlated with organochlorine/TOC [179]. Triclosan addition to agricultural soil from Fairfax, Minnesota, USA, without previous exposure to this substrate increased abundance of *Dehalococcoides*-like *Chloroflexi* in soil microcosms [230]. Furthermore, recently the enrichment of a TCE respiring consortium from garden soil collected from Cuzdrioara, Cluj County, Romania, was reported [231]. In spite of the presence of *D. mccartyi* and *tceA*, *vcrA* and *bvcA* genes, dechlorination stalled at *cis*-DCE even after repeated biostimulation with lactate. The authors hypothesized that this might be a consequence of the intrinsic competition for electron donor (H₂) in soils and sediments, driven by a variety of electron acceptors such as nitrate, Fe (III), sulfate, and bicarbonate [231].

Besides the abundant OHRB belonging to *Chloroflexi*, important roles played by the OHRB belonging to *Firmicutes* were reported in terrestrial soil environments. Geographic distribution of *Desulfitobacterium hafniense* PCP-1 and other *Desulfitobacterium* spp. was studied in 44 soil samples from Quebec, Canada, using a PCR method showing ubiquitous presence of *Desulfitobacterium* spp. [232, 233]. A survey of 65 soil samples from chloroethene-contaminated and pristine soils in Japan showed PCE to *cis*-DCE dechlorination in 16 out of 44 chloroethene-contaminated soils and three out of 21 pristine soils [173]. PCE dechlorination was mostly associated with detection of *Desulfitobacterium* spp. and probably *Dehalobacter* spp. rather than *D. mccartyi* [173]. Complete pentachlorophenol (PCP) to phenol degradation was noted in an enrichment culture obtained from paddy field soil located at Yatomi-cho, Aichi prefecture, Japan [234]. The involvement of Gram-positive spore-forming bacteria belonging to the phylum *Firmicutes* was proposed, although *Desulfitobacterium* spp. were not detected by PCR using a specific primer set [234]. The same soil samples were later shown to be conducive to dechlorination of fthalide where *Dehalobacter* sp. strains FTH1 and FTH2 were identified as potential OHRB [55]. Involvement of novel *Firmicutes* referred to as Gopher group was also proposed in the dechlorination of two chlorinated xanthenes, analogues of natural organochlorines in pristine forest soil from New Jersey and pristine sediment samples collected from lake sediment in Minnesota, USA [235]. The Gopher group was shown to be distinct from but related to *Dehalobacter* and *Desulfitobacterium* [235].

Factors impacting environmental distribution of OHRB

Of particular importance for the distribution and fate of OHRB (and hence organohalides) in the environment are redox conditions, availability of micronutrients, the presence of suitable electron donors and acceptors and the interaction with chemical species.

Redox conditions

With the exception of *Comamonas* sp. 7D-2 as a strict aerobic bacterium capable of OHR [151] and *Anaeromyxobacter dehalogenans* as a facultative anaerobe [119], the currently known OHRB are generally strictly anaerobic bacteria and sensitive to oxygen exposure. This is of high importance for the more delicate OHRB such as *Dehalococcoides* and *Dehalobacter* spp. that require strict anoxic techniques and presence of reducing chemicals in laboratory cultures. Lacking the genes necessary for direct utilization of oxygen, these OHRB are highly dependent on an array of natural shields in the environment. Recent findings showed that in spite of stringent requirement for anoxic conditions in axenic cultures,

members of *Dehalococcoides* might be well protected in their natural habitats such as riverbed sediments [226]. The organic rich matrix of riverbed sediments limits oxygen penetration to the surficial sediment layers. Moreover, sediment aggregates can host anoxic microniches inside sediment-associated biofilms. Presence of facultative aerobic bacteria colonizing the outer layers of sediment biofilms, which rapidly consume oxygen, can protect the strict anaerobes such as OHRB in core microniches [226]. In line with this, a former study on distribution and localization of *D. mccartyi* 195 and methanogenic populations in an enrichment culture using fluorescence *in situ* hybridization (FISH) showed that methanogens reside in the exterior layer of bioflocs, whereas *D. mccartyi* 195 cells were more evenly distributed within bioflocs as well as between biofloc and planktonic phases [236]. A comparative metagenomic study on three different *Dehalococcoides*-containing dechlorinating microbial consortia indicated the presence of several genes related to oxygen tolerance and scavenging from acetogens, fermenters and methanogens commonly found to co-exist with OHRB in enrichment cultures and natural environments [237]. Activity of such genes in non-dehalogenating community members would enforce oxygen scavenging mechanisms, collaterally protecting the OHRB along-side.

Micronutrients

OHRB also depend on other non-dehalogenating community members for the supply of electron donor(s) and growth factors such as vitamins. Fermentation intermediates such as acetate, formate, and H₂ are common products of anaerobic hydrocarbon degradation [238], and are used by other community members such as iron reducers, SRB, OHRB and methanogens. This overlapping nutritional niche caused controversial discussions in the field of OHR. Early reports considered hydrogenotrophic SRB and methanogens as competitors of OHRB for reducing equivalents [239, 240], however, the (predicted) supply of nutrients by SRB and methanogens [237] points towards interactive networks between these microbial guilds rather than pure competition for the shared resources. Indeed, the importance of symbiotic interactions between SRB and OHRB for effective OHR in their co-cultures was previously reported and was attributed to interspecies H₂ transfer [241, 242] or supply of unknown growth factors by the SRB partner [243]. Recent dedicated studies showed that supply of corrinoid cofactors by SRB and methanogens sustained OHR by *D. mccartyi* 195, although corrinoid modification and remodelling by strain 195 was essential to add 5',6'-dimethylbenzimidazole (DMB) as the lower ligand to cobamide precursors and produce cobalamin as the preferred corrinoid cofactor [93-95]. Similarly, *Geobacter lovleyi* strain SZ

was shown to fulfil *Dehalococcoides*' corrinoid requirements by the exchange of DMB [93]. Given the fact that all *D. mccartyi* strains for which genome sequences are available lack the capability of *de novo* cobalamin synthesis [96], and all share the same putative genes involved in corrinoid-remodelling pathways [94], it is tempting to assume that OHRB exploited the biotopes shared with non-dechlorinating community members and have lost the costly but still key metabolic trait of *de novo* cobalamin synthesis. In line with this, recent studies on corrinoid-auxotrophy of *Dehalobacter restrictus* showed that in spite of presence of both anaerobic cobalamin biosynthesis and cobinamide-salvaging pathways, one important protein, CbiH (precorrin-3B C17-methyltransferase), was missing from the upper cobalamin biosynthesis pathway, which was believed to be due to frame-shift mutations [65]. Supply of the OHRB with corrinoids in their natural environment or even during long-term enrichments in the lab may enforce such a genetic trade-off that disables the unused trait. This may explain why OHRB and in particular those who rely on OHR as the sole mode of metabolism have shared/overlapping niches with non-dechlorinators that are capable of *de novo* corrinoid biosynthesis. Experimental evidence also suggest that OHRB are mostly enriched in methanogenic and sulfate-reducing conditions. For example, a recent batch-scale biostimulation of TCE dechlorination showed incomplete TCE dechlorination in microcosms where methanogens were absent [199]. In another study, inhibition of methanogenesis negatively impacted community members such as *Desulfovibrio* [244] that are known to synthesize corrinoids *de novo*.

In natural habitats such as cell aggregates and biofilms where cells form close physical association, OHRB and methanogens may play complementary roles in alleviating thermodynamic constraints for other syntrophic microorganisms. The degradation of fatty acids such as propionate and butyrate only takes place in a thermodynamically interdependent lifestyle where degradation intermediates, usually acetate, formate, and H_2 , are maintained at very low concentrations [238]. Such metabolic/physical networks may also explain why most fastidious OHRB like *D. mccartyi* strains do not submit readily to growth in the laboratory as they need metabolites, signals and structure provided by the community. Further, the structured associations would be more successful in retaining metabolites such as H_2 that would otherwise be lost by diffusion. However, in spite of the central role of microbe-microbe interactions, this subject has received surprisingly little attention in studies involved in OHR. Such information would highly assist in understanding the ecophysiology of OHRB and facilitate design of effective and sustainable bioremediation strategies.

Electron acceptors

The presence of the right terminal electron acceptor is vital for the success of this process as not all OHRB are dedicated to OHR. It is not known whether facultative OHRB would prefer other electron acceptors over organohalides in their natural environment although some general assumptions can be made. At higher redox potential the facultative OHRB such as proteobacterial OHRB may prefer electron acceptors like nitrate, Mn(IV), and Fe(III). A recent study on correlations between environmental variables and bacterial community structures indicated that both VC and Fe(III) reduction were most important but antagonistic terminal electron-accepting processes [245]. Once highly reducing conditions prevail, organohalides may act as electron acceptors. However, the organohalides in natural habitats may not be as readily accessible as in well-homogenised lab cultures. For example, higher degree of halogen substitution can reduce bioavailability of organohalides. This is particularly important in soil and sediment matrices that contain strong sinks of organohalides such as amorphous organic carbon and black carbon [246]. In such environments, highly halogenated and hydrophobic organohalides such as dioxins and PCBs adsorb strongly to organic matter, reducing their bioavailability to OHRB [247]. It should however be noted that such a reduced bioavailability will also lower the acute toxicity of organohalides to OHRB, hence playing a double-edged sword function. Besides, not all types of organohalides are degraded by all OHRB. The facultative OHRB known to date do not dechlorinate PCE past DCE. Even within *D. mccartyi* strains, except for genomic evidence for the strain BTF08 [248], no other isolate is capable of complete PCE dechlorination [40]. However, multiple OHRB may reside in close proximity and co-exist in the same habitat, which is necessary for their robust OHR activity by complementary dehalogenation activity. For example, the concomitant presence of bacteria belonging to *D. mccartyi* and *Desulfitobacterium* groups was previously reported in chloroethene-dechlorinating microbial communities [191]. Another study with an enrichment culture showed that *Sulfurospirillum*-affiliated bacteria were involved in PCE dechlorination to *cis*-DCE whereas *Dehalococcoides*-affiliated bacteria mainly dechlorinated *cis*-DCE to ethene [249].

Interaction with chemical species

Interaction of OHRB with abiotic factors is highly important for their fate in environment. The electrophilic nature of organohalides and reductive nature of the OHR process demands efficient electron transfer mechanisms. Naturally occurring electron shuttles and redox mediators that can be reversibly oxidised and reduced have the capacity to serve as external

electron carriers in redox processes such as OHR [250]. Humic substances and their quinoid analogues are among the best studied natural redox mediators in promoting OHR [251, 252]. Under oxygen-depleted conditions in the subsurface, these redox mediators can be reduced not only by combustion of organic substrates, but also by chemical reactions with reductants common to anaerobic soils and sediments such as Fe(II), sulphide, and cysteine. The electron-withdrawing organohalides can in turn be reduced in subsequent OHR thereby re-oxidising the mediators. Hence, a cocktail of biotic and abiotic reactions could indirectly stimulate OHRB. The organohalides were also shown to be abiotically degraded by naturally-occurring minerals such as pyrite, magnetite and green rusts [253]. Biogenic abiotic catalysts such as palladium nanoparticles can also effectively remove organohalides such as TCE [254]. The abiotic reductive dechlorination reaction could be complementary to OHR at places where the condition is less favourable for OHRB and hence compensate for their drawbacks.

Conclusions

The currently known OHRB belong to distinct phylogenetic groups of deeply branching *Chloroflexi*, Gram-negative *Proteobacteria* and Gram-positive *Firmicutes*. The ubiquitous documentation of OHR potential in aquatic as well as terrestrial environments indicates that the current collection of OHRB is only the tip of the iceberg. It is likely that a considerable portion of OHRB has to date been ignored during conventional culturing techniques. Recent advances in high-throughput culturing approaches should pave the way for isolation of novel OHRB, bypassing many obstacles of conventional cultivation. Moreover, most studies to date were performed using model chlorinated compounds of different haloaliphatic and haloaromatic classes. This limited spectrum of substrates in part is due to simplicity of handling these rather “classical” organohalides and the fact that most of complex naturally occurring organohalides are not commercially available. The known OHRB show moderate traits with regards to extremes of temperature (except for the psychrophilic marine bacterium, *Shewanella sediminis* [147]), pH, and salinity and up to now, there has been no report on OHRB isolated from (poly)extreme environments. Moreover, the OHR capacity has been restricted to the bacterial domain of life and no archaeon has been reported to be associated with OHR in axenic cultures. This is despite the fact that they have been repeatedly found in close association with OHRB in enrichment cultures and environmental samples.

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Primers that target functional genes of organohalide- respiring bacteria

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Abstract

Halogenated organic hydrocarbons are problematic environmental pollutants that can be reductively dehalogenated by organohalide-respiring bacteria (OHRB) in anoxic environments. This energy-conserving process is mediated by reductive dehalogenases (RDase). To amplify the diversity of reductive dehalogenase-encoding genes, degenerate primers have been designed, most of which target the conserved regions of the encoded protein sequences of the catalytic subunit, RdhA. In addition, specific primer sets have been developed and widely used to quantify and characterize OHRB and the reductive dehalogenase homologous (*rdh*) genes in the environment. The specific primers have been applied to multiple molecular techniques including regular and quantitative PCR, Southern blot hybridization, terminal restriction fragment length polymorphism (T-RFLP), and reverse transcriptase PCR (RT-PCR). The hunt for novel *rdhA* genes has benefited greatly from next generation sequencing techniques, including primer-dependent amplicon sequencing and primer-independent metagenomic analyses. This chapter provides an overview of most primers targeting RDase-encoding genes described to date and their applications, and it discusses the developing trend of leveraging primer-(in)dependent techniques for better understanding of OHRB and their RDase gene pools.

Introduction

Halogenated organic compounds (organohalides) are problematic environmental chemicals that over the last century have been widely produced and used for industrial applications and chemical manufacturing. As a result of accidental or indiscriminate disposal, organohalides are among the most abundant soil and groundwater contaminants. Microorganisms have evolved different strategies to take advantage of organohalides, greatly impacting the compounds' environmental fates. Under anaerobic conditions, organohalides can be reductively dehalogenated, which, depending on the degree of dehalogenation, can lead to their detoxification or render them susceptible for further (bio)transformation [10]. During this process, known as organohalide respiration (OHR), the organohalides are used as terminal electron acceptors in an energy-conserving respiratory metabolism. The chemically stable halogen-carbon bond is unlocked by replacing the halogen atom with hydrogen and liberating it as a halide [46]. This process is mediated by organohalide-respiring bacteria (OHRB), some of which are extreme niche specialists with OHR as their only metabolism. This group of obligate OHRB comprises the organohalide-respiring members of *Chloroflexi* (including strains of *Dehalococcoides mccartyi*, *Dehalogenimonas*, and *Dehalobium*) and *Firmicutes* (strains of *Dehalobacter*) [13, 46]. It should however be noted that recent studies showed fermentative growth of a few *Dehalobacter* spp. on chloromethane [42, 43]. Moreover, single cell genomic studies of marine *Dehalococcoidetes* did not reveal any evidence for catabolic reductive dehalogenation, indicating that microorganisms closely related to known obligate OHRB do not rely on OHR for energy conservation, but rather utilize organic matter degradation pathways [44, 45]. Compared to obligate OHRB, facultative OHRB have versatile metabolisms encoded by relatively large genomes, and are capable of using a broad variety of electron acceptors for respiratory growth that include but are not limited to organohalides. This group comprises phylogenetically diverse proteobacterial OHRB such as members of the genera *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter*, *Desulfomonile*, *Desulfovibrio* and *Sulfurospirillum*, and also the *Desulfitobacterium* spp. belonging to the *Firmicutes* [13, 46].

The key enzymes of OHR are reductive dehalogenases (RDase). For several of these enzymes the catalytic subunits have been purified and biochemically characterized, which are referred to as RDase catalytic subunit (as opposed to RdhA if only predicted based on sequence homology) [64, 78, 101, 255-259]. These studies, combined with PCR-based [64, 99-101, 260] and genomic analyses [20, 92, 261], have revealed a conserved operon structure for RDase-

encoding genes that consist of *rdhA*, coding for the catalytic subunit RdhA; *rdhB*, coding for a small putative membrane anchor (RdhB) that (presumably) locates the A subunit to the outside of the cytoplasmic membrane; and a variable set of accessory genes, the majority of which has been predicted to code for proteins involved in regulation and maturation of Rdh synthesis [46]. In general, the catalytic subunits (RdhAs) are characterized by two iron sulfur-clusters and a cobamide (corrinoid cofactors) as cofactors in the active holoenzyme. As an exception to this rule, the chlorobenzoate RDase of *Desulfomonile tiedjei* contains a heme cofactor [255], and *Sulfospirillum multivorans* was shown to specifically require “norpseudo-B₁₂” as the corrinoid cofactor [145]. Another conserved feature among the catalytic subunits is the presence of an N-terminal Tat (twin-arginine translocation) signal peptide. After cofactor incorporation and folding of the cytoplasmic precursors of an RdhA enzyme, the Tat signal peptide is necessary for secretion of the mature protein through the cell membrane where it is located at the exocyttoplasmic face of the cytoplasmic membrane [262].

Phylogenetic context of *rdhA*

The genomes of OHRB encode either few (1-3) or many (10-40) distinct RDase gene operons. In general, larger numbers of *rdhA* genes are encoded on genomes of obligate OHRB, with fewer present on genomes of microorganisms with versatile metabolism [13]. The expansion of the gene family through gene duplication has led to multiple non-identical *rdhA* genes per genome and across diverse, unrelated genera resulting in a complicated evolutionary history for *rdhA*. A tree of RdhA protein sequences from known microorganisms (not including sequences retrieved from environmental samples) shows limited correlation between microorganism taxonomy and RDase phylogeny (Figure 3.1). Most phyla with multiple OHRB do not form monophyletic clades on the RdhA tree, and, even within clades comprised of a single phylum or group, the branching order of the RdhA does not usually follow the corresponding 16S rRNA-based taxonomy tree. In addition, microorganisms within the same genus often do not have the same RdhA homologs in their genomes. Taken together, the evolutionary relationships between the *rdhA* genes can only be explained using a combination of vertical inheritance, gene duplication, and lateral gene transfer [46].

Most interesting from a functional perspective is the placement of the biochemically characterized RDases, which are distributed across the tree with little apparent relationship between proteins with shared substrate specificities when they are present in different phyla. The five characterized tetrachloroethene-reducing enzymes (PceA) are located in four distinct clades (Figure 3.1), indicating their low sequence similarity despite shared specificity. In

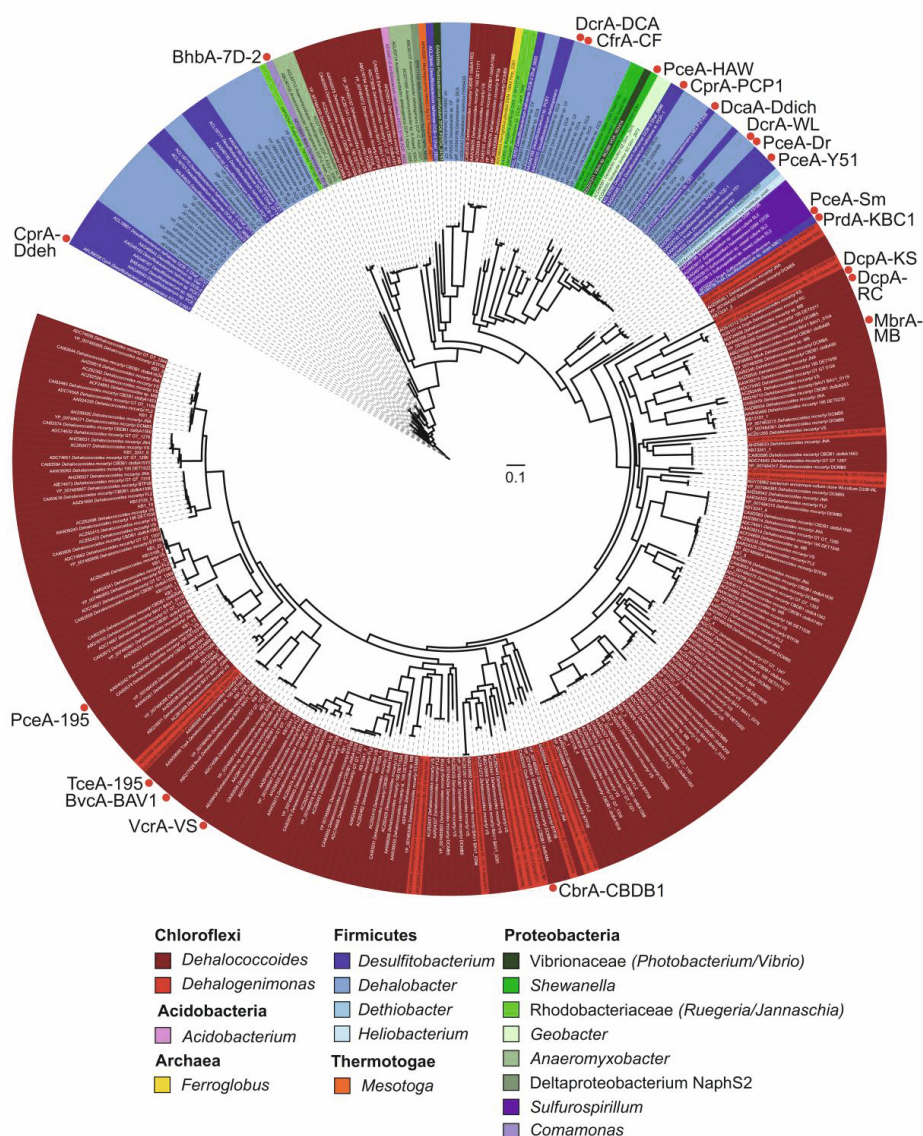


Figure 3.1 Maximum likelihood tree of RdhA amino acid sequences from known organisms. RdhAs are coloured by genus affiliation of the source organism or, in two cases, order affiliation. Functionally characterized RdhA are highlighted with red circles and named according to Table 3.1. The tree was constructed from 354 full-length RdhA sequences. Sequences were aligned using MUSCLE [263], and the alignment was iteratively refined using HMMER 3.0 [264] for ten iterations. Alignment columns were removed if they contained >90% gaps, and the ends of the alignment trimmed to remove trailing ends. The tree was generated using RAXML [265, 266] under the PROTGAMMAWAG model of evolution and visualized using iTOL [267, 268]. Microorganism information and protein NCBI accession numbers are included in each RdhA gene name on the tree.

contrast, dichloroethane RDase DcrA and the chloroform RDase CfrA from *Dehalobacter* strains DCA and CF, respectively, share extremely high sequence identity (98 %) but do not have overlapping substrate specificities (Figure 3.1) [269]. The lack of correlation between sequence similarity and substrate specificity hinders the ability to predict substrates for novel genes, as well as to design primers targeting specific functions of interest.

Molecular characterisation of RDase encoding genes

Initial identification of *rdh* gene sequences was performed by classical reverse genetics approaches based on N-terminal amino acid sequences obtained from purified catalytic subunits [78, 260]. Subsequent availability of RDase encoding gene sequences has made it possible to design degenerate primers for PCR-based *rdh* sequence retrieval. This has been particularly helpful for strains of *D. mccartyi* whose low biomass yields have hindered

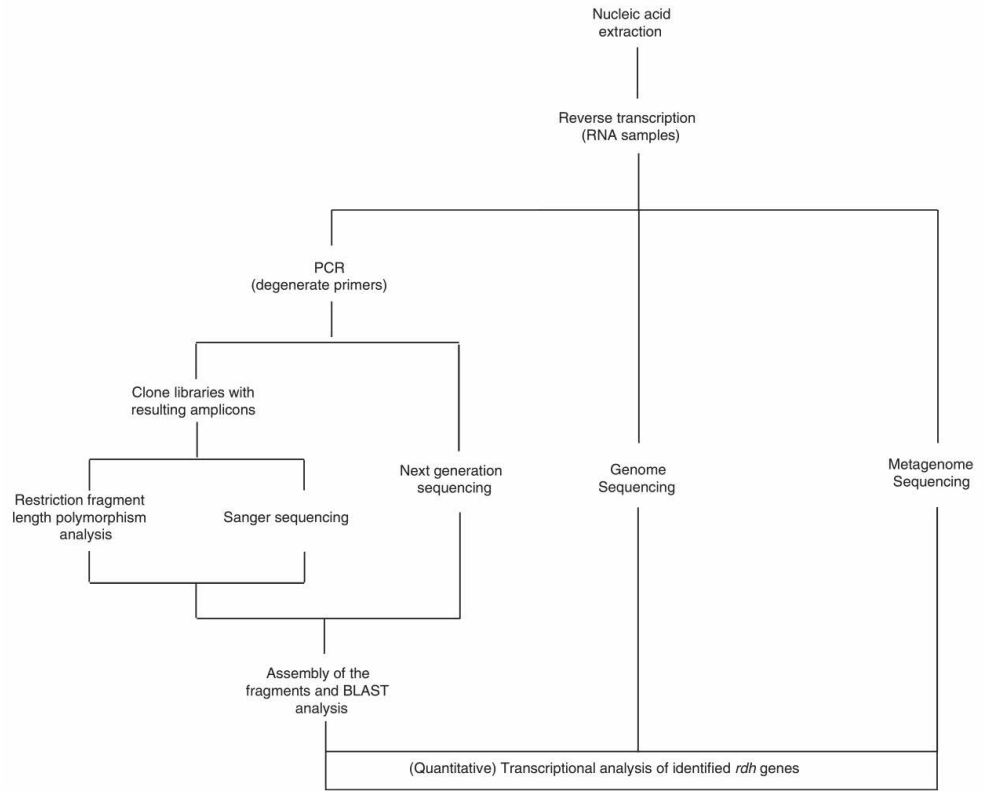


Figure 3.2 Overview of the methods used for molecular characterisation of RDase-encoding genes.

protein-based identification in the past. In recent years genomic- and metagenomic-based approaches have also been applied for sequence-based retrieval and characterization of *rdh* genes (Figure 3.2).

Degenerate primers for characterization of RDase genes

Degenerate primers are primer mixes with degenerate positions instead of a single sequence with specified bases. The primer degeneracy allows amplification of multiple loci simultaneously from different microorganisms, targeting sequences with similar but not identical motifs. Degenerate primers are widely used to amplify *rdhA* genes, especially when the aim is to amplify genes from uncultivated OHRB for which genomic information is not available. Degenerate primers are usually designed based on conserved regions of RDase catalytic subunit encoding genes by aligning amino acids sequences found in sequence databases like GenBank [270], and using specific approaches including Consensus-Degenerate Hybrid Oligonucleotide Primer (CDHOP) [271]. The first set of degenerate primers (primer pair 1, Table 3.2) for an RDase gene was designed based on internal peptides in 1998, targeting a 1200 bp-long region of the PCE dehalogenase from *Dehalospirillum multivorans* [260] (later reclassified as *Sulfurospirillum multivorans* [142] (Table 3.2). In the following years, with increased availability of homologous sequences of RDase encoding genes, multiple degenerate primer sets were designed and applied for *rdh* detection, mostly targeting conserved sequences of *rdhA* [64, 79, 98, 101, 258] (Figure 3.3, Table 3.2). von Wintzingerode, Schlötelburg [272] developed primers (primer pair 13-16, Table 3.2) binding to sequence motifs encoding the conserved regions of PceA (*Sulfurospirillum multivorans*) and CprA (*Desulfitobacterium dehalogenans*) (Figure 3.3), which enabled amplification of *cprA*-like gene fragments from *Desulfitobacterium hafniense*, *Desulfitobacterium* sp. strain PCE1, and *Dehalobacter restrictus*. Expanding this technique further, Smidt [273] designed multiple sets of highly degenerate oligonucleotide primers (primer pairs 17-20, Table 3.2) based on twin-arginine signal peptides, iron-sulfur clusters, and five additional highly conserved sequence motifs (Figure 3.3), which were used to target *rdhA* genes from different *ortho*-chlorophenol- and chloroethene-dechlorinating OHRB. Of all primers, the RRF2 and B1R primers (primer pair 4, Table 3.2) became one of the most popular sets [100, 274]. Originally used to identify the *bvcAB* gene from *D. mccartyi* BAV1, this primer set targets the RRXFXK motif of the Tat signal peptide (RRF2) (Figure 3.3), and the WYEW motif internal to the downstream associated *rdhB* gene (B1R), yielding amplicons of 1.5-1.7 kb containing almost complete *rdhA* genes (Table 3.2). Using this primer set, Krajmalnik-Brown [100]

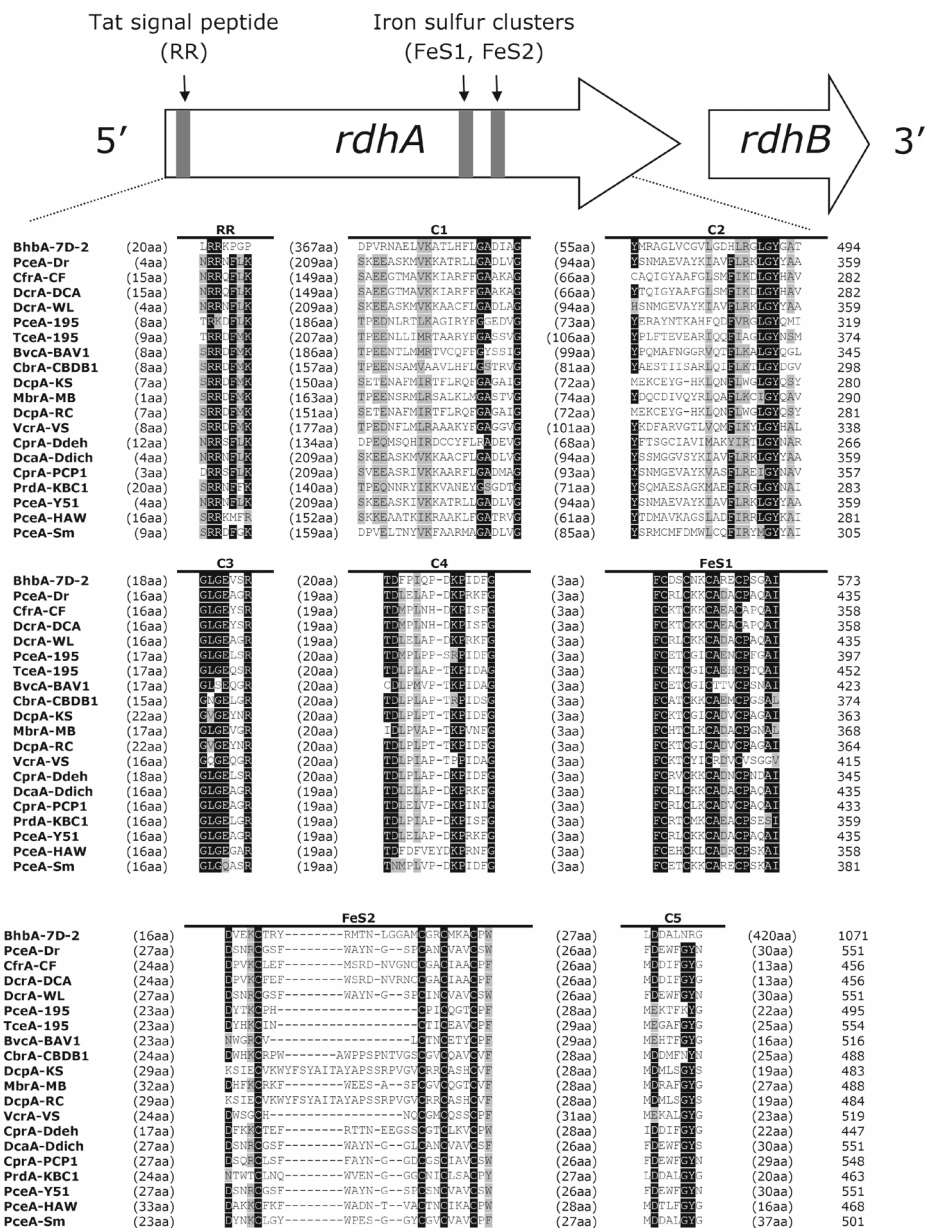


Figure 3.3 Physical map of *rdhAB* operon (top) and primary sequence alignment of known RdhAs (bottom). Note that in some cases, the order of genes in the operon is reversed (i.e. *rdhBA*, rather than *rdhAB*). Clustal W [275] multiple sequence alignment was conducted by using BioEdit version 7.2.5 (<http://bioedit.software.informer.com/>). The conserved sequence motifs among experimentally characterized reductive dehalogenases (RR, two Fe-S and C1-C5)

are indicated with black backgrounds. Functionally characterized RdhAs are named according to Table 3.1.

Table 3.1 Functionally characterized RDase catalytic subunits.

Code	Protein	Microorganism	Protein NCBI accession numbers	Reference
BhbA-7D-2	BhbA	<i>Comamonas</i> sp. 7D-2	AFV28965.1	[151]
PceA-Dr	PceA	<i>Dehalobacter restrictus</i> PER-K23	CAD28790.2	[64]
CfrA-CF	CfrA	<i>Dehalobacter</i> sp. strain CF	AFQ20272.1	[269]
DcrA-DCA	DcrA	<i>Dehalobacter</i> sp. strain DCA	AFQ20273.1	[269]
DcrA-WL	DcrA	<i>Dehalobacter</i> sp. strain WL	ACH87594.1	[276]
PceA-195	PceA	<i>Dehalococcoides mccartyi</i> 195	AAW40342.1	[97]
TceA-195	TceA	<i>Dehalococcoides mccartyi</i> 195	AAF73916.1	[98]
CbrA-CBDB1	CbrA	<i>Dehalococcoides mccartyi</i> CBDB1	CAI82345.1	[103]
DcpA-KS	DcpA	<i>Dehalococcoides mccartyi</i> KS	AGS15112.1	[104]
MbrA-MB	MbrA	<i>Dehalococcoides mccartyi</i> MB	ADF96893.1	[99]
DcpA-RC	CbrA	<i>Dehalococcoides mccartyi</i> RC	AGS15114.1	[104]
VcrA-VS	VcrA	<i>Dehalococcoides mccartyi</i> VS	AAQ94119.1	[101]
CprA-Ddeh	CprA	<i>Desulfitobacterium dehalogenans</i> JW/IU-DC1	AAD44542.1	[78, 97]
DcaA-Ddich	DcaA	<i>Desulfitobacterium dichloroeliminans</i> DCA1	CAJ75430.1	[83]
CprA-PCP1	CprA	<i>Desulfitobacterium hafniense</i> sp. PCP-1	AAQ54585.2	[77]
PrdA-KBC1	PrdA	<i>Desulfitobacterium</i> sp. strain KBC1	BAE45338.1	[80]
PceA-Y51	PceA	<i>Desulfitobacterium</i> sp. strain Y51	AAW80323.1	[79]
PceA-HAW	PceA	<i>Shewanella sediminis</i> HAW-EB3	ABV38373.1	[277]
PceA-Sm	PceA	<i>Sulfurospirillum multivorans</i> DSM 12446	AAC60788.1	[260]

found seven *rdhA* genes in *D. mccartyi* BAV1. Later, the RR2F/B1R pair was widely used to detect *rdhA*'s from *D. mccartyi* isolates and enrichments, including 13 *rdh* loci in strain CBDB1 [274], 14 in strain FL2, 14 in the mixed culture KB1 [278], 8 in the enrichment culture TUT2264 [279] and 4 from TCE-contaminated groundwater samples [194]. Although the RR2F/B1R primer set was designed based on 17 *rdhAB* genes in the genome of *D. mccartyi* 195 [100], the primer set fails to amplify *tceA*-like genes [99]. Furthermore, it should be noted that the *rdh-A* and *-B* genes are not always oriented in an A-B direction in the *rdh* operon [96]. To circumvent this shortcoming, Chow [99] designed a

complimentary primer set RDH F1C/RDH R1C (primer pair 7, Table 3.2) targeting the coding region of the twin arginine motif and conserved PIDDG motif, respectively (Figure 3.3, Table 3.2). With a product size of 1200 bp, this primer pair targeted only the catabolic subunit gene (*rdhA*), and (at the time of design) could cover ~ 90% of the known *rdhAs* in *D. mccartyi* populations. Using this primer set, the authors detected seven putative *rdhA* genes from *D. mccartyi* strain MB [99]. Another potential problem with the RR2F/B1R primer pair is that, although the N-terminal Tat signal peptide motif is highly conserved among RdhAs, it is also found in numerous other exported proteins, and as such is not unique to this class of proteins [262]. Meanwhile, Regeard [271] had designed seven degenerate primers (primer pairs 23-28, Table 3.2) based on four conserved regions of chloroethene RDase catalytic subunit encoding amino acid sequences from *Desulfitobacterium* sp. PCE-1, *S. multivorans* and *D. restrictus*, and chlorophenol RDase catalytic subunit encoding amino acid sequences from *Desulfitobacterium dehalogenans*, *D. hafniense* strain DCB-2 and *Desulfitobacterium chlororespirans* (Table 3.2). This suite of degenerate primers did not utilize the Tat signal sequence, reducing non-specific amplification, but also resulted in shorter amplicons than the primers developed by Chow [99]. The primers were originally used to estimate the diversity of *rdhA* from tetra- and trichloroethene dechlorinating enrichments [271] and later to detect *rdhA* from *Desulfitobacterium dichloroeliminans* DCA1 [83, 280], *Dehalobacter* sp. WL [276] and sub-seafloor sediments [178].

It is evident from the alignment of *rdhA* genes that one primer pair cannot cover all possible sequences. Accordingly, Futagami [178] used an array of degenerate primer sets designed to target different *rdhAs* [100, 271, 272, 274]. Using this approach, 32 putative *rdhA* phylotypes were detected from marine subsurface sediments [178]. In a further step, Wagner *et al.* (2009) designed 13 degenerate primer pairs to monitor the expression of all 32 *rdhA* genes present in the genome of *D. mccartyi* CBDB1 [281]. Although ideal in order to cover a diversity of different *rdhAs*, application of such an array of degenerate primers would dramatically increase the work-load in subsequent clone library construction. Besides, most of the degenerate primers were designed based on the limited number of RDase sequences available at the time, which could potentially overlook distantly related novel *rdhAs*. To adequately cover the full diversity of *rdhA* homologous sequences displaying lower similarity, a suite of

Table 3.2 Degenerate primer sets targeting RDase genes.

Primer pair	Targeted RDase genes	Name	Primer sequence (5'- 3')	Targeted region	Annealing T (°C)	Size (bp)	Targeted OHRB
1	<i>pceA</i>	unnamed [260]	GGIGAGGTIAAGCCITGGTT	Internal peptides: 'GEVKPWF ^a	45	1,200	<i>Sulfurospirillum multivorans</i> [260]
		unnamed [260]	GTCCCAIACYTCIGTDATRTT	Internal peptides: 'NITEVWD ^a			
2	<i>iceA</i>	TFRO [98]	GCIAAYAARGTIAAAYAAAYCA YCCNTGGTGGG	N-terminal: 'ANKVNNHPWW ^a	60	491	<i>Dehalococcoides mccartyi</i> 195 [98]
		TREV [98]	CCYTCCCAYTTIGGRTARTTN GTNGT	Internal peptide: 'TTNYPKWE ^a			
3	<i>vcrA</i>	Nterm13F [101]	ACVAARGAYCARCCDTGGTA	N-terminal: 'TKDQPWY ^a	47	292	<i>Dehalococcoides mccartyi</i> VS [101]
		Intern4R [101]	GGYTGBGTIACKGCRTARAA ^b	Internal peptide: 'FYAVTQP ^a			
4	<i>bvcA</i>	RRF2 [100]	SHMGBMGWGATTYYATGAA RR	Twin-arginine motif: 'RRXFXK'	48	1,500	<i>Dehalococcoides mccartyi</i> [100]
	<i>bvcB</i>	B1R [100]	CHADHAGCCAYTCRTACCA	Associated anchoring protein motif: 'WY ^a	48	1,700	<i>Dehalococcoides mccartyi</i> CBDB1 and FL2 [274]
					48	~1,500	<i>Dehalococcoides</i> -containing mixed culture KB1 [278]
					48	~1,700	environmental sample containing <i>Dehalococcoides</i> spp. [194]
					-	>1,520	enrichment culture TUT2264 [279]
					48	1,500-1,700	<i>Dehalococcoides</i> spp. [99]
5	<i>rdhA</i>	fdehal [274]	CARGGXACXCCXGARGA	Internal peptides: 'QGTP ^e (D/E) ^e	-	500	<i>Dehalococcoides mccartyi</i> CBDB1 [274]

(continued)

		rdhA [274]	RSXCCRAARTCXATXGG	Internal peptide of C4 region: 'P(I/M)DFG(P/L/A/ V) ^c			~1,000	<i>Dehalococcoides mccartyi</i> CBDB1 [274]
6	<i>rdhA</i>	mem2 [274]	NNNTTYCAYGAYYTNAGAYGA M	Upstream of rRR motif: 'XFHD(F/L)D (D/E) ^c	-			
7	<i>rdhA</i>	mem5 [274]	NCCNGCRTCDATNGGNNNN N	Internal peptide of C4 region: 'XXPIDAG ^c	47		1,200	<i>Dehalococcoides</i> spp. [99]
8	<i>pceA</i>	RDH FIC [99]	TTYMVIGAYITIGAYGA	Conserved twin arginine motif				
		RDH RIC [99]	CCIRMRTYIRYIGG	Conserved motif: 'PIDD'				
8	<i>pceA</i>	NTERM [79]	CIGAYATIGTIGCICCIAT	N-terminal: 'ADIVAPI' ^a	46		1,000	<i>Desulfitobacterium</i> sp. Y51 [79]
9	<i>pceC</i>	INT14 [79]	YTTRTCIGGIGCIARYTC	Internal peptide of C4 region: 'VYTDLELAPDK'				
		unnamed [258]	GCIGARGTITAYAAAYAARGA	N-terminal: 'AEVYNKD' ^a	43		81	Coculture DPH-1 [258] (containing <i>Desulfitobacterium hafniense</i> strain JH1 [282])
		unnamed [258]	GTRTCRTTISIRAAARTATG	N-terminal: 'HYFSDDA' ^c				
10	<i>pceA</i>	DR3f [64]	GAYATIGTIGCICCIATAC	N-terminal: 'DIVAPIT'	50		1,126	<i>Dehalobacter restrictus</i> PER-K23 [64]
		DR4r [64]	CCRAARTCIATIGGYTTRTCIGG	Internal peptide of C4 region: 'PDKPIDFG'				
11	<i>rdh</i>	DebfrdhF [58]	ATGGGNGARATHAAAYMG	Peptide: 'MGEIN(S/R) ^c	-		~1,100	<i>Dehalobacter</i> sp. TCP-1 [58]
		DebfrdhR [58]	TCNGRCRCAYTTYTTRCA	Iron-sulfur cluster binding motif: 'CKKCA(D/E) ^c				

(continued)

12	<i>rdh</i>	RRF2 [100]	SHMGBMGWGATTYYATGAAR R	Twin arginine motif: 'RRXFYK' ^e	48	1,000	WL cultures (containing <i>Dehalobacter</i> sp. WL) [276]
13	<i>cprA/peaA</i> - like gene	RD7r [271] dehaloF3 [272] dehaloR2 [272]	AANGGRCAIACIGCIWCRCA ATCGWTSRMRGGTAT TYTGTACCATAGCC	Iron-sulfur cluster peptide: 'CV(E)AVCP' Internal peptide 'I(D/V)(A/Q)GI' ^e C-terminal: 'GYGT(K/E)' ^e	50	~600	<i>Desulfitobacterium dehalogenans</i> , <i>Sulfurospirillum multivorans</i> , <i>Desulfitobacterium</i> sp. PCE1 and <i>Dehalobacter restrictus</i> [272]
14	<i>cprA/peaA</i> - like gene	dehaloF4 [272] dehaloR3 [272]	YMTKGGTTAYWATGC AGCCMAANRYATCATC	Internal peptide of C2 region: 'LGYNA' ^e Internal peptide of C5 region: 'DD(A/I)(L/F)G' ^e	50	~450	DCP-dechlorinating batch culture [272]
15	<i>cprA/peaA</i> - like gene	dehaloF5 [272] dehaloR4 [272]	GGTTGCATTGCYGTCTAT TGCTTYATGGAAACCAGG	Internal peptide of C2 region: 'GCIAVI' ^e C terminal: 'SWFHQA' ^e	50	425	<i>Desulfitobacterium dehalogenans</i> , <i>Desulfitobacterium hafnense</i> , <i>Desulfitobacterium</i> sp. PCE1 and <i>Dehalobacter restrictus</i> [272]
16	<i>cprA/peaA</i> - like gene	dehaloF4 [272] dehaloR2 [272]	YMTKGGTTAYWATGC TYTGTACCATAGCC	Internal peptide of C2 region: 'LGYNA' ^e C-terminal: 'GYGTX' ^e	50	~500	<i>Desulfitobacterium dehalogenans</i> , <i>Sulfurospirillum multivorans</i> , <i>Desulfitobacterium</i> sp. PCE1 and <i>Dehalobacter restrictus</i> [272]
17	<i>cprA</i>	D1 [273] D8 [273]	AAYMGVMGVAAYYTYCTBAA GCRCAYTTYTTRCA	Twin arginine motif: 'NRRNFLK' Iron-sulfur cluster binding motif: 'CKKCA'	35-40	-	<i>Desulfitobacterium dehalogenans</i> and <i>Desulfitobacterium</i> sp. PCE-1 [273]

(continued)

18	<i>rdhA</i>	D2 [273] D7 [273]	GCIGAYATHGTIGCICC ATIGGYTTRTCIGG	N-terminal: 'ADIVAP' Internal peptide of C4 region: 'PDKPI'	35–40	-	<i>Desulfitobacterium hafnense</i> , <i>Desulfitobacterium frappieri</i> strains PCP-1 and TCE1 [273]
19	<i>rdhA</i>	D4 [273] D7 [273]	GGIGCIGAYYTIGTIGG ATIGGYTTRTCIGG	Internal peptide of C1 region: 'GADLVG' Internal peptide of C4 region: 'PDKPI'	35–40	-	<i>Desulfitobacterium dehalogenans</i> , <i>Desulfitobacterium</i> sp. PCE-1 and <i>Sulfurospirillum multivorans</i> [273]
20	<i>rdhA</i>	D4 [273] D8 [273]	GGIGCIGAYYTIGTIGG GRCAYTTYTTRCA	Internal peptide of C1 region: 'GADLVG' Iron–sulfur cluster binding motif: 'CKKCA'	35–40	-	<i>Desulfitobacterium dehalogenans</i> , <i>Desulfitobacterium</i> sp. PCE-1 and <i>Sulfurospirillum multivorans</i> [273]
21	<i>cprA</i>	DHU1080F [283]	TWGYCCYRMYAARCCYATY GA	Internal peptide of C4 region: 'LXPX(N/S)P(S/L)' Internal peptide of C5 region: '(M/I)D(D/E)I(F/L) GYG ^{ce}	55	450	2-bromophenol-degrading consortium [283]
22	<i>rdhA</i>	DHAR1000 F [283] DHU1350R [283]	GWAGCAGCTYTRGGASAA CCRTAGCCVAAKATWTCATC MAT	Internal peptide of C2 region: '(V/E)AGLG(Q/E) ^{ce} Internal peptide of C5 region: '(M/I)D(D/E)I(F/L) GYG ^{ce}	55 46	350 no amplicon	2-bromophenol-degrading consortium [283] Tidal Flat <i>Chloroflexi</i> Cluster [49]
23	<i>pceA</i>	ceRD2Lf /ceRD2Sf [271]	GCAGCACGCCTTTTGGIGCIK MIYTNGTIGG/ GCAGCACGCCTTTTGGIGCIK MIWSIGTIGG	Internal peptide of C1 region: 'AARLFGA(D/S)(L /S)VG'	47	750– 800	<i>Desulfitobacterium</i> sp. PCE-1, <i>Sulfurospirillum multivorans</i> and <i>Dehalobacter restrictus</i> [271]

(continued)

			RD7r [271]	AANGGRCAIACIGCIWCRCA	Iron-sulfur cluster peptide: 'C(V/E)AVCP'	47	800- 1,150	1,2-DCA enriched culture 6VS (<i>Desulfotobacterium dichloroeliminans</i> DCA1) [83] [280] WL cultures (containing <i>Dehalobacter</i> sp. WL) [276]
24	<i>pceA</i>		RD5f [271]	CCIRMIAARCCIIATIRAIKYNGG	Internal peptide of C4 region:'P(D/T)KPI(D/K)(A /F)'G'	47	200 (expect ed), no amplific ation	<i>Desulfotobacterium</i> sp. PCE-1, <i>Sulfurospirillum multivorans</i> and <i>Dehalobacter restrictus</i> [271]
25	<i>pceA</i>		RD7r [271]	AANGGRCAIACIGCIWCRCA	Iron-sulfur cluster peptide: 'C(V/E)AVCP'	50	350 (expect ed), no amplific ation	<i>Desulfotobacterium</i> sp. PCE-1, <i>Sulfurospirillum multivorans</i> and <i>Dehalobacter restrictus</i> [271]
26	<i>pceA</i>		ceRD2Lf /ceRD2Sf [271]	GCAGCACGCCTTTTGGIGCIK MIYTNGTIGG/ GCAGCACGCCTTTTGGIGCIK MIWSIGTIGG	Internal peptide of C1 region:'AARLFGA(D/S)(L /S)'VG'	50	450 (expect ed), no amplific ation	<i>Desulfotobacterium</i> sp. PCE-1, <i>Sulfurospirillum multivorans</i> and <i>Dehalobacter restrictus</i> [271]
27	<i>pceA</i>		RD4f [271]	CKIGAIGCYTSICCIARNCC GGNYTIGGISARGCITCIMG	Internal peptide of C3 region:'GLG(E/Q)ASR' Internal peptide of C3 region: Conserved sequence:'GLG(E/Q)ASR'	50	150 (expect ed), no amplific ation	<i>Desulfotobacterium</i> sp. PCE-1, <i>Sulfurospirillum multivorans</i> and <i>Dehalobacter restrictus</i> [271]

(continued)

		RD5r [271]	CCIRMIYIATIGGYTTIKYNGG	Internal peptide of C4 region: 'P(D/T)KPI(D/K)(A /F)G'			
28	<i>pceA</i>	ceRD2Lf /ceRD2Sf [271]	GCAGCACGCCCTTTTGGIGCIK MIYTNGTIGG/ GCAGCACGCCCTTTTGGIGCIK MIWSIGTIGG	Internal peptide of C1 region: 'AARLFGA(D/S)(L /S)VG'	51	550	<i>Sulfurospirillum multivorans</i> and <i>Dehalobacter restrictus</i> [271]
		RD5r [271]	CCIRMIYIATIGGYTTIKYNGG	Internal peptide of C4 region: 'P(D/T)KPI(D/K)(A /F)G'			
29	<i>pceA</i>	unnamed [284]	GAYAAARCCNATHGAYTTYGG	Internal peptide of C4 region: 'PDKPIDFG' ^c	-	330	environmental DNA [284]
		unnamed [284]	CCRTANCCNARNGCRTCRTC	Internal peptide of C5 region: 'DDALGYG' ^c			

Abbreviations of degenerate nucleotides: I = inosine; R = A or G; K = G or C; S = C or G; W = A or T; Y = C or T; B = C, G, or T; D = A, G, or T; V = A, C, or G; H = A, C, or T; N or X = A/C/G/T.

^aThe internal peptides published as the binding motif for primer design in the original publication are adjusted to the actual length of the primer.

^bThe sequence of the Intern4R primer in the original publication by Müller [101], represents the coding strand, rather than the reverse complement, and is therefore not correct as confirmed by the author. The primer sequence given in the table 3.2 is therefore the reverse complement of the originally published sequence.

^cThe binding motif of the targeted internal peptide was not shown in the original publication. The amino acid sequences shown here are the (possible) binding motifs according to genetic code translation of primer sequence and amino acid sequences alignment of reductive dehalogenases in Figure 3.3.

degenerate PCR primers (comprising 44 phylogeny-derived groups) targeting 255 RDase catalytic subunit genes was recently designed and applied to environmental and enrichment culture samples [285]. Subsequent application of Illumina HiSeq next-generation sequencing identified a much broader diversity of *rdhA* gene sequence than was previously accounted for [285].

Specific primers and application potential

In addition to degenerate primers, a broad range of specific primers targeting selected RDase genes have been designed (Table 3.3). Usually, specific primers are developed and used for different purposes than the more exploratory degenerate primer sets, including analysis of gene expression in *S. multivorans* [260] and *Desulfitobacterium* sp. Y51 [79], preparation of probes for Southern blot hybridization [83], detection of *Sulfurospirillum rdhA* genes by T-RFLP [286], and detection of known chloroethene RDase genes [271]. Additionally, specific primers were used for putative *rdhA* detection from various OHRB encoding *tceA* [98], *bvcA* [100], *vcrA* [101], *cfrA* [269], *dcrA* [269], and *dcpA* [104] (Table 3.3). Some primer sets have been designed to obtain complete *rdhAB* sequences of specific reductive dehalogenases, including *pceAB* [64, 260, 273], *vcrAB* [101] and *cprAB* [77, 273].

To connect the presence of specific *rdhAs* with OHR activity, multiple primer sets were developed for *rdhA* quantification (Table 3.4). For instance, by using *D. mccartyi*-specific primer sets targeting *bvcA* (BVC925F/1017R), *vcrA* (Vcr1022F/1093R), and *tceA* (TceA1270F/1336R), Ritalahti [195] were able to link abundance and identity of *D. mccartyi* populations to different organohalide electron acceptors. From this, the authors were able to predict the composition of a *D. mccartyi* community using the quantitative results of *rdhA* and 16S rRNA gene-targeted quantitative PCR (qPCR). qPCR assays with *rdhA* targeted primers have also been used for monitoring of bioremediation in polluted sites [83, 104, 195]. Building upon this knowledge, microfluidics-based, moderately to massively parallel qPCR approaches were recently developed for covering much of the known *rdhA* sequence space. The method was helpful in quantitative analysis of *rdhA* repertoires and identification of closely related populations of OHRB [287, 288]. It should be noted that to date all known RDase and 16S rRNA genes identified in the genomes of known *D. mccartyi* strains occur as single copies (unlike *Desulfitobacterium* spp. and *Dehalobacter* spp. strains with multiple copies of 16S rRNA genes), suggesting that qPCR data can be converted from gene copy

Table 3.3 Non-degenerate primer sets designed for specific RDase genes.

Target gene	Target OHRB	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Application potential	Comments	Reference
<i>pceA</i>	<i>Sulfurospirillum multivorans</i>	unnamed (ATAGACCATG GAAAAAGAAAAAAG CCTGAACCTCTC)	unnamed (GCAAGGATC CTCATGATTTTAAAC CCTATCCTTTCTAAA GC)	-	Expression of RDase		[260]
<i>pceAB</i>	<i>Sulfurospirillum multivorans</i>	unnamed (ATAGACCATG GAAAAAGAAAAAAG CCTGAACCTCTC)	unnamed (AGCTGGATCC TTAACGCTTAAAGCTTT TCCCATAAAATATAT G)	-	Expression of RDase		[260]
<i>tceA</i>	<i>Dehalococcoides mccarthy</i> 195	797F (ACGCCAAAAGTGC GAAAAGC)	2490R (TAATCTATTCCAT CCTTTCTC)	1,693	Entire <i>tceA</i> sequencing		[98]
<i>cprAB</i>	<i>Desulfotobacterium dehalogenans</i>	PS1 (CAITTOCTTGGATC CTTTTGACTACGG)	PS2 (CCGGAATTTCGATTA TC'TGCCTTAGG)	1,884	<i>cprAB</i> identification		[273]
<i>pceAB</i>	<i>Sulfurospirillum multivorans</i>	PS3 (TGGAAAAAGAAAA AAAAGCCTGAACTC)	PS4 (CTTCATCAGCGCTTT GAATTCACCTCC)	1,874	<i>pceAB</i> identification		[273]
<i>pceA</i>	<i>Desulfotobacterium</i> sp. Y51	PCEAF (GGCGGGGATC CAATGGGAGAAATCA AC)	PCEAR (GGCGGGTCGACT TGTTTTATAGACTCAG)	1,700	Expression of RDase		[79]
<i>pceAB</i>	<i>Dehalobacter restrictus</i> PER-K23 and <i>Desulfotobacterium</i> spp. strains TCE1 and PCE-1	PCE1f (ATGCAATTAATTA TTAAGGAGGAAAG)	PCE2r (AGCAGAAATAGTA TCCGAACT)	-	Entire <i>pceAB</i> sequencing	1656 bp for <i>pceA</i> and 318 bp for <i>pceB</i>	[64]
<i>pceA</i>	<i>Dehalobacter restrictus</i> PER-K23 and <i>Desulfotobacterium</i> spp. strains TCE1 and PCE-1	SpDr11f (TTGGATGAGGC CTTGAAACGC)	SpDr9r (GCGCTGCATAATA GCCAAGC)	618	Known CE- RDases ^a detection		[271]
<i>pceA</i>	<i>Desulfotobacterium</i> spp. strains TCE1 and PCE-1	SpDr1f (CGTTGGACCTAT TCCACCTG)	SpDr1r (CAAGAACGAAAG CAATCACA)	199	Known CE- RDases ^a detection		[271]

(continued)

<i>peaA</i>	<i>Sulfurospirillum multivorans</i>	SpSm1f (TCGTTGCAGGTA TCGCTATG)	SpSm1r (TTCAACAGCAAA GGCAACTG)	194	Known CE- RDases ^a detection	[271]
<i>tceA</i>	<i>Dehalococcoides ethenogenes</i> 195	SpDe1f (GCTTTGGCGGTG ATGATAAG)	SpDe1r (GTTATAGCCAAG GCCTGCAA)	194	Known CE- RDases ^a detection	[271]
<i>rdhA</i>	<i>Sulfurospirillum multivorans</i>	SpSmAL3f (CCCCCTGAGTA TGGACCAATG)	SpSmAL3r (GTAGCGGCCA TTTGCAATTAT)	199	Known CE- RDases ^a detection	[271]
<i>rdhA</i>	<i>Desulfotobacterium</i> sp. strains PCE1 (Forward) and <i>Dehalobacter restrictus</i> PER-K23 (Reverse)	SpDPAS9f (TGA AAAAGCG CCTTATGCTA)	SpDPAS9r (CAATTCCGAA ACCATTCAGG)	199	Known CE- RDases ^a detection	[271]
<i>vcrAB</i>	<i>Dehalococcoides mccartyi</i> VS	unnamed (CTATGAAGGCC TCCAGATG)	unnamed (GTAAACAGCCCC AATATGCCAAGTA) ^b	-	<i>vcrAB</i> identification	[101]
<i>bvcA</i>	<i>Dehalococcoides mccartyi</i> BAV1	bvcA F (TGCCTCAAGTACA GGTGGT)	bvcA R (ATTGTGGAGGAC CTACCT)	839	Putative <i>bvcA</i> gene identification	[100]
<i>cprAB</i>	<i>Desulfotobacterium hafniense</i> strain PCP-1	HafRdA1-G (TGCGGCACCTT TTCTTGATCGC)	HafRdA1 (GTCTTAAGCAAA TGACGCAGC)	2,300	<i>cprAB</i> sequencing	[77]
<i>rdhA1</i>	<i>Dehalococcoides</i> - containing mixed culture KB1.	rdhA1_246f (ATCGGAGCTG CACAAAGTAGG)	rdhA1_336r (TCTTGTGAGCG GTGTCCTTG)	-	Another 13 primer pairs targeting <i>rdhA2-rdhA14</i>	[278]
<i>dcaA</i>	<i>Desulfotobacterium dichloroelminans</i> DCA1	Dea1F (CAGGCAAGAAAGA TACGG)	DHL-REV (CAGGCTCATTA GCTATTCA)	282	Putative <i>rdhA</i> identification	[83]
<i>rdhA</i>	<i>Sulfurospirillum</i> spp.	Sul-rdhA-f (TTGTGTRGGTGT GCAAGATT)	Sul-rdhA-r (CTGTCTCTAAAC CTGCTTC)	-	Southern hybridization	[286]

(continued)

<i>rdhA</i>	ACT-3 enrichment culture (containing <i>Dehalobacter</i> sp.)	<i>rdhA</i> _23f (AAGAGATTGTAG AAGCAGCGG)	<i>rdhA</i> _1383r (CTTAGTAAATG GGCAAGCAGC)	1,371	Sanger sequencing	[269]
<i>cfrA</i>	ACT-3 enrichment culture (containing <i>Dehalobacter</i> sp.)	<i>cfrA</i> -413f (CCCGAACCTCT AGCACTTGTAG)	<i>cfrA</i> -531r (ACGGCAAAAGCT TGCACGA)	-	Putative <i>rdhA</i> identification	[269]
<i>derA</i>	ACT-3 enrichment culture (containing <i>Dehalobacter</i> sp.)	<i>derA</i> -424f (AGCAC TCAGAG AGCGTTTGC)	<i>derA</i> -533r (CAACGGCCACG CTTGCAT)	-	Putative <i>rdhA</i> identification	[269]
<i>dcpA</i>	<i>Dehalococcoides mccartyi</i> RC and KS, <i>Dehalogenimonas lykanthroporepellens</i> BL-DC-9	<i>dcpA</i> -360F (TTGCGTGATCA AATTGGAGCCTGG)	<i>dcpA</i> -1449R (TTTAAACAGC GGCAGGTACTGGT)	1,089	Putative <i>rdhA</i> identification	[104]

^aCE-RDases=chloroethene reductive dehalogenases.

Table 3.4 Specific primer sets designed for quantitative PCR (qPCR) and RT-PCR (reverse transcriptase PCR).

Target gene	Target OHRB	Forward primer (5' - 3')	Reverse primer (5' - 3')	Product size (bp)	Application potential	Probe	Reference
<i>cprA</i>	<i>Desulfotobacterium dehalogenans</i>	BG475 (GGCAGGTCTGGGAGA ATTG)	BG476 (GTCTCTGTACC ATAGCCAAAG)	-	RT-PCR	-	[97]
<i>cprA</i>	<i>Desulfotobacterium dehalogenans</i> and <i>Desulfotobacterium</i> sp. PCE-1	D5(CTBGCNCCBGAYAARCC)	D9 (TCCAVGGRCANAC)	-	RT-PCR	-	[273]
<i>rddA</i>	<i>Desulfotobacterium</i> sp. PCE-1	D4 (GGIGCIGAYYTIGTIGG)	D7 (ATIGGYTTRTCIGG)	-	RT-PCR	-	[273]
	<i>Dehalococcoides mccartyi</i> VS						
	<i>Dehalococcoides mccartyi</i> 195, FL2 [101]; mixed culture YK-TCE1, PM-VC1, RC-VC2 [192]	verAf (TGCTGGTGGCGTTG GTGCTCT)	verAr (TGCCCGTCAAAA GTGCTAAAG)	441	RT-PCR	-	[101]
<i>tceA</i>	TCE enriched ANAS culture containing <i>Dehalococcoides</i>	TeeA1270F (ATCCAGATTA TGACCCTGGTGAA)	TeeA1336R(GCGGCATA TATTAGGGCATCTT)	66	qPCR and RT-PCR	TGGCTATG GCGACCGC AGG	[289]
<i>vcrA</i>	TCE enriched ANAS culture containing <i>Dehalococcoides</i>	unnamed (CTCGGCTACCG AACGGATT)	unnamed (GGGCAGGAG GATTGACACAT)	-	qPCR	CGCACTGGTTATG GCAACCACTC	[290]
<i>bvcA</i>	TCE enriched ANAS culture containing <i>Dehalococcoides</i>	unnamed (GGTGCCGCGAC TTCAGTT)	unnamed (TCGGCACTAG CAGCAGAAATT)	-	qPCR	TGCCGAATTT CACGACTTG GATGAAG	[290]
<i>bvcA</i>	<i>Dehalococcoides mccartyi</i> BAV1	Bvc925F (AAAAGCACTTGG CTATCAAGGAC)	Bvc1017R (CCAAAAAGCAC CACCAGGTC)	92	qPCR	TGGTGGCGA CGTGGC TATGTGG	[195]
<i>vcrA</i>	<i>Dehalococcoides mccartyi</i> GT and VS	Vcr1022F (CGGGCGGATGC ACTATTT)	Vcr1093R (GAATAGTCCG TGCCCTTCTC)	71	qPCR	CGCAGTAACTC AACCAATTCC TGGTAGTGG	[195]

(continued)

<i>dca4</i>	1,2-DCA enriched culture 6VS	DHL F1 (GGACCTCGTTGGA CTCC)	DHL R1 (GGCAAATCCCAT GGCATTAA)	383	RT-PCR	-	[83]
<i>dca4</i>	1,2-DCA enriched culture 6VS	DHL F2 (GTTAAAAAGGACGCTGTT)	DHL R1 (GGCAAATCCCAT GGCATTAA)	130	RT-PCR	-	[83]
<i>dcaB</i>	1,2-DCA enriched culture 6VS	DHL F1 (GGACCTCGTTGGA CTCC)	DcaB rev (TGGTATTACAG CTCCGA)	1462	RT-PCR	-	[83]
<i>dca4</i>	<i>Desulfitobacterium dichloroeliminans</i> strain DCA1	DH3F (ATTGGGAGAAGCAT GCAGGT)	DH3R (GACCACCGTTATAG GCCCAGA)	526	qPCR	-	[83]
<i>rdhA1</i>	WL cultures (containing <i>Dehalobacter</i> sp.)	WLrdhA1f (GCAGGAAGATT CTAAAAACCTTG)	WLrdhA1r (CACCGAGGTAC TGGAAATGA)	484	qPCR and RT-PCR	-	[276]
<i>rdhA2</i>	WL cultures (containing <i>Dehalobacter</i> sp.)	WLrdhA2f (AGTTTTTGGAG AGGATGTTGAA)	WLrdhA2r (GGCCAAAAGGAAT ACTCAATTGA)	236	qPCR	-	[276]
<i>rdhA3</i>	WL cultures (containing <i>Dehalobacter</i> sp.)	WLrdhA3f (ATTAACCTGCGT AATGGGTGG)	WLrdhA3r (CAAGGTTTTTAGA ATCCTCCTGC)	352	qPCR	-	[276]
<i>rdhAB1</i>	WL cultures (containing <i>Dehalobacter</i> sp.)	WLrdhA1f (GCAGGAAGAT TCTAAAAACCTTG)	WLrdhB1r (CTTTCAGATACC GCCAGATTCT)	-	RT-PCR	-	[276]
<i>cprA</i>	<i>Dehalobacter</i> sp. TCP-1	debeprAF (CATCAGCTGTG CCAATGGAA)	debeprAR (CGGATACAGCTC GCGTCITT)	60	qPCR	-	[58]
<i>rdh1</i>	<i>Shewanella sediminis</i>	rdh1_F (GCAAGCCATCTTA CCCATGT)	rdh1_R (CCGTGTCGGTATCG CTAAAT)	200	qPCR	-	[277]
<i>rdh2</i>	<i>Shewanella sediminis</i>	rdh2_F (GTTCACGCTGGA TCGTTAT)	rdh2_R (CCCGGTTACCAGCA GATAGA)	200	qPCR	-	[277]
<i>rdh3</i>	<i>Shewanella sediminis</i>	rdh3_F (GTCCACGCTTTA TCACGTT)	rdh3_R (TTGCTGGCATGGAA TGAATA)	200	qPCR	-	[277]

(continued)

<i>rdh4</i>	<i>Shewanella sediminis</i>	rdh4_F (CGCGGTTGGTTAT TCAACTT)	rdh4_R (CACTTCTGGCGTCA TCAAGA)	200	qPCR	-	[277]
<i>rdh5</i>	<i>Shewanella sediminis</i>	rdh5_F (GGGTCCGATTAAAG CCGGGCG)	rdh5_R (CATCGCGCGCCATCC GGTTCA)	200	qPCR	-	[277]
<i>dcpA</i>	<i>Dehalococcoides mccartyi</i> RC and KS, <i>Dehalogenimonas</i> <i>lykantiroporepellens</i> BL- DC-9	1257F (CGATGTGCCAGCCA TTGTGCTTT)	1449R (TTTAAACAGCGGGCA GGTACTGGT)	192	qPCR and RT-PCR	ACGTCATCTC AGATGAAGGC AGAGCT	[104]

Abbreviations: R = A or G; Y = C or T; B = C, G, or T; V = A, C, or G.

numbers to cell numbers, albeit with the restriction that actively growing cultures might contain multiple genomes per cell.

Primers have additionally been designed for reverse transcription, which, in conjunction with qPCR, was applied to quantify specific *rdh* transcripts from microbial samples. From this, genes encoding the A and B subunits of reductive dehalogenases were found to be co-transcribed [83, 276, 291], confirming previous Northern blot hybridization and gene-spanning RT-PCR examinations of *Desulfotobacterium dehalogenans* transcripts [97]. Nevertheless, the accuracy of RT-PCR is limited by inefficiencies in reverse transcription and loss during sample processing. To account for this, Johnson [289] introduced an exogenous internal reference mRNA for normalization of RT-PCR, thereby improving the accuracy of quantification and allowing quantification of mRNA loss during specific steps, including RNA extraction, RT-PCR and qPCR.

Conclusions

Degenerate primers have been valuable tools for discovery of new *rdhA* sequences. Their application is not costly and does not need complicated bioinformatics data analysis. Specific primers, on the other hand, have been successfully applied for quantitative analysis of OHRB and their biomarkers and functionality proofs of the identified genes. The hunt for new *rdhAs* was moved to a high-throughput approach by combined application of primer-dependent analysis and next-generation amplicon sequencing [285]. Additionally, primer-independent metagenomic surveys are expected to further broaden the diversity of currently known *rdh* genes, as has already been demonstrated in marine subsurface sediments [216]. Combined application of amplicon-based or metagenomic surveys with high-throughput quantitative analysis methods [288] should be instrumental in obtaining a comprehensive understanding of OHRB and their catabolic reductive dehalogenation gene pools.

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**Functional genomics of corrinoid
starvation in the organohalide-
respiring bacterium *Dehalobacter
restrictus* strain PER- K23**

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Abstract

De novo corrinoid biosynthesis represents one of the most complicated metabolic pathways in nature. Organohalide-respiring bacteria (OHRB) have developed different strategies to deal with their need of corrinoid, as it is an essential cofactor of reductive dehalogenases, the key enzymes in OHR metabolism. In contrast to *Dehalococcoides mccartyi*, the genome of *Dehalobacter restrictus* strain PER-K23 contains a complete set of corrinoid biosynthetic genes, of which *cbiH* appears to be truncated and therefore non-functional, explaining the corrinoid auxotrophy of this obligate OHRB. Comparative genomics within *Dehalobacter* spp. revealed that one (operon-2) of the five distinct corrinoid biosynthesis associated operons present in the genome of *D. restrictus* appeared to be present only in that particular strain, which encodes multiple members of corrinoid transporters and salvaging enzymes. Operon-2 was highly up-regulated upon corrinoid starvation both at the transcriptional (346-fold) and proteomic level (46-fold on average), in line with the presence of an upstream cobalamin riboswitch. Together, these data highlight the importance of this operon in corrinoid homeostasis in *D. restrictus* and the augmented salvaging strategy this bacterium adopted to cope with the need for this essential cofactor.

Introduction

Corrinoids are essential cofactors for a wide variety of enzymes that facilitate reactions including rearrangements, methyl group transfers, and reductive dehalogenation [292]. A recent bioinformatic study has revealed that while 76% of 540 sequenced bacterial genomes contain corrinoid-dependent enzymes, only 39% of these genomes encode the complete corrinoid biosynthesis pathway, suggesting that the salvage of corrinoids from the environment is an important process for many bacteria [293]. Both aerobic and anaerobic corrinoid biosynthesis pathways have been described showing few but significant differences, notably in tetrapyrrole ring contraction and the step at which cobalt is inserted into the ring [294, 295]. This pathway is complex and consists of approximately 30 reactions (see [294] for a recent review).

Organohalide respiration (OHR) is an anaerobic bacterial respiration process of environmental interest, as many anthropogenic halogenated organic compounds can be used as terminal electron acceptors by organohalide-respiring bacteria (OHRB) [7]. OHRB are capable to remove the halogens and therefore contribute to bioremediation of environments polluted with these compounds [10]. The key enzyme in OHR is the reductive dehalogenase (RDase) [46], which strictly depends on corrinoid cofactors for the dehalogenation reaction. Although the reaction mechanism has not yet been fully understood, RDases represent a particular family of corrinoid enzymes as they catalyze electron transfer rather than methyl transfer. Moreover the absence of corrinoid binding motif in RDase sequences reflects the base-off/his-off conformation of the corrinoid in the enzyme [78, 296]. In recent years, corrinoid biosynthesis and salvaging in OHRB regained substantial interest in the scientific community as exemplified by the following studies: an unusual corrinoid cofactor (norpseudo-B₁₂) has been identified in the tetrachloroethene (PCE) RDase of *Sulfurospirillum multivorans* [297]; the lack of exogenous corrinoid had an effect on the RDase activity of *Desulfitobacterium hafniense* when cultivated with an alternative electron acceptor [298]; many essential corrinoid biosynthetic genes have been found on a plasmid in *Geobacter lovleyi* [128]; the involvement of the bacterial community accompanying members of *Dehalococcoides mccartyi* for corrinoid supply has been highlighted [93, 95, 237, 299]; modifying the lower ligand of the corrinoid had a severe effect on the activity of the PCE RDase of *S. multivorans* [145].

Contrasting situations have been observed regarding the ability of OHRB to produce corrinoid cofactors *de novo*. Both genome analysis and physiological studies have shown that the obligate OHR *D. mccartyi* is strictly dependent on exogenous corrinoid supply and that 5,6-dimethylbenzimidazole can serve as nucleotide loop in corrinoid cofactors [40, 94, 95, 299, 300]. On the contrary, the facultative OHRB *S. multivorans* strain K and *D. hafniense* strains encode the full corrinoid biosynthetic pathway in their genome and have been shown to grow without any supply of corrinoid in the medium [20, 74, 301, 302].

Dehalobacter restrictus strain PER-K23 is an obligate OHRB only able to grow by dechlorinating tetra- and trichloroethene (PCE and TCE, respectively). It was first isolated from Rhine river sediment and since then always cultivated in the presence of exogenous vitamin B₁₂ (cyanocobalamin) [52]. The PCE RDase (PceA) of *D. restrictus* has been extensively studied and revealed a 60-kDa enzyme containing a corrinoid cofactor and two 4Fe-4S clusters with estimated redox potential of -350 mV (Co^{1+/2+}) and -480 mV (4Fe-4S^{2+/1+}), respectively, and a specific dechlorination activity of 250 nkat/mg. Spectrophotometric analysis of the corrinoid extracted from *D. restrictus* PceA with cyanide has shown a spectrum resembling the one of cyanocobalamin [296], although this method does not allow identifying corrinoid unambiguously. Analysis of the newly published genome of *D. restrictus* revealed the presence of a complete set of corrinoid biosynthetic genes where one gene, *cbiH*, is truncated due to a 101-bp deletion, likely responsible for the corrinoid auxotrophy of *D. restrictus* [67, 261] (Figure 4.1).

This present study aims to explore in detail the effect of corrinoid starvation on *D. restrictus* with a combination of comparative genomics, as well as transcription and proteome analysis.

Materials and methods

Bacteria, plasmids, and growth conditions

Dehalobacter restrictus strain PER-K23 (DSM 9455) was cultivated as described earlier [52, 64, 67]. Anaerobic serum flasks of 500 mL were supplemented with hydrogen as electron donor, inoculated with 2 % (v/v) inoculum, and finally 1 % (v/v) of 2 M PCE dissolved in hexadecane was added as electron acceptor. Batch cultures of *D. restrictus* were cultivated in 300 mL medium at 30°C under gentle agitation (100 rpm), and chloride release was used as an indicator of growth. Chloride concentration was measured with a Chlor-o-counter (Flohr Instrument, Nieuwegein, The Netherlands) as described earlier [249]. Cultures for proteomic

analysis were prepared in triplicate with high, mid and low concentration of cyanocobalamin corresponding to 250, 50 and 10 µg/L, respectively.

Escherichia coli DH5α was cultivated on liquid or solid LB medium containing 100 µg/L ampicillin after transformation with derivatives of the pGEM-T Easy vector (Promega, Duebendorf, Switzerland).

Sequence retrieval and genome analysis

All sequences mentioned in this study were taken from the recently published genome of *D. restrictus* strain PER-K23 [261] and from other *Dehalobacter* spp. genomes including *Dehalobacter* sp. E1 [66], *Dehalobacter* sp. DCA and sp. CF [303], *Dehalobacter* sp. FTH1 (RefSeq PRJNA199134, JGI genome project), *Dehalobacter* sp. UNSWDHB [304]. The original annotation of *D. restrictus* gene loci obtained in collaboration with the Joint Genome Institute (JGI project #402027) was used here as the present study is the follow-up study of two previous reports where the JGI annotation was used [67, 261]. Another version of *D. restrictus* genome was recently annotated by the automatic pipeline of the NCBI database and is available under accession number CP007033. Corresponding loci from both databases are given for the selected corrinoid proteome in Table S4.1.

The annotation of selected genes was verified using a manual search with BLAST [305]. Protein sequences were aligned using ClustalX v.2.0 [306]. Sequence maximum likelihood tree analysis was done with MEGA5 [307]. Cobalamin riboswitches (Cbl-RS) were identified using Rfam [308] and initially aligned using ClustalX and then corrected manually as described earlier [301]. Comparative genome analysis was performed using the Artemis Comparison Tool [309].

Transcription analysis

RNA was extracted using the TRIzol method according to [310] with the following modification. The DNaseI treatment was stopped by adding the DNase stop solution and incubating for 10 min at 65°C. RNA concentration was estimated using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Ecublens, Switzerland). Reverse transcription was performed as described before [67].

Primers targeting each gene present immediately downstream of the five Cbl-RS of *D. restrictus* were designed. PCRs and cloning using the pGEM-T Easy vector, cloning, clone selection, sequencing and quantitative PCR were performed as described earlier [67]. Primer

sequences, amplicon sizes, plasmids and quantitative PCR specifications are given in Table S4.2.

Protein extraction and SDS-PAGE

Cells were harvested by 10 min centrifugation at $12000 \times g$, washed twice with 25 mL 20 mM Tris-HCl (pH 7.5), and then flash-frozen in liquid nitrogen. All biomass samples were stored at -80°C until use. Cell pellets were resuspended in 0.5 mL lysis buffer (100 mM Tris/HCl, pH 7.5, 4% sodium dodecyl sulfate, and 0.1 M dithiothreitol) and then transferred to 2-mL protein LoBind tubes (Eppendorf, Hamburg, Germany). Protein extraction was done as described earlier [67]. Protein concentration was determined with the Qubit® protein assay kit (Invitrogen, Eugene, OR, USA) following the manufacturer's instructions. Protein samples were stored at -20°C until use. SDS-PAGE was done following standard procedures [311]. In brief, 15 μg of proteins from each sample were loaded in separate lanes in gels containing 10% SDS. Gels were stained with Coomassie brilliant blue R250 (Merck, Darmstadt, Germany) and scanned using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA). The Quantity One basic software package was used to quantify the intensity of lanes. Series of gels were prepared and analyzed until less than 5% differences in the intensity between any lanes were achieved.

Gel digestion and peptides purification

In-gel digestion of proteins and purification of peptides were done following a modified version of the protocol described earlier [67]. Disulphide bridges in proteins were reduced by covering whole gels with reducing solution (10 mM dithiothreitol, pH 7.6, in 50 mM NH_4HCO_3), and the gels were incubated at 60°C for 1 h. Alkylation was performed for 1 h by adding 25 mL of iodoacetamide solution (10 mM iodoacetamide in 100 mM Tris-HCl, pH 8.0). Gels were thoroughly rinsed with dd H_2O water in between steps. Each lane of SDS-PAGE gels was cut into three equally sized slices, and each slice was cut into approximately 1 mm^3 cubes and transferred to separate 0.5 mL protein LoBind tubes (Eppendorf, Hamburg, Germany). Enzymatic digestion was done by adding 50 μL of trypsin solution (5 $\text{ng}/\mu\text{L}$ trypsin in 50 mM NH_4HCO_3) to each tube, and incubating at room temperature overnight with gentle shaking. Extraction of peptides was performed with manual sonication in an ultrasonic water bath for 1 s before the supernatant was transferred to a clean protein LoBind tube. Additional peptides were recovered by adding 25 μL of 2.5% (v/v) trifluoroacetic acid to the gel pieces, which were sonicated for 2 s before the supernatant was combined with the first supernatant obtained. Peptides were purified with a C18 Empore disk as previously described

[312]. Acetonitrile in the samples was removed by using a concentrator vacuum centrifuge. Finally, sample volume was adjusted to 50 μ L with 0.1% (v/v) formic acid.

nLC-MS/MS and data analysis

Peptides derived from extracted and digested proteins were analyzed by nLC-MS/MS (Biqualys, Wageningen, The Netherlands) as described earlier [313]. MaxQuant v.1.3.0.5 [314] with default settings for the Andromeda search engine [314] in the label free quantitation mode was used to analyze MS and MS/MS spectra, except that extra variable modifications were set as described before [67]. A protein database of *D. restrictus* was generated from the whole genome sequence [261] using the Artemis genome browser (release 15.0.0). Also, a contaminant database including sequences of common contaminants like trypsin, BSA and human keratins [67, 315] was used. Further filtering and bioinformatics analysis was performed with Perseus software v. 1.3.0.4 as described before [316]. Also, protein groups with a logarithmic label-free quantitation (LFQ) intensity of zero for all treatments were deleted from the MaxQuant result table. Subsequently, remaining Log LFQ zero values were replaced by 5 (slightly below the lowest value measured) in order to make sensible ratio calculations possible. Student's T-test was used to identify significant differences in the proteome when comparing logarithmic LFQ values obtained from two culture conditions.

Results

Corrinoids are essential as a growth factor for *D. restrictus* [52]. The corrinoid present in the PCE reductive dehalogenase (PceA) of *D. restrictus* is presumably similar to the type added to the medium, i.e. cobalamin [52]. Detailed analysis of the genome of *D. restrictus* revealed a seemingly complete corrinoid biosynthesis pathway. Compared to other *Dehalobacter* genomes, however, a 101-bp fragment was found to be missing in the *cbiH* gene of *D. restrictus* [67, 261] (Figure S4.1). The present study aimed specifically at obtaining a better understanding of the corrinoid metabolism in *D. restrictus*.

Growth of *D. restrictus* under corrinoid-limiting conditions

The full corrinoid biosynthetic pathway was described earlier [67]. A modified and extended version of it is depicted in Figure 4.1. Briefly, the pathway can be divided in two branches, namely the upper corrinoid biosynthesis (UCB) and the nucleotide loop assembly (NLA), which are connected at the level of ado-cobyric acid.

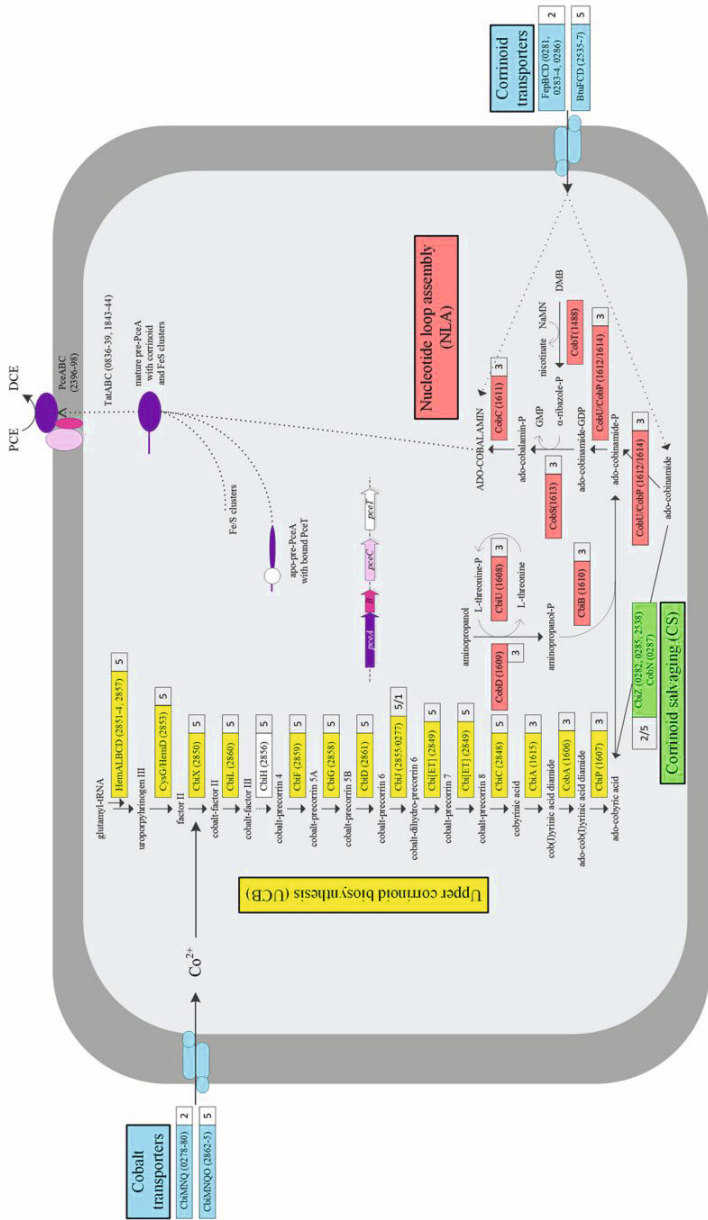


Figure 4.1 Predicted corrinoid biosynthesis pathway of *D. restrictus*. Based on the genome annotation, a map of the corrinoid biosynthesis was predicted including four pathways: the upper corrinoid biosynthesis (UCB, in yellow), the nucleotide loop assembly (NLA, in red), cobalt and corrinoid transporters (in blue) and corrinoid salvage (in green). The PCE reductive dehalogenase (PeeA and associated proteins PeeB and PeeC) is also depicted. The enzymes catalyzing each reaction are given colour boxes indicating the protein name (when available) and corresponding gene loci (with the Dehre # prefix removed). CblH in *D. restrictus* is likely to be non-functional as the corresponding gene is truncated (yellow box with white background). Note: the details of CblH catalysed reaction can be found in [294].

In the present study, batch cultures were cultivated with addition of 250 µg/L cyanocobalamin to the growth media. An experiment was performed to assess the effects of lowering the initial corrinoid concentration in the medium (250, 50, 10, 1 µg/L and no corrinoid) on dechlorination, which for this obligate OHRB is also a good estimation for growth (Figure 4.2) [52]. The extent of PCE dechlorination was the same in cultures provided with 50 or 250 µg/L corrinoid demonstrating that the former was enough to reach the maximum dechlorination capacity. In contrast, the chloride release was only half of the maximum in cultures supplemented with 10 µg/L corrinoid, implying that availability of corrinoids was a limiting factor. Further lowering the corrinoid concentration to 1 or 0 µg/L resulted in negligible levels of dechlorination, and therefore growth was assumed to be abolished in these cultures.

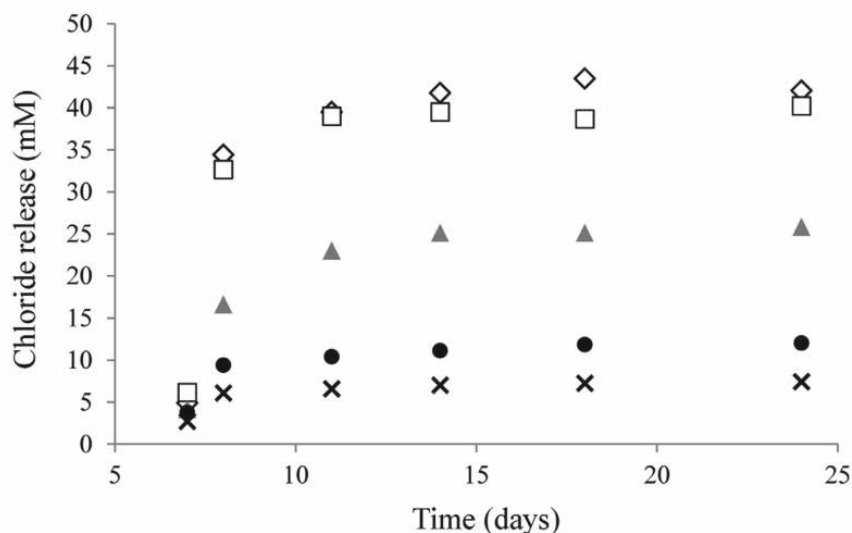


Figure 4.2 Corrinoid starvation effect on PCE dechlorination by *D. restrictus* by lowering the initial concentration of corrinoid supplemented into the medium. Corrinoid concentration: 250 µg/L (white diamonds), 50 µg/L (white squares), 10 µg/L (grey triangles), 5 µg/L (black circles) and 1 µg/L (black crosses).

Corrinoid metabolic gene arrangement in *D. restrictus*

The complete corrinoid biosynthetic and uptake pathway is genetically encoded in *D. restrictus*. These genes can be divided into four functional groups depending on the part of the pathway they encode for (Figure 4.1). The first group denoted as the upper corrinoid

biosynthesis (UCB) pathway genes contains genes required to synthesize ado-cobyric acid. The second group consists of genes required for synthesis and the nucleotide loop assembly (NLA) of corrinoids, and the third functional group comprises the corrinoid salvaging (CS) pathway, i.e. genes involved in remodeling corrinoid intermediates salvaged from the environment into ado-cobyric acid (*cbiZ* gene family). The fourth group harbors both cobalt and corrinoid transporter encoding genes (CT).

Most genes associated with corrinoid metabolism are arranged in the genome of *D. restrictus* in five gene clusters (referred to as operon-1 to -5), which are roughly organized according to the function they play in corrinoid biosynthesis (Figure 4.3). Most proteins involved in the UCB pathway are encoded in operon-5 with the exception of the three last steps that are catalyzed by the product of genes present in operon-3. This latter operon also codes for the enzymes involved in the NLA pathway. Within operon-3, the locus Dehre_1608 was initially annotated as a phosphoglycerate mutase, but shows also sequence similarity with archaeal-type homoserine kinase (with conserved domain TIGR02535) involved in the synthesis of threonine. Here, we propose it could act as an L-threonine kinase (in analogy to PduX in *Salmonella* [317]), which might therefore be involved in the production of aminopropanol-phosphate. No *pduX* homolog could be identified in *D. restrictus*, suggesting that this function is fulfilled by the gene product of Dehre_1608. Hence, we propose to name it *cbiU*. Operon-1 contains a homolog of *cbiJ* (besides the *cbiJ/cysG* gene, Dehre_2855, present in the conserved biosynthesis operon-5), and a set of genes coding for the energy-coupling factor-type CbiMNQ cobalt transporter. An additional, albeit different *cbiMNQO* gene cluster is also present at the 5'-end of operon-5 together with the genes for the UCB pathway. Operon-2 harbors a combination of genes coding for transporters (with sequence similarity to FepBCD/BtuCDE ABC-type transporters) likely involved in corrinoid transport, the genes for two different salvaging enzyme (CbiZ) paralogues (Dehre_0282 and _0285), a gene cluster encoding the cobaltochelate CobN (Dehre_0287), and several subunits of a magnesium chelate complex. All proteins encoded in operon-2 share between 50 and 77% sequence identity with homologous proteins present in the non-dechlorinating *Firmicute* *Acetobacterium woodii*. Finally, operon-4 contains a gene cluster coding for an ABC-type corrinoid transporter (BtuFCD) and another copy of *cbiZ* (Dehre_2538). Two additional genes potentially involved in corrinoid biosynthesis (*cobT*/Dehre_1488 and *cobB/cobQ*, Dehre_2360) are located elsewhere in the genome and not in one of the five operons.

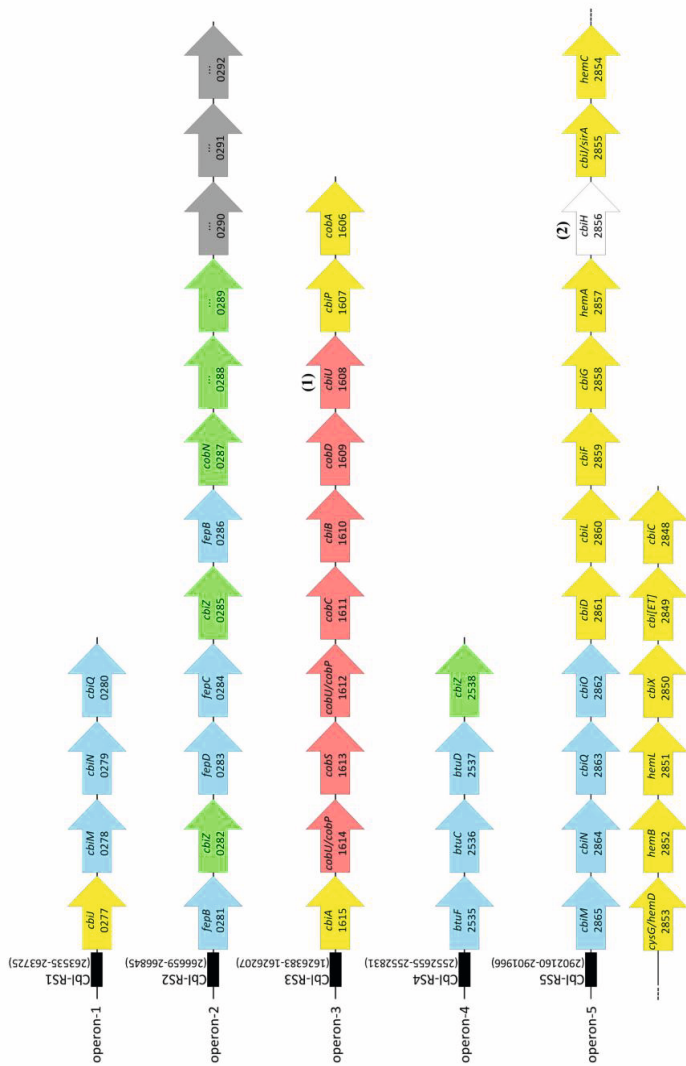


Figure 4.3 Arrangement of genes associated with corrinoid biosynthesis and uptake in the genome of *D. restrictus*. Genes involved in corrinoid metabolism are grouped in five operons (operon-1 to -5) located in different places of the genome. All five operons are predicted to be regulated by cobalamin riboswitches (Cbl-RS1 to -5, indicated by a black box) located directly upstream of the first gene of each operon. Cbl-RS coordinates are given based on their identification by Rfam. Genes are depicted as arrows in colours corresponding to four distinct pathways (see also Figure 4.1): upper corrinoid biosynthesis (UCB, in yellow), nucleotide loop assembly (NLA, in red), cobalt and corrinoid transporters (in blue) and corrinoid salvage (in green). Grey arrows depict genes that have no clear function in corrinoid biosynthesis. Gene names and corresponding gene loci (with the *Dehre_#* prefix removed) are given when available. Notes: (1) *cbiU* was newly annotated as a possible L-threonine kinase encoding gene. (2) a 101-bp deletion makes *cbiH* non-functional in *D. restrictus*.

Comparative genomics of corrinoid operons in *Dehalobacter* spp.

The genome of *D. restrictus* was compared with newly available genomes of *Dehalobacter* spp. strains DCA, CF, E1, FTH1, and UNSWDHB with regard to the organization of corrinoid operons (Table 4.1 and Table S4.1). Synteny maps for operon-1 and -2 (Figure S4.2), and for operon-3, -4 and -5 (Figure S4.3 to S4.5, respectively) are given as Supplementary materials. Operon-1 is conserved in *D. restrictus* and *Dehalobacter* sp. E1 but absent in all other genomes. Operon-2, which is directly following operon-1 in *D. restrictus*, is lacking in all other *Dehalobacter* spp. for which genome sequences are available to date. However, a detailed analysis of *Dehalobacter* sp. E1 suggests that operon-2 was lost in that strain as the sequence conservation with *D. restrictus* is extended slightly beyond operon-1 but is readily interrupted within the homolog of Dehre_0281 (the first gene of operon-2 in *D. restrictus*). This deletion in strain E1 includes all of the remaining genes operon-2 and beyond, as a 5'-truncated version of Dehre_0297 is again found in strain E1 (Figure S4.2, panel C). *D. restrictus* operon-3 to -5 are fully conserved in all *Dehalobacter* spp. with the exception of another deletion in the proximal region of operon-5 in strain E1 (Figure S4.5).

Table 4.1 Comparative genomics of corrinoid operons in *Dehalobacter* spp.

Operon	<i>Dehalobacter restrictus</i>	<i>Dehalobacter</i> sp. E1	<i>Dehalobacter</i> sp. DCA	<i>Dehalobacter</i> sp. CF	<i>Dehalobacter</i> sp. FTH1	<i>Dehalobacter</i> sp. UNSWDH B
1	+	+	-	-	-	-
2	+	-	-	-	-	-
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	partial	+	+	+	+
<i>cbiH</i>	deletion	intact	intact	intact	intact	intact

In *Desulfitobacterium hafniense* in contrast, the corrinoid biosynthesis genes are organized in two operons. The major operon (corresponding to DSY4057-4072 in *D. hafniense* strain Y51) encodes proteins of the UCB pathway and part of the NLA pathway, while a 3-gene operon (DSY2114-2116) encodes for the remaining NLA proteins [74, 301]. Some proteins encoded by *D. restrictus* operon-2 have their counterpart in other OHRB. For example, corrinoid transporters are present in most OHRB, however, corrinoid producers such as *Desulfitobacterium hafniense* and *Sulfurospirillum multivorans* do not harbor any *cbiZ* homologous gene. In *Dehalococcoides mccartyi* in contrast, multiple *cbiZ* genes are present in the genomes (Figure S4.6).

All proteins encoded in operon-2 of *D. restrictus* share between 50 and 77% sequence identity with homologous proteins present in the non-dechlorinating *Firmicute* *Acetobacterium woodii*. A high level of genetic synteny was further identified between operon-2 of *D. restrictus* and a part of the genome of *A. woodii* (GenBank NC_016894.1, [318]) (Figure S4.7). Significant sequence similarity of individual proteins of operon-2 was mostly found with homologs of some other members of *Chlostridia* and a few δ -*Proteobacteria* (data not shown).

Identification of cobalamin riboswitches in *D. restrictus*

Upstream of each of the five corrinoid biosynthesis-related operons in *D. restrictus* a distinct cobalamin riboswitch (Cbl-RS) was identified using Rfam. These five sequences were manually refined in a similar way as done previously for the Cbl-RS sequences of *Desulfitobacterium hafniense* [301]. The alignment of structurally conserved regions of *D. restrictus* riboswitches (Cbl-RS01 to -RS05) was compared to *E. coli* *btuB* Cbl-RS (Figure S4.7). In contrast to *E. coli* Cbl-RS, which is regulated at the level of translation [319], all five *D. restrictus* Cbl-RS sequences end with a predicted transcriptional terminator, suggesting that the regulation operates at the level of transcription.

Transcriptional analysis of corrinoid biosynthesis operons in *D. restrictus*

The transcription of genes located directly downstream of the Cbl-RS in *D. restrictus* was analyzed for cells cultivated in the presence of high (250 μ g/L) and low (10 μ g/L) corrinoid concentration, and after corrinoid replenishment from low to high concentrations (Figure 4.4). Quantitative PCR was applied on complementary DNA targeting the first gene located directly downstream of each cobalamin riboswitch. Analysis of corrinoid-starved *D. restrictus* RNA revealed a higher transcription level of these genes, confirming an active regulation of the respective riboswitches at transcriptional level. Two hours after corrinoid replenishment, transcription of all selected genes was again repressed to the same level as observed under high corrinoid concentration. However, individual responses were significantly different. Indeed, the most pronounced effect was observed for two genes, namely *Dehre_0277* (73-fold repression) and *_0281* (346-fold), corresponding to the first genes in operon-1 and -2 in *D. restrictus*, respectively.

Proteome analysis of corrinoid starvation in *D. restrictus*

Comparative whole-proteome analysis was done on *D. restrictus* PER-K23 cells cultivated in the presence of 250 (high), 50 (mid) or 10 (low) μ g/L cyanocobalamin. A total of 1195 proteins were detected, corresponding to 42% of the predicted 2826 proteins encoded on the

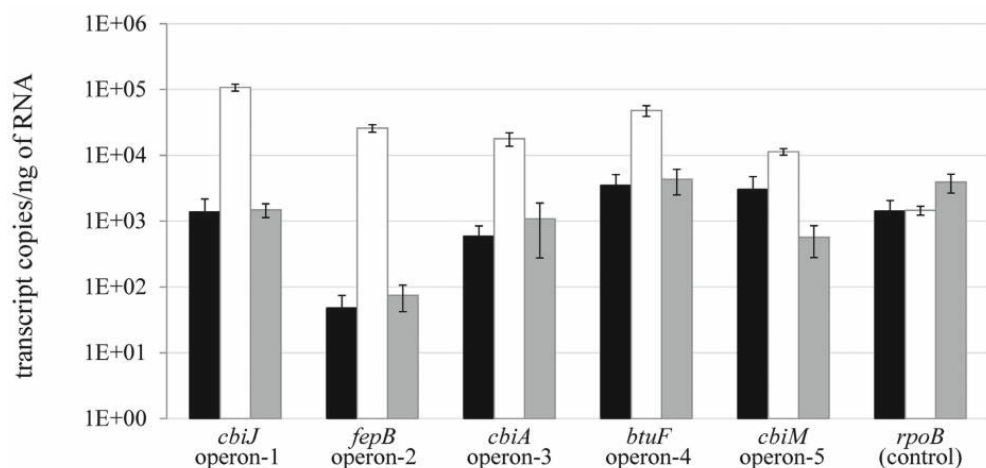


Figure 4.4 Transcriptional analysis of cobalamin riboswitch-dependent genes in *D. restrictus*. The transcription of the genes located directly downstream of the cobalamin riboswitches in operon-1 to -5 was analyzed. The housekeeping gene *rpoB* was used as unregulated control. The black bars indicate the transcriptional level under corrinoid standard conditions (250 µg/L), the white bars under corrinoid starvation conditions (10 µg/L), and the grey bars show the transcriptional level 2 h after replenishment of the latter cultures with 250 µg/L cyanocobalamin. The data show the mean of triplicate cultures with standard deviation.

genome [261]. The majority of the detected proteins (1175) were identified in cells from all the tested cyanocobalamin concentrations (Table S4.4). Normalized LFQ protein intensities were used to compare relative abundances of proteins between different cyanocobalamin treatments. A minimal change of 3-fold in LFQ protein intensity was considered throughout the study. The abundance of 44 proteins showed significant difference ($P < 0.01$) between high (250 µg/L) and low (10 µg/L) corrinoid concentration, and the relative abundance of another 29 proteins showed more than 10-fold changes, albeit not significant due to high variation between triplicates (Figure S4.8). The results for protein abundance ratios between high and mid, and between mid and low are in the same range (see Table S4.3).

Proteins associated with cobalamin biosynthesis were further analyzed. A complete *de novo* corrinoid biosynthesis pathway was predicted in the genome of *D. restrictus* starting from glutamyl-tRNA to cobalamin (Figure 4.1) [67, 261]. All proteins required for biosynthesis of ado-cobyric acid from cobalt-precorrin 5B were identified in proteomic data including CbiD (cobalamin biosynthesis protein, Dehre_2861), an alternative CbiJ (precorrin-6x reductase, Dehre_0277) and Cbi[ET] (precorrin-6Y methyltransferase, Dehre_2849), which were not detected in a previously analyzed proteome from *D. restrictus* [67]. However, CbiH

(precorrin-3B C17-methyltransferase, Dehre_2856), CbiG (cobalamin biosynthesis protein, Dehre_2858) and CbiJ/CysG (precorrin-6x reductase, Dehre_2855) belonging to the UCB pathway and CobS (cobalamin 5'-phosphate synthase, Dehre_1613) of the NLA pathway were not found in the current proteome analysis. The lack of CbiH in the proteome is in line with the observation of a 101-bp deletion in *cbiH* likely leading to a non-functional gene (Figure S4.1), thus likely to explain why *D. restrictus* requires exogenous corrinoids supply to the growth medium.

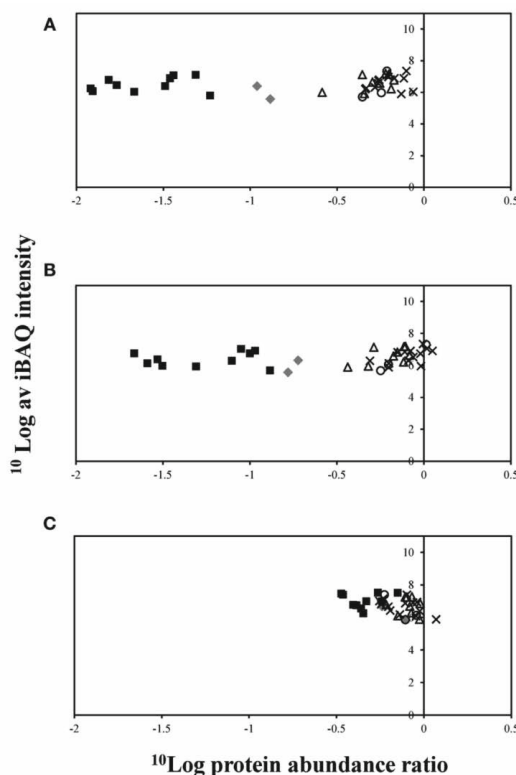


Figure 4.5 Proteins associated with corrinoid biosynthesis and uptake, extracted from *D. restrictus* cells cultivated in the presence of different cyanocobalamin concentrations. (A) Proteomics analysis of cells cultivated in the presence of high (250 µg/L) vs. low (10 µg/L) corrinoid concentrations; (B) high (250 µg/L) vs. mid (50 µg/L) corrinoid concentrations; (C) mid (50 µg/L) vs. low (10 µg/L) corrinoid concentrations. Proteins encoded by corrinoid operon-1 (grey diamonds), operon-2 (black squares), operon-3 (white triangles), operon-4 (white circles) and operon-5 (black crosses) are shown. Logarithmic average of iBAQ (Intensity based absolute quantitation) value is plotted against the log value of protein abundance ratio based on LFQ value. CbiM (Dehre_0278) was left out because it could not be identified in two replicates of cultures under low corrinoid concentration.

Previously, the presence of one ABC-type cobalt transporter (Dehre_0850-0852) and two energy-coupling factor-type cobalt transporters (Dehre_0278-0280 and Dehre_2862-2865) was predicted in the genomic study of *D. restrictus* [67]. Here, we identified CbiM (Dehre_0278), CbiQ (Dehre_0280), CbiO (Dehre_2862) and CbiN (Dehre_2864) in the proteome dataset obtained in this study (Table S4.4).

Relative abundance of proteins associated with corrinoid biosynthesis and salvaging pathways was further analyzed. Interestingly, nearly all proteins associated with corrinoid biosynthesis and salvaging pathways were up-regulated under corrinoid limiting growth conditions (Figure 4.5 and Table S4.5). As expected the overall largest change in the abundance of proteins related to corrinoid biosynthesis and salvaging pathways was observed when comparing the proteome of cells cultivated at high vs. low concentration of cyanocobalamin (Figure 4.5A). The corrinoid metabolism differed more strongly when comparing cells cultivated in the presence of high vs. mid than mid vs. low concentrations (Figure 4.5B and 5C).

Proteins encoded by operon-2 showed the largest change in protein abundance ratios with on average 46-fold up-regulation when comparing cells cultivated at low vs. high corrinoid concentrations (Figure 4.5A). Operon-2 encodes proteins predicted to be involved in corrinoid salvaging or corrinoid transport. Among these are two predicted CbiZ proteins, amidohydrolases required for salvaging the corrinoid precursor cobinamide, which were up-regulated 80-fold (Dehre_0285) and 58-fold (Dehre_0282) under corrinoid starvation, respectively (Table S4.4). Furthermore, proteins encoded in operon-1 including an energy-coupling factor-type cobalt transporter (Dehre_0278-0280) which is likely to be involved in the cobalt uptake process, and a precorrin-6x reductase (Dehre_0277), were on average 8-fold up-regulated when comparing cells cultivated in the presence of low vs. high corrinoid concentrations. Fewer changes were found for proteins encoded by the three remaining corrinoid-related operons under the different corrinoid conditions.

Discussion

Corrinoid biosynthesis of *D. restrictus* and other OHRB

In the present study, ≥ 50 $\mu\text{g/L}$ of cyanocobalamin was required for *D. restrictus* to reach its maximum PCE dechlorination, in line with previous observation that this organism depends on externally supplemented corrinoids [52]. The genome of *D. restrictus* encodes a complete set of corrinoid biosynthesis genes, with the exception of a non-functional *cbiH* gene, suggesting that tetrapyrrole ring contraction does not occur here and represents a dead-end in

the biosynthesis pathway. Comparative genomic analysis among other *Dehalobacter* spp. revealed that an intact *cbiH* gene is present in all other genomes. However, little is known about the capacity of other members of this genus to *de novo* synthesize corrinoids. Indeed they only have been studied under growth conditions with external addition of cyanocobalamin or in co-cultures [55, 59, 61, 66, 304]. Similarly, strains of *Dehalococcoides mccartyi*, which are also obligate OHRB, are corrinoid-auxotroph [40]. Unlike *D. restrictus*, the corrinoid auxotrophy in *D. mccartyi* strains is due to the lack of the complete biosynthetic pathway. Instead they rely on uptake of extracellular corrinoids via the salvaging pathway and on remodelling of incomplete or non-functional corrinoids in the presence of appropriate free lower ligands, among which 5,6-dimethylbenzimidazole plays a key role [93, 94, 299, 300, 320]. Interestingly, most facultative OHRB such as *Sulfurospirillum multivorans* [297], *Desulfitobacterium hafniense* [74, 301] or *Geobacter lovleyi* [128] are capable of *de novo* biosynthesis of corrinoids.

The genome of *D. restrictus* encodes five well-organized operons containing most of the corrinoid biosynthesis-associated genes. Comparing the genomes of the sequenced *Dehalobacter* spp. revealed that *D. restrictus* harbours an extra set of genes (operon-2) coding for putative corrinoid transporters and salvaging enzymes (CbiZ and cobaltochelateses), suggesting an augmented capacity for corrinoid uptake and remodelling compared to other *Dehalobacter* spp. The importance of *cbiZ* genes in remodelling corrinoids has been already demonstrated for *D. mccartyi* [91, 92, 300]. The role of operon-2 in *D. restrictus* was evidenced by the significant up-regulation of the corresponding enzymes when corrinoid concentration in the medium was lowered. This result clearly showed that *D. restrictus* has developed a unique strategy to cope, at least partially, with its lack of corrinoid biosynthesis under unfavourable corrinoid conditions. The presence of additional *cbiZ* genes in operon-2 raises the questions of the functional redundancy vs. specificity of multiple CbiZ proteins within a single strain, and of the origin of the additional *cbiZ* genes present in *D. restrictus*. While *in vitro* biochemical investigations would be required to answer the first question, a detailed analysis of CbiZ sequence homology (Figure S4.6) revealed that the two additional CbiZ proteins in *D. restrictus* show a high level of sequence identity with CbiZ homologues present in *Acetobacterium woodii*, a corrinoid-producing bacterium [321], which has been well-characterized for the Wood-Ljungdahl pathway that also requires corrinoids as an essential cofactor [322]. The high degree of genomic synteny identified between the corrinoid operon-2 of *D. restrictus* and *A. woodii* suggests that *D. restrictus*, but not the other members

of the *Dehalobacter* genus, most probably acquired operon-2 by horizontal gene transfer and successfully exploited this operon to partially alleviate the loss of a functional *cbiH* gene.

Effect of corrinoid starvation on *D. restrictus* metabolism

Reduction of cyanocobalamin amendment in the growth medium strongly inhibited PCE dechlorination by *D. restrictus*. It also had a profound effect on *D. restrictus* corrinoid metabolism both at the level of transcription and at the proteome level. While changing from high, (250 µg/L) corrinoid concentration to an intermediate concentration (50 µg/L), *D. restrictus* responded by up-regulating proteins associated with corrinoid transport and salvaging pathways encoded in operon-1 and -2, allowing the strain to reach the same PCE dechlorination level as observed during high corrinoid concentration. Decreasing the corrinoid concentration even further to 10 µg/L showed, however, that, while the extent of PCE dechlorination was strongly affected, the amount of corrinoid-associated proteins did not notably change when compared to cells cultivated in the presence of 50 µg/L. This indicates that at corrinoid concentrations as low as 10 µg/L, *D. restrictus* was not able to compensate the lack of externally provided corrinoids by increased corrinoid transport and salvaging.

The presence of cobalamin riboswitches directly upstream of the five corrinoid operons in *D. restrictus* already suggested an active repression at the level of transcription by cyanocobalamin. Similar to transcriptional studies on *D. mccartyi* [323] and *D. hafniense* [301], the cobalamin riboswitches of *D. restrictus* responded to addition of excess cyanocobalamin, and the level of repression of the gene located directly downstream of the riboswitches correlated well with the proteomic data, showing the strongest effect for *cbiJ* (Dehre_0277, operon-1) and for *fepB* (Dehre_0281, operon-2). The sequence of individual cobalamin riboswitches is likely responsible for their differential responsiveness towards cobalamin concentration, as both their affinity to cobalamin and the strength which the expression platform exerts on transcriptional repression are sequence dependent. Such effects have already been shown for a few cobalamin riboswitches in *D. hafniense* [301],

D. mccartyi strain 195, another corrinoid-auxotroph, requires a concentration of 25 µg/L cyanocobalamin to support optimal TCE dechlorination rates and growth yield [324], a value that is similar to what was observed for *D. restrictus*. Therefore, and in addition to the ecogenomic biomarkers defined by Maphosa *et al.* [13], one could consider the physiological threshold of corrinoid concentration as a possible diagnostic tool to delineate the reductive dechlorination potential by corrinoid-auxotrophic OHRB in anaerobic environments.

Meanwhile, the production of the PCE reductive dehalogenase (PceA, Dehre_2398) in *D. restrictus* showed no significant change under different corrinoid concentrations, which strongly suggests that the amount of available corrinoid and not of the apo-enzyme represents the main limiting factor for PCE dechlorination.

Taken altogether, our results support the hypothesis that, besides the partial deletion of *cbiH* in *D. restrictus* [67, 261], which already represents a crucial checkpoint in the corrinoid biosynthesis pathway, the energetic cost of *de novo* corrinoid biosynthesis might explain why *D. restrictus* has developed enhanced corrinoid transport and salvaging strategies. *D. restrictus* corrinoid metabolism represents an intermediate situation between the true corrinoid-auxotrophic and obligate organohalide-respiring *D. mccartyi*, which lacks the corrinoid biosynthesis pathway completely [93, 300, 324, 325], and the facultative OHRB able to produce corrinoids *de novo*.

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**Dehalogenation of three
tetrachlorobenzene isomers by
contaminated harbor sludge-derived
enrichment cultures follows
thermodynamically favorable reactions**

This chapter has been submitted for publication:
Yue Lu, Javier Ramiro-Garcia, Pieter Vandermeeren, Steffi Herrmann,
Danuta Cichocka, Dirk Springael, Siavash Atashgahi and Hauke Smidt.
Dechlorination of three tetrachlorobenzene isomers by contaminated
harbor sludge-derived enrichment cultures follows
thermodynamically favorable reactions. Under review.

Abstract

Dechlorination patterns of the three tetrachlorobenzene isomers 1,2,3,4-, 1,2,3,5- and 1,2,4,5-TeCB were studied in anoxic microcosms derived from a contaminated harbor. The removal of doubly, singly and un-flanked chlorine atoms was noted in 1,2,3,4- and 1,2,3,5-TeCB fed microcosms, whereas only singly flanked chlorine was removed in 1,2,4,5-TeCB microcosms. Di- and/or mono-chlorobenzene were observed as the main end products of the reductive dechlorination of all three isomers. Among various dechlorinating pathways of TeCB isomers, the thermodynamically more favorable reactions were selectively followed by the enriched cultures. Based on quantitative PCR analysis targeting 16S rRNA genes of known organohalide-respiring bacteria, the growth of *Dehalococcoides* was found to be associated with the reductive dechlorination of all three isomers, while growth of *Dehalobacter*, another known TeCB dechlorinator, was only observed in one 1,2,3,5-TeCB enriched microcosm among biological triplicates. Numbers of *Desulfitobacterium* and *Geobacter* as facultative dechlorinators were rather stable suggesting that they are not directly involved in TeCB dechlorination. Overall bacterial community profiling suggested bacteria belonging to the phylum *Bacteroidetes* and the order *Clostridiales* as well as sulfate-reducing members of the class *Deltaproteobacteria* as putative stimulating guilds that provide electron donor and/or organic cofactors to fastidious dechlorinators. Overall, our results provide a better understanding of thermodynamically preferred TeCB dechlorinating pathways in harbor environments and microbial guilds enriched and active in anoxic TeCB dechlorinating microcosms.

Introduction

Chlorobenzenes (CBs) are aromatic chlorinated compounds with a benzene ring substituted with one to six chlorine atoms. CBs are important industrial intermediates in pesticides synthesis and manufacturing of other chemicals [326, 327]. Anthropogenic production and uncontrolled release of CBs in addition to their high persistence and bioaccumulation has resulted in broad distribution of these pollutants in the environment [326-328]. Partial dechlorination of highly chlorinated benzenes has been shown in a broad range of sub-oxic/anoxic environments such as soil [329, 330], river sediment [331-333], sewage sludge [334, 335] and drainage ditch [57] with lower chlorinated benzenes as end products. Even though monochlorobenzene (MCB) was considered to be recalcitrant to biotransformation in anoxic environments [8], several studies reported further dechlorination of MCB to benzene in anaerobic enrichments originated from freshwater sediments [333, 336, 337]. Organohalide respiration (OHR), i.e. energy conservation from respiratory reductive dechlorination of organohalides as the final electron acceptors, is currently the only known microbial process for the partial/complete dechlorination of highly chlorinated benzenes like hexachlorobenzene (HCB), pentachlorobenzene (QCB) and tetrachlorobenzenes (TeCBs) under anoxic conditions [8, 338]. Therefore, the presence and activity of organohalide-respiring bacteria (OHRB) is pivotal for bioremediation of anoxic CB contaminated environments.

TeCBs have previously been reported as intermediates of HCB dechlorination, and include three isomers, i.e., 1,2,3,4-, 1,2,3,5- and 1,2,4,5-TeCB. TeCBs can be anaerobically biotransformed following diverse pathways [8, 338]. Preference for thermodynamically more favorable CB dechlorination pathways was shown before in an anaerobic microbial consortium originating from lake sediment [339], but this selectivity was not observed in other microcosms derived from contaminated freshwater sediments [336, 340]. Currently known bacterial isolates able to dechlorinate TeCB isomers belong to *Dehalococcoides mccartyi* in the phylum *Chloroflexi* [159, 335] and *Dehalobacter* in the phylum *Firmicutes* [57]. *D. mccartyi* strains CBDB1 [159], 195 [335] and DCMB5 [27] dehalogenate CB congeners with four or more chlorine substituents to trichlorobenzenes (TCBs) or dichlorobenzenes (DCBs). Besides, a broad TeCB dechlorination spectrum was demonstrated for *Dehalobacter* spp. strains 12DCB1 and 13DCB1 and a highly enriched culture containing *Dehalobacter* sp. 14DCB1, all of which were able to dechlorinate highly chlorinated CBs to DCBs and MCB [57]. In addition to these obligate organohalide-respiring isolates that are restricted to OHR as the sole metabolism to yield energy, growth of members of metabolically

more versatile OHRB in the presence of CBs was also studied. Bacteria of the genus *Geobacter* were found associated with HCB dechlorination in an anaerobic consortium originating from contaminated river sediment as determined by genus-specific quantitative PCR (qPCR), suggesting they also may serve as CB dechlorinators [336]. Furthermore, a recently reported bacterial community analysis with 454 pyrosequencing of 16S ribosomal RNA (rRNA) gene fragments conducted on a range of anaerobic CB-dechlorinating enrichments from contaminated river sludge revealed a high relative abundance (21.38 % - 55.07%) of populations closely related to *Desulfitobacterium*, while *Dehalococcoides* and *Dehalobacter* together represented less than 0.53% of the bacterial community [340].

Up to date, the fate of TeCBs in marine and estuarine environments is still not well known. 1,2,4-TCB dechlorination in sediment collected from Ise Bay (Japan) revealed that DCB production rates were related to anaerobic plate counts and activity of sulfate reducing bacteria, although there was no solid evidence showing their direct involvement in 1,2,4-TCB dechlorination [341]. Furthermore, dechlorination of CBs was reported in enrichments derived from estuarine sediments collected in Tsurumi river (Japan) [342] and Bayou d'Inde (USA) [343], but the microorganisms responsible for dechlorination were not identified. To date, the only reported CB-dechlorinating bacterium isolated from an estuarine environment (Charleston Harbor, USA) is *Dehalobium chlorocoercia* [40], which was shown to dechlorinate HCB via 1,2,3,5-TeCB to 1,3,5-TCB [115]. This pathway was also proposed as the main pathway of dechlorination of HCB and QCB in estuarine sediments with very low levels of other CBs detected [115, 343]. Overall, dechlorination of CBs and in particular of TeCB in marine and estuarine sediments remains poorly explored, and better understanding of the activity of CB-dehalogenating microbes and the microbial context they interact with in those habitats is required for their *in situ* bioremediation [344].

The aim of this study was to assess i) the TeCB dechlorination potential of microbial communities present in an estuarine environment ii) microbial community dynamics during enrichment and iii) the preferred TeCB dechlorinating pathways. To this end, we characterized TeCB-fed anoxic microcosms derived from contaminated harbor sludge, and a combination of bacterial community profiling and qPCR were applied to gain insight into TeCB dechlorinators and co-existing, non-dechlorinating bacterial guilds that may be important for sustained dechlorination by providing electron donors and/or organic cofactors required by OHRB.

Materials and methods

Chemicals

CBs, benzene, and 1,3,5-tribromobenzene (TBrB) of analytical grade and high purity were purchased from Sigma Aldrich. Benzene, MCB, 1,2-DCB, 1,4-DCB, 1,2,3-TCB, 1,2,4-TCB, 1,3,5-TCB and 1,2,3,5-TeCB had a purity of 99%; 1,3-DCB, 1,2,4,5-TeCB and 1,3,5-TBrB: 98%; and 1,2,3,4-TeCB: 96%.

Microcosm Setup

Harbor sludge from Zeebrugge, Belgium was sampled (Figure 5.1, step 1) to prepare anoxic microcosms. All samples were collected in September 2008, and stored for three months at 4 °C until they were used in this study. The microcosms were set up as described elsewhere [340] with 10 g of sludge and 40 ml of an anoxic medium [52], and 20 mM lactate as electron donor and carbon source (Figure 5.1, step 2). Three TeCB isomers (1,2,3,4-, 1,2,3,5- and 1,2,4,5-TeCB) were added individually from acetone stocks to final concentrations of 50 µM for each isomer. Abiotic controls were prepared by addition of 0.5 ml of 37% formaldehyde. Initial microcosms were set up in triplicates. Two spikes of 50 µM of TeCB isomer were supplied separately to the cultures during 93 days of non-shaking cultivation at 25 °C in the dark (Figure 5.1, step 2). Cultures were subsequently kept for five years at 4 °C, after which a subset (single 1,2,3,4- and 1,2,4,5-TeCB enrichment cultures and triplicate 1,2,3,5-TeCB cultures) was transferred to Wageningen, the Netherlands, for further characterization of TeCB dechlorination. To revive TeCB dechlorination in the original cultures, 30 ml of fresh anoxic medium was added to the original bottles and 20 mM lactate was supplied. Bottles were sealed with a viton stopper, and the headspace was exchanged with N₂/CO₂ (80:20 v/v). Spikes of the respective TeCBs (50 µM/each) were added at the beginning and on day 35, 96 and 134 of incubation, and the cultures were maintained actively dechlorinating for over 200 days without shaking at 25 °C in the dark (Figure 5.1, step 3).

To study the dynamics of degradation pathways and microbial communities, and to obtain sediment-free TeCB-enriched cultures, serial transfers were performed. A 5% inoculum from the original TeCB-fed microcosms was transferred into fresh anoxic bottles containing growth medium, fed with 50 µM of the same TeCB isomer as the initial inoculum and cultivated for 66 days (Figure 5.1, step 4). Further transfers were conducted with the 1,2,4,5-TeCB dechlorinating culture enriched from harbor sludge (culture A in Figure 5.1, step 5), and their performance was compared to that of transfers of two sediment-free 1,2,4,5-TeCB-enriching

cultures (culture B and C in Figure 5.1, step 5) that were previously obtained from chlorinated aromatics-contaminated river sludge collected in Kanaal Ieper-Ijzer, Belgium (50°52'14"N 2°52'31"E) [340]. A 5% inoculum was taken for serial transfer (Figure 5.1, step 6).

Effects of vancomycin addition and/or vitamin B12 starvation on the dechlorination activity of the sediment-free 1,2,4,5-TeCB-fed enrichments were investigated with a 2×2 factorial design experiment (Figure 5.1, step 7) that was set up in duplicate. 12 µg/l of vancomycin and 0.03 µM of vitamin B12 were added where required.

Detection of benzene and chlorinated benzenes

Benzene and CBs were quantified on a GC-2000 equipped with flame ion detector (FID) (Shimadzu, Japan). 0.2 ml headspace samples were used for benzene measurement as described before [345], and 1 ml liquid samples were used for the detection of CBs. Liquid samples were extracted with 400 µl hexane by overnight shaking at 600 rpm followed by 100 µl hexane phase transfer to HPLC vials containing 5 µl of 1 mM 1,3,5-tribromobenzene as the internal standard. The column temperature program was 60 °C hold for 4 min, followed by a gradient of 5 °C min⁻¹ to 180 °C and another hold for 3 min. The carrier gas was nitrogen at a flow of 1 ml min⁻¹, a spit flow of 12 ml min⁻¹ with a split ratio of 1:10. Injection was performed by an auto-sampler (Shimadzu, Japan) injecting 2 µl of the sample into a split injector held at 250 °C. Standards with a mixture of benzene and CBs at 10, 20, 40, 60, 100, 200 µM were set up in 125 ml serum bottles with the same headspace-liquid phase ratio of treatment cultures.

Sampling for molecular analysis and DNA extraction

Cultures were sampled for molecular analysis as shown in Figure 5.1, and samples taken before adding TeCBs to the original bottles were indicated as 'Before reviving' (Figure 5.1, step 3). Two ml slurry/liquid samples were withdrawn from each bottle, centrifuged (5417R, Eppendorf, Germany) at 6010 rcf for 5 min at 4 °C, and pellets were stored at -20 °C. DNA isolation was performed using the PowerSoil DNA isolation kit (MO-BIO, USA) according to the manufacturer's instructions except that three 45 s bead beating (5.5 m/s) steps using a Fastprep Instrument (MP Biomedicals, USA) were included instead of the horizontally vortexing step suggested in the manual. The quality and quantity of DNA was checked using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis.

Illumina MiSeq sequencing and data analysis

A 2-step PCR strategy was used to generate barcoded amplicons from the V1-V2 region of the 16S rRNA gene. The first PCR (50 µl) contained 1× HF buffer (Thermo Scientific™, The Netherlands), 1 µl dNTP Mix (10 mM; Promega, Leiden, The Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific™), 500 nM of 27F-DegS forward primer, 500 nM of 338R I and II reverse primers (Table S5.1), and 1 µL template DNA (15-20 ng/µl). The forward and reverse primers were appended at the 5' end with 18 bp Universal Tag (Unitag) 1 and 2, respectively (Table S5.1). PCR conditions were 98°C for 30 s, 25 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 20 s and elongation at 72°C for 20 s, and a final extension at 72°C for 10 min. The PCR product size was examined by gel electrophoresis. The second PCR (100 µl) contained 1× HF buffer, 2 µl dNTP Mix 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward and reverse primer equivalent to the Unitag1 and Unitag2 sequences, respectively, that were each appended with an 8 nt sample specific barcode [346] at the 5' end, and 5 µl PCR product of the first reaction. PCR conditions were similar to those used for the first PCR except for an annealing temperature of 52°C and a reduced number of 5 cycles. The PCR product size was examined by gel electrophoresis, purified with HighPrep™ (Magbio Genomics, Rockville, MD, USA) and quantified using a Qubit in combination with the dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA). Purified PCR products were pooled, underwent adaptor ligation and sequenced on a MiSeq platform (GATC-Biotech, Konstanz, Germany). Sequence analysis was performed with NG-Tax, an in-house pipeline [346] in which operational taxonomic units (OTUs) were assigned to taxonomy using uclust [347] in an open reference approach against the SILVA 16S rRNA gene reference database [348]. Finally a biom file was generated using Quantitative Insights Into Microbial Ecology (QIIME) v1.2 [349].

Quantitative PCR

The abundance of the 16S rRNA genes of *Dehalococcoides*, *Dehalobacter*, *Desulfotobacterium*, *Geobacter* and total bacteria was determined by qPCR performed on a CFX384 Real-Time system with C1000 Thermal Cycler (Bio-Rad Laboratories, USA) with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, USA). Each DNA sample was analysed in triplicates. The primers and qPCR amplification program used in this study have been described before for *Dehalobacter* [226] and other targets [352]. The relative abundance of *Dehalococcoides* in qPCR data was calculated by dividing the number of *Dehalococcoides* 16S rRNA gene copies by the copy number of bacterial 16S rRNA gene copies, multiplied by

Nucleotide sequences

16S rRNA gene sequences have been deposited in the European Nucleotide Archive (ENA) with accession number ERS1082969-ERS1083005 under study PRJEB13024.

Results

TeCB dechlorination in sediment microcosms

During 93 days of anaerobic cultivation (Figure 5.1, step 2), complete depletion of two spikes of 50 μ M of corresponding TeCBs (1,2,3,4-, 1,2,3,5-, 1,2,4,5-TeCB) was observed in all sediment microcosms (data not shown). In contrast, no decrease of TeCBs was observed and no dechlorination products were detected in the abiotic controls (data not shown), indicating that biological dechlorination via OHR occurred.

After these microcosms had been stored for five years at 4 °C, new spikes of TeCBs were added to recover the dechlorination activity. After a short lag phase of 1-8 days, dechlorination of TeCBs was observed in all microcosms (Figure 5.2). 1,2,4-TCB was observed as a transient intermediate during the dechlorination of all three TeCB isomers, whereas 1,3,5- TCB was only detected as an intermediate product of 1,2,3,5-TeCB-dechlorination. All microcosms showed MCB accumulation except for the 1,2,4,5-TeCB-fed microcosm in which 1,4-DCB was detected as the main end product (Figure 5.2e). Production of 1,2,3-TCB, 1,2-DCB or benzene was not detected in any of the microcosms during 161 days of incubation.

1,2,4,5-TeCB dechlorination in sediment-free cultures

To achieve sediment-free TeCB-enriched cultures, a 5% inoculum was directly transferred from original TeCB-fed microcosms (Figure 5.1, step 4). Dechlorination was only observed in the transferred 1,2,4,5-TeCB-fed enrichments (culture A in Figure 5.1, step 5) after 66 days of anaerobic cultivation (data not shown).

In the following, the dechlorination performance of the newly obtained transfer culture derived from the estuarine harbor environment was compared with another two sediment-free 1,2,4,5-TeCB-enriching cultures that were previously enriched from river sludge in Kanaal Ieper-Ijzer (Belgium) [340]. During incubation of the second transfer, three spikes of 1,2,4,5-TeCB were completely depleted in all transferred enrichments, concomitant with the

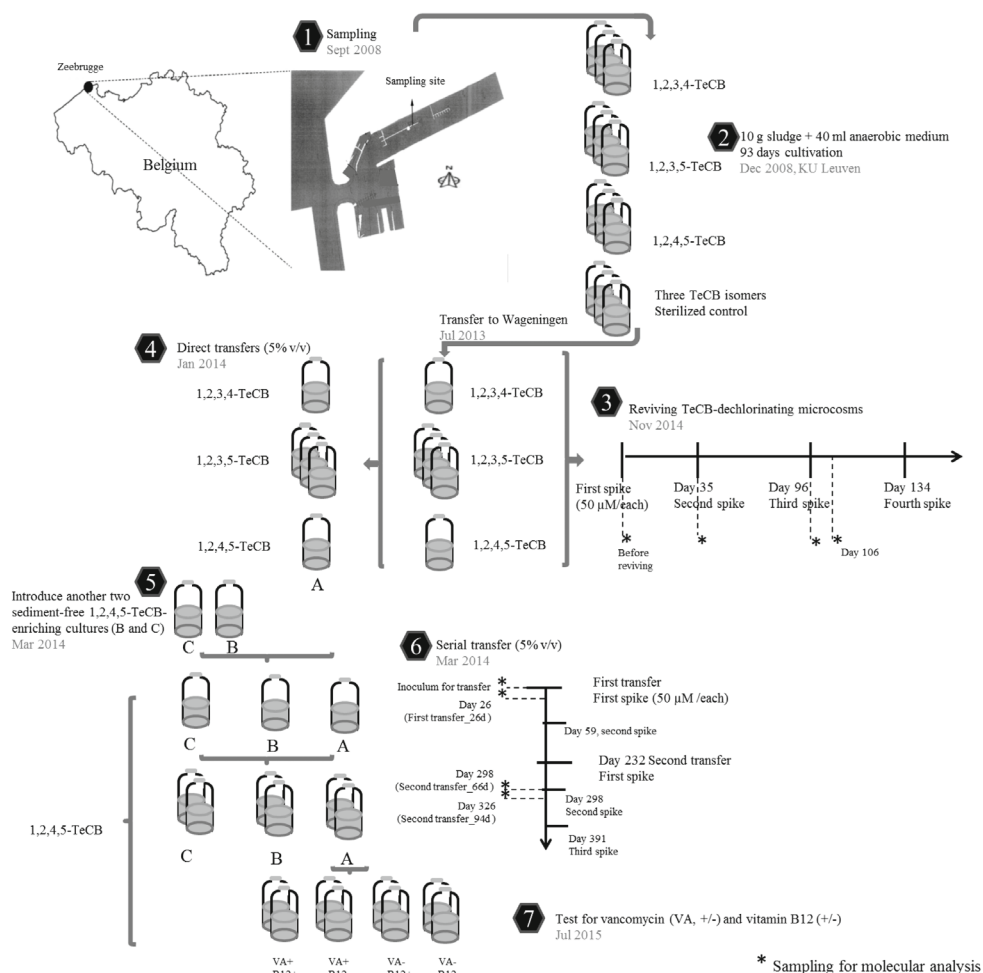


Figure 5.1 Schematic representation of the experiment.

production of 1,2,4-TCB, 1,4-DCB and 1,3-DCB (Figure 5.3). 1,4-DCB was the dominant end product in harbor derived enrichments (Figure 5.3a), which was in line with the observation in the original 1,2,4,5-TeCB-fed microcosm (Figure 5.2e), whereas slightly more 1,3-DCB was detected in river sludge-derived enrichments (Figure 5.3b&c).

Dechlorination pattern of TeCB isomers and selectivity of the reactions

The reductive removal of doubly, singly and non-flanked chlorine atoms was noted in the 1,2,3,4- and 1,2,3,5-TeCB-fed microcosms derived from contaminated harbor sludge. In contrast only singly flanked, but not the non-flanked chlorine, was removed during the

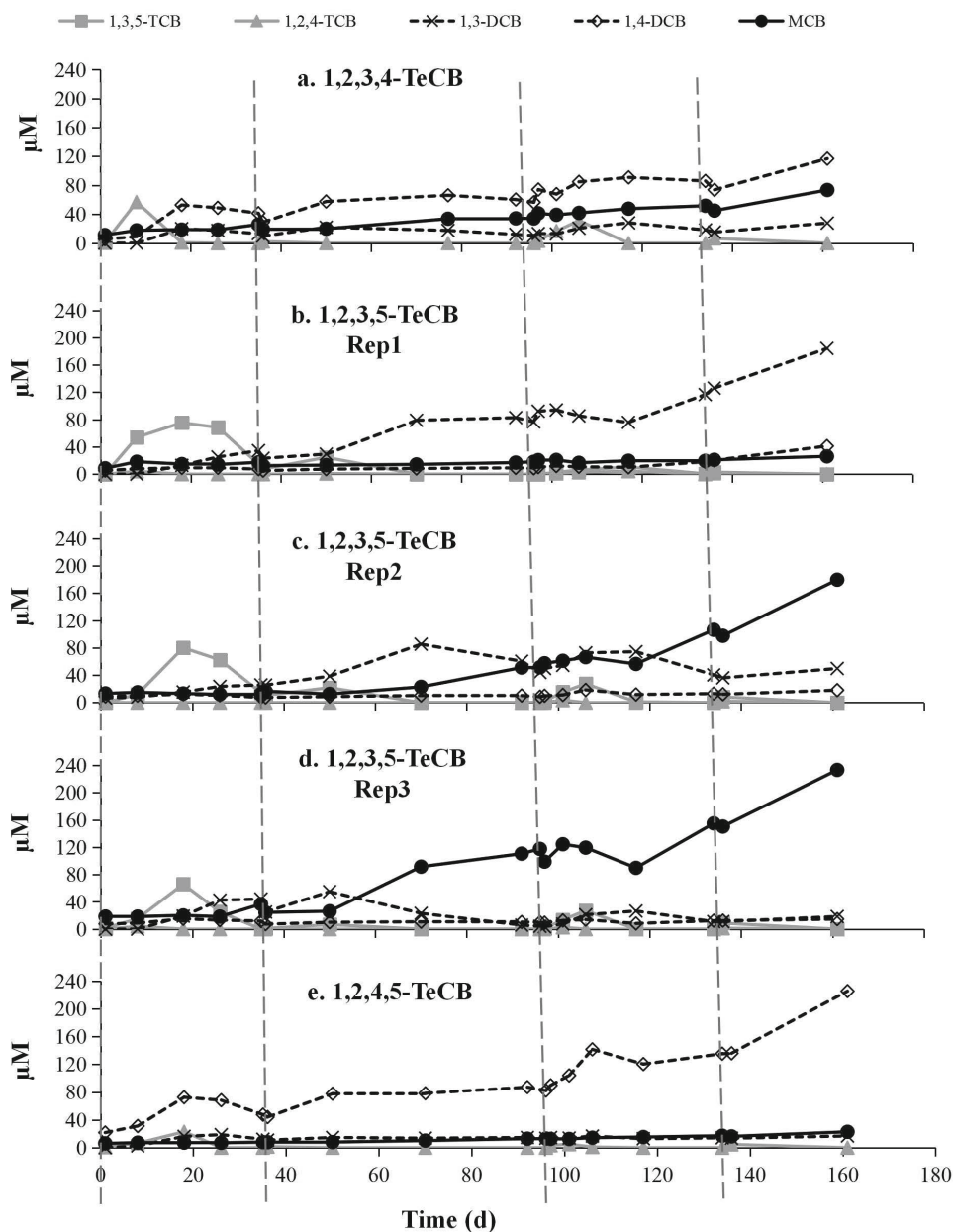


Figure 5.2 Dechlorination of 1,2,3,4-TeCB (a), 1,2,3,5-TeCB (b-d, triplicate bottles), and 1,2,4,5-TeCB (e) in original microcosms derived from harbor sludge. 50 μM spikes of the respective TeCBs are indicated by vertical dashed lines. Concentrations of TeCBs are not shown for the clarity of figure.

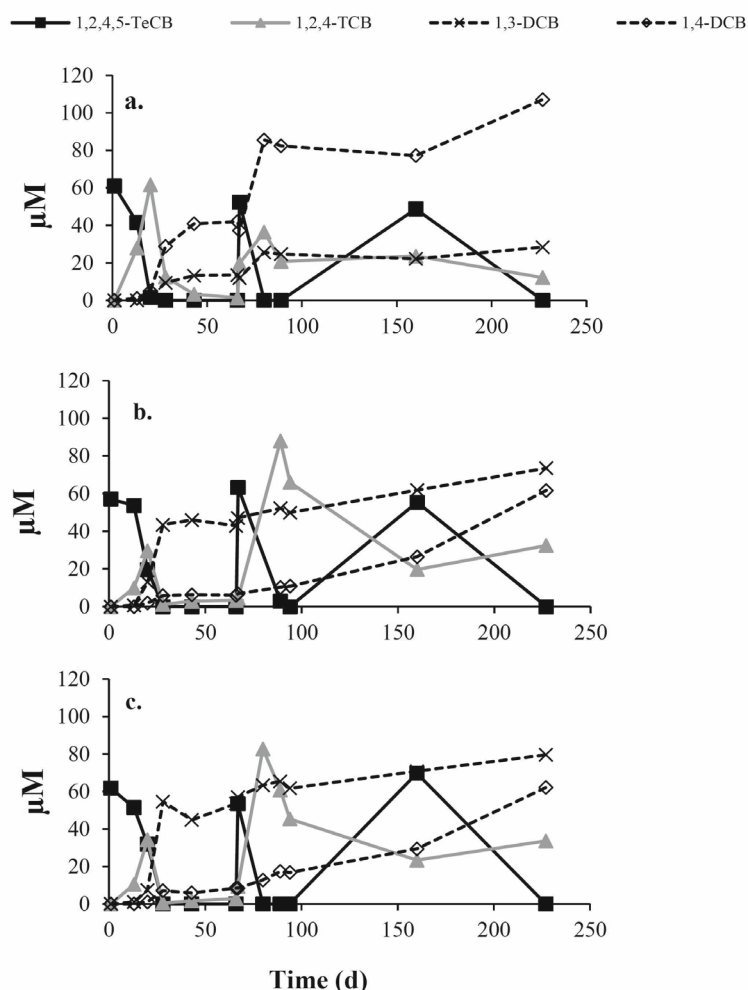


Figure 5.3 Dechlorination of 1,2,4,5-TeCB in second transfers (Figure 5.1, step 6) from the 1,2,4,5-TeCB dechlorinating enrichment derived from harbor sludge (a) and 1,2,4,5-TeCB dechlorinating sediment-free enrichments (culture B and C in Figure 5.1) derived from river sludge (b-c). Data from an individual bottle is shown here as a representative of duplicate cultures, which both followed the same overall dechlorination pattern.

dechlorination of 1,2,4,5-TeCB. Based on Gibbs free-energies ($\Delta G^{0'}$ under standard condition, kJ/mol) taken from Dolfig and Keith Harrison [353] calculated with hydrogen as electron donor, the harbor sludge-derived microcosms showed a preference to follow the thermodynamically most favorable dechlorination pathways. For example, 1,2,3,5-TeCB was mainly dechlorinated to 1,3,5-TCB as an intermediate rather than 1,2,4-TCB, and 1,2,3-TCB was not detected, in line with the fact that this reaction theoretically yields the least negative

free energy change (Figure 5.4). A similar pattern was observed for the dechlorination of 1,2,3,4-TeCB to 1,2,4-TCB instead of 1,2,3-TCB and further dechlorination of 1,2,4-TCB to 1,4-DCB instead of 1,3-DCB as the main product (Figure 5.4).

Effect of vancomycin addition and vitamin B12 starvation

Vancomycin, an inhibitor of the peptidoglycan synthesis of Gram-positive bacteria, was added to harbor sludge-derived, sediment-free 1,2,4,5-TeCB enrichments to test its effect on TeCB dechlorination. After 21 days of anaerobic cultivation, the amended 1,2,4,5-TeCB was completely depleted in control microcosms (Figure S5.1a) with 1,4-DCB and 1,3-DCB as the main products. In contrast, in vancomycin treated microcosms, only 36-59% of the 1,2,4,5-TeCB was dechlorinated within the same incubation time, with 1,2,4-TCB as the main product and trace amounts of 1,4-DCB and 1,3-DCB (Figure S5.1b). The delayed dechlorination in enrichments supplied with vancomycin suggested (in)direct involvement of vancomycin-sensitive Gram-positive bacteria in 1,2,4,5-TeCB dechlorination. Additionally, vitamin B12 (cofactor of reductive dehalogenase enzymes) starvation was investigated in the presence or absence of vancomycin (Figure S5.1c&d). No effect, however, on TeCB dechlorination activity was observed, suggesting that the 1,2,4,5-TeCB-enriched microbial communities tested here were capable of *de novo* vitamin B12 biosynthesis.

Dynamics of bacterial communities

During 161 days reviving of original TeCB microcosms, *Dehalococcoides* as a potential TeCB dechlorinator was detected at relative abundances below 2% in 1,2,3,4- and 1,2,4,5-TeCB fed microcosms and one of the triplicate 1,2,3,5-TeCB fed microcosms (Figure 5.5a). The detected sequence of *Dehalococcoides* was blast in NCBI and showed 100% identity with that of *D. mccartyi* strains CBDB1 and 195. Additionally, diverse non-dechlorinating bacteria were found in the bacterial community of TeCB-fed microcosms, especially from the phyla *Bacteroidetes*, *Fibrobacteres* and the *Deltaproteobacteria* class (Figure 5.5a). *Bacteroidetes* and *Deltaproteobacteria* were detected in all the microcosms in the range of 10-56% and 10-47%, respectively. *Fibrobacteres* was mainly enriched in the 1,2,4,5-TeCB-fed microcosm and two of three replicate 1,2,3,5-TeCB fed microcosms (Figure 5.5a), whereas *Bacteroidetes* was observed with higher relative abundance in the other 1,2,3,5-TeCB fed microcosm with no *Fibrobacteres* detected during cultivation (Figure 5.5a). Differences of bacterial community composition observed among three replicate 1,2,3,5-TeCB-fed microcosms may have been caused by the heterogeneity of inocula sediments.

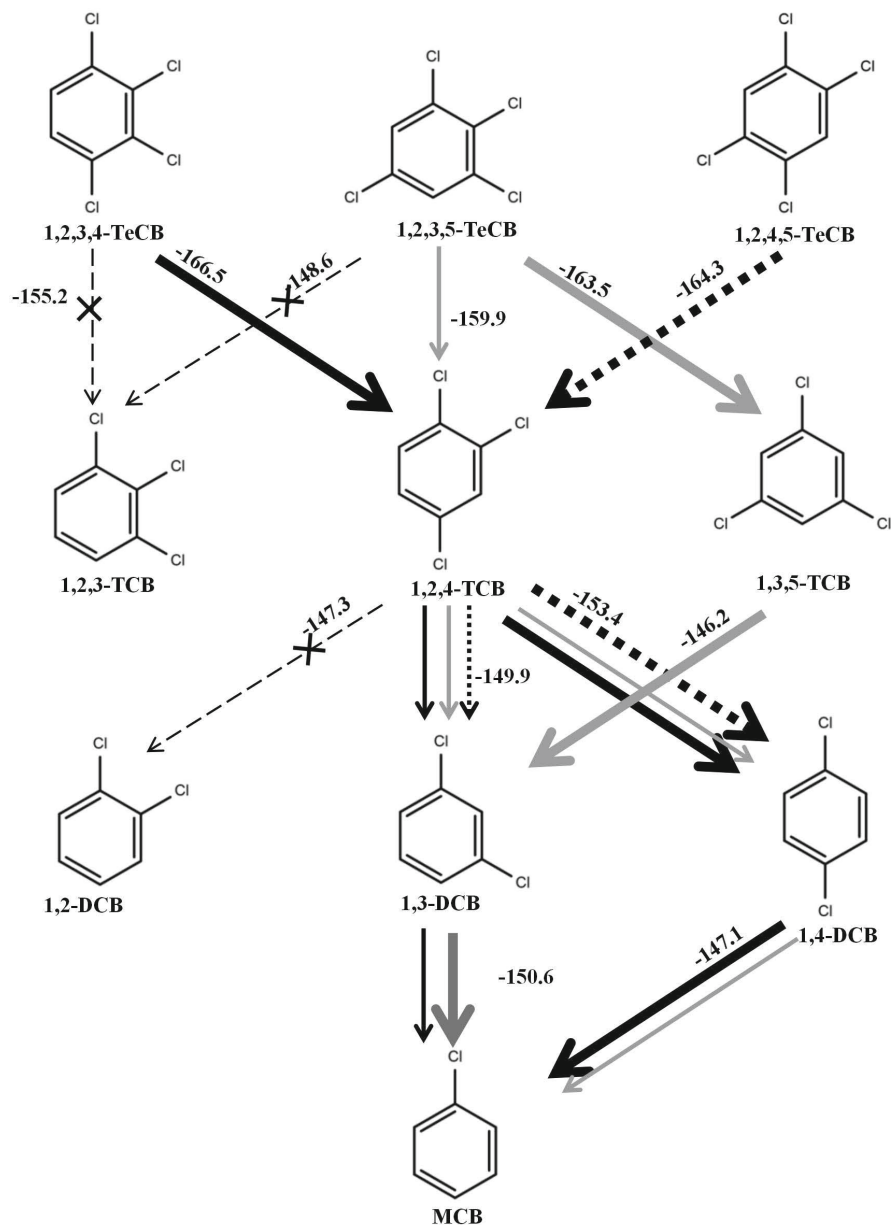


Figure 5.4 Dechlorination patterns of 1,2,3,4- (black solid line), 1,2,3,5- (grey solid line) and 1,2,4,5- (black dotted line) TeCB in microcosms derived from harbor sludge. Thin arrows represent reactions that were detected but were not dominant. Reactions catalyzed preferentially are indicated with bold arrows, and reactions not observed in our study are indicated by dashed lines with a cross. The $\Delta G^{\circ'}$ (kJ/mol) indicated for each dechlorination reaction is based on previously reported calculations with hydrogen as the electron donor [353].

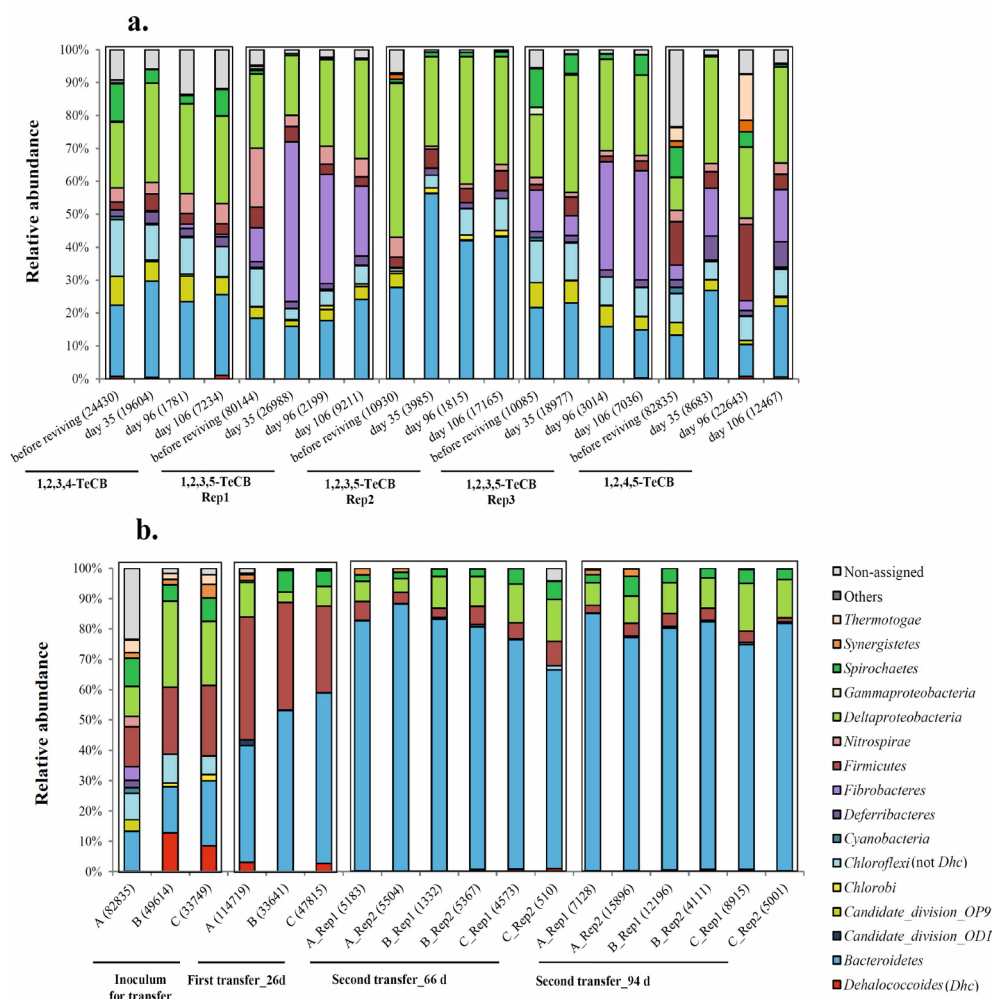


Figure 5.5 Bacterial 16S rRNA gene-based community dynamics of TeCB-fed microcosms during 161 days of reviving (**a**) and 1,2,4,5-TeCB dechlorinating enrichments during serial transfers (**b**). **a.** Rep1, Rep2 and Rep3 are triplicate original bottles fed with 1,2,3,5-TeCB. Sampling times are labelled as in Figure 5.1 (step 3). **b.** Rep1 and 2 are duplicates. Inoculum and sampling time are labeled as in Figure 5.1 (step 6). Data are shown at phylum level, except *Delta*- and *Gammaproteobacteria* that are presente at the class level and the genus *Dehalococcoides* that is shown separately from its corresponding phylum (*Chloroflexi*). The number of reads of each sample is given in brackets. Phyla with relative abundance lower than 1% in all samples are summed up as ‘Others’.

During serial transfers of the different 1,2,4,5-TeCB-enriched cultures, members of the *Bacteroidetes* phylum became strongly enriched with an average relative abundance of 79.7% (SD=3.8, Table S5.2) at the end of the second transfer (Figure 5.5b). *Bacteroidetes* detected in the the MiSeq dataset were mainly from the classes *Bacteroidia* (especially from the genus *Petrimonas*) and *Sphingobacteriia* (Table S5.2). *Petrimonas* was not detected in the TeCB-enriched cultures derived from harbor sludge, in which the *Sphingobacteriia* dominated the bacterial community (Table S5.2).

Quantification of potential dechlorinators during TeCB dechlorination

Growth of total bacteria, as measured by bacterial 16S rRNA gene-specific qPCR, was observed in all TeCBs fed microcosms (Figure 5.6). Interestingly, in one out of three 1,2,3,5-TeCB fed microcosms, the abundance of the genus *Dehalobacter* increased considerably from none-detectable levels before reviving to $2.59\text{E}+06$ (SD= $3.48\text{E}+05$) 16S rRNA gene copies/ml rRNA genes with $7.41\text{E}+04$ copies/ml (SD= $1.74\text{E}+04$). This indicates that in this microcosm, *Dehalobacter* rather than *Dehalococcoides* is the main TeCB dechlorinator. In all other microcosms, a clear increase of the *Dehalococcoides* counts during reviving was observed with an average abundance in the range of $3.93\text{E}+05$ to $1.12\text{E}+07$ copies/ml at the end of the cultivation (Figure 5.6). In contrast, 16S rRNA gene copy numbers of *Desulfitobacterium* and *Geobacter* remained stable (Figure 5.6). In all the 1,2,4,5-TeCB-fed transferred cultures, *Dehalococcoides* was detected with abundances that were more than three orders of magnitude higher than those of *Dehalobacter* (Figure S5.2), suggesting a more important role of *Dehalococcoides* in 1,2,4,5-TeCB dechlorination in these enrichments. Furthermore, the relative abundance of *Dehalococcoides* calculated from qPCR data generally matched with the results derived from the MiSeq dataset, although in several samples higher relative abundance of *Dehalococcoides* was detected via MiSeq (Figure 5.7). Both qPCR and MiSeq results showed decreased relative abundance of *Dehalococcoides* during transfer, which was mainly caused by the increase of average bacterial numbers from $3.05\text{E}+08$ copies/ml in inoculum to $1.98\text{E}+09$ copies/ml at 94 day of second transfer, while the absolute numbers of *Dehalococcoides* remained around $1.0\text{E}+07$ copies/ml medium during serial transfer (Figure S5.2).

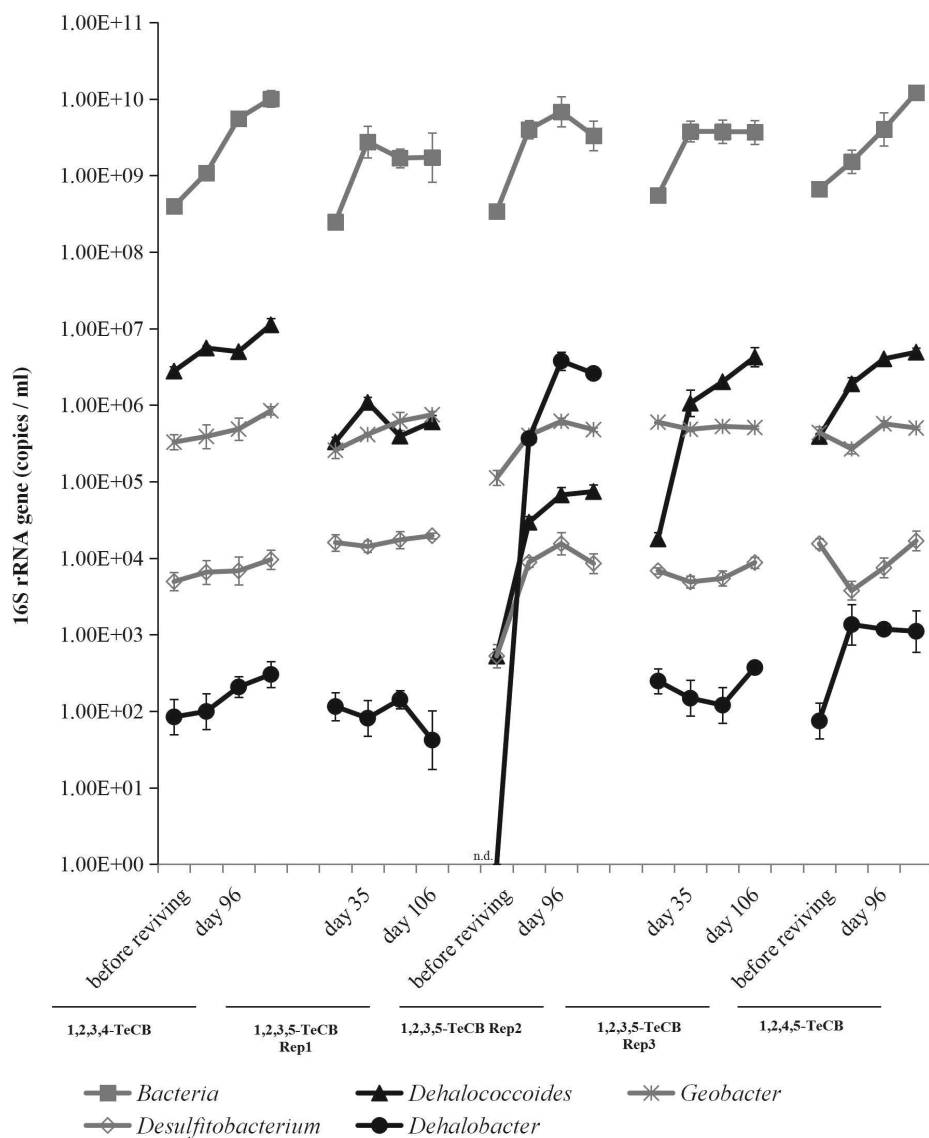


Figure 5.6 Quantitative PCR (qPCR) targeting 16S rRNA genes of *Dehalobacter*, *Desulfitobacterium*, *Geobacter* and *Dehalococcoides*, and of total bacteria during 161 days reviving of TeCB-fed microcosms derived from harbor sludge. Microcosm set up and sampling times are labelled as Figure 5.1 (step3). Rep1, Rep2 and Rep3 are triplicate original bottles fed with 1,2,3,5-TeCB. Error bars indicate standard deviations of qPCR technical triplicates. n.d. nondetectable.

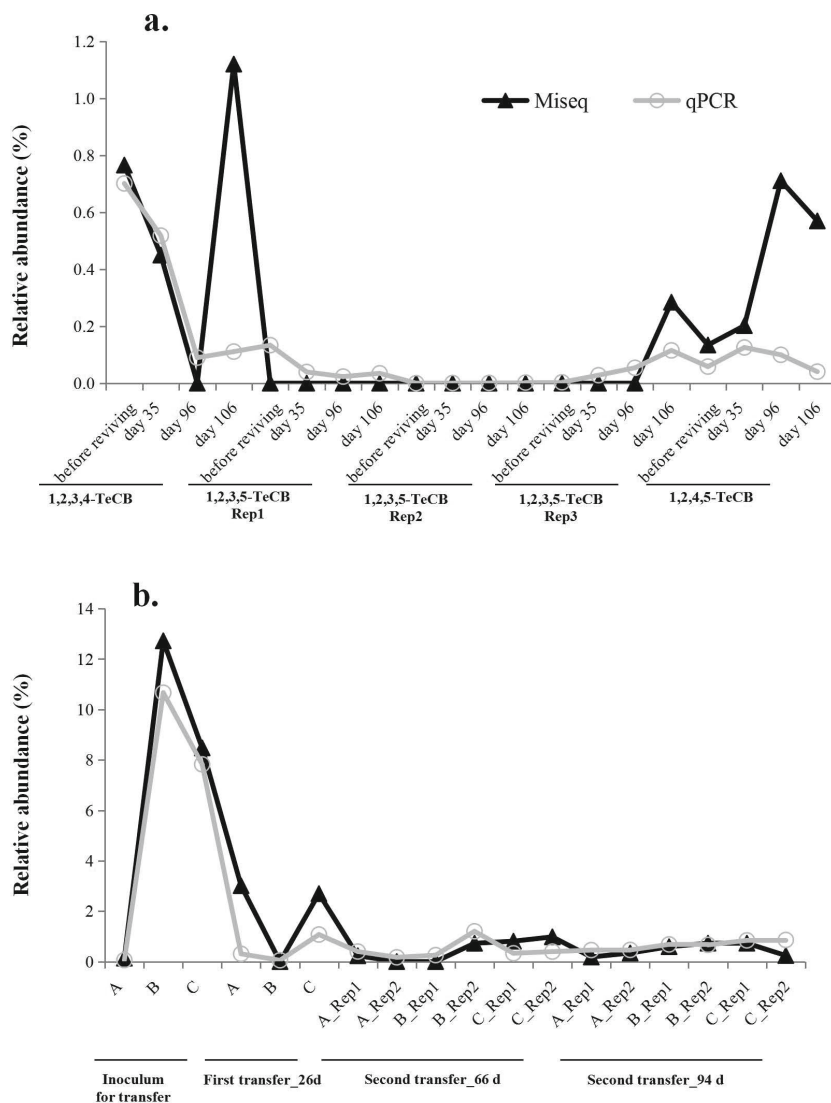


Figure 5.7 Relative abundance of *Dehalococcoides* in TeCB-fed microcosms during 161 days of reviving (a) and 1,2,4,5-TeCB dechlorinating enrichments during serial transfers (b). **a.** Rep1, Rep2 and Rep3 are triplicate original bottles fed with 1,2,3,5-TeCB. Sampling times are labelled the same as Figure 5.1 (step 3). **b.** Rep1 and 2 are duplicates. Inoculum and sampling time are labeled as in Figure 5.1 (step 6).

Discussion

Dechlorination of 1,2,3,4-, 1,2,3,5- and 1,2,4,5-TeCB isomers to DCB and/or MCB was observed in anoxic microcosms derived from harbor sludge, and enriched consortia showed selectivity in mediating thermodynamically more favorable steps in the reductive dechlorination of TeCB isomers (Figure 5.4). Such preference for thermodynamically more favorable CB dechlorination pathways was shown before in an anaerobic microbial consortium originating from lake sediment [339], but in that report, the TeCB isomers were dechlorinated with 1,3,5-TCB or 1,2,4-TCB as the end products. In our study, 1,2,3,5-TeCB was mainly dechlorinated to 1,3,5-TCB with the removal of doubly flanked chlorine, and subsequent removal of the unflanked *meta*-substituted chlorines to produce 1,3-DCB (Figure 5.2b) and MCB (Figure 5.2c&d). This implies that the enriched consortia from harbor sludge may host multiple CB dechlorinators with complementary dechlorination function. The dechlorination of HCB via 1,2,3,5-TeCB was found to predominate in cultures derived from estuarine sediment with 1,3,5-TCB [115] and 1,3-DCB [343] as the end products. Additionally, 1,3,5-TCB production from 1,2,3,5-TeCB was reported before for *D. mccartyi* strains [27, 159, 335], however, no further dechlorination of 1,3,5-TCB was observed with those isolates and the underlying reason is still not clear. A different dechlorination pattern of 1,2,3,5-TeCB, with even less favourable thermodynamics, was found in *Dehalobacter* sp. 13DCB1, which predominantly dechlorinated singly flanked *ortho*-substituted chlorine with 1,2,4-TCB as the dominant intermediate leading to the formation of 1,4-DCB as the main end product and small amounts of MCB [57]. 1,2,4-TCB and 1,4-DCB were also detected in our 1,2,3,5-TeCB amended microcosms, albeit not as dominant dechlorination products (Figure 5.4). Up to date, the dechlorination of 1,2,3,5-TeCB with the removal of unflanked chlorine to produce 1,2,3-TCB has not been reported.

1,2,3-TCB was shown to be the only product of 1,2,3,4-TeCB dechlorination by *Dehalobacter* strain 12DCB1 and 14DCB1 [57] and could be further dechlorinated to 1,3-DCB by *D. mccartyi* strain DCMB5 [27]. However, those metabolites were not detected in our study (Figure 5.2a), probably because 1,2,3-TCB production was thermodynamically less favourable relative to the reductive removal of doubly flanked chlorine from 1,2,3,4-TeCB that leads to 1,2,4-TCB formation (Figure 5.4).

Furthermore, 1,2,4-TCB was the only TCB detected in the 1,2,4,5-TeCB amended microcosms that was predominantly dechlorinated to 1,4-DCB with slightly larger negative

change of Gibbs free energy than formation of 1,3-DCB by the microcosm originated from contaminated harbor sludge (Figure 5.4). In contrast, more 1,3-DCB than 1,4-DCB was produced via 1,2,4-TCB in the transferred cultures derived from Kanaal Ieper-Ijzer sludge (Figure 5.3b&c), indicating the impact of inoculum source on the preference for the prevailing biotransformation pathway. The formation of 1,2-DCB from 1,2,4-TCB with the removal of unflanked meta-chlorine and lowest energy release was neither observed in our microcosms (Figure 5.4) nor in other reported enrichment cultures [330, 336, 340, 354] or in studies using CB dechlorinating isolates [27, 57, 159, 335].

In our study, *Dehalococcoides* and *Dehalobacter* were detected in all TeCB-fed microcosms as putative TeCB dechlorinators. In most of the microcosms, the growth of *Dehalococcoides* during 161 days of cultivation was associated with the addition of new spikes of the different TeCB substrates and subsequent TeCB dechlorination (Figure 5.2 and 6). However, no *Dehalococcoides* isolates have been previously reported to dechlorinate 1,2,3,5-TeCB to 1,4-DCB via 1,2,4-TCB or to dechlorinate any DCBs to MCB. Hence, the detected 1,2,4-TCB and 1,4-DCB in one of the 1,2,3,5-TeCB-fed microcosm among triplicates and the MCB increase in all the 1,2,3,4- and 1,2,3,5-TeCB-fed microcosms were possibly due to the dechlorination activity of *Dehalobacter* or other unknown dechlorinators. Besides, *Dehalococcoides* and *Dehalobacter* both use hydrogen as the sole electron donor for reductive dehalogenation and are corrinoid auxotroph [40, 52, 57, 65]. Lack of a negative effect of vitamin B12 starvation on the 1,2,4,5-TeCB dechlorination capability of the transferred cultures indicated the presence of corrinoid producers in the microbial community that fulfill nutritional requirements of the TeCB dechlorinators. Additionally, *Dehalococcoides* are Gram-negative [40] and members of *Dehalobacter* were found resistant to vancomycin before [62], which might explain that vancomycin treatment did not fully abolish dechlorination activity. Nevertheless, strong inhibition of vancomycin on dechlorination activity was observed in our study, and the 1,2,4,5-TeCB dechlorination pathway was changed in transferred cultures (Figure S5.1). This sensitivity to vancomycin was observed before in HCB and 1,3,5-TCB fed microcosms derived from anoxic sediments [355], and therefore indicated the (in)direct involvement of vancomycin-sensitive bacteria in 1,2,4,5-TeCB dechlorination in transferred cultures.

Bacteria belonging to the genera *Desulfitobacterium* and *Geobacter* were also detected in the TeCB-fed microcosms by qPCR (Figure 5.6). Although high abundance of *Geobacter* was previously detected by qPCR in HCB dechlorinating consortia [336], and *Desulfitobacterium*

predominated CBs-dechlorinating bacterial communities [340], these two genera have not been previously reported to dechlorinate CB, and hence no conclusion can be drawn on their (direct) role in dechlorination of TeCB isomers and corresponding daughter products. Moreover, *Desulfitobacterium* and *Geobacter* are known as facultative OHRB with versatile metabolism [68, 124] that can use lactate as electron donor and other electron acceptors like nitrate and sulfite supplied as media composition in our study for their growth and maintain their populations. Additionally, *Desulfitobacterium* spp. were reported to be capable of *de novo* corrinoid biosynthesis [298], and many essential genes that are involved in cobalamin synthesis and possible corrinoid transporters were found in the genome of *Geobacter lovleyi* [128], suggesting that these organisms may serve as corrinoid producers in the enrichments studied here.

In our study, members of the phylum *Fibrobacteres* were detected in all TeCB-fed microcosms, except one of three 1,2,3,5-TeCB-fed microcosms, with relative abundances ranging between 0 and 49.5% (Figure 5.5). Members of this phylum are known as plant polymer-degrading bacteria and were reported before from anaerobic cellulose-fed digesters and herbivore guts [356]. Even though no evidence has been shown for their (direct) involvement in reductive dechlorination of organohalide pollutants, they were detected before in contaminated sites like a hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) contaminated vadose zone [357, 358] and soils contaminated with cadmium [359] and crude oil [360]. High relative abundance of sulfate-reducing *Deltaproteobacteria*, mainly from the *Desulfobacteraceae* family, with relative abundances ranging between 5 and 25.9% was detected in all TeCB-fed microcosms during 161 days of cultivation (Figure 5.5a). Sulfate reducing members of the *Deltaproteobacteria* were suggested to support *Dehalococcoides* by providing essential corrinoids [237] and therefore may have a stimulatory effect on dechlorination [352]. Besides, syntrophic association between OHRB and co-existing, non-dechlorinating microbes (i.e. sulfate-reducing bacteria) was discovered before via providing substrate to carbon monoxide-metabolizing anaerobes [37].

Increase of *Bacteroidetes* abundance was reported before after *in situ* bioaugmentation to treat trichloroethene (TCE)-contaminated groundwater, which indicated a potential syntrophy and/or synergistic relationship between *Bacteroidetes* and chlorinated ethene dechlorinators [361]. *Bacteroidetes* were also found highly enriched in our study, especially from the genus *Petrimonas* and the class *Sphingobacteriia*. Members of the genus *Petrimonas* are known as anaerobic fermentative bacteria and were previously isolated from a biodegraded oil reservoir

and confirmed as lactate fermentator [362]. Furthermore, *Petrimonas* was found predominant in a laboratory upflow anaerobic sludge blanket (UASB) reactor treating TCE containing wastewater supplied with glucose and lactate as substrates, and *Petrimonas* showed a positive correlation with TCE removal efficiency [363]. In our study, *Petrimonas* was mainly detected in the transferred cultures derived from river sludge collected in Kanaal Ieper-Ijzer (Table S5.2), but not from transfers derived from the harbor sludge, in which *Sphingobacteriia* dominated the bacterial community (Table S5.2). *Sphingobacteriia* were reported before to use lactate as carbon source [364], which might explain their considerable growth found in our study. Bacterial clones affiliated with *Sphingobacteriia* were detected before in a TCE-degrading community after *in situ* lactate biostimulation [365] and sequences of *Sphingobacteriia* (in family WCHB1-69) were detected in a dioxin-dehalogenating enrichment derived from contaminated freshwater sediment with acetate as carbon source [366] and in RDX-contaminated groundwater after acetate biostimulation [367]. In our study, a strong increase of the *Bacteroidetes* population in the transferred cultures suggested a potential link (e.g. syntrophy and/or synergy) between lactate-utilizing *Bacteroidetes* and CBs dechlorinators. Additionally, *Firmicutes*, all from the *Clostridiales* order, were detected in the transferred cultures especially in the first transfer (Figure 5.5b). In addition to known organohalide-respiring *Clostridiales* belonging to the genera *Dehalobacter* and *Desulfitobacterium*, other *Clostridiales* were reported at high relative abundances in microcosms enriched with organohalides such as CBs [340], chlorinated ethane [368], chlorinated ethene [369] and chlorinated phenols [370] as electron acceptor and lactate as the electron donor, which suggested their role as important co-existing microbial guilds in organohalide-contaminated environments and a potential partnership between non-dechlorinating corrinoid-producing *Clostridiales* and CBs dechlorinators [244]. One of the genera within the *Clostridiales* order found in this study, *Sedimentibacter*, was previously reported in co-culture with *Dehalobacter* sp. E1 and was postulated to support β -hexachlorocyclohexane (HCH) dechlorination by providing essential co-factors (e.g. corrinoid) [63, 66]. *Sedimentibacter* alone was not able to survive in HCH-fed media [63], therefore, OHRB as detoxicator of chlorinated contaminants may have stimulating impact on the growth of other anaerobic community members.

In summary, in this study we report the reductive dechlorination of three isomers of TeCB in anoxic microcosms enriched from contaminated harbor. Thermodynamically favorable dechlorinating steps were found to be selectively followed by the TeCB-fed microcosms.

Additionally, we confirmed the important roles of *Dehalobacter* and *Dehalococcoides* in CB dechlorination. Bacterial community profiling showed diverse non-dechlorinating bacteria, some of which may have stimulated TeCB dechlorination by providing essential electron donor and cofactors. Overall, these results provide further insight into the potential CBs dechlorination in contaminated harbor environments and the interaction between TeCB dechlorinators and co-existing non-dechlorinators.

Acknowledgements

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Supplementary materials

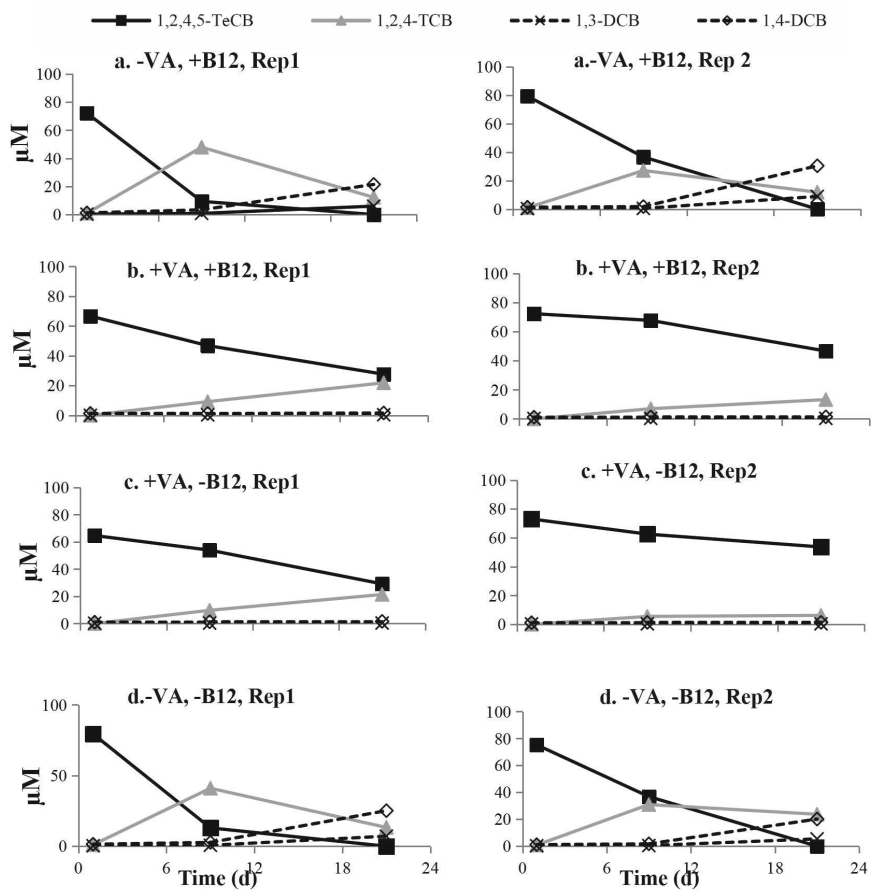


Figure S5.1 The effects of vancomycin addition and/or vitamin B12 starvation on the dechlorination activity of sediment-free 1,2,4,5-TeCB-enriched cultures derived from harbor sludge. Rep1 and 2 are replicates. +/- represents addition/deficiency of certain component in the medium.

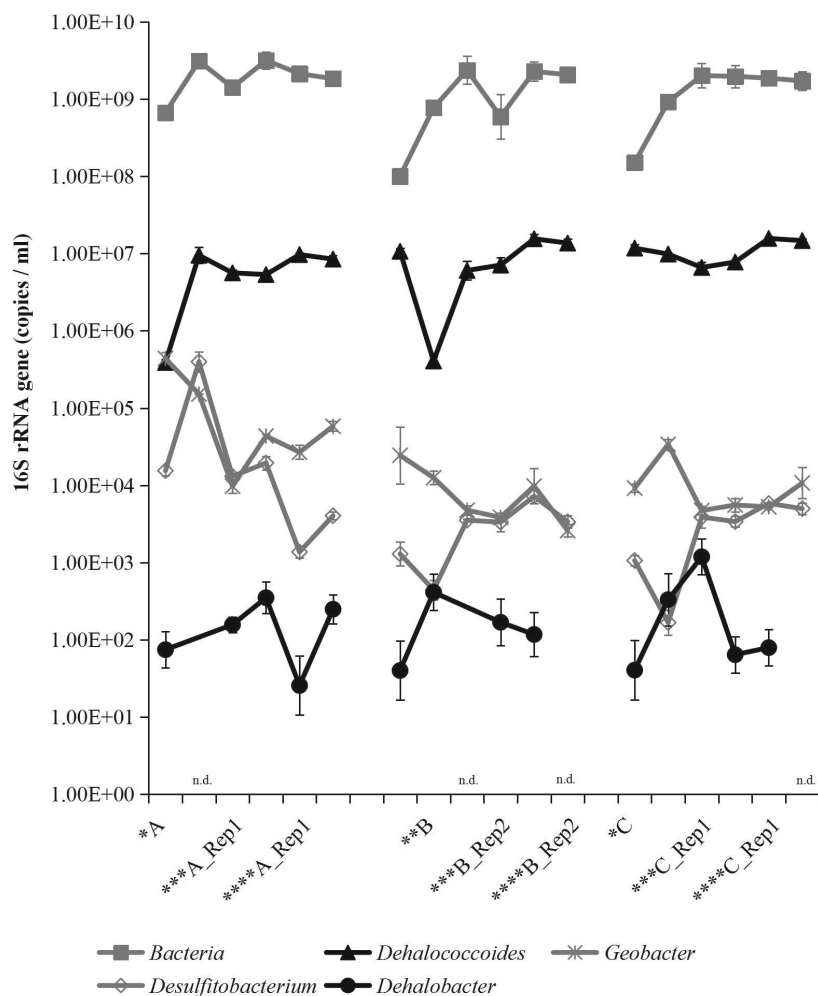


Figure S5.2 Quantitative PCR (qPCR) targeting 16S rRNA genes of *Dehalobacter*, *Desulfitobacterium*, *Geobacter*, *Dehalococcoides* and total *Bacteria* during serial transfers of 1,2,4,5-TeCB-enriched cultures. A, is the 1,2,4,5-TeCB-enriched culture derived from harbor sludge; B and C are 1,2,4,5-TeCB-enriched sediment-free cultures derived from river sludge. Symbols for sampling times are: Inoculum for transfer (*), First transfer_26d (**), Second transfer_66d (***) and Second transfer_94d (****). Inoculum and sampling time are indicated as in Figure 5.1 (step 6). Rep1 and 2 are duplicates. Error bar is the standard deviation of technical triplicates of qPCR. n.d.: nondetectable.

Table S5.1 Overview of the sequences of the MiSeq primers and Unitags.

Primer ^a	Oligonucleotide sequence (5'-3') ^b	Reference
27F-DegS	GTTYGATYMTGGCTCAG	[371]
338R-I	GCWGCCTCCCGTAGGAGT	[372]
338R-II	GCWGCCACCCGTAGGTGT	[372]
Unitag1	GAGCCGTAGCCAGTCTGC	[373]
Unitag2	GCCGTGACCGTGACATCG	[373]

^a Primer names may not correspond to original publication

^b M = A or C; R = A or G; W = A or T; Y = C or T

Table S5.2 Miseq sequences assigned to the phylum *Bacteroidetes* as observed in bacterial 16S rRNA gene-based community composition data of 1,2,4,5-TeCB-enriched cultures during serial transfers. A is the 1,2,4,5-TeCB-enriched culture derived from harbor sludge; B and C are 1,2,4,5-TeCB-enriched sediment-free cultures derived from river sludge. Inoculum and sampling time are labeled as in Figure 5.1 (step 6). Rep1 and 2 are duplicates. Taxa with relative abundance lower than 3% in all samples is sum up and shown as 'rest'. n.d.: nondetectable.

Taxon_Bacteroidetes			Inoculum for serial transfer			First transfer_26d			Second transfer_66 d						Second transfer_94 d					
Class	Family	Genus	A	B	C	A	B	C	A	Rep1	A	Rep2	B	Rep1	B	Rep2	C	Rep1	C	Rep2
<i>Bacteroidia</i>	<i>Porphyromonadaceae</i>	<i>Petrimonas</i>	n.d.	2.6	7.3			19.6	n.d.	n.d.			20.9	29.8	19.8	n.d.	n.d.	20.2	19.3	27.8
			n.d.	1.2	1.6		4.2	2.6	n.d.	n.d.			0.4	1.2	1.6	n.d.	n.d.	0.4	0.5	1.0
		<i>Proteiniphilum</i>	n.d.	6.8	7.9		38.3	35.4	80.3	84.1			46.0	52.2	37.5	34.5	68.7	49.4	53.1	39.3
<i>Sphingobacteriia</i>	WCHB1-69	-	12.0	2.7	3.6	0.3	0.6	0.8	2.1	4.1	8.9	5.4	5.3	3.1	2.7	8.1	9.0	7.4	5.0	6.8
<i>vandiniHA17</i>	-	-	1.1	1.8	1.1	n.d.	3.1	2.5	n.d.	n.d.	0.5	1.0	1.9	6.5	n.d.	n.d.	0.6	1.3	0.9	1.7
total			13.1	15.2	21.5	38.5	53.1	56.2	82.3	88.2	83.1	79.9	75.6	65.5	84.8	76.8	79.6	81.5	74.1	81.6
<i>Bacteroidetes</i>																				

Geochemical and microbial analysis of groundwater at a site with biostimulated reductive dechlorination

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Abstract

Biostimulation is widely used to enhance reductive dechlorination of chlorinated ethenes in contaminated aquifers. However, the knowledge on corresponding biogeochemical responses is limited which hampers designing effective bioremediation strategies. In this study glycerol was injected in an aquifer contaminated with *cis*-dichloroethene (cDCE) and geochemical and microbial shifts were followed for 265 days. Highly reducing conditions prevailed in impacted wells after biostimulation. Sulfate reduction was the predominant electron-accepting process. MiSeq 16S rRNA gene sequencing revealed temporarily increased relative abundance of *Firmicutes*, *Bacterioidetes* and sulfate reducing *Deltaproteobacteria* with positive correlation to organic rich and reducing conditions. Dechlorination was observed towards the end of the field experiment with concomitant enrichment of ^{13}C cDCE and increased *Dehalococcoides mccartyi* (*Dcm*) numbers. However, with fading organic-rich conditions towards the end of the experiment, dechlorination stalled mostly at vinyl chloride. This was concurrent with i) reduced relative abundances of fermenting and sulfate reducing bacteria that have been suggested to support *Dcm* growth by providing electron donor and key cofactors, ii) increased relative abundance of *Epsilonproteobacteria* and *Deferribacteres* as putative oxidizers of reduced sulfur compounds but not previously described as supporters of *Dcm* growth. Our results imply the importance of syntrophic interactions to sustain robust dechlorination after biostimulation.

Introduction

The widespread use of chlorinated ethenes (CEs) as solvents has resulted in severe groundwater contamination [181]. The incomplete transformation of CEs such as tetrachloroethene (PCE) and trichloroethene (TCE) in aquifers leads to the accumulation of *cis*-dichloroethene (cDCE) and vinyl chloride (VC) [374], the latter known as a human carcinogen [375]. *In situ* bioremediation via enhanced reductive dechlorination (ERD) has become a widely applied remediation approach [376], and is achieved by biostimulation and in some cases bioaugmentation to activate microbial reductive dechlorination by organohalide-respiration (OHR) [188, 189, 197, 377]. Organohalide-respiring bacteria (OHRB) conserve energy by OHR [7] and belong to distinct bacterial genera distributed among the phyla *Chloroflexi*, *Firmicutes*, and *Proteobacteria* [378]. While many OHRB can only dechlorinate PCE and TCE to cDCE, *Dehalococcoides mccartyi* (*Dcm*) is capable of dechlorinating CEs to VC and nontoxic ethene. *Dcm* has been recognized as a unique key to a broad range of organohalides chemically locked for mineralization. This is due to its highly specialized metabolism restricted to OHR, rich repertoire of reductive dehalogenase genes (*rdhAB*) ranging from 10 to 36 copies in a single genome [40] and widespread distribution in pristine and contaminated sites [90]. *Dcm* growth and dechlorination is more robust in mixed microbial communities as it is a fastidious microbe and depends on other organisms to provide hydrogen and acetate as electron donor and carbon source, respectively [40], to produce organic cofactors [320], to scavenge oxygen [237] and to remove the carbon monoxide that it produces but cannot tolerate [37].

Following addition of an electron donor in contaminated aquifers, OHRB will depend on composition and activity of the resident microbial community, the local geochemical and hydrological conditions and the interactions between these factors. Aquifers are biogeochemically and hydrologically highly heterogeneous/diverse and understanding biostimulation-induced subsurface feedback demands insight in microbial community dynamics and physiology and their interactions with geochemical and hydrological parameters across space and time. This knowledge is crucial to guide design and optimization of ERD efforts but remains limited. To date, ERD related microbial monitoring efforts have mainly focused on quantitative PCR (qPCR) based tracking of key OHRB and their *rdhA* genes [197, 379-382] or occasionally of selected non-dechlorinating microbial guilds [361]. While application of qPCR as a specific assay has highly refined diagnostic power and guided bioremediation efforts, it normally assays a limited number of targets and hence provides only

fragmented information regarding microbial community composition, interactions between community members and community dynamics in response to ERD. Former studies on biogeochemical trajectories of ERD either applied low resolution microbial community analysis methods such as clone libraries and terminal restriction fragment length polymorphism of PCR-amplified 16S rRNA genes [189, 365, 383] or were not complemented with detailed analysis of geochemical parameters and CE concentration dynamics [384]. Some studies have addressed microbial community dynamics [385, 386] and interactions with geochemical parameters [387-391] at CE contaminated sites undergoing natural attenuation without ERD. However, unlike the steady-state conditions under natural attenuation, biostimulation induces perturbations in the aquifer ecosystem that can reshape both living systems and the abiotic environment [392].

In this study an *in situ* glycerol injection experiment was conducted to stimulate reductive dechlorination of cDCE and VC as products of a former TCE contamination at a site in an industrial area in Vilvoorde, Belgium [199]. Before and after injection groundwater geochemical parameters and bacterial community composition were analyzed over a period of 265 days. In parallel, the progress of ERD was followed by enumeration of *Dcm* and relevant *rdhA* genes using qPCR and monitoring of cDCE and VC isotopic signatures using compound specific isotope analysis (CSIA). This way, we aimed to gain an integrated geochemical and microbial insight that can be used to guide the design and optimization of ERD. Results showed how temporal succession of the predominant microbial guilds governed by their known/predicted physiology and aquifer geochemical feedback promoted and/or precluded ERD. Data presented here support the notion that microbial guilds known to sustain robust *Dcm* growth/dechlorination by syntrophic interactions seem pivotal for the success of ERD.

Materials and methods

Site description, treatment and sampling procedures

At the contaminated site, a 1.4 km-wide groundwater plume mainly contaminated with cDCE flows towards the Zenne River in a northwesterly direction (Figure S6.1). Four injection wells were used to inject a single pulse of a glycerol:water mixture (1 : 3 v/v) by sonic drilling injection, at 20 L per vertical meter from 7 to 13 meters below surface (mbs) at each injection point. Eight monitoring wells were used for groundwater sampling and monitoring. Seven wells were located downstream of the injection points (wells 1-7) and one upstream (well 0, designated as the control well). All monitoring wells contained a shallow filter at 7-9 mbs (A-

filters) and a deep filter at 11-13 mbs (B-filters), except the control well that only contained a shallow filter. Groundwater was sampled from the control well and the wells impacted by glycerol injection (wells 2, 3, 5, 7A; Figure S6.1) on day 0 (March 15th 2011) before glycerol injection, and 37, 72, 195 and 265 days after injection as outlined in Supplementary materials.

DNA extraction and qPCR

For DNA extraction, 2 L groundwater samples were vacuum-filtered over a 0.22 µm membrane filter (Millipore, USA), and filters were stored at -80 °C. Filters were cut into small strips for DNA extraction by the FastDNA Spin Kit for Soil (MP Biomedicals). qPCRs were performed in triplicate in 25-µL reactions in an iQ5 iCycler using the iQ SYBR Green Supermix kit (Bio-Rad, Veenendaal, the Netherlands). A list of target genes, primers and thermal cycling conditions for qPCRs is shown in Table S6.1. Standard curves were obtained using serial dilutions of a known concentration of plasmid DNA containing a suitable fragment of the target genes.

Bacterial community analysis

A 2-step PCR strategy was used to generate barcoded amplicons from the V1-V2 region of the 16S rRNA gene (primers and thermal cycling conditions shown in Table S6.2) as shown in chapter 5. The PCR product was purified with HighPrepTM (Magbio Genomics, Rockville, MD, USA) and quantified using a Qubit 2.0 Fluorometer (Life Technologies, Darmstadt, Germany). Purified PCR products were pooled and sequenced on a MiSeq platform (GATC-Biotech, Konstanz, Germany).

Analysis of the MiSeq data

NG-Tax, an in-house pipeline [346] was used for the analysis of the 16S rRNA gene sequencing data. In brief, paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes, and those barcodes were used to separate reads by sample. Finally operational taxonomic units (OTUs) were assigned using an open reference approach and a customized SILVA 16S rRNA gene reference database [348]. Bacterial composition plots were generated using a workflow based on Quantitative Insights Into Microbial Ecology (QIIME) v1.2 [349].

Chemical and carbon isotope analysis:

Concentrations of CEs, ethene, ethane, and methane in groundwater samples were determined on a Varian GC-FID (CP-3800) as described previously [226]. Sulfate concentrations were analyzed by ion chromatography using a Dionex DX-120 ion chromatograph equipped with a

Dionex AS14A column (Dionex, Sunnyvale, CA). Concentrations of aqueous Fe(II), total Fe and NO₃-N were determined using HACH kits (HACH, USA) according the manufacturer's instructions. Dissolved organic carbon (DOC) was determined from samples as the difference between total dissolved carbon and dissolved inorganic carbon, measured with a Shimadzu TOC-5000 analyser equipped with an ASI-5000 auto-sampler. The stable carbon isotope composition of CEs in the groundwater samples were determined as described in Supplementary materials. Concentration-weighted average values of the isotope signature for CEs ($\Sigma(\text{EC})$) was calculated by weighting compound-specific values $\delta^{13}\text{C}$ weighted by their molar fractions (x) [393]:

$$\delta^{13}\text{C}_{\Sigma(\text{CE})} = \delta^{13}\text{C}_{\text{DCE}}x_{\text{DCE}} + \delta^{13}\text{C}_{\text{VC}}x_{\text{VC}}$$

Statistical analysis

In order to compare microbial communities observed at different times and locations, different statistical approaches were used based on Bray–Curtis distances. PERMANOVA (Permutational Multivariate Analysis of Variance) [394] and ANOSIM (Analysis of Similarity) [395] were performed using the Fathom Toolbox for Matlab [396]. We used 10⁴ permutations to assess significance of observed differences. MixOmics R package [397, 398] was used to integrate microbiota abundance data and geochemical parameters and to perform regression analysis. Sparse partial least squares was used for simultaneous variable selection and integration to avoid selection of spurious associations. Geochemical (microbiota) data were set as dependent (independent) variables. Interchanging both sets did not significantly impact the results. An additional threshold on correlation values was imposed by retaining only the 20% most relevant associations in the final network.

Nucleotide sequences

Nucleotide sequence data reported are available at the European Bioinformatics Institute under accession number PRJEB13312.

Results

Geochemical characterisation

Geochemical parameters were stable in the control well throughout the experiment. After biostimulation, DOC in the impacted filters was significantly ($P<0.05$) higher than before biostimulation over the experiment duration (Figure S6.2, Table S6.3). The opposite pattern was observed for dissolved oxygen (DO) and oxidation-reduction potential (ORP) that declined and were significantly ($P<0.05$) lower throughout the experiment relative to pre-

biostimulation values. ORP and DO slightly increased towards the end of the experiment. Nitrate was not detected. Fe(II) concentrations varied between day 0 and 72 in the down-gradient wells but showed a significant ($P < 0.05$) reduction by day 195 and 265 relative to pre-biostimulation concentrations. This was accompanied by appearance of black precipitate indicative of ferrous sulfide formation. In most samples throughout the experiment, the Fe(II)/Fe ratio was close to 1 indicating lack of reducible Fe(III). Starting from a background concentration of 463 ± 133 mg/l, sulfate steadily decreased to 212 ± 116 mg/l by day 195 in down-gradient wells and rebounded in most wells by day 265 reaching 267 ± 180 mg/l (Figure S6.2, Table S6.3).

Reductive dechlorination of CEs, CSIA and qPCR analysis

The pre-stimulation carbon isotope values of cDCE (-19.3 ‰ on average) and the sum of carbon isotope values of all CEs weighted by their molar fractions ($\delta^{13}\text{C}_{\Sigma(\text{CE})}$, -21.4 ‰ on average) were highly consistent in the seven filters later impacted by biostimulation ($\text{SD} < 1$ ‰). By day 195, there was clear onset of cDCE dechlorination with concomitant increase in its $\delta^{13}\text{C}$ values and increased VC concentration in most filters. $\delta^{13}\text{C}$ of VC remained more negative compared to cDCE in shallow filters of 2A, 3A and 7A and VC was not further transformed to ethene. Further degradation of cDCE to VC was observed at day 265 with progressive enrichment of cDCE in ^{13}C . In addition, a significant increase (3‰) in the resultant $\delta^{13}\text{C}_{\Sigma(\text{CE})}$ relative to the control well provided evidence for incipient dechlorination of VC in filters 2A, 2B and 7A between day 195 and 265 (Figure 6.1, Table S6.4) [399]. The evidence from CE concentrations and isotopic signatures was further corroborated by over 1-2 orders of magnitude increased abundances of *Dcm* and *vcrA* and *bvcA* genes in the impacted wells relative to the control well. Among the *rdh* genes, *bvcA* became dominant in most filters, whereas *vcrA* was dominant in well 2 reaching above 10^7 copies/L by day 265. No ERD was noted in well 5 and the control well, in which cDCE concentration as well as isotopic and qPCR signatures were rather stable (Figure 6.1, Table S6.5).

Impact on bacterial community alpha-diversity

Biostimulation significantly affected bacterial community alpha-diversity. The post-biostimulation samples (seven samples at each sampling time point, excluding control well samples) showed significantly higher ($P < 0.05$) predicted (Chao 1) and observed OTU

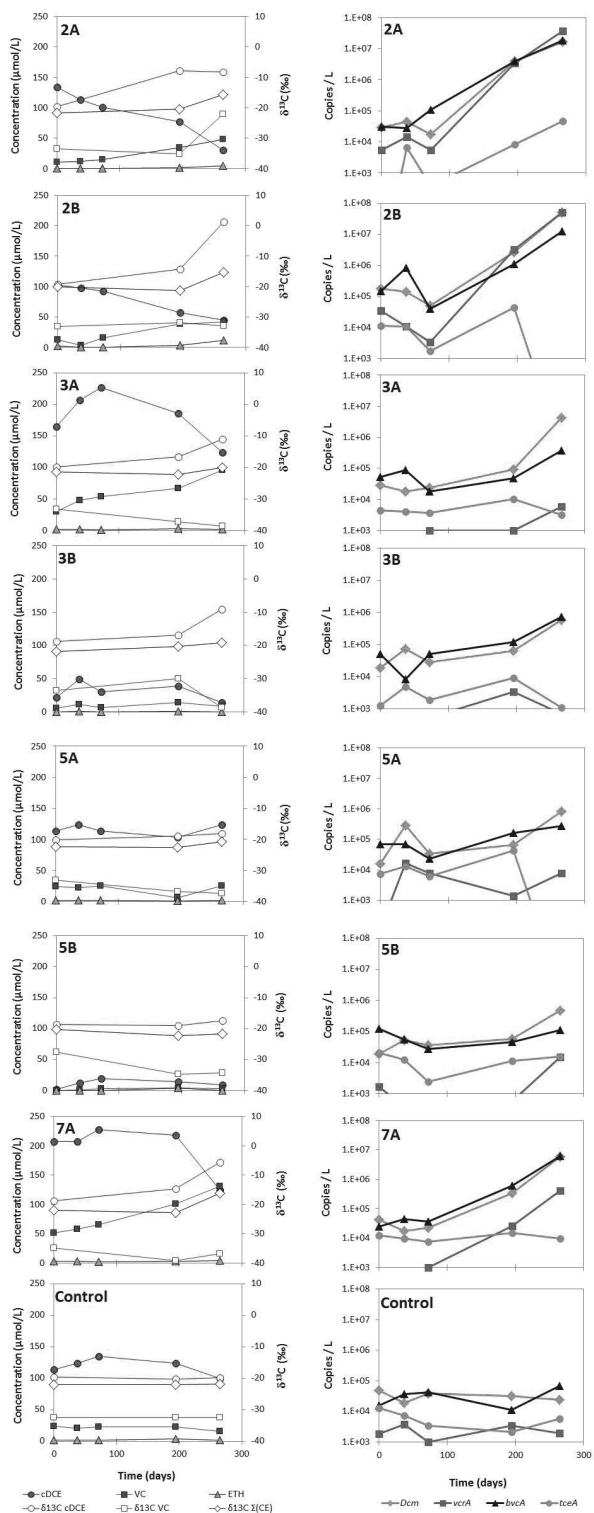


Figure 6.1 Evolution of reductive dechlorination over time. Left: concentrations of cDCE, VC, ethene, $\delta^{13}\text{C}$ of cDCE and VC and concentration-weighted average of CE $\delta^{13}\text{C}$ values ($\delta^{13}\text{C}_{\Sigma(\text{CE})}$). Right: qPCR quantification of 16S rRNA gene copy numbers of *Dcm* and reductive dehalogenase encoding *vcrA*, *bvcA* and *tceA* genes. Each qPCR value represents the average of triplicate reactions. ETH: ethene.

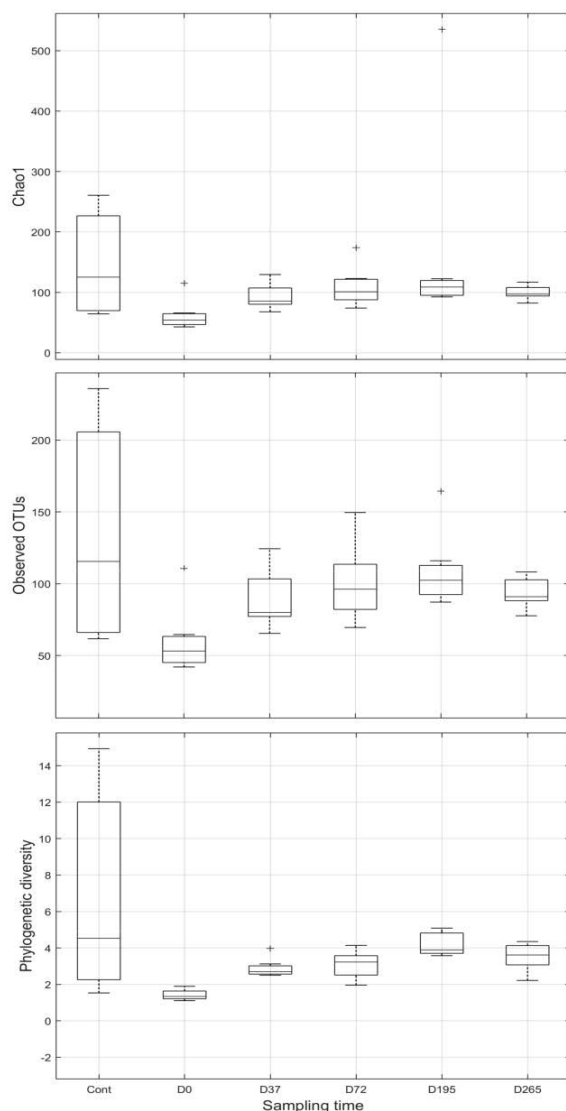


Figure 6.2 Predicted (Chao 1) and observed OTU richness, and phylogenetic diversity in the control (five samples) and impacted filters at each sampling campaign (seven samples). Cont: control well.

richness, and phylogenetic diversity (PD) relative to the pre-biostimulation samples (Figure 6.2). No significant difference was found between the post-biostimulation sampling dates, except for the PD at day 195 that was significantly higher ($P<0.05$) than at day 37 and 72. The control well showed progressively increasing alpha-diversity over time until day 195 (Figure S6.3).

Bacterial community succession

The pre-biostimulation bacterial community composition showed high between-sample similarity including the control samples (Figure S6.4), and was significantly different from all post-biostimulation communities (Table S6.7). Remarkably, among the post-biostimulation communities, samples of day 265 were not significantly different from those taken at the well (Figure S6.4, Table S6.6). At day 0, the bacterial community was dominated by *Campylobacteriales* (*Epsilonproteobacteria*) followed by *Flavobacteriales* (*Bacteroidetes*), representing 75% and 18% of the average relative abundance (ARA), respectively (Figure 6.3, Table S6.6). Recorded *Campylobacteriales* comprised members of *Sulfuricurvum*, *Sulfurospirillum* and *Arcobacter* genera, while *Flavobacterium* was the main genus within *Flavobacteriales* (Figure S6.5, Table S6.6). Following biostimulation, clear shifts in the bacterial community structure were noted. Except in the 2B and 7A filters that did not show DOC change at day 37 (Figure S6.2, Table S6.4), *Campylobacteriales* and *Flavobacteriales* were greatly reduced in ARA relative to day 0 and replaced by fermentative members of the *Firmicutes* (*Clostridia* and *Bacilli* orders) and *Bacteroidetes* (*Bacteroidia* order) and by sulfate reducing *Deltaproteobacteria* (*Desulfobacteriales* order) (Figure 6.3, Table S6.6). Within the *Campylobacteriales* members of the genus *Sulfurospirillum* became predominant whereas *Sulfuricurvum*, *Arcobacter* and *Flavobacterium* showed drastic reduction in ARA. Members of the genus *Trichococcus* (*Bacilli*) within the *Firmicutes* increased substantially in relative abundance in 5A and 5B filters (Figure S6.5, Table S6.6) that received the highest DOC input, whereas *Clostridia* genera of *Acetobacterium*, *Clostridium* and *Pelosinus* were noted in most filters. By day 72, *Campylobacteriales* and *Flavobacteriales* dropped to below 20 and 1% ARA, respectively in impacted filters. In contrast, the sulfate reducing *Desulfobacteriales* dominated by *Desulfobulbus* genus flourished followed by *Bacteroidia*, *Clostridia* and *Bacilli*. *Desulfobacteriales* continued to dominate communities observed in day 195 samples while *Bacteroidia*, *Clostridia* and *Bacilli* were fading. Interestingly, at day 195 and day 265, the *Campylobacteriales* became predominant again in 5A and 5B filters, reaching 60% and 30% in relative abundance, respectively. Instead, *Desulfobacteriales* were reduced in

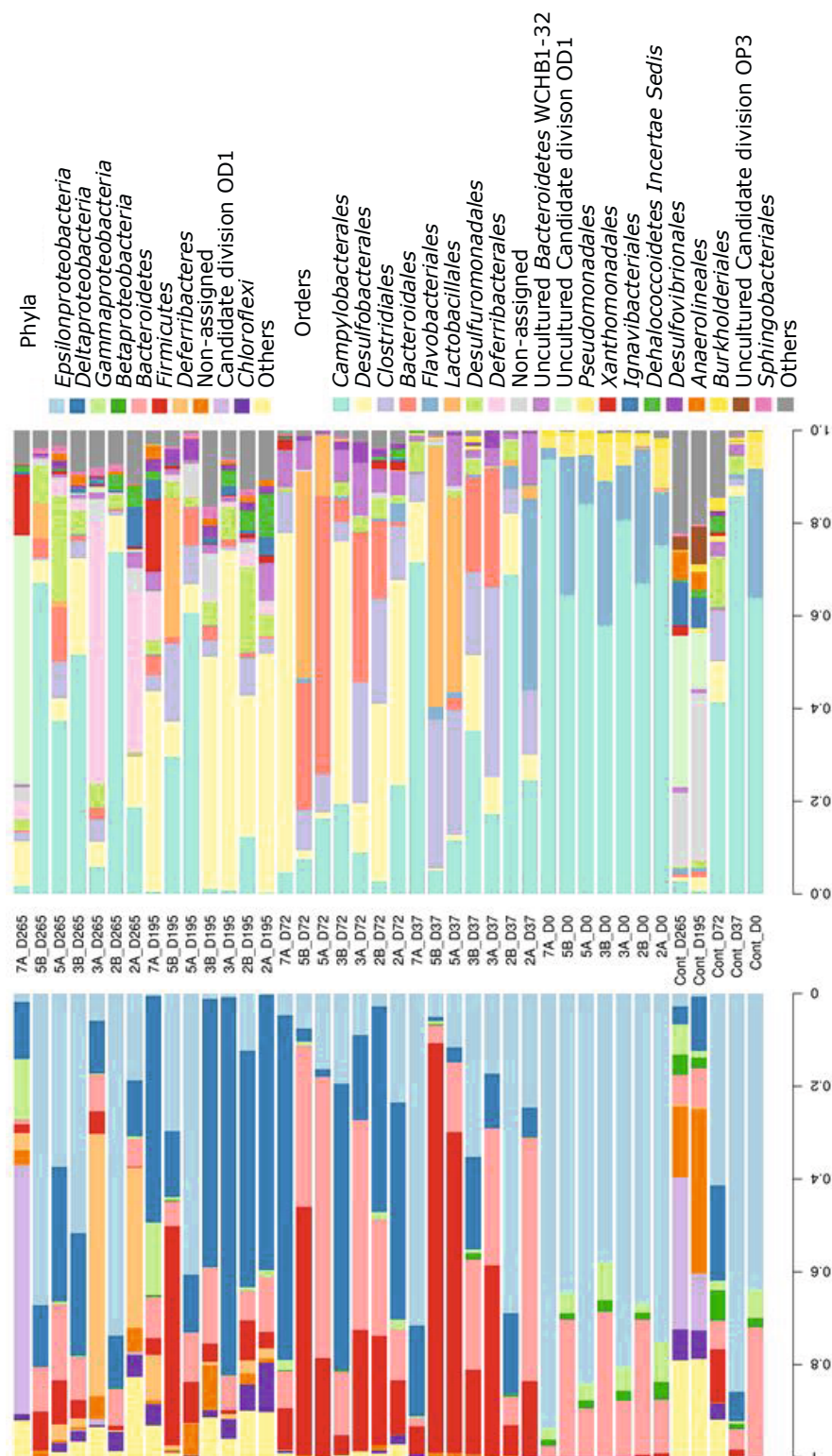


Figure 6.3 Distribution of the most abundant phyla (left) and orders (right) in the control and impacted filters. Cont: control well.

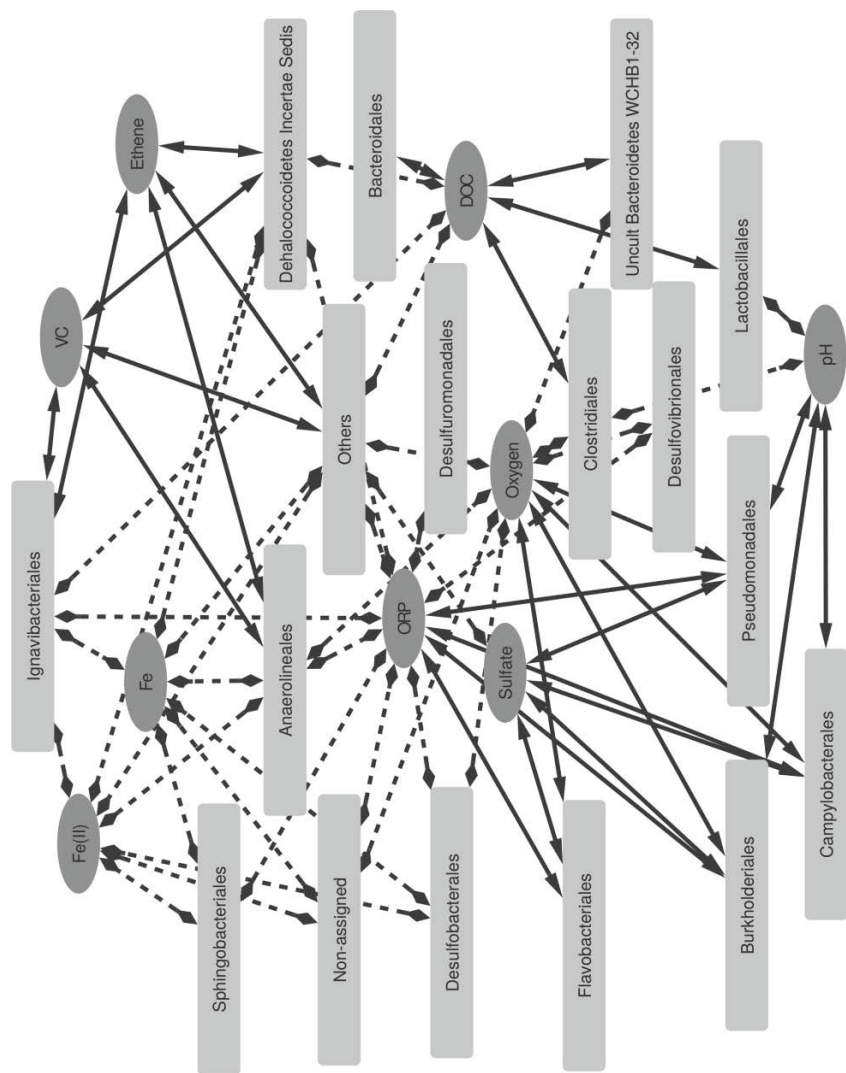


Figure 6.4 Association network of geochemical and microbial interactions. Rectangular (lighter) and elliptic (darker) nodes represent orders (Table S6.6) and geochemical parameters (Table S6.3-4), respectively. Edges represent positive (solid lines with arrowheads) and negative (dashed lines with diamond-shape heads) correlations between an order and a geochemical parameter. Note that the correlation between *Bacteroidales* and DOC is positive.

ARA at day 265. *Dehalococcoidetes* and *Geobacter* did not surpass 1% of the community at any time point, and other OHRB including *Dehalobacter*, *Desulfotobacterium*, *Desulfomonile*, *Desulfuromonas* were not detected. Furthermore, there was notable emergence of sequences associated with *Deferribacterales* (*Deferribacteres*) and the candidate phylum OD1 by day 195 and 265, which were not observed in pre-biostimulation samples. The bacterial community showed a distinct succession pattern in the control well compared to stimulated wells. While *Campylobacteriales* was the most predominant taxon at day 0 and 37, their relative abundance dropped to 41% by day 72 and below 2% by days 195 and 265. The community in the control well became more diverse over time (Figure 6.2) with increased abundance of non-assigned reads and candidate phylum OD1 (Figure 6.3, Table S6.6).

Geochemical and microbial interactions

The relative abundances of the orders *Campylobacteriales*, *Flavobacteriales*, *Burkholderiales* and *Pseudomonadales* were positively correlated with DO, ORP and sulfate (Figure 6.4 and S7.6). Putative fermenters belonging to *Clostridiales*, *Lactobacillales*, *Bacteroidales* and uncultured *Bacterioidetes* WCHB1-32 showed a positive correlation with DOC whereas DO was inversely correlated with the relative abundance of *Clostridiales* and uncultured *Bacterioidetes* WCHB1-32. A negative correlation was noted between Fe(II)/Fe and *Dehalococcoidetes*.

Discussion

There is growing interest in using the capacities of indigenous microbial communities to remediate CE contaminated aquifers by ERD. In order to improve CE contaminated site bioremediation, the biogeochemical shifts induced by biostimulation must be understood, and the abundance and activity of key OHRB must be placed into the context of interlinked microbial networks, including the supporting/competing electron donor/acceptor processes.

Geochemical and microbial dynamics: pre-biostimulation phase

The almost complete removal of TCE and the dominance of cDCE before biostimulation indicated naturally occurring reductive dechlorination and hence TCE depletion (Table S6.4) formerly reported to be present at this site [199]. This could be an explanation for the presence of *Sulfurospirillum* as non-obligate dechlorinator of TCE to cDCE [142] in the pre-biostimulation samples that was also reported from other TCE contaminated aquifers [365, 383, 400]. Some members of this genus are capable of chemolithoautotrophic growth by coupling nitrate and oxygen reduction to the oxidation of sulfide, sulfur and thiosulfate [142,

401] and therefore, their growth using sulfur released into groundwater from soils, rocks and minerals [402] coupled to oxygen reduction cannot be excluded. *Sulfuricurvum* as another predominant genus currently contains only one characterised isolate, *Sulfuricurvum kujiense* strain YK-1, an obligate chemolithoautotroph [403] that grows by oxidation of reduced sulfur compounds coupled to nitrate and oxygen (microaerophilic condition) respiration (Kodama and Watanabe, 2003). Although dechlorination or syntrophic interactions with OHRB by this strain are not reported, *Sulfuricurvum* was detected at TCE contaminated sites [404, 405] and in 1,2-dichloroethane dechlorinating enrichment cultures [368]. Another predominant genus in pre-biostimulation samples was *Flavobacterium* whose members play an important role in decomposition of organic materials by hydrolysing organic polymers such as proteins and polysaccharides produced from cell debris in oligotrophic environments [406, 407].

Geochemical and microbial dynamics: post-biostimulation phase

Glycerol injection induced cDCE concentration reduction accompanied by a shift of carbon isotope values. The most dynamic period was after day 72 during which the average $\delta^{13}\text{C}$ cDCE values increased by +3.9 ‰ at day 195 and by +5.7‰ at day 265 (Figure 6.5). This development was reflected by a successional change in groundwater bacterial community composition. There was a sharp increase in *Firmicutes* (*Clostridiales* and *Lactobacillales*) ARA followed by *Bacteroidetes* (*Bacteroidales*). These taxa that also showed positive correlation to DOC (Figure 6.4) had low initial ARA and decreased again towards the end of the field experiment. Other studies at CE contaminated sites reported a similar transient peak of these microbes upon biostimulation [361, 365, 384]. The emergence of members of the genera *Trichococcus*, *Clostridium* and *Pelosinus* following biostimulation (Figure S6.5, Table S6.6) is likely due to their glycerol fermentation capacity [408-411]. Moreover, *Pelosinus* strains were shown to sustain growth of *Dcm* by providing the necessary corrinoids [95, 300]. The observed putative acetogenic bacteria belonging to *Acetobacterium*, *Clostridium* and *Spirochaetaceae* (Figure S6.5, Table S6.6) have been proposed to stimulate *Dcm* growth by production of acetate and corrinoid cofactors [324, 361, 365].

Sulfate reduction appeared to be the prevalent electron-accepting process at the site. Between day 37 and 195, *Desulfobulbaceae* flourished that can perform incomplete oxidation of propionate coupled to sulfate reduction [412] yielding acetate and sulfide that can stimulate and inhibit *Dcm* growth, respectively. On the other hand, both glycerol and its degradation product 1,3-propanediol can be converted to acetate using sulfate as electron acceptor by members of the genus *Desulfovibrio* [413, 414] that were present during the same period.

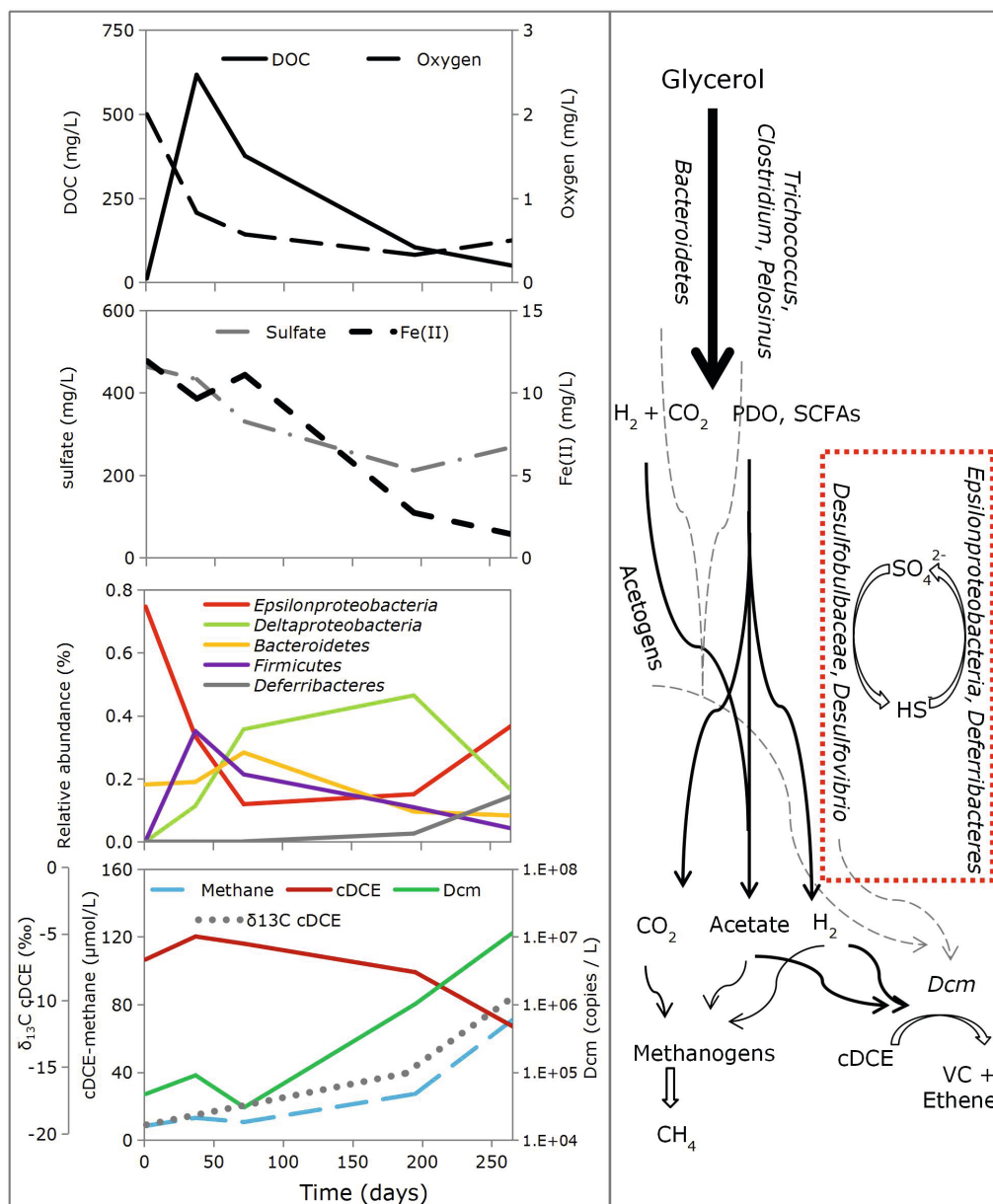


Figure 6.5 Summary of major geochemical and microbial evolution before and after biostimulation (left), and a conceptual model of glycerol degradation based of the observed taxa from MiSeq analysis and their known/putative physiology (right). The proposed sulfur cycle is shown in red dotted square and the putative corrinoid supply to *Dcm* is shown by dashed gray arrows. All values are averages of the seven impacted filters. Error bars are not shown for clarity. PDO: 1,3-propanediol, SCFA: short chain fatty acids.

Further, *Desulfovibrio* strains are known to sustain *Dcm* growth by providing hydrogen, acetate and corrinoid cofactors [325]. However, sulfate at >500 mg/l as observed in 3B and 7A filters (Figure S6.2, Table S6.3) might be inhibitory to *Dcm* due to electron donor exhaustion [415], though this seems unlikely on the basis of the observed dechlorination at these filters (Figure 6.1). Methanogenesis as a sink of hydrogen and acetate [240] did not seem to be a major concern for OHR except at filters 5A and 5B that showed the highest DOC levels upon biostimulation and enhanced methanogenesis by day 265. This can be an explanation for lack of dechlorination at these filters indicating a competitive advantage for dechlorination over methanogenesis in other filters with lower DOC levels [239].

Geochemical and microbial dynamics: the control well

Despite stable geochemical parameters in the up-gradient control well, the bacterial community observed at days 195 and 265 highly diverged from the original state. This could be due to the high hydraulic conductivity of 1-3 m/day at this site [222] facilitating a rapid dispersion and community succession [416]. Accordingly, the pre-biostimulation bacterial community in the stimulated wells was significantly different from the control well (Table S6.7) whereas the geochemical parameters were similar between the pre-biostimulation and control well samples and clustered closely in the PCA plot (Figure S6.7). On the other hand, the bacterial community in the stimulated wells at day 265 was significantly different from the pre-biostimulation community but not from the control well (Table S6.7). This indicates that the resident taxa in the impacted wells exploiting the stimulated conditions were also exposed to the arriving immigrant taxa brought by groundwater flow, in line with what has previously been suggested by Shade and co-workers [392]. However, it was only towards the end of the experiment (and hence fading stimulation) that the immigrant taxa became established in stimulated wells which can be described by the niche occupation concept outlined in Figure S6.8. The observed geochemical stability but compositional instability of the control well over time indicates the necessity of previous knowledge of community behavior in relation to site geochemistry/hydrology as baseline prior to biostimulation to better guide intensity, duration and location of post-biostimulation sampling.

Putative sulfur cycle following biostimulation

After the dominance of sulfate reducers between day 37 and 195 and concurrent sulfate depletion, sulfate concentrations increased again in most wells at day 265. This was accompanied by a substantial reduction of *Desulfobulbaceae* ARA, re-establishment of initially abundant *Sulfuricurvum* and *Sulfurospirillum*, and appearance of putative sulfur

oxidizers i.e. *Deferribacteres* and the candidate phylum OD1, which were barely detectable in pre-biostimulation samples (Figure 6.3). The chemolithoautotrophic oxidation of the reduced sulfur compounds by *Sulfuricurvum* and *Sulfurospirillum* coupled to reduction of DO that was slightly increasing during the late phase may explain the increased sulfate levels. Accordingly, the epsilonproteobacterial *Campylobacterales* showed positive correlation with DO and ORP (Figure 6.4). Enrichment of *Epsilonproteobacteria* as the successors of *Deltaproteobacteria* was previously reported during biostimulation of aquifers contaminated with uranium [161, 417] and CEs [384]. The resulting reduced sulfide levels can in turn support reductive dechlorination by decreasing the toxicity of sulfide to *Dcm* [161, 415].

Emergence of candidate phylum OD1

Members of the candidate phylum OD1 have mostly been connected with suboxic and anoxic pristine sulfur-rich environments [418-420]. However, OD1 [421] and another candidate division, OP11, [384] also appeared at a later stage during aquifer biostimulation for CE and uranium bioremediation possibly due to ample presence of reduced sulfur compounds (as products of sulfate reducers) and the sulfur oxidation capacity of these candidate phyla [419, 422]. Besides, owing to their obligatory fermentative lifestyle, the production of hydrogen and organic acids (e.g. acetate) from complex organic materials such as decaying biomass from microbial blooms by these candid phyla can fuel respiratory processes with nitrate, sulfate and Fe(III) [422, 423] and perhaps CEs as terminal electron acceptors. Interestingly, OD1 has not been reported as member of CE dechlorinating communities in enrichment cultures [129, 154, 236, 237, 352, 424-429] that usually receive constant supply of easily accessible electron donors, presumably circumventing the services provided by this phylum and hence exterminating it from the dechlorinating enrichment cultures.

Resilient and sensitive taxa

Epsilonproteobacterial *Sulfuricurvum* and *Sulfurospirillum* recovered in ARA towards the end of the experiment, though remarkably only in stimulated wells. Resilience of the members of *Sulfurospirillum* spp. was previously reported in permanganate treated enrichment cultures [352]. In contrast, the initially predominant genera *Arcobacter* and *Flavobacterium* did not recover, which could be due to their high sensitivity to biostimulation-induced perturbation. However, they were also barely detectable from the up-gradient control well towards the end of the experiment indicating a role of other factors such as hydrology in controlling community succession.

Conclusions and perspectives

Based on the observed geochemical, isotopic and bacterial patterns, a conceptual model of the metabolic interactions within the microbial foodweb during biostimulation was proposed (Figure 6.5). cDCE dechlorination was only noted by day 195 and it was not fully converted to ethene likely due to the fading organic-rich condition towards the end of the experiment. Remarkably, during the same period, the relative abundance of non-dechlorinating fermenters and sulfate reducers decreased that is considered to stimulate robust *Dcm* dechlorination by providing organic cofactors such as the key vitamin B12 [237, 325]. The increased relative abundance of *Deferribacteres* and *Epsilonproteobacteria* by the end of the field experiment likely supported the *Dcm* population by reducing sulfide level/toxicity. However, to our knowledge, these taxa are not known to provide the more important organic cofactors such as vitamin B12 needed by *Dcm*, hence they are not likely “sufficient” as companions to sustain *Dcm* growth. In line with this, other studies showed the importance of particular syntrophic partners belonging to *Firmicutes* and *Deltaproteobacteria* in providing the “right” cobamide lower base of vitamin B12 that is vital for *Dcm* dechlorination [95, 300] but cannot be provided even by closely related microbes [93, 299].

In case of incomplete dechlorination, biostimulation is performed together with bioaugmentation with dechlorinating enrichment cultures containing *Dcm* populations but also their non-dechlorinating partners [361, 383]. In fact, sustained presence of non-dechlorinating guilds that support *Dcm* growth might be the key to success of bioaugmentation as opposed to the initial biostimulation. Accordingly, a recent field biostimulation failed to induce complete dechlorination while dechlorination to ethene was achieved after bioaugmentation. This was concurrent with enrichment of *Bacteroidetes* while *Dcm* and *vcrA* concentrations were rather stable [361]. Therefore, in addition to monitoring dechlorinating guilds, future ERD monitoring efforts must consider composition and successional patterns of supportive non-dechlorination community members as well as geochemical factors controlling them to ensure robust *Dcm* growth and activity. This knowledge is pivotal to establish and maintain the required syntrophic relationships for OHR under challenging field conditions.

Acknowledgements

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Supplementary materials

Sampling procedure

All samples were collected using a peristaltic pump in polyethylene tubes (Eijkelkamp, Giesbeek, The Netherlands), following purging until electrical conductivity, pH, dissolved oxygen (DO), temperature and oxidation-reduction potential (ORP) parameters stabilized. Those parameters were measured using a flow-through cell (Eijkelkamp, Giesbeek, The Netherlands), equipped with a multimeter (MultiLine F/SET3, WTW, Weilheim, Germany) and suitable electrodes for determining temperature and electrical conductivity (TetraCon 325, WTW, Weilheim, Germany), pH (Sen Tix 41, WTW, Weilheim, Germany), DO (CellOx 325, WTW, Weilheim, Germany) and ORP (Oxitrode Platinum Hamilton, Bonaduz, Switzerland). Samples for stable carbon isotope analysis were collected in 250 mL amber bottles without leaving headspace, and immediately treated with NaOH pellets to reach a pH above 11 to stop microbial activity.

Carbon isotope analysis

The stable carbon isotope composition of chlorinated ethenes (CEs) in the groundwater samples were determined on a gas chromatograph-combustion-isotope ratio mass spectrometer (TRACE GC Ultra, GC combustion interface, MAT 253, all from Thermo Fisher Scientific, Bremen, Germany). Each sample was analyzed in duplicate via purge-and-trap, cryofocussing and subsequent analysis on a VOCOL 60 m x 0.25 mm ID. Authentic laboratory standards were used for identification of chlorinated compounds and to improve the accuracy of $^{13}\text{C}/^{12}\text{C}$ analyses by linear correction of raw isotope values. Isotope ratios (R) of samples are expressed in the delta notation ($\delta^{13}\text{C}$) in per mil [‰] relative to the international Vienna Pee Dee Belemnite standard (VPDB, $^{13}\text{C}/^{12}\text{C} = (11237.2 \pm 2.9) \times 10^{-6}$) according to:

$$\delta^{13}\text{C}_{\text{sample}} [\text{‰}] = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000$$

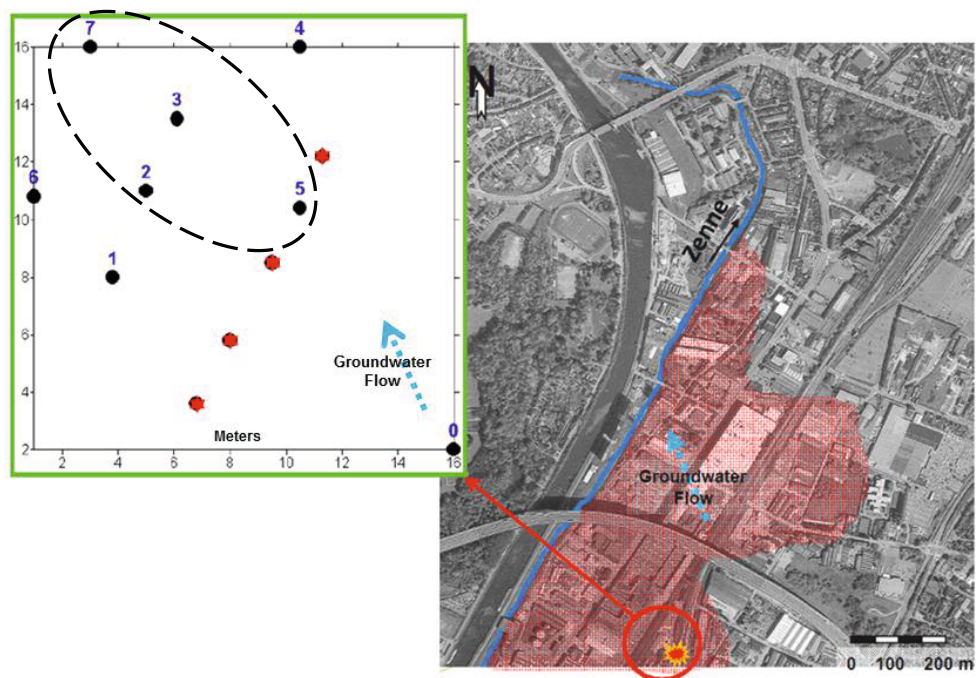


Figure S6.1 Schematic presentation of the study site close to the Zenne River. Monitoring wells (black circles) and injection wells used for glycerol delivery (red stars) at the site are indicated. Well 0, located upstream of the injection wells, is taken as control. Samples were taken from the monitoring wells impacted by glycerol injection (shown in the dashed oval) i.e. well 2, 3, 5 and 7 (only shallow filter, 7A) as well as for the control well throughout the study.

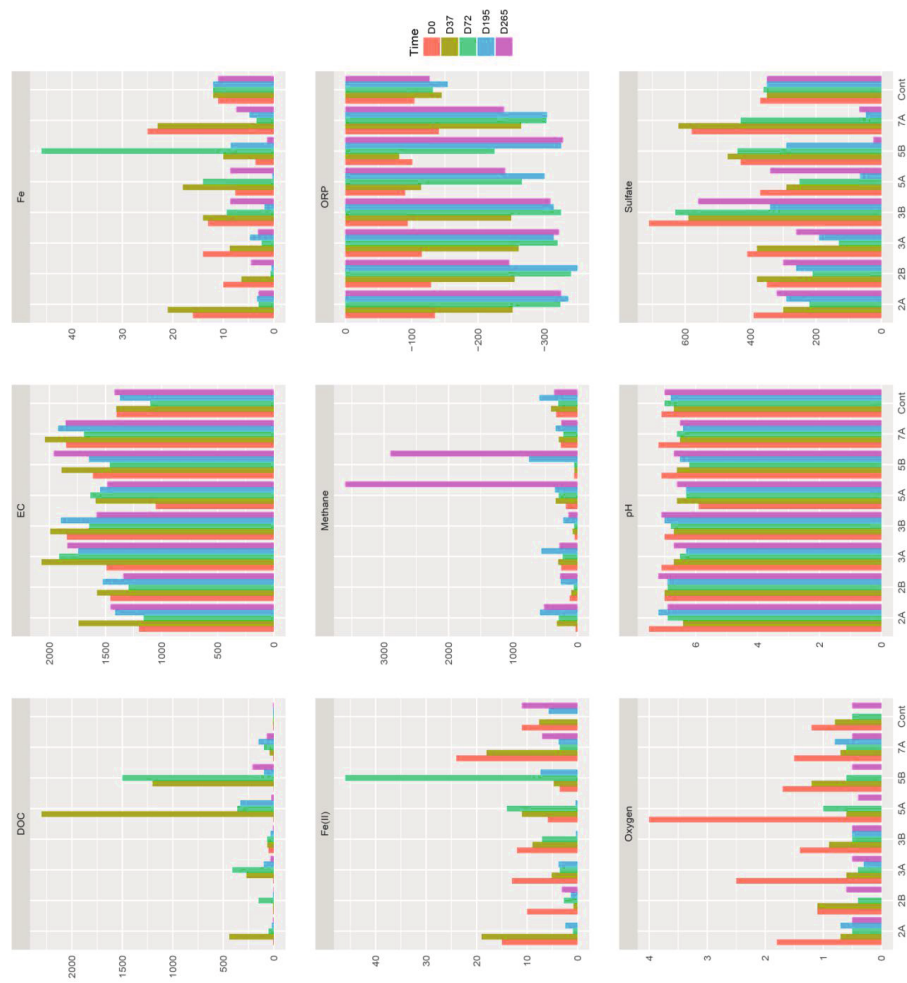


Figure S6.2 Groundwater geochemical parameters. Scales on Y-axes are: dissolved organic carbon (DOC) (mg/L), electrical conductivity (EC) (μS/cm), Fe (mg/l), Fe(II) (mg/l), methane (μg/l), oxidation-reduction potential (ORP) (mV), oxygen (mg/l), sulfate (mg/l), Cont: control well.

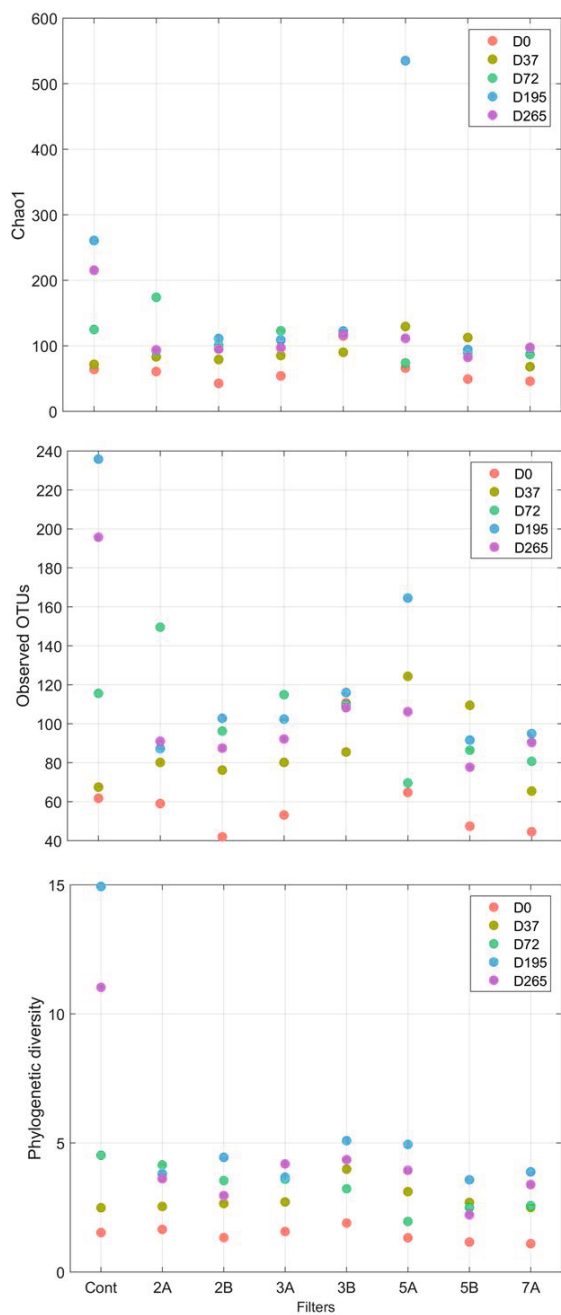


Figure S6.3 Predicted (Chao 1) and observed OTU richness, and phylogenetic diversity at each filter over time (five samples per filter). Cont: control well.

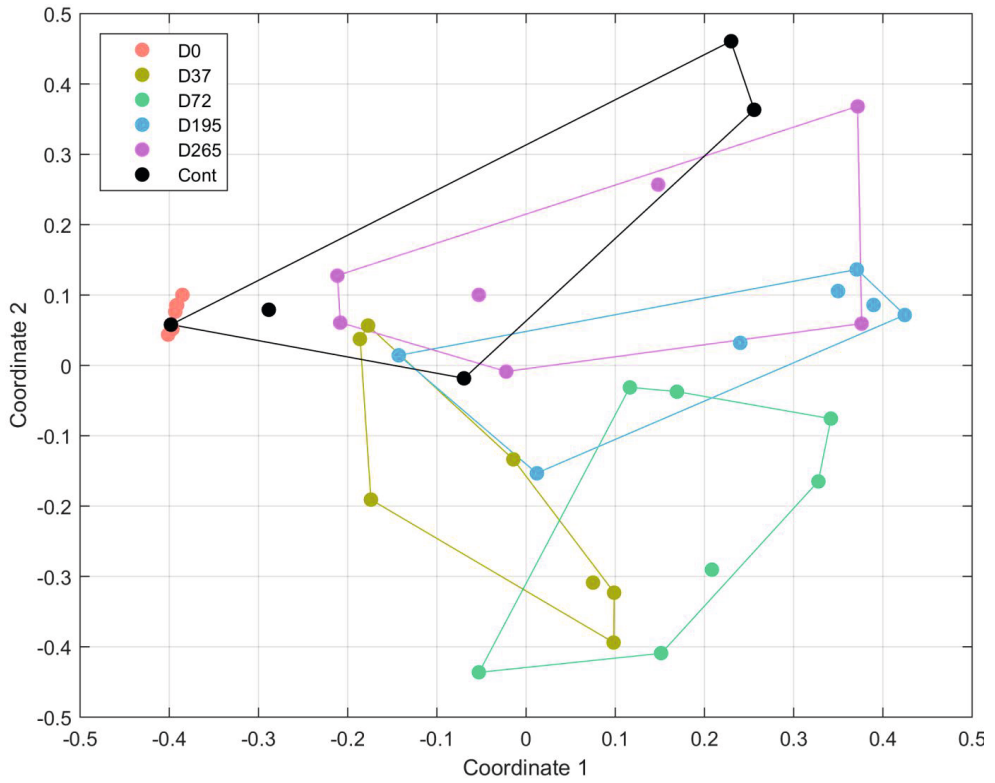
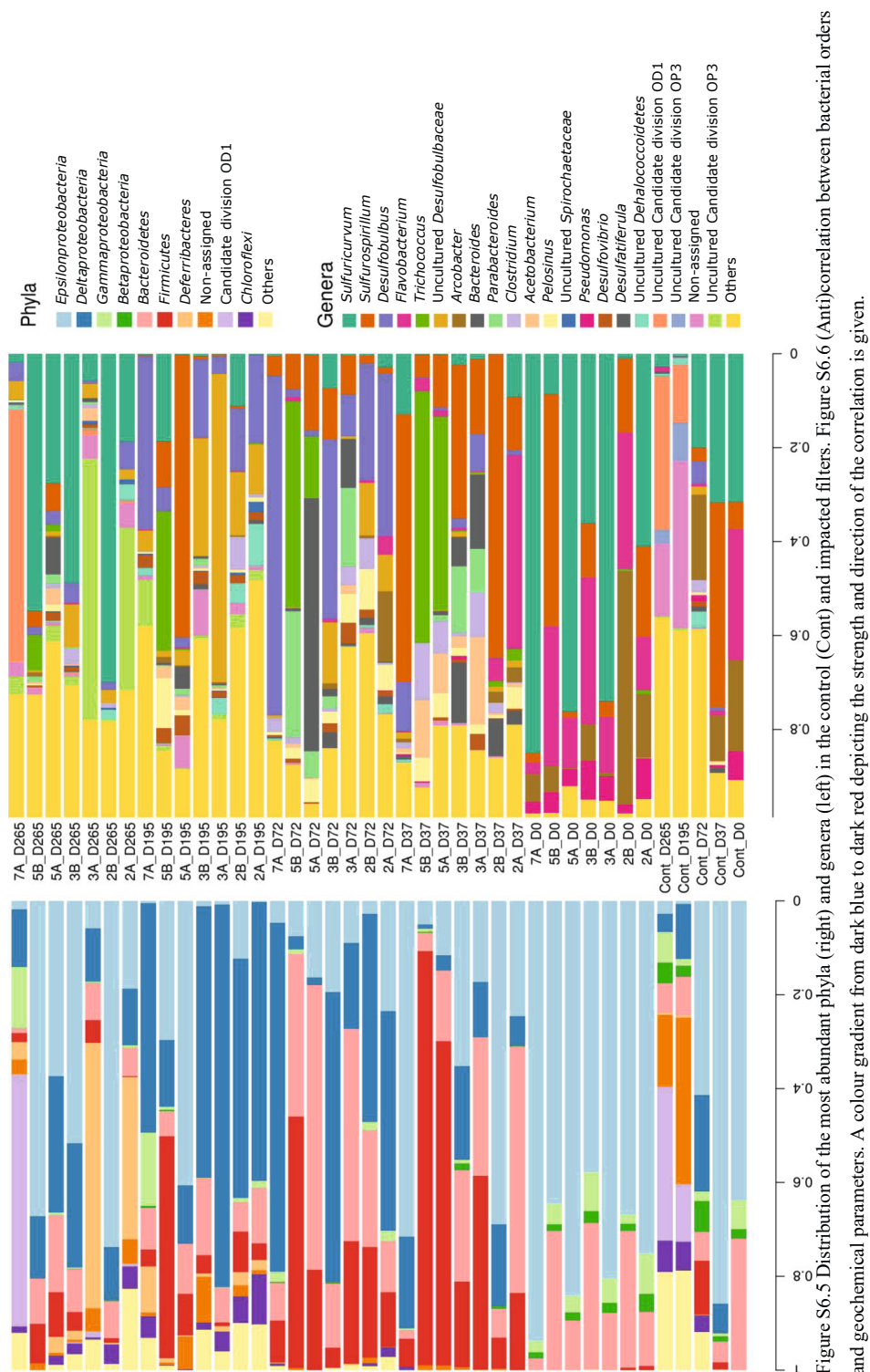


Figure S6.4 Ordination of community composition by nonmetric multidimensional scaling (NMDS) based on Bray–Curtis distances. Cont: control well.



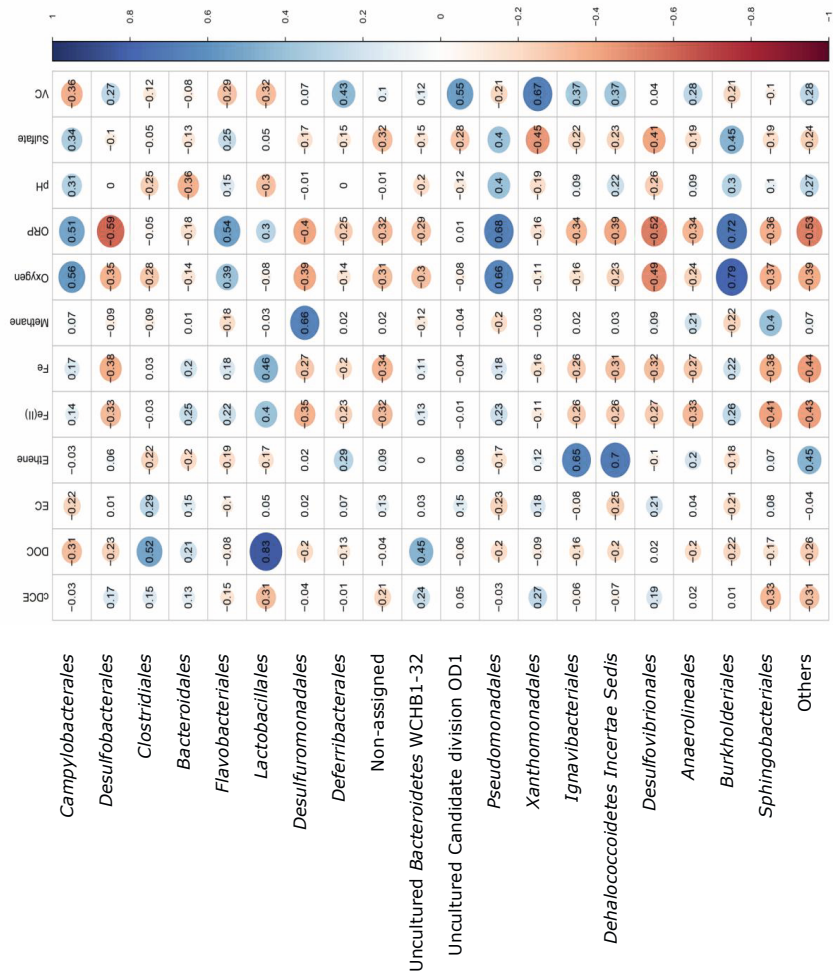


Figure S6.6 (Ant)correlation between bacterial orders and geochemical parameters. A colour gradient from dark blue to dark red depicting the strength and direction of the correlation is given.

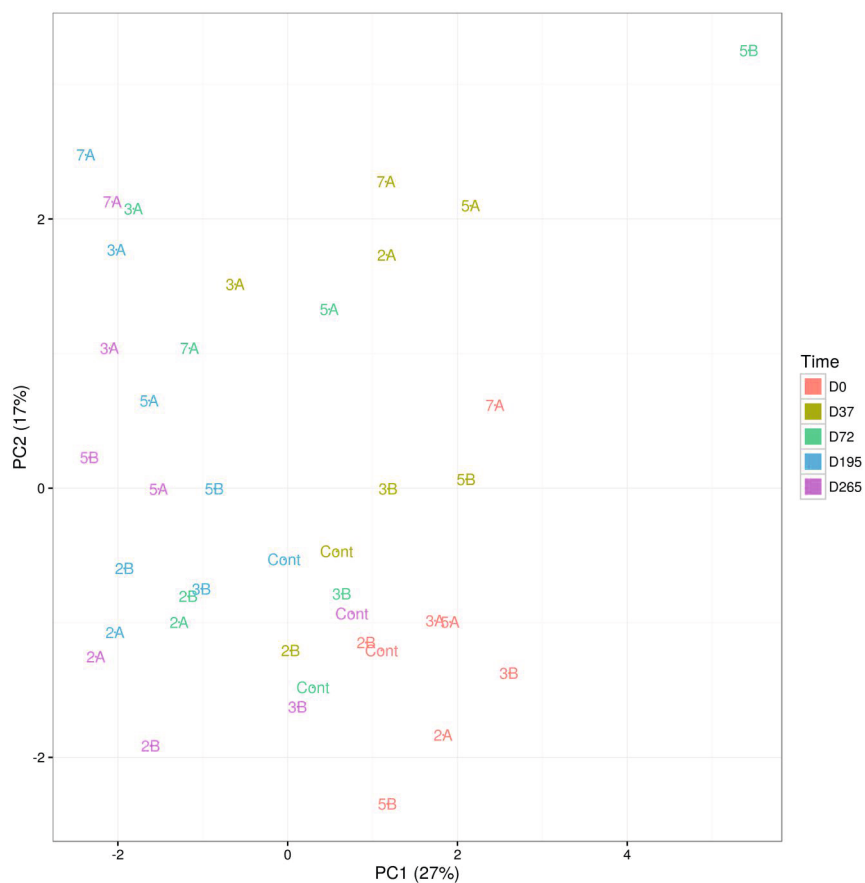


Figure S6.7 Principal component analysis (PCA) of geochemical parameters in the control (Cont) and impacted filters.



Figure S6.8 Niche occupation concept. According to phylogenetic instability in the control well (well 0 in left figure), groundwater brings in immigrant microbes (the arrow from the control well towards the stimulated zone or active growth zone). The resident taxa in the stimulated wells exploit the stimulated conditions and occupy the available niches in the active growth zone. However, they were exposed to the newly arriving taxa brought by natural groundwater flow but it is only towards the end of the experiment (and hence fading stimulation) that the immigrants pass the threshold after day 195 (dashed line in right figure) and become established in the stimulated wells by day 265.

Table S6.1 Overview of primer sequences and thermal cycling conditions used for qPCR in this study.

Primer	Oligonucleotide sequence (5'-3')	Thermal profile	Number of cycles	Target	Reference
Dec728F	AAGCGGTTTCTAGGTTGTCAC	95 °C 10 min	1	<i>Dcm</i>	[196]
Dec944R	CTTCAATGCATGTCAAAT	95 °C 15 s, 50 °C 30 s, 72 °C 30 s	40		
Ver1022F	CGGGCGGATGCACATATTT	95 °C 10 min	1	<i>verA</i>	[195]
Ver1093R	GAATAGTCCTGCGCTTCCTC	95 °C 15 s, 58 °C 60 s	40		
Bvc925F	AAAAGCAC'TTGGCTATCAAGGAC	95 °C 10 min	1	<i>bvcA</i>	[195]
Bvc1017R	CCAAAACACACACAGGTC	95 °C 15 s, 58 °C 60 s	40		
TeeA1270F	ATCCAGATTATGACCCCTGGTGAA	95 °C 10 min	1	<i>tceA</i>	[195]
TeeA1336R	ATCCAGATTATGACCCCTGGTGAA	95 °C 15 s, 60 °C 60 s	40		

Table S6.2 Overview of MiSeq primers and Unitags and thermal cycling conditions.

Primers and Unitags		Thermal cycling conditions	
Primer ^a	Oligonucleotide sequence (5'-3') ^b	Reference	Thermal profile
27F-DegS	GTTYGATYMTGGCTCAG	[430]	98 °C for 30 s
338R-I	GCWGCCTCCCGTAGGAGT	[431]	25 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 20 s and elongation at 72 °C for 20 s
338R-II	GCWGCCACCCGTTAGGTGT	[431]	final extension at 72 °C for 10 min
Unitag1	GAGCCGTAGCCAGTCTGC	[373]	98 °C for 30 s
Unitag2	GCCGTGACCGTGACATCG	[373]	5 cycles of denaturation at 98 °C for 10 s, annealing at 52 °C for 20 s and elongation at 72 °C for 20 s final extension at 72 °C for 10 min

^a Primer names may not correspond to original publication.

^b M = A or C; R = A or G; W = A or T; Y = C or T.

Table S6.3 Groundwater geochemical parameters (a-i). Con: control well.

a.

Filters	Dissolved organic carbon (DOC) (mg/l)				
	Day 0	Day 37	Day 72	Day 195	Day 265
2A	6.5	440	51	17	7.5
2B	6.5	6.8	150	8	4.1
3A	5.9	270	410	99	32
3B	51	63	65	31	6.8
5A	3.9	2300	360	330	23
5B	2.7	1200	1500	96	210
7A	5.5	40	98	150	69
Cont	6	6.9	6	6.2	7.3

b.

Filters	Electrical conductivity (EC) ($\mu\text{S}/\text{cm}$)				
	Day 0	Day 37	Day 72	Day 195	Day 265
2A	1201	1741	1160	1414	1455
2B	1456	1576	1295	1524	1341
3A	1492	2070	1913	1744	1840
3B	1844	1992	1646	1897	1579
5A	1051	1588	1635	1548	1483
5B	1612	1892	1463	1647	1960
7A	1850	2040	1692	1922	1855
Cont	1401	1404	1100	1370	1419

c.

Filters	Oxidation-reduction potential (ORP)				
	Day 0	Day 37	Day 72	Day 195	Day 265
2A	-135	-252	-324	-336	-325
2B	-129	-255	-340	-350	-247
3A	-115	-261	-320	-314	-322
3B	-94	-250	-325	-314	-309
5A	-90	-114	-266	-300	-241
5B	-101	-81	-225	-325	-328
7A	-141	-265	-303	-304	-239
Cont	-104	-145	-132	-154	-127

d.

Filters	Oxygen (mg/l)				
	Day 0	Day 37	Day 72	Day 195	Day 265
2A	1.8	0.7	0.5	0.7	0.5
2B	1.1	1.1	0.4	0	0.6
3A	2.5	0.6	0.4	0.3	0.5
3B	1.4	0.9	0.5	0.5	0.5
5A	4	0.6	1	0	0.4
5B	1.7	1.2	0.6	0	0.5
7A	1.5	0.7	0.6	0.8	0.5
Cont	1.2	0.8	0.5	0	0.5

(continued)

f.

Filters	Sulfate (mg/l)				
	Day 0	Day 37	Day 72	Day 195	Day 265
2A	390	300	220	290	320
2B	350	380	210	260	300
3A	410	380	130	190	260
3B	710	590	630	340	560
5A	370	290	250	65	340
5B	430	470	440	290	24
7A	580	620	430	47	67
Cont	370	350	360	350	350

h.

Filters	Fe (mg/l)				
	Day 0	Day 37	Day 72	Day 195	Day 265
2A	16	21	3	3.3	3
2B	10	6.4	0.63	0.43	4.5
3A	14	8.7	2.4	4.7	3.1
3B	13	14	9.3	1.8	8.6
5A	7.6	18	14	0.26	8.6
5B	3.6	10	46	8.5	1.3
7A	25	23	3.4	4.8	7.4
Cont	11	12	12	12	11

(continued)

e.

Filters	Methane (µg/l)				
	Day 0	Day 37	Day 72	Day 195	Day 265
2A	29	320	290	580	520
2B	120	97	58	260	270
3A	250	300	230	560	280
3B	45	77	52	220	140
5A	180	340	290	350	3600
5B	53	46	53	750	2900
7A	260	290	220	340	250
Cont	330	410	300	590	360

g.

Filters	Fell (mg/l)				
	Day 0	Day 37	Day 72	Day 195	Day 265
2A	15	19	0.9	2.4	0
2B	10	0.79	2.7	1.3	3.1
3A	13	5.1	3.5	3.7	0
3B	12	8.9	7	0.29	0
5A	5.9	11	14	0.37	0
5B	3.5	4.7	46	7.3	0
7A	24	18	3.5	3.7	7
Cont	11	7.6	0	5.7	11

i.

pH					
Filters	Day 0	Day 37	Day 72	Day 195	Day 265
2A	7.5	6.4	6.9	7.2	6.9
2B	7	7	6.9	6.9	7.2
3A	7.1	6.7	6.5	6.3	6.7
3B	7	6.7	6.8	7	7.1
5A	5.9	6.6	6.3	6.3	6.6
5B	7.1	6.6	6.2	6.5	6.7
7A	7.2	6.5	6.6	6.4	6.5
Cont	7.1	6.7	7	6.8	7

Table S6.4 Concentrations of TCE, cDCE, VC, ethene and $\delta^{13}\text{C}$ of cDCE and VC and concentration-weighted average values of the isotope signature ($\delta^{13}\text{C}_{\Sigma\text{CE}}$).

a.

Filters	Concentrations	Day 0	Day 37	Day 72	Day 195	Day 265
2A	TCE ($\mu\text{mol/L}$)	0.5	0.3	0.0	0.0	0.0
	cDCE ($\mu\text{mol/L}$)	134.2	113.5	101.1	77.4	29.9
	VC ($\mu\text{mol/L}$)	24.0	24.0	27.2	65.6	48.0
	ETH ($\mu\text{mol/L}$)	1.9	2.0	1.4	7.1	10.7
	$\delta^{13}\text{C}$ cDCE (‰)	-19.7	NA	NA	-7.9	-8.2
	$\delta^{13}\text{C}$ VC (‰)	-33.5	NA	NA	-35	-22
	$\delta^{13}\text{C}_{\Sigma\text{CE}}$ (‰)	-21.8	NA	NA	-20.5	-15.7
2B	TCE ($\mu\text{mol/L}$)	0.0	0.0	0.0	0.0	0.0
	cDCE ($\mu\text{mol/L}$)	103.2	98.0	92.9	56.8	44.4
	VC ($\mu\text{mol/L}$)	12.5	3.7	16.0	38.4	41.6
	ETH ($\mu\text{mol/L}$)	2.4	0.5	0.5	3.0	11.4
	$\delta^{13}\text{C}$ cDCE (‰)	-19.3	NA	NA	-14.4	1.3
	$\delta^{13}\text{C}$ VC (‰)	-27	NA	NA	-32	-33.0
	$\delta^{13}\text{C}_{\Sigma\text{CE}}$ (‰)	-20.2	NA	NA	-21.4	-15.2
3A	TCE ($\mu\text{mol/L}$)	0.5	0.4	0.0	0.0	0.0
	cDCE ($\mu\text{mol/L}$)	165.1	206.4	227.0	185.8	123.8
	VC ($\mu\text{mol/L}$)	30.4	48.0	54.4	67.2	96.0
	ETH ($\mu\text{mol/L}$)	2.4	2.4	1.6	3.4	1.8
	$\delta^{13}\text{C}$ cDCE (‰)	-20.0	NA	NA	-16.6	-11.1
	$\delta^{13}\text{C}$ VC (‰)	-30	NA	NA	-37.2	-38.5
	$\delta^{13}\text{C}_{\Sigma\text{CE}}$ (‰)	-21.5	NA	NA	-22.1	-20.0
3B	TCE ($\mu\text{mol/L}$)	0.0	0.0	0.0	0.0	0.0
	cDCE ($\mu\text{mol/L}$)	21.7	49.5	29.9	39.2	14.4
	VC ($\mu\text{mol/L}$)	5.6	11.0	6.9	14.2	8.5
	ETH ($\mu\text{mol/L}$)	0.0	0.5	0.2	1.1	0.3
	$\delta^{13}\text{C}$ cDCE (‰)	-18.8	NA	NA	-16.9	-9.1
	$\delta^{13}\text{C}$ VC (‰)	-33.6	NA	NA	-29.9	-38.7
	$\delta^{13}\text{C}_{\Sigma\text{CE}}$ (‰)	-21.8	NA	NA	-20.3	-19.2
5A	TCE ($\mu\text{mol/L}$)	1.0	0.9	0.0	0.0	0.6
	cDCE ($\mu\text{mol/L}$)	113.5	123.8	113.5	103.2	123.8
	VC ($\mu\text{mol/L}$)	24.0	22.4	25.6	6.1	25.6
	ETH ($\mu\text{mol/L}$)	1.7	1.8	1.6	0.8	1.9
	$\delta^{13}\text{C}$ cDCE (‰)	-20.2	NA	NA	-18.8	-18.2
	$\delta^{13}\text{C}$ VC (‰)	-33.0	NA	NA	-36.7	-37.4
	$\delta^{13}\text{C}_{\Sigma\text{CE}}$ (‰)	-22.4	NA	NA	-22.5	-20.8
5B	TCE ($\mu\text{mol/L}$)	0.0	0.0	0.0	0.7	0.0
	cDCE ($\mu\text{mol/L}$)	2.1	12.4	19.6	14.4	9.6
	VC ($\mu\text{mol/L}$)	0.4	1.6	3.5	4.0	3.4
	ETH ($\mu\text{mol/L}$)	0.4	0.2	0.1	3.9	0.5
	$\delta^{13}\text{C}$ cDCE (‰)	-18.6	NA	NA	-18.9	-17.4

(continued)

	$\delta^{13}\text{C}$ VC (‰)	-27.6	NA	NA	-34.6	-34.3
	$\delta^{13}\text{C}_{\Sigma\text{CE}}$ (‰)	-20.2	NA	NA	-22.3	-21.6
7A	TCE ($\mu\text{mol/L}$)	0.1	0.3	0.0	0.0	0.0
	cDCE ($\mu\text{mol/L}$)	207.3	207.3	228.0	217.6	124.4
	VC ($\mu\text{mol/L}$)	51.2	57.6	65.6	100.8	131.2
	ETH ($\mu\text{mol/L}$)	2.5	2.4	1.9	3.1	3.5
	$\delta^{13}\text{C}$ cDCE (‰)	-18.7	NA	NA	-14.7	-5.6
	$\delta^{13}\text{C}$ VC (‰)	-34.9	NA	NA	-40.2	-36.8
	$\delta^{13}\text{C}_{\Sigma\text{CE}}$ (‰)	-21.9	NA	NA	-22.8	-16.2
Control	TCE ($\mu\text{mol/L}$)	2.2	2.5	2.4	1.9	1.1
	cDCE ($\mu\text{mol/L}$)	113.5	123.8	134.2	123.8	100.1
	VC ($\mu\text{mol/L}$)	24.0	20.8	22.4	22.4	16.0
	ETH ($\mu\text{mol/L}$)	1.9	2.1	1.6	3.4	1.9
	$\delta^{13}\text{C}$ cDCE (‰)	-19.9	NA	NA	-20.5	-20.0
	$\delta^{13}\text{C}$ VC (‰)	-32.4	NA	NA	-31	-32
	$\delta^{13}\text{C}_{\Sigma\text{CE}}$ (‰)	-22.1	NA	NA	-22.1	-21.9

Table S6.5 qPCR quantification of 16S rRNA gene copy numbers of *Dcm*, and *verA*, *bvcA* and *tceA* genes. Each qPCR value represents the average value obtained from triplicate reactions.

Filters	Genes	Day 0		Day 37		Day 72		Day 195		Day 265	
		Copies/L	Standard deviation	Copies/L	Standard deviation	Copies/L	Standard deviation	Copies/L	Standard deviation	Copies/L	Standard deviation
2A	<i>Dcm</i>	3.0E+04	8.5E+03	4.5E+04	1.8E+04	1.8E+04	1.5E+03	3.8E+06	1.2E+06	1.6E+07	1.3E+06
	<i>verA</i>	5.5E+03	1.8E+03	1.4E+04	5.1E+03	5.4E+03	6.7E+02	3.5E+06	6.6E+05	3.6E+07	1.7E+06
	<i>bvcA</i>	3.1E+04	1.9E+03	2.8E+04	1.4E+03	1.1E+05	7.8E+03	3.9E+06	4.2E+05	1.9E+07	2.0E+06
	<i>tceA</i>	0.0E+00	0.0E+00	6.5E+03	9.1E+03	0.0E+00	0.0E+00	8.2E+03	6.7E+02	4.7E+04	3.1E+04
2B	<i>Dcm</i>	1.8E+05	2.8E+04	1.4E+05	4.1E+04	5.1E+04	3.5E+04	2.7E+06	1.1E+06	5.1E+07	1.8E+07
	<i>verA</i>	3.6E+04	8.8E+03	1.1E+04	3.2E+03	3.5E+03	2.1E+03	3.3E+06	1.3E+05	5.0E+07	1.0E+06
	<i>bvcA</i>	1.5E+05	1.7E+04	8.5E+05	9.9E+03	4.0E+04	2.5E+04	1.1E+06	1.0E+05	1.2E+07	1.0E+06
	<i>tceA</i>	1.1E+04	1.0E+04	1.1E+04	4.3E+03	1.8E+03	9.6E+03	4.4E+04	3.9E+04	0.0E+00	0.0E+00
3A	<i>Dcm</i>	2.9E+04	1.3E+03	1.8E+04	4.4E+03	2.3E+04	1.6E+03	9.3E+04	2.6E+03	4.2E+06	1.0E+05
	<i>verA</i>	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.0E+03	5.36E+02	1.0E+03	5.9E+02	5.8E+03	1.6E+03
	<i>bvcA</i>	5.2E+04	6.7E+03	8.9E+04	1.4E+03	1.8E+04	8.2E+03	4.8E+04	9.2E+03	3.7E+05	5.0E+04
	<i>tceA</i>	4.3E+03	6.1E+03	4.0E+03	3.0E+03	3.6E+03	3.7E+02	1.0E+04	8.9E+03	3.3E+03	4.6E+03
3B	<i>Dcm</i>	1.9E+04	6.2E+03	7.3E+04	1.8E+03	2.8E+04	1.8E+04	6.4E+04	1.1E+03	5.9E+05	3.1E+04
	<i>verA</i>	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	3.4E+03	2.8E+03	0.0E+00	0.0E+00
	<i>bvcA</i>	5.2E+04	4.1E+03	8.4E+03	2.4E+01	5.1E+04	1.8E+03	1.2E+05	1.5E+04	7.4E+05	8.3E+04
	<i>tceA</i>	1.3E+03	1.0E+03	4.9E+03	3.5E+03	1.9E+03	1.4E+02	9.1E+03	1.0E+04	1.1E+03	1.6E+03
5A	<i>Dcm</i>	1.6E+04	3.5E+03	2.8E+05	5.1E+04	3.4E+04	1.3E+03	6.5E+04	1.6E+04	8.3E+05	2.6E+05
	<i>verA</i>	0.0E+00	0.0E+00	1.7E+04	1.9E+03	7.7E+03	1.27E+02	1.4E+03	8.6E+02	7.7E+03	1.7E+02
	<i>bvcA</i>	7.0E+04	1.3E+04	7.0E+04	4.6E+03	2.3E+04	7.0E+03	1.6E+05	8.3E+04	2.7E+05	3.8E+04
	<i>tceA</i>	7.3E+03	6.5E+03	1.3E+04	9.8E+03	6.0E+03	4.3E+03	4.4E+04	6.2E+04	0.0E+00	0.0E+00

(continued)

5B	<i>Dem</i>	2.0E+04	2.8E+03	5.3E+04	3.0E+03	3.6E+04	4.4E+03	5.8E+04	1.5E+03	4.8E+05	1.1E+05
	<i>vrA</i>	1.7E+03	2.4E+03	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.5E+04	1.5E+03
	<i>bvcA</i>	1.2E+05	6.4E+04	5.6E+04	6.2E+02	2.8E+04	2.4E+03	4.6E+04	1.5E+03	1.1E+05	1.8E+04
	<i>tceA</i>	2.1E+04	1.8E+04	1.3E+04	9.9E+03	2.5E+03	9.7E+02	1.1E+04	1.6E+04	1.6E+04	7.8E+03
7A	<i>Dem</i>	4.3E+04	1.3E+04	1.8E+04	4.7E+03	2.2E+04	1.8E+03	3.4E+05	1.1E+05	6.0E+06	2.0E+05
	<i>vrA</i>	0.0E+00	0.0E+00	0.0E+00	0.0E+00	1.0E+03	7.4E+02	2.6E+04	4.2E+02	4.0E+05	4.4E+03
	<i>bvcA</i>	2.5E+04	2.9E+03	4.4E+04	2.6E+03	3.6E+04	5.6E+03	6.1E+05	4.3E+04	6.3E+06	8.5E+04
	<i>tceA</i>	1.2E+04	9.6E+03	9.6E+03	3.7E+03	7.6E+03	6.1E+03	1.5E+04	7.6E+03	9.4E+03	3.8E+02
Control	<i>Dem</i>	4.8E+04	6.7E+03	1.9E+04	8.5E+03	3.8E+04	1.1E+03	3.1E+04	4.4E+02	2.4E+04	4.4E+03
	<i>vrA</i>	1.8E+03	1.6E+03	3.7E+03	2.6E+03	1.0E+03	6.2E+02	3.3E+03	7.4E+02	1.9E+03	1.8E+04
	<i>bvcA</i>	1.6E+04	8.8E+03	3.6E+04	1.4E+03	4.2E+04	1.4E+03	1.1E+04	1.1E+03	6.9E+04	1.0E+03
	<i>tceA</i>	1.3E+04	3.7E+03	7.3E+03	7.0E+03	3.3E+03	2.8E+03	2.1E+03	3.0E+03	5.8E+03	4.3E+03

Table S6.6 Distribution of the most abundant phyla (a, b and c), orders (d, e and f) and genera (g, h and i) in the control and impacted filters.

a.

Phyla	D0 Cont	D37 Cont	D72 Cont	D195 Cont	D265 Cont	D0 2A	D0 2B	D0 3A	D0 3B	D0 5A	D0 5B	D0 7A
<i>Epsilonproteobacteria</i>	0.638	0.859	0.414	0.007	0.028	0.751	0.669	0.805	0.579	0.841	0.646	0.937
<i>Deltaproteobacteria</i>	0.000	0.063	0.206	0.117	0.039	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Gammaproteobacteria</i>	0.061	0.006	0.020	0.014	0.065	0.086	0.019	0.052	0.083	0.036	0.043	0.025
<i>Betaproteobacteria</i>	0.020	0.012	0.066	0.023	0.044	0.038	0.015	0.021	0.025	0.018	0.013	0.013
<i>Bacteroidetes</i>	0.281	0.044	0.061	0.082	0.064	0.115	0.292	0.122	0.313	0.106	0.297	0.025
<i>Firmicutes</i>	0.000	0.015	0.114	0.000	0.000	0.009	0.005	0.000	0.000	0.000	0.000	0.000
<i>Deferribacteres</i>	0.000	0.000	0.000	0.005	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Non-assigned	0.000	0.000	0.002	0.355	0.154	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Candidate division	0.000	0.000	0.000	0.123	0.327	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Chloroflexi</i>	0.000	0.000	0.035	0.061	0.067	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Others	0.000	0.001	0.081	0.212	0.209	0.000	0.000	0.000	0.001	0.000	0.000	0.000

b.

Phyla	D37 2A	D37 2B	D37 3A	D37 3B	D37 5A	D37 5B	D37 7A	D72 2A	D72 2B	D72 3A	D72 3B	D72 5A	D72 5B	D72 7A
<i>Epsilonproteobacteria</i>	0.246	0.689	0.173	0.352	0.116	0.050	0.715	0.235	0.028	0.090	0.194	0.163	0.075	0.047
<i>Deltaproteobacteria</i>	0.063	0.174	0.118	0.200	0.032	0.009	0.196	0.468	0.443	0.183	0.617	0.016	0.028	0.743
<i>Gammaproteobacteria</i>	0.000	0.000	0.000	0.008	0.000	0.007	0.004	0.022	0.017	0.000	0.000	0.000	0.009	0.022
<i>Betaproteobacteria</i>	0.002	0.006	0.000	0.014	0.000	0.002	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.002
<i>Bacteroidetes</i>	0.525	0.061	0.295	0.237	0.151	0.039	0.018	0.109	0.249	0.452	0.137	0.606	0.347	0.081
<i>Firmicutes</i>	0.164	0.066	0.413	0.182	0.691	0.883	0.060	0.117	0.235	0.261	0.043	0.214	0.535	0.089
<i>Deferribacteres</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.002
Non-assigned	0.000	0.003	0.001	0.004	0.010	0.010	0.000	0.001	0.012	0.001	0.000	0.000	0.005	0.000
Candidate division	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
OD1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

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<i>Chloroflexi</i>	0.000	0.000	0.000	0.001	0.000	0.000	0.007	0.020	0.007	0.000	0.000	0.000	0.000	0.005
Others	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.028	0.000	0.011	0.009	0.005	0.000	0.009

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Phyla	D195_2A	D195_2B	D195_3A	D195_3B	D195_5A	D195_7A	D265_2A	D265_2B	D265_3A	D265_3B	D265_5A	D265_5B
<i>Episilonproteobacteria</i>	0.003	0.123	0.008	0.011	0.606	0.297	0.005	0.187	0.089	0.516	0.374	0.672
<i>Deltaproteobacteria</i>	0.594	0.509	0.815	0.578	0.124	0.142	0.489	0.120	0.113	0.265	0.290	0.133
<i>Gammaproteobacteria</i>	0.015	0.009	0.002	0.002	0.000	0.007	0.156	0.005	0.003	0.002	0.006	0.000
<i>Betaproteobacteria</i>	0.000	0.000	0.000	0.000	0.000	0.003	0.004	0.000	0.002	0.001	0.000	0.000
<i>Bacteroidetes</i>	0.118	0.063	0.074	0.163	0.107	0.053	0.089	0.060	0.079	0.092	0.163	0.097
<i>Firmicutes</i>	0.035	0.086	0.008	0.039	0.088	0.473	0.036	0.002	0.010	0.039	0.095	0.084
<i>Deferribacteres</i>	0.021	0.028	0.011	0.007	0.003	0.010	0.098	0.346	0.004	0.020	0.034	0.000
Non-assigned	0.009	0.024	0.000	0.097	0.069	0.004	0.009	0.052	0.000	0.005	0.006	0.015
Candidate division OD1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.003	0.000	0.000
<i>Chloroflexi</i>	0.107	0.057	0.043	0.016	0.003	0.002	0.046	0.048	0.005	0.023	0.020	0.000
Others	0.098	0.101	0.040	0.086	0.000	0.010	0.069	0.173	0.065	0.034	0.011	0.000

d.

Orders	D0_Cont	D87_Cont	D72_Cont	D195_Cont	D265_Cont	D0.2A	D0.2B	D0.3A	D0.3B	D0.5A	D0.5B	D0.7A
Campylobacterales	0.638	0.859	0.414	0.007	0.028	0.751	0.669	0.805	0.579	0.841	0.646	0.937
Desulfobacterales	0.000	0.022	0.088	0.030	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Clostridiales	0.000	0.015	0.109	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
Bacteroidales	0.000	0.000	0.004	0.012	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Flavobacteriales	0.279	0.010	0.005	0.008	0.013	0.114	0.290	0.119	0.313	0.106	0.297	0.025
Lactobacillales	0.000	0.000	0.000	0.000	0.000	0.008	0.005	0.000	0.000	0.000	0.000	0.000
Desulfuromonadales	0.000	0.040	0.106	0.015	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000

(continued)

<i>Deferribacteriales</i>	0.000	0.000	0.000	0.000	0.005	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Non-assigned	0.000	0.000	0.000	0.002	0.355	0.154	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Uncultured																	
<i>Bacteroidales</i>	0.000	0.024	0.031		0.009	0.012	0.001	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
WCHB1-32																	
Uncultured Candidate	0.000	0.000	0.000	0.000	0.123	0.327	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
division OD1																	
<i>Pseudomonadales</i>	0.061	0.006	0.013		0.009	0.000	0.086	0.019	0.052	0.083	0.036	0.043	0.000	0.000	0.025		
<i>Xanthomonadales</i>	0.000	0.000	0.007		0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
<i>Ignavibacteriales</i>	0.000	0.000	0.000		0.066	0.094	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
<i>Dehalococcoidetes</i>																	
<i>Incertae Sedis</i>	0.000	0.000	0.035		0.016	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
<i>Desulfotribionales</i>	0.000	0.002	0.012		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
<i>Anaerolineales</i>	0.000	0.000	0.000		0.039	0.061	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
<i>Burkholderiales</i>	0.018	0.006	0.028		0.016	0.002	0.021	0.009	0.019	0.019	0.018	0.010	0.010	0.013			
Uncultured Candidate																	
division OP3	0.000	0.000	0.000		0.082	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
<i>Springobacteriales</i>	0.000	0.003	0.001		0.005	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
Others	0.003	0.013	0.145		0.203	0.223	0.017	0.006	0.005	0.007	0.000	0.004	0.000	0.000	0.000		

c.

Orders	D37.2A	D37.2B	D37.3A	D37.3B	D37.5A	D37.5B	D37.7A	D72.2A	D72.2B	D72.3A	D72.3B	D72.5A	D72.5B	D72.7A
<i>Campylobacteriales</i>	0.246	0.689	0.173	0.352	0.116	0.050	0.715	0.235	0.028	0.090	0.194	0.163	0.075	0.047
<i>Desulfobacteriales</i>	0.055	0.131	0.080	0.163	0.012	0.004	0.130	0.442	0.382	0.106	0.566	0.012	0.019	0.731
<i>Clostridiales</i>	0.139	0.053	0.409	0.180	0.269	0.321	0.060	0.117	0.227	0.261	0.043	0.082	0.087	0.089
<i>Bacteroidales</i>	0.000	0.001	0.254	0.203	0.025	0.000	0.003	0.009	0.168	0.324	0.048	0.600	0.275	0.000
<i>Flavobacteriales</i>	0.413	0.048	0.002	0.006	0.013	0.027	0.003	0.041	0.005	0.000	0.007	0.000	0.010	0.000
<i>Lactobacillales</i>	0.025	0.013	0.003	0.003	0.421	0.561	0.000	0.000	0.000	0.000	0.000	0.132	0.445	0.000
<i>Desulfuromonadales</i>	0.004	0.042	0.000	0.030	0.014	0.003	0.066	0.015	0.043	0.033	0.031	0.000	0.000	0.008
<i>Deferribacteriales</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.002

(continued)

Non-assigned	0.000	0.003	0.001	0.004	0.010	0.010	0.000	0.000	0.001	0.012	0.001	0.000	0.000	0.005	0.000
Uncultured															
<i>Bacteroidetes</i>	0.113	0.012	0.039	0.018	0.109	0.001	0.012	0.052	0.051	0.114	0.069	0.005	0.063	0.081	
WCHB1-32															
Uncultured Candidate	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
division OD1															
<i>Pseudomonadales</i>	0.000	0.000	0.000	0.008	0.000	0.002	0.004	0.002	0.000	0.000	0.000	0.000	0.000	0.000	
<i>Xanthomonadales</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.017	0.000	0.000	0.000	0.000	0.022	
<i>Ignavibacteriales</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.002	0.000	0.000	0.000	0.000	0.000	
<i>Dehalococcoides</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	
<i>Incertae Sedis</i>	0.000	0.000	0.000	0.001	0.000	0.000	0.007	0.020	0.007	0.000	0.000	0.000	0.000	0.004	
<i>Desulfotribionales</i>	0.004	0.002	0.038	0.007	0.007	0.000	0.000	0.011	0.018	0.044	0.019	0.004	0.009	0.004	
<i>Anaerolineales</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	
<i>Burkholderiales</i>	0.000	0.000	0.000	0.012	0.000	0.002	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	
Uncultured Candidate															
division OP3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
<i>Sphingobacteriales</i>	0.000	0.000	0.000	0.001	0.000	0.004	0.000	0.000	0.002	0.000	0.006	0.000	0.000	0.000	
Others	0.002	0.006	0.000	0.012	0.004	0.012	0.000	0.029	0.038	0.025	0.015	0.002	0.013	0.011	

f.

Orders	D195.2A	D195.2B	D195.3A	D195.3B	D195.5A	D195.5B	D195.7A	D265.2A	D265.2B	D265.3A	D265.3B	D265.5A	D265.5B	D265.7A
<i>Campilobacteriales</i>	0.003	0.123	0.008	0.011	0.606	0.297	0.005	0.187	0.738	0.059	0.516	0.374	0.672	0.018
<i>Desulfobacteriales</i>	0.516	0.304	0.733	0.500	0.062	0.075	0.432	0.109	0.078	0.054	0.208	0.047	0.048	0.096
<i>Clostridiales</i>	0.033	0.082	0.008	0.036	0.084	0.169	0.036	0.002	0.010	0.048	0.037	0.080	0.006	0.019
<i>Bacteroidales</i>	0.003	0.009	0.016	0.031	0.079	0.013	0.042	0.001	0.004	0.025	0.012	0.117	0.041	0.004
<i>Flavobacteriales</i>	0.000	0.001	0.000	0.001	0.003	0.000	0.002	0.000	0.000	0.000	0.004	0.000	0.000	0.000
<i>Lactobacillales</i>	0.002	0.002	0.000	0.000	0.004	0.302	0.000	0.000	0.000	0.000	0.000	0.015	0.077	0.000
<i>Desulfuromonadales</i>	0.045	0.184	0.070	0.050	0.018	0.035	0.030	0.007	0.032	0.052	0.051	0.225	0.081	0.025
<i>Deferribacteriales</i>	0.021	0.028	0.011	0.007	0.003	0.010	0.098	0.346	0.004	0.565	0.020	0.034	0.000	0.037
Non-assigned	0.009	0.024	0.000	0.097	0.069	0.004	0.009	0.052	0.000	0.050	0.005	0.006	0.015	0.031

(continued)

Uncultured <i>Bacteroidetes</i> WCHB1-32	0.082	0.010	0.013	0.019	0.008	0.013	0.042	0.034	0.000	0.014	0.009	0.007	0.006	0.006
Uncultured Candidate division OD1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.012	0.003	0.000	0.000	0.537
<i>Pseudomonadales</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Xanthomonadales</i>	0.015	0.009	0.002	0.002	0.000	0.007	0.156	0.005	0.000	0.003	0.000	0.006	0.000	0.131
<i>Ignavibacteriales</i>	0.040	0.008	0.019	0.012	0.000	0.002	0.043	0.086	0.005	0.009	0.009	0.001	0.000	0.006
<i>Dehalococcoidetes</i> <i>Incertae Sedis</i>	0.094	0.043	0.035	0.001	0.001	0.002	0.018	0.046	0.034	0.003	0.001	0.004	0.000	0.012
<i>Desulfotribionales</i>	0.017	0.018	0.012	0.027	0.045	0.033	0.027	0.000	0.000	0.008	0.007	0.018	0.005	0.002
<i>Anaerolineales</i>	0.013	0.013	0.007	0.014	0.002	0.000	0.029	0.002	0.008	0.002	0.021	0.015	0.000	0.002
<i>Burkholderiales</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Uncultured Candidate division OP3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Springobacteriales</i>	0.000	0.015	0.004	0.025	0.011	0.005	0.000	0.001	0.015	0.015	0.007	0.016	0.013	0.000
Others	0.107	0.127	0.062	0.166	0.006	0.035	0.031	0.117	0.073	0.081	0.090	0.034	0.037	0.073

g-

Genera	D0:Cont	D37:Cont	D72:Cont	D195:Cont	D265:Cont	D0.2A	D0.2B	D0.3A	D0.3B	D0.5A	D0.5B	D0.7A
<i>Sulfuricurvum</i>	0.316	0.317	0.200	0.007	0.028	0.410	0.010	0.740	0.360	0.761	0.085	0.849
<i>Sulfurospirillum</i>	0.058	0.435	0.029	0.000	0.000	0.193	0.158	0.033	0.116	0.013	0.495	0.021
<i>Desulfotulbus</i>	0.000	0.008	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Flavobacterium</i>	0.279	0.010	0.005	0.001	0.010	0.114	0.290	0.119	0.313	0.106	0.297	0.025
<i>Trichococcus</i>	0.000	0.000	0.000	0.000	0.000	0.008	0.005	0.000	0.000	0.000	0.000	0.000
Uncultured <i>Desulfotulbacaceae</i>	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Acrobacter</i>	0.194	0.098	0.182	0.000	0.000	0.136	0.497	0.008	0.078	0.004	0.056	0.059
<i>Bacteroides</i>	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Parabacteroides</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

(continued)

<i>Clostridium</i>	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Acetobacterium</i>	0.000	0.002	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Pelosinus</i>	0.000	0.006	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Uncultured <i>Spirochaetaceae</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Pseudomonas</i>	0.061	0.006	0.013	0.000	0.000	0.000	0.086	0.019	0.052	0.083	0.036	0.043	0.025						
<i>Desulfotribrio</i>	0.000	0.002	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Desulfatiferula</i>	0.000	0.010	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Uncultured <i>Dehalococcoides</i>	0.000	0.000	0.035	0.016	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Uncultured Candidate division OD1	0.000	0.000	0.000	0.123	0.327	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Uncultured Candidate division OP3	0.000	0.000	0.000	0.082	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Non-assigned Uncultured <i>Deferribacteriales</i> SAR406	0.000	0.000	0.002	0.355	0.154	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Others	0.000	0.000	0.000	0.005	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	0.093	0.107	0.414	0.411	0.437	0.052	0.022	0.049	0.051	0.080	0.023	0.021							

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Genera	D37.2A	D37.2B	D37.3A	D37.3B	D37.5A	D37.5B	D37.7A	D72.2A	D72.2B	D72.3A	D72.3B	D72.5A	D72.5B	D72.7A
<i>Sulfuricurvum</i>	0.093	0.001	0.011	0.024	0.003	0.003	0.129	0.029	0.004	0.004	0.074	0.004	0.000	0.005
<i>Sulfurospirillum</i>	0.112	0.646	0.160	0.326	0.112	0.048	0.570	0.014	0.016	0.082	0.109	0.160	0.075	0.042
<i>Desulfobulbus</i>	0.011	0.001	0.078	0.020	0.007	0.003	0.104	0.345	0.249	0.089	0.382	0.012	0.017	0.723
<i>Flavobacterium</i>	0.413	0.048	0.002	0.006	0.013	0.027	0.003	0.040	0.005	0.000	0.007	0.000	0.010	0.000
<i>Trichococcus</i>	0.025	0.013	0.003	0.003	0.413	0.536	0.000	0.000	0.000	0.000	0.000	0.132	0.438	0.000
Uncultured <i>Desulfobulbacae</i>	0.014	0.011	0.001	0.010	0.002	0.000	0.013	0.077	0.112	0.005	0.130	0.000	0.002	0.005
<i>Arcobacter</i>	0.032	0.023	0.000	0.002	0.000	0.000	0.008	0.150	0.006	0.001	0.012	0.000	0.000	0.000
<i>Bacteroides</i>	0.000	0.000	0.159	0.061	0.009	0.000	0.000	0.002	0.000	0.104	0.016	0.539	0.007	0.000

(continued)

<i>Bacteroides</i>	0.000	0.000	0.004	0.006	0.047	0.005	0.000	0.000	0.000	0.000	0.009	0.000	0.079	0.000	0.002
<i>Parabacteroides</i>	0.000	0.000	0.005	0.014	0.015	0.003	0.000	0.000	0.000	0.000	0.004	0.004	0.019	0.012	0.000
<i>Clostridium</i>	0.007	0.069	0.000	0.005	0.003	0.000	0.003	0.000	0.000	0.010	0.009	0.032	0.011	0.000	0.000
<i>Acetobacterium</i>	0.000	0.002	0.004	0.002	0.028	0.026	0.000	0.000	0.000	0.000	0.027	0.003	0.035	0.003	0.000
<i>Pelosinus</i>	0.009	0.005	0.002	0.005	0.011	0.105	0.003	0.000	0.000	0.000	0.000	0.000	0.013	0.003	0.004
Uncultured <i>Spirochaetaceae</i>	0.023	0.006	0.002	0.001	0.000	0.000	0.003	0.010	0.004	0.004	0.007	0.004	0.003	0.000	0.003
<i>Pseudomonas</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>Desulfovibrio</i>	0.017	0.018	0.012	0.027	0.043	0.033	0.027	0.000	0.000	0.000	0.008	0.007	0.018	0.005	0.002
<i>Desulfatiferula</i>	0.008	0.000	0.002	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000
Uncultured <i>Dehalococcoides</i>	0.090	0.043	0.035	0.001	0.001	0.002	0.018	0.034	0.021	0.003	0.001	0.001	0.004	0.000	0.010
Uncultured Candidate division ODI	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.012	0.003	0.000	0.000	0.537
Uncultured Candidate division OP3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Non-assigned	0.009	0.024	0.000	0.097	0.069	0.004	0.009	0.052	0.000	0.000	0.050	0.005	0.006	0.015	0.031
Uncultured <i>Deferribacterales</i> SAR406	0.021	0.028	0.011	0.007	0.003	0.010	0.098	0.346	0.004	0.004	0.556	0.020	0.034	0.000	0.037
Others	0.518	0.417	0.221	0.393	0.115	0.155	0.420	0.284	0.218	0.221	0.294	0.294	0.387	0.274	0.275

Table S6.7 Significance tests of the differences of microbial communities at order level. The groups with no significant differences ($P>0.05$) are shown in bold.

Sample groups	PERMANOVA ^a		ANOSIM ^b	
	<i>F</i>	<i>P</i>	<i>R</i>	<i>P</i>
Control vs Day 0	2.0930	0.0090	0.3862	0.0101
Control vs Day 37	1.5131	0.0480	0.2535	0.0404
Control vs Day 72	2.0912	0.0007	0.5631	0.0013
Control vs Day 195	1.8299	0.0218	0.3991	0.0215
Control vs Day 265	1.1284	0.2397	0.1152	0.1301
Day 0 vs Day 37	3.0799	0.0001	0.5607	0.0006
Day 0 vs Day 72	4.8358	0.0004	0.9485	0.0006
Day 0 vs Day 195	4.6262	0.0007	0.8387	0.0006
Day 0 vs Day 265	2.9800	0.0001	0.5267	0.0006
Day 37 vs Day 72	1.4551	0.0717	0.1633	0.0793
Day 37 vs Day 195	2.0512	0.0200	0.3848	0.0192
Day 37 vs Day 265	1.6617	0.0180	0.2459	0.0146
Day 72 vs Day 195	1.3986	0.1152	0.1118	0.1166
Day 72 vs Day 265	2.1443	0.0004	0.4956	0.0006
Day 195 vs Day 265	1.529	0.0651	0.1681	0.0810

^aPermutational multivariate analysis of variance using distance matrices. Significance tests were performed by *F* test based on sequential sums of squares from permutations of the raw data.

^bAnalysis of similarities. Statistic *R* is based on the difference of mean ranks between groups and within groups.

Organohalide respiration in pristine hypersaline lakes

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Abstract

Despite compiling evidence for the presence of organohalide-respiring bacteria in pristine environments, there is lack of knowledge about their distribution and potential contribution to halogen cycle, especially in pristine environments with harsh pH and salinity such as hypersaline lakes. Biotic formation of chloromethane and chloroform was reported from Lake Strawbridge. Assuming that natural occurrence of organohalides can prime development of organohalide respiration (OHR), we prepared anoxic microcosms from Lake Strawbridge sediments. Dechlorination of amended chloroform and tetrachloroethene (PCE) to dichloromethane and trichloroethene was observed. Known chloroform and/or PCE dechlorinators were not detected in sediment microcosms and transfer cultures using MiSeq 16S ribosomal RNA gene analysis and quantitative PCR, suggesting the presence of yet unknown OHRB. To our knowledge, this is the first report on OHR occurrence in extreme pristine environments. Combined with formerly documented biotic organohalogen formation, this suggests potential interdependencies between microbial chlorinators and dechlorinators in local chlorine cycle.

The long-held view that organohalides exclusively originate from anthropogenic sources was changed following the discovery of ubiquitous naturally produced organohalides [432]. Such ample production of organohalides in pristine environments can feed organohalide-respiring bacteria (OHRB) that use organohalides as electron acceptors and grow via organohalide respiration (OHR) [47, 49, 179, 433]. OHRB isolated to date have moderate traits with regards to pH and salinity [378], but it can be anticipated that they should also present in extreme environments such as hypersaline lakes that have been reported to emit C1/C2 organohalides including but not restricted to chloromethane [39], chloroform [39, 434, 435], and tetrachloroethene (PCE) [434]. Nevertheless, to our knowledge, there is no report on the occurrence of OHRB in such extreme environments where these organisms could potentially contribute to local halogen cycles. The aim of this study was to assess OHR potential of hypersaline lake sediments and to identify the responsible OHRB.

We sampled duplicate sediment cores from two hypersaline lakes in Western Australia, i.e. Lake Strawbridge and Lake Whurr (Figure S7.1). Biotic formation of chloroform and chloromethane was previously reported from Lake Strawbridge [39], but not from Lake Whurr. Sediment cores were cut into two parts, i.e. the top layer (0-12 cm) and bottom layer (>12 cm). Geochemical properties such as salinity, total organic carbon (TOC), and pH were different between the two layers and between the samples of different lakes (Table 7.1). A detailed description of materials and methods used in this study is given in Supplementary Materials.

Microcosms were prepared using sediments from the two sampled lakes in two different anoxic media i.e. MGM and DBCM2 (Table S7.1, Dyll-Smith [436]). These media were previously used for the isolation of haloarchaea [436] and were selected based on our keen interest to investigate whether archaea can mediate OHR in these extreme environments. Sediment microcosms were prepared at the same salinity and pH as measured in the corresponding sediment layer used as inocula (Table S7.1). Chloroform or PCE were added at 1.25 μmol to each bottle. Chloromethane was not used in this study due to its lower oxidation state and hence poor capacity as an electron acceptor for OHR. Chloroform was dechlorinated to dichloromethane in all microcosms derived from Lake Strawbridge sediments (Figure S7.2) but not in microcosms obtained from Lake Whurr sediments (data not shown), which is in line with the lack of natural OH production in the latter lake. Dechlorination of chloroform was faster in microcosms derived from bottom as compared to top layer sediments (Figure S7.2), the former being characterized by lower TOC and salinity and higher pH (Table 7.1).

Furthermore, dechlorination was faster in incubations using MGM than DBCM2 medium, with the former containing peptone and yeast extract but no separate addition of trace elements and vitamins. To our surprise, PCE was dechlorinated to trichloroethene (TCE) after a long lag phase in microcosms prepared from Lake Strawbridge bottom layer sediment, but only in DBCM2 medium (Figure S7.2). It is possible that PCE was naturally formed in Lake Strawbridge but not detected by Rücker [39], or that OHRB carrying reductive dehalogenases with a broad substrate spectrum were present in the sediment and adapted to use PCE as the electron acceptor after incubation in the lab.

The microcosms derived from Lake Strawbridge bottom layer sediments and cultivated in MGM media were selected for further enrichment using chloroform. Dechlorination activity was stably maintained during three sequential transfers with dichloromethane as the end product (Figure 7.1). No dechlorination was observed in abiotic controls (data not shown) indicating OHR-mediated dechlorination.

Higher abundance of bacteria than archaea was detected in original microcosms and transfers (Figure S7.3). The archaeal abundance was decreased considerably by the third transfer, which makes their involvement in OHR unlikely. 16S ribosomal RNA (rRNA) genes of canonical chloroform- and/or PCE-dechlorinating OHRB such as *Desulfitobacterium*, *Dehalobacter*, *Dehalococcoides mccartyi*, *Geobacter* and *Sulfurospirillum* were not detected in any of the anoxic microcosms and transfers derived from Lake Strawbridge sediments (data not shown) using genus-specific qPCRs (Table S7.2). Principal coordinate analyses (PcoA) of 16S rRNA gene-based community patterns (MiSeq) revealed separate clustering of original sediment samples of Lake Whurr from samples of Lake Strawbridge (Figure S7.4). Besides, the initial sediment-derived microcosms and transfer cultures grouped together but distinctly separated from the original lake sediment samples, indicating their divergence from the original sediments used as inocula and convergence to a stable bacterial community during transfers (Figure S7.4). Twenty-eight of the 47 operational taxonomy units (OTUs) with relative abundance of above 2.5% in at least one sample showed higher relative abundance in enrichments and transfers compared to the original sediments inocula (Figure S7.5, Table S7.3). Twenty-four OTUs (mainly halophilic *Firmicutes*, *Proteobacteria* and *Bacteroidetes*) were not detected in sediment inocula (Table S7.3). Three of these OTUs were assigned to the genus of *Desulfovibrio* (Figure S7.6, Table S7.3). Some members of this genus are known halophenol-respirers such as *Desulfovibrio* sp. TBP-1 [137] and *D. dechloracetivorans* SF3 [138], but their ability to dechlorinate chloroform and PCE have not been reported.

Furthermore, OTU719, assigned as uncultured *Clostridiales*, was the most enriched OTU in both LS1 and LS2 transfers (Figure S7.5, Table S7.3), indicating that this novel *Clostridiales* phylotype likely is involved in chloroform dechlorination and/or played an important role in the chloroform-dechlorinating community.

To our knowledge, OHRB have until now only been detected/isolated from habitats with neutral or acidic pH, but not from alkaline environments like Lake Strawbridge. Our study is also the first report suggesting the presence of OHR in a pristine environment with high salinity. Hypersaline environments like salt lakes/marshes are widespread in the world, and areas affected by high salt concentrations will increase in the next few decades due to global sea level rise (coastal areas) and secondary salinization in inland environments. Those hypersaline environments seem to play an important role in natural OH emission [434, 437-439]. OHRB in salt lakes might take advantage from the products of biotic chlorination as electron acceptors. Future functional (meta)genomics, further enrichment and isolation and compound specific isotope analysis will be necessary to reveal microbial players of chlorination and dechlorination, their strategy to adapt to alkaline pH and high salinity, and their (presumed) respective roles and interactions within local chlorine cycles.

Acknowledgements

We would like to thank Laura A. Hug and Pascal Weigold for expert advice and technical assistance and Christoph Tubbesing for the copyright of sediment photos. We acknowledge the China Scholarship Council for the support to Yue Lu and Peng Peng. Furthermore, Siavash Atashgahi, Detmer Sipkema and Hauke Smidt received support by a grant of BE-Basic-FES funds from the Dutch Ministry of Economic Affairs. Andreas Kappler and Alexander Ruecker received funding from the research unit 763 “Natural Halogenation Processes in the Environment, Atmosphere and Soil” funded by the German Research Foundation (DFG).

Table 7.1 Geochemical properties of Lake Strawbridge and Lake Whurr sediments.

Properties	Lake Strawbridge (LS)				Lake Whurr (LW)			
	LS1 Top	LS2 Top	LS1 Bottom	LS2 Bottom	LW1 Top	LW2 Top	LW1 Bottom	LW2 Bottom
pH ^b	8.2	8.3	8.5	8.5	5.4	5.4	4.5	4.6
Water content (%)	37.3	27.3	16.7	15.4	26.0	25.7	24.2	23.0
Salinity (%)	17	14	5	5	15	20	11	11
TOC (g/kg)	21	15	5	5	12	14	6	6
Na (mg/g)	57.0	48.5	17.5	18.1	55.0	71.1	35.0	35.8
Ca (mg/g)	0.7	0.8	0.1	0.2	6.8	4.2	0.3	0.3
K (mg/g)	2.0	2.0	1.0	0.9	1.7	1.8	1.1	1.2
Mg (mg/g)	2.8	2.9	1.1	1.1	4.5	4.6	3.5	3.4
Total Fe (mg/g)	6.5	6.3	2.2	1.9	1.5	3.2	0.3	0.6
Cl ⁻ (mg/g)	101.3	84.7	31.9	33.1	93.1	123.5	64.8	64.0
SO ₄ ²⁻ (mg/g)	3.9	3.6	1.5	1.8	19.6	14.8	4.3	4.4
NO ₃ ⁻ (mg/g)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ClO ₃ ⁻ (mg/g)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Duplicates sediment cores from each hypersaline lake are labelled as LS1&LS2 and LW1&LW2. Top :0-12 cm depth, Bottom: >12 cm depth.

^aDetected as single measurement.

^bMeasured in 0.01 M CaCl₂ after 2 h.

^cCalculated based on the NaCl concentration as described before [440].

n.d. not detected.

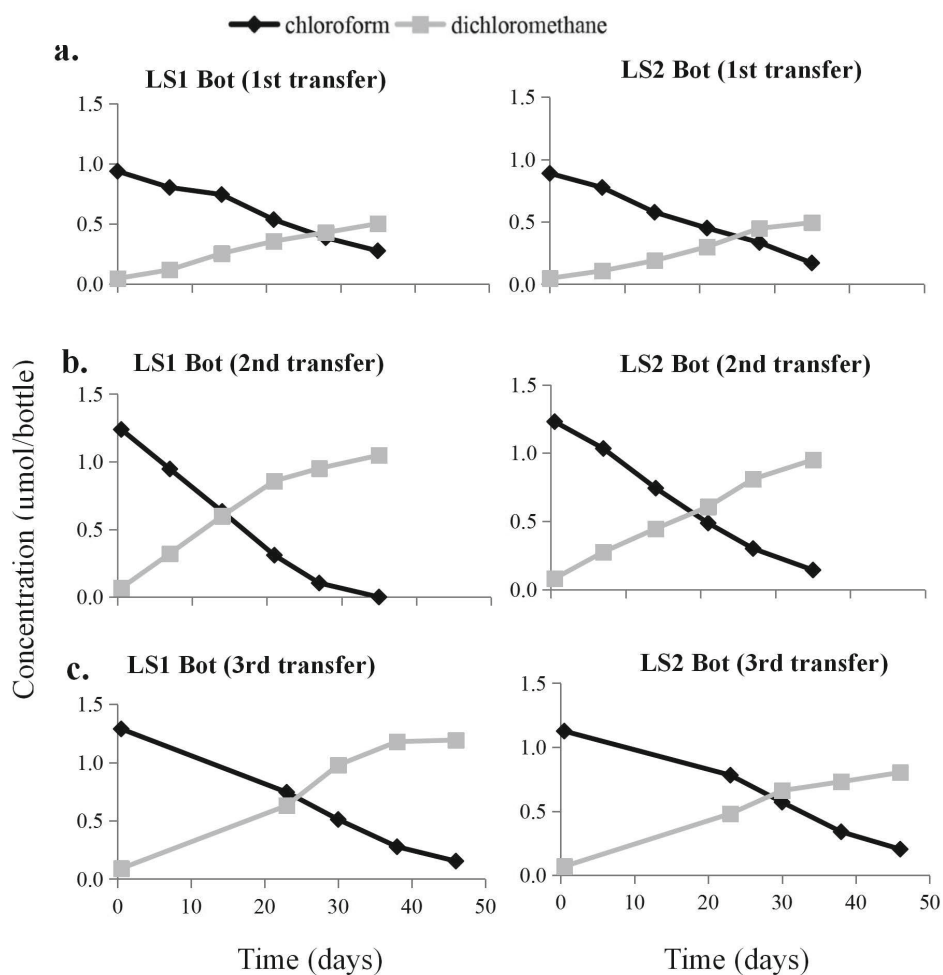


Figure 7.1 Dechlorination of chloroform in transfers of anaerobic enrichments derived from the microcosms prepared from the bottom layer (Bot, >12 cm depth) sediment collected from Lake Strawbridge. Duplicates sediment cores are labelled as LS1 and LS2. **a**, **b**, **c** are the first, second and third transfers, respectively.

Supplementary materials

Sampling site

Sediment samples were collected from Lake Strawbridge (LS, 32.84°S, 119.40°E) and Lake Whurr (LW, 33.04°S, 119.01°E), two salt lakes located in the Western Australian wheat belt (Figure S7.1). Duplicates sediment cores of approximately 24 cm length and 4 cm internal diameter were sampled from close locations within each of the two lakes and transported to the Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands. In an anoxic chamber filled with an atmosphere of N₂/H₂ (96%:4 %, v/v), sediment cores were cut into two parts, i.e. the top layer (0-12 cm) and bottom layer (>12 cm). Subsamples from each sediment layer were homogenized removing big pieces of vegetation before geochemical analysis and set-up of anoxic microcosms. Aliquots of the remainder were stored in Eppendorf tubes at -80 °C for further molecular analysis.

Geochemical analysis

Water content was determined by weighing wet samples and subsequently drying at 105 °C overnight to a constant weight and cooling down to room temperature in a desiccator. The weight loss in percentage was calculated as the water content. pH was measured with air-dried sediments suspended in 0.01 M CaCl₂ solution, and the pH was measured immediately and again after two hours. Sediment total organic carbon (TOC) was measured using the Kurmies method [441] in the chemical biological soil laboratory (CBLB) of Wageningen University. Low crystalline “bioavailable” iron was extracted from 0.5 g wet sediment for one hour in the dark using 25 ml of 0.5 M anoxic HCl [442], and concentrations of dissolved Fe(II) and Fe(III) were quantified using the spectrophotometric Ferrozine assay (Stookey, 1970). Major anions including Cl⁻, SO₄²⁻, NO₃⁻ and ClO₃⁻, were analyzed on a Thermo Scientific Dionex™ ICS-2100 Ion Chromatography System (Dionex ICS-2100). Major cations including Ca²⁺, K⁺, Mg²⁺ and Na⁺ were measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES, Varian, The Netherlands). Salinity was calculated based on the NaCl concentration as described before [440].

Microcosm set-up and transfers

Hypersaline sediments from top and bottom layers of Lake Strawbridge and Lake Whurr were used for microcosm set-up. Anoxic microcosms were prepared in 50 ml serum bottles with 2.5 g wet sediment and 25 ml of either MGM or DBCM2 medium (Table S7.1, [436]. The components of the SL10 trace element solutions (per liter) were 10 ml of 25% HCl;

$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 1.5 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 190 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 100 mg; ZnCl_2 , 70mg; H_3BO_3 , 6 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 36 mg; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 24mg; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 2 mg. The components of the Vit10 vitamin solution (per liter) were 4-aminobenzoate, 13 mg; d-(+)-biotin, 3 mg; nicotinic acid, 33 mg; hemicalcium D-(+)-pantothenate, 17 mg; pyridoxamine hydrochloride, 50 mg; thiamine chloride hydrochloride, 33 mg; cyanocobalamin, 17 mg; D,L-6,8-thioctic acid, 10 mg; riboflavin, 10 mg; folic acid, 4 mg. Bottles were sealed with a viton stopper, and the headspace was exchanged with N_2 gas (140 kPa). Salinity and pH in the medium were adjusted to the same values as observed in inocula sediments (Table 7.1, Table S7.1). Chloroform or PCE were added at 1.25 μmol to each bottle. A second spike (1.25 μmol) of chloroform was added at day 46 to MGM medium microcosms derived from LS bottom sediments that had completely dechlorinated the first chloroform amendment. All microcosms were set up in duplicate and cultivated without shaking at 37 °C in the dark.

A 10% (v/v) inoculum was taken from MGM medium microcosms derived from duplicate LS bottom sediments for three consecutive serial transfers using the same medium and chloroform dosing. Transfer cultures were set up as single bottles and an abiotic control was prepared by adding sterilized (autoclaving at 121 °C for 30 min) inocula. All cultures were cultivated without shaking at 37 °C in the dark.

Detection of chloroform and dichloromethane

Chlorinated methanes and ethenes were quantified by taking 0.5 ml headspace samples of the cultures and analyzed using a gas chromatograph equipped with a flame ionization detector (GC-FID, Shimadzu 2010) as described before for chlorinated methanes [436] and chlorinated ethenes [30]. A Stabilwax column (Restek Corporation, U.S) was used with nitrogen as carrier gas at a flow of 1 ml min^{-1} . A split flow of 12 ml min^{-1} with split ratio of 1:10 was set in the injector held at 250 °C. Standards were set up with the same headspace-liquid phase ratio as treatment cultures.

DNA extraction

To avoid the interference of high salinity of samples with the DNA extraction procedure [440], 0.5 g wet sediment was washed three times with 1.5 ml of 10 mM TE buffer (pH 7.0), and the washing buffer was collected and filtered through a 0.22 μm Millipore membrane filter (MP, USA). DNA loss during washing was anticipated, but with limited sediment material this was the only solution found to ensure enough DNA for further analysis. Washed sediment and the Millipore membrane filter were extracted separately using the PowerSoil DNA isolation kit

(MO-BIO, USA) following the manufacturer's instructions. DNA extracts from sediment and filter were combined for further molecular analysis as described before [443]. DNA isolation was conducted from three independent samples from each sediment layer. In total, DNA was extracted from 24 samples (two hypersaline lakes \times duplicates sediment cores from each lake \times two sediment layers \times technical triplicates). Two ml of anoxic medium were sampled from anoxic microcosms and transferred cultures for DNA extraction using the same method but without the initial washing step.

Illumina MiSeq sequencing and data analysis

A 2-step PCR strategy was applied to generate barcoded amplicons from the V1-V2 region of the bacterial 16S ribosomal RNA (rRNA) genes, and the PCR products were purified and sent for sequencing on a MiSeq platform (GATC-Biotech, Konstanz, Germany) as described in chapter 5. Sequence analysis was performed with NG-Tax, an in-house pipeline [346]. Principal coordinate analyses (PcoA) plot of bacterial 16S rRNA gene-based community composition was done by using Quantitative Insights Into Microbial Ecology (QIIME) v1.2 [349]. Heatmap visualisation of microbial composition was performed using R 3.2.3 [444] and the following libraries: gplots, vegan and RColorBrewer. The Bray method implemented in the “vegan” library was used to create a dissimilarity matrix and derived dendrograms of samples and operational taxonomic units (OTUs).

Phylogenetic Analysis

Phylogenetic analysis of selected 16S rRNA gene sequences derived from the bacterial MiSeq dataset was performed via Mega 4 program [350]. The Neighbour-joining method was used to construct a phylogenetic tree.

Quantitative PCR

The abundance of 16S rRNA genes of total bacteria, archaea, and several canonical OHRB including *Desulfitobacterium*, *Dehalobacter*, *Dehalococcoides mccartyi*, *Sulfurospirillum* and *Geobacter* were determined by quantitative real time PCR (qPCR). Assays were performed in triplicates on a CFX384 Real-Time system in C1000 Thermal Cycler (Bio-Rad Laboratories, USA) with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, USA). The primers and qPCR programs used in this study are listed in Table S7.2.

Nucleotide sequences

Nucleotide sequences were deposited in the European Nucleotide Archive (ENA) with accession number ERS1165096-ERS1165117 under study PRJEB14107.

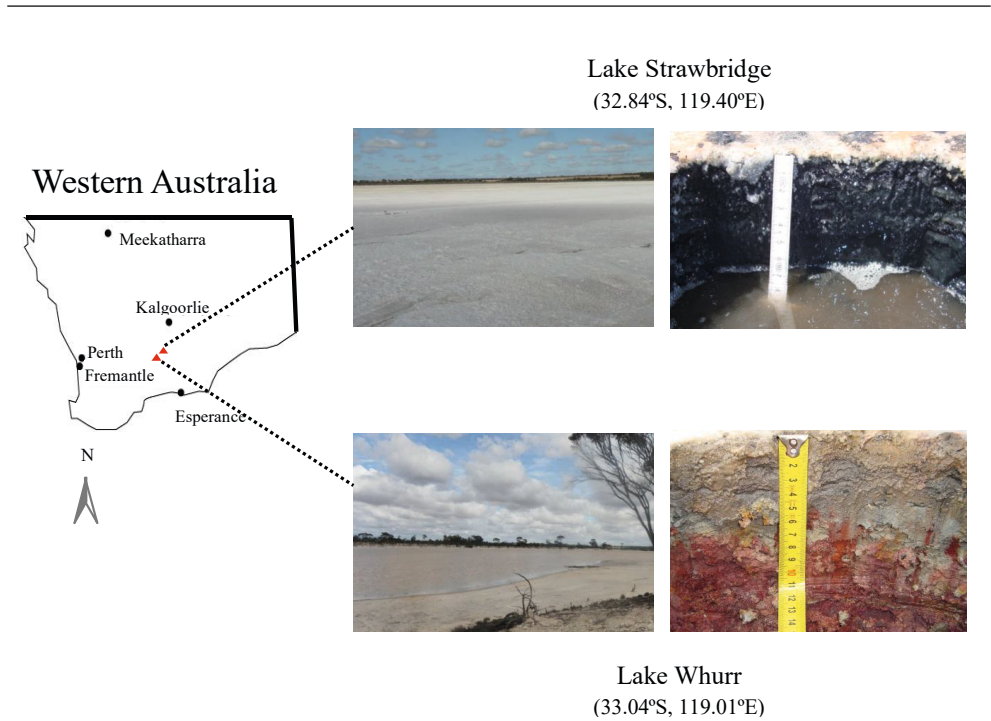


Figure S7.1 Location of Lake Strawbridge and Lake Whurr and coordinates of the sampling points.

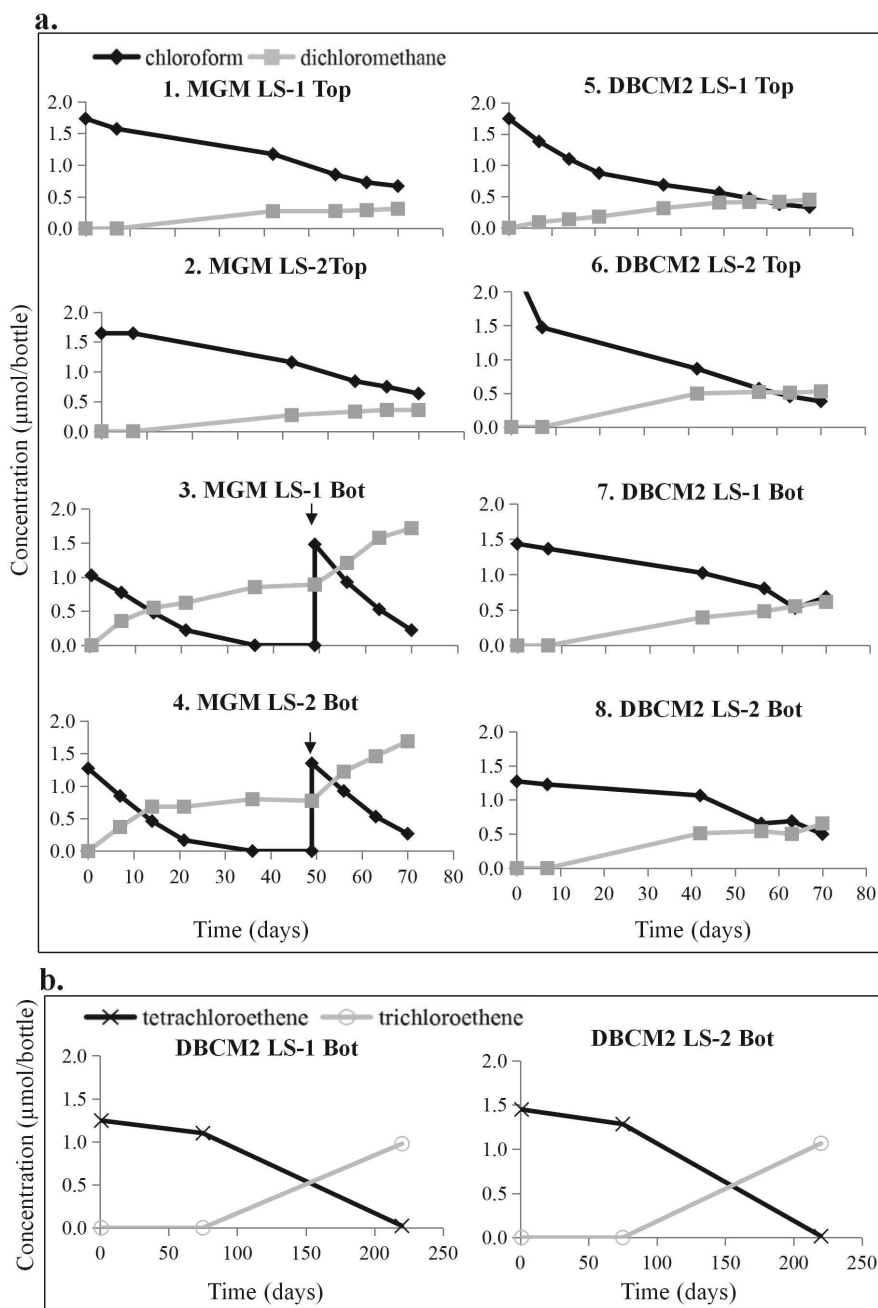


Figure S7.2 Dechlorination of chloroform (**a**) and tetrachloroethene (**b**, no analysis between day 75 and 220) in microcosms derived from the Lake Strawbridge (LS) sediment and cultivated in MGM (**a1-a4**) or DBCM2 (**a5-a8**, **b1-b2**) media. Abbreviation: Top, top layer (0-12 cm dph); Bot, bottom layer (>12 cm depth). Duplicates sediment cores are labelled as LS1 and LS2. Addition of second spike of chloroform is indicated by arrows.

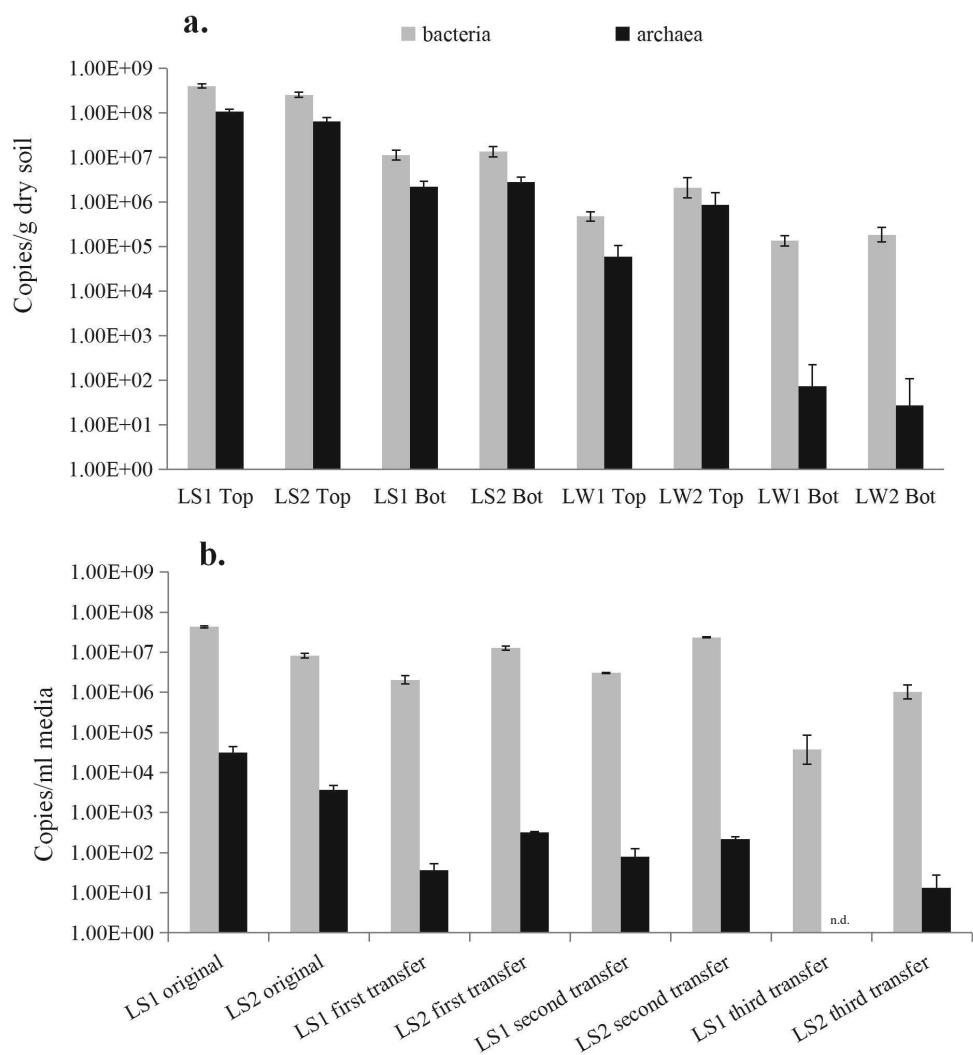


Figure S7.3 Quantitative PCR (qPCR) targeting total bacterial and archaeal 16S rRNA genes in hypersaline lake sediments (a) and during transfers (b). Abbreviation: LS, Lake Strawbridge; LW, Lake Whurr; Top, top layer (0–12 cm depth); Bot, bottom layer (>12 cm depth). Duplicate sediment cores from each lake are labelled as LS1&LS2 and LW1&LW2. Error bars in: a. represent standard deviations of three independent DNA extractions, and qPCR technical triplicates are conducted for each DNA sample and b. standard deviations of qPCR technical triplicates from the same DNA. n.d. nondetectable.

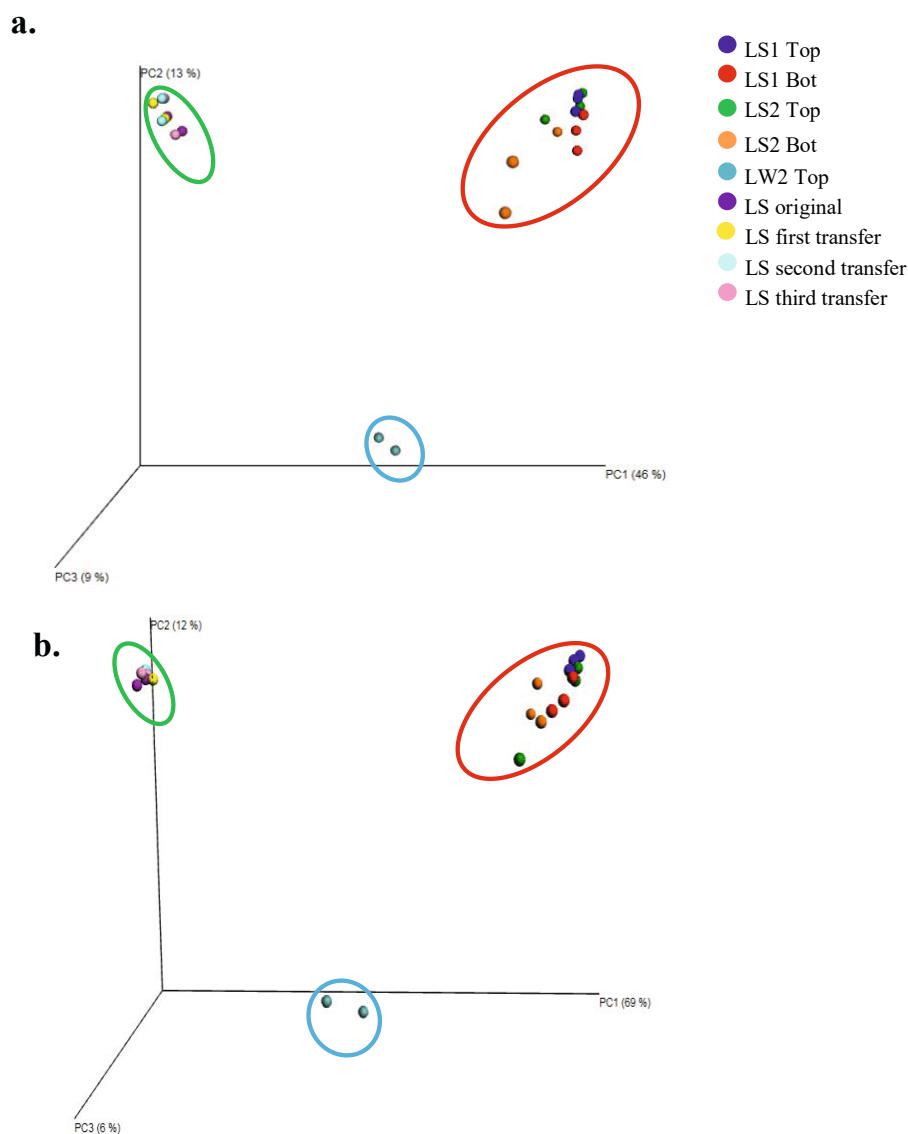


Figure S7.4 Unweighted (**a**) and weighted (**b**) principal coordinate analyses (PcoA) plot of bacterial OTUs in hypersaline lake sediments and anaerobic enrichments derived from the bottom layer sediments in Lake Strawbridge. Abbreviation: LS, Lake Strawbridge; LW, Lake Whurr; Top, top layer (0-12 cm depth); Bot, bottom layer (>12 cm depth). Duplicate sediment cores from each lake are labelled as LS1&LS2 and LW1&LW2. No data from LW1 samples are available because of low DNA yield. Original sediment samples from Lake Whurr and Lake Strawbridge are shown within solid blue and red ovals, respectively. Anaerobic enrichments derived from bottom layer sediments in Lake Strawbridge and transfers are shown within solid green ovals.

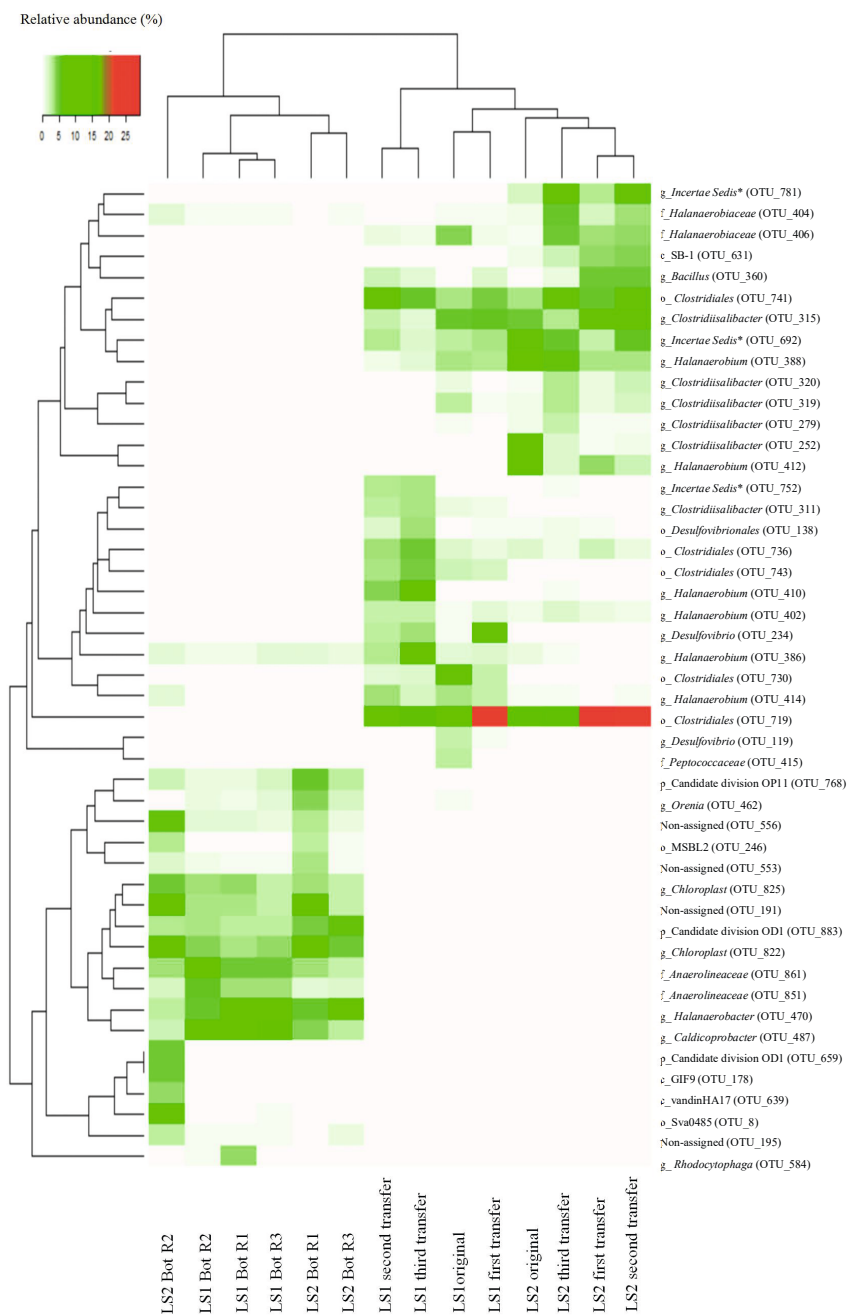


Figure S7.5 Heatmap of OTUs (relative abundance in at least one sample > 2.5%) derived from bacterial 16S rRNA gene-based community composition (MiSeq) in sediments of Lake Strawbridge (LS) and during transfers of chloroform-dechlorinating enrichments (enrich) derived from bottom layer (Bot, >12 cm depth) sediments in LS. Duplicates sediment cores are labelled as LS1 and LS2. R1-3 are three independent DNA extractions. OTUs

are defined at a threshold of 99% (maximum one nucleotide mismatch is allowed). OTUs that could be assigned to class, order, family and genus level are indicated with c_, o_, f_ and g_ prefixes, respectively. *OTU_781, OTU_692 and OTU_752 belong to the families of *Ruminococcaceae*, *Peptostreptococcaceae* and *Erysipelotrichaceae*, respectively.

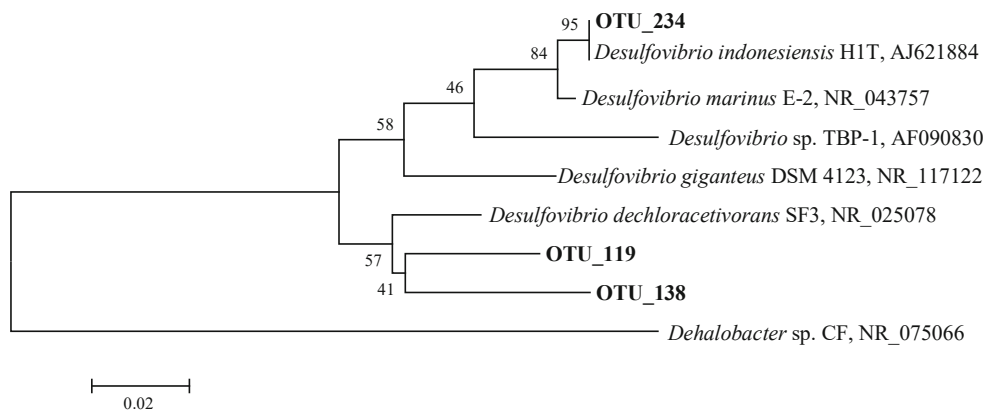


Figure S7.6 Phylogenetic tree of *Desulfovibrionales* 16S rRNA gene sequences derived from MiSeq dataset. Phylogenetic tree is constructed using the neighbour-joining method in the MEGA 4 program. Reference sequences are downloaded from NCBI and trimmed for sequence alignment with the concatenated sequences (200 bp) from merging pair-end reads (MiSeq). OTUs found in this study are shown in bold. The scale bar represents 2% sequence divergence. The 16S rRNA gene sequence of the chloroform-degrading *Dehalobacter* sp. CF is used as the root.

Table S7.1 Anaerobic media components.

Medium type (Salinity %)	Salt water ^a (ml/L)	Demi water (ml/L)	NH ₄ Cl (g/L)	Peptone (g/L)	Yeast Extract (g/L)	Pyruvate (g/L)	Resazurin (g/L)	Na ₂ S·9H ₂ O (g/L)	SL10 trace elements ^a (ml/L)	Vit10 vitamin solution ^a (ml/L)	pH ^b	Cultivated sediment
MGM (20%)	660	240	0	5	1	0	0.005	0.48	0	0	5.4	LW2 Top
MGM (17%)	560	340	0	5	1	0	0.005	0.48	0	0	8.1	LS1 Top
MGM (15%)	500	400	0	5	1	0	0.005	0.48	0	0	5.5	LW1 Top
MGM (14%)	460	440	0	5	1	0	0.005	0.48	0	0	8.3	LS2 Top
MGM (11%)	360	540	0	5	1	0	0.005	0.48	0	0	4.6	LW1&2 Bot
MGM (5%)	160	740	0	5	1	0	0.005	0.48	0	0	8.5	LS 1&2 Bot
DBC2 (20%)	660	240	0.5	0	0	1.1	0.005	0.48	1	3	5.4	LW2 Top
DBC2 (17%)	560	340	0.5	0	0	1.1	0.005	0.48	1	3	8.1	LS1 Top
DBC2 (15%)	500	400	0.5	0	0	1.1	0.005	0.48	1	3	5.5	LW1 Top
DBC2 (14%)	460	440	0.5	0	0	1.1	0.005	0.48	1	3	8.3	LS2 Top
DBC2 (11%)	360	540	0.5	0	0	1.1	0.005	0.48	1	3	4.6	LW 1&2 Bot
DBC2 (5%)	160	740	0.5	0	0	1.1	0.005	0.48	1	3	8.5	LS 1&2 Bot

Duplicates sediment cores from each hypersaline lake are labelled as LS1&LS2 and LW1&LW2. Top :0-12 cm depth, Bot: >12 cm depth.

^aSalt water, SL10 trace elements and Vit10 vitamin solution were prepared as described before [436].

^bpH was adjusted to the pH in the corresponding sediment layer using 1M Tris-base and/or 1M acetic acid.

Table S7.2 qPCR conducted in this study.

Target	Name ^a	Oligonucleotide sequence (5'-3') ^b	Application	Reference for primer	Reference for qPCR program
Bacterial 16S	27F-DegS	GTTYGATYMTGGCTCAG	Miseq	[371]	-
	338R-I	GCWGCCTCCGTAGGAGT	Miseq	[372]	-
	338R-II	GCWGCCACCCGTAGGTGT	Miseq	[373]	-
	Unitag1	GAGCCGTAGCCAGTCTGC	Miseq		
Bacteria 16S	Unitag2	GCCGTGACCGTGACATCG	qPCR	[445]	[226]
	Eub341F	CCTACGGGAGGCAGCAG	qPCR	[446]	[446]
	Eub534R	ATTACCGCGCTGCTGGC	qPCR	[196]	[196]
Archaea 16S	ARC787F	ATTAGATACCCSBGTAGTCC	qPCR	[196]	[226]
	ARC1059R	GCCATGCACCCWCCCTCT	qPCR	[196]	[226]
<i>Desulfotobacterium</i>	Dsb406F	GTACGACGAAAGGCCTTCGGGT	qPCR	[196]	[226]
	Dsb619R	CCCAGGGTTGAGCCCTAGGT	qPCR	[196]	[226]
<i>Dehalococcoides</i>	Deo728F	AAGGCGGTTTCTAGTGTGTCAC	qPCR	[196]	[226]
	Deo944R	CTTCATGCATGTCAAAT	qPCR	[196]	[226]
<i>Dehalobacter</i>	Dre441F	GTTAGGGAAAGAACGGCATCTGT	qPCR	[447]	[448]
	Dre645R	CCTCTCTGTCTCAAGCCATA	qPCR	[129]	[352]
<i>Geobacter</i>	Geo196F	GAATATGCTCTGATTC	qPCR		
	Geo535R	TAAATCCGAAACAACGCTT	qPCR		
<i>Sulfurospirillum</i>	Sulfuro114F	GCTAACCTGCCCTTTAGTGG	qPCR		
	Sulfuro421R	GTTTACACACCGAAATGCGT	qPCR		

^a Primer names may not correspond to original publication.^b M = A or C; R = A or G; W = A or T; Y = C or T.

Table S7.3 Overview of OTUs (relative abundance in at least one sample > 2.5%) derived from bacterial 16S rRNA gene-based community composition (MiSeq), which have higher relative abundance in enrichments and transferred cultures than inocula. Samples are from sediments of Lake Strawbridge (LS, inocula) and during transfers of chloroform-dechlorinating enrichments (original) derived from bottom layer (Bot, >12 cm depth) sediments in LS.

OTU ^a	Number of MiSeq reads										Assignment				
	LS1					LS2					phylum	class	order	family	genus
	inocula ^b	origi- nal	1 st transfer	2 nd transfer	3 rd transfer	inoc- ula ^b	origi- nal	1 st transfer	2 nd transfer	3 rd transfer					
119	0	4342	399	0	0	0	0	0	0	0	Pro- teobacteria	Delta- proteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
138	0	223	408	2838	6595	0	1530	216	130	479	Pro- teobacteria	Delta- proteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
234	0	538	7211	5070	6640	0	0	0	0	0	Pro- teobacteria	Delta- proteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
252	0	0	0	0	0	0	2897	299	505	935	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridialisibacter
279	0	508	264	0	0	0	2339	328	378	1424	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridialisibacter
311	0	1773	641	5344	6239	0	0	0	0	0	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridialisibacter
315	0	8871	5542	4766	2513	0	1562	4748	5125	1650	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridialisibacter
319	0	4544	391	0	0	0	1965	723	1504	1755	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridialisibacter
320	0	1898	0	0	0	0	1488	625	1579	1749	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridialisibacter
360	0	0	1676	4074	2518	0	0	3356	3679	509	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus
386	167	2342	1543	5811	15390	60	3177	0	0	308	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	Halanaerobium
388	0	5950	3219	1632	2586	0	3839	2363	2558	3316	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	Halanaerobium
402	0	500	1434	4935	4454	0	2065	735	473	964	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	Halanaerobium
404	64	666	320	228	0	25	2774	1288	2828	2871	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	Halanaerobium
406	0	7354	859	2082	1226	0	1631	2463	2854	2748	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	Halanaerobium
410	0	204	278	8312	12814	0	0	0	0	189	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	Halanaerobium

(continued)

412	0	0	0	0	0	0	0	0	0	2990	2847	1678	895	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	Halanaerobium
414	9	5686	2574	7156	3516	4	1100	0	221	249				Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	Halanaerobium
415	0	4832	0	0	0	0	839	0	0	0				Firmicutes	Clostridia	Clostridiales	Peptococcaceae	-
631	0	0	0	0	0	0	2061	2792	3136	1240				Bacteroidetes	SB-1	-	-	-
692	0	4671	3647	5619	2765	0	2115	1703	4066	2893				Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Incertae Sedis
719	0	2398	23641	22437	27696	0	5361	20509	17344	9023				Firmicutes	Clostridia	Clostridiales	-	-
730	0	1105	2731	2280	2887	0	0	0	0	0				Firmicutes	Clostridia	Clostridiales	-	-
736	0	2733	951	6760	8519	0	5132	1456	656	394				Firmicutes	Clostridia	Clostridiales	-	-
741	0	5518	4695	13226	9225	0	1057	3574	4743	3439				Firmicutes	Clostridia	Clostridiales	-	-
743	0	3537	2093	6128	8453	0	0	0	0	0				Firmicutes	Clostridia	Clostridiales	-	-
752	0	0	154	6026	5921	0	0	0	162	282				Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Incertae Sedis
781	230	0	0	0	0	170	5862	2102	4746	3701				Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Incertae Sedis
Total reads	182	1716	105063	190739	181897	5741	3239	70406	74897	55494								

^aThe OTUs are defined at a threshold of 99% (maximum one mismatch of nucleotide is allowed).

^bCumulative read numbers for three independent DNA extractions are given.

General discussion

Isolation of organohalide-respiring bacteria (OHRB) and their environmental distribution

OHRB are a special group of bacteria which are able to couple anaerobic respiration of halogenated organic compounds to energy conservation in a process termed as organohalide respiration (OHR) [7, 10]. Traditional culture-based microbiological methods, e.g. selective cultivation and strain isolation, have provided fundamental insights into physiology, biochemistry and genetics of this intriguing bacterial group. Up to date, 75 organohalide-respiring isolates belonging to 14 different genera in three bacterial phyla, i.e. *Chloroflexi*, *Firmicutes* and *Proteobacteria*, have been reported, of which 73 have been summarized in **Chapter 2**. Two new organohalide-respiring isolates were published after July 2015. One of these is *Dehalococcoides* sp. UCH007 derived from contaminated groundwater in Japan [449]. This bacterium dechlorinates trichloroethene, *cis*-1,2-dichloroethene (DCE) and vinyl chloride to non-toxic ethene [449]. Another isolate is *Dehalobacter* sp. UNSWDHB that was obtained from a contaminated aquifer near Sydney, Australia, and was shown to dechlorinate chloroform and chlorinated ethanes [450]. This new isolate added another continent from which OHRB have been isolated to date, further supporting their ubiquitous occurrence discussed in **Chapter 2**. Nevertheless, currently available isolates still represent only a very small fraction of the total diversity of OHRB that exist in the environments. High-throughput culturing approaches that aim at better mimicking *in situ* conditions, including the iChip [451] and the Microdish culture chip [452], have more recently been exploited to cultivate and/or isolate previously ‘uncultivable’ microbes. Considering that OHRB are fastidious microbes and mostly strict anaerobes, however, it remains to be demonstrated in future if such innovative cultivation approaches will allow improved cultivation and isolation of OHRB.

Up to date, most of the OHRB have been isolated from organohalide-contaminated environments (**Chapter 2**), however, an increasing number of reports show that OHRB are widely present in pristine environments, where they are believed to use naturally-occurring organohalides as electron acceptors. For instance, organohalide-respiring *Chloroflexi* were detected in 116 pristine soil samples collected in the USA [179]. Furthermore, members of the so-called Tidal Flat Clusters within the *Dehalococcoidia* that are capable of tetrachloroethene (PCE) dechlorination were enriched from marine sediments in Jade Bay, Germany [49]. More recently, bacteria belonging to the class of *Dehalococcoidia* were detected in deep anoxic waters of a meromictic crater lake, Lake Pavin in France [433]. However, to our knowledge, OHRB in pristine habitats with more extreme conditions such as those characterized by

acidic/alkaline pH and high salinity have not been studied before. To fill this knowledge gap, in **Chapter 7**, we reported reductive dechlorination of chloroform and PCE in anoxic microcosms prepared from sediments collected from the hypersaline Lake Strawbridge, Western Australia. This provided for the first time evidence for the presence of yet unknown OHRB in pristine hypersaline lakes. Considering the previously reported biological organohalide formation in such ecosystems [39, 434, 435], they provide an intriguing example of habitats potentially hosting interdependent biological chlorination and dechlorination in local halogen cycles and can serve as an interesting, yet untapped source for isolation of novel OHRB.

Detecting uncultured OHRB from environments

As summarized in **Chapter 2**, the application of culture-independent methods has highly facilitated the investigation of potential OHRB and their environmental distribution. This has led to the discovery of a broad range of potentially novel OHRB taxa. For instance, uncultured Lahn Clusters [48] and Tidal Flat Clusters [49] were both identified as PCE dechlorinators via combined application of RNA-based stable isotope probing and T-RFLP analysis, and were found only distantly affiliated to cultivated *Dehalococcoidia*, namely *Dehalococcoides mccartyi*, *Dehalogenimonas* spp. and *Dehalobium chlorocoercia*. Those discoveries have broadened our knowledge of the phylogenetic diversity of OHRB. Nevertheless, these and similar microbial ecological studies such as those described in this thesis (**Chapter 5-7**) used approaches that rely on the characterization of the microbial 16S rRNA (gene) pool. Such approaches have biases due to lack of universal coverage of primers that might overlook poorly/un-amplified bacterial groups. Therefore, primer-independent molecular approaches, including various ‘omics’ methods [13], have been applied for the detection and characterization of potential OHRB in environmental samples. For example, single-cell genomic studies on marine *Dehalococcoidia* [44, 45] did not reveal OHR associated genes, implying alternative metabolic modes for otherwise metabolically restricted *Dehalococcoidia*. It cannot be excluded, however, that because complete genomes are hardly retrieved by single-cell genome sequencing, genes might have been missed using this approach [44, 45]. In contrast, using single-cell sorting and subsequent single cell genome analysis, a recent study identified an *rdhA* from marine *Anaerolineales* suggesting that OHR is not limited to members of the *Dehalococcoidia* within the *Chloroflexi* [453]. Metagenomics was also applied to study OHRB, and to examine the metabolic processes and putative interactions within defined mixed cultures and dechlorinating microbial community [66, 237,

244, 303]. As with single-cell genomics-based approaches, to achieve complete genomes of OHRB, additional experimental efforts such as primer walking and subsequent amplicon sequencing are required to clarify ambiguities and to achieve gap closure and genome polishing [303]. Therefore, combined application of primer-dependent techniques, single-cell- and metagenomic surveys is instrumental in gaining a comprehensive understanding of the physiological and genomic characteristics of OHRB and their environmental distribution.

Organohalide substrate spectrum and biotransformation pathways

Even though diverse organohalides are present in the environment, most studies so far have focussed on biotransformation of organochlorine compounds and to a lesser extent on organobromine, organofluorine and organoiodine compounds. Also in our studies (**Chapter 4-7**), only OHR of chlorinated aliphatic (chlorinated ethenes, chloroform) and aromatic (chlorinated benzenes, CBs) compounds was investigated. Based on genome information different OHRB, a broad substrate spectrum with respect to OHR has been suggested [23]. However, only limited types of organohalides can be commercially purchased for research. Furthermore, most OHRB are strict anaerobes and grow with low biomass yields, which makes it difficult to test all available organohalides via traditional culture-based microbiological methods, and hence known OHRB have in most cases been only tested for a limited spectrum of potential substrates. In addition, the lack of correlation between sequence similarity of reductive dehalogenases (RDase) encoding genes and substrate specificity, i.e. the lack of robust structure-function relationships, has hindered the ability to confidently predict possible substrate spectra for novel RDase genes as discussed in **Chapter 3**. Interestingly several recent studies provided new leads towards possible structure-function relationships that might be useful for the prediction of organohalide substrate spectra for OHRB and their preferred biotransformation pathways. Elucidation of the crystal structures of two RDases for the first time indicated that substrate size might be an important factor, as a ‘letterbox’ with limited space and deeply buried corrinoid cofactor restrict the range of substrates able to enter and bind to the RDase active site [152, 454]. Furthermore, phylogenetic clustering of novel RDase amino acid sequences with those of characterized RDases was suggested as a useful tool to predict their functional specificity [12]. Another factor that can affect the substrate range and associated pathways is toxicity caused by the hydrophobic nature of substrates as revealed in the study of dehalogenation of bromophenol blue and tetrabromobisphenol A by *Dehalococcoides mccartyi* CBDB1 [455], and thermodynamic properties of substrates [456]. In line with the latter, thermodynamically more

favorable reactions were found to be selectively followed by CBs-dechlorinating microcosms as described in **Chapter 5**.

Corrinoid auxotrophy

Corrinoid is an essential cofactor for RDase, the key enzymes mediating microbial reductive dehalogenation. Corrinoid co-factors were found in all studied reductive dehalogenases except for the chlorobenzoate RDase functionally characterized from *Desulfomonile tiedjei* that harbors a heme cofactor [255]. Some facultative OHRB such as *Sulfurospirillum multivorans* [297], *Geobacter lovleyi* [128] and *Desulfitobacterium hafniense* [74, 301] are capable of *de novo* corrinoid biosynthesis. In contrast, *Dehalobacter restrictus* as an obligate OHRB is known as a corrinoid auxotroph. In **Chapter 4**, we confirmed the corrinoid biosynthetic pathway in *D. restrictus* PER-K23^T that is non-functional due to a 101-bp truncation in *cbiH* gene [67, 261]. Cobalamin is routinely supplied in the growth media during the isolation and maintenance of *D. restrictus* PER-K23^T in the laboratory, and it is tempting to speculate that the frameshift mutation in *cbiH* gene arose during long-term cultivation under cobalamin-rich conditions, and decay of the corrinoid biosynthesis pathway as an unused but costly trait. As this mutation is not found in other *Dehalobacter* spp. genomes, it would be interesting to study their response to corrinoid starvation. This has, however, not been investigated to date since sufficient corrinoid is always supplied during cultivation [55, 63, 269, 450].

Among the important elements in controlling corrinoid uptake and metabolism, five cobalamin riboswitches (Cbi-RS) were identified in *D. restrictus* PER-K23^T, which regulated the corrinoid metabolism at the transcription level (**Chapter 4**). The specificity of Cbi-RS to a particular corrinoid derivative was observed before in *Desulfitobacterium hafniense* [301]. It would be interesting to further investigate the substrate specificity of Cbi-RS found in *D. restrictus* PER-K23^T, which might help to reveal their interaction with particular corrinoid derivatives biosynthesized by co-existing microbes. The importance of gaining additional information on the *in situ* functionality of *Dehalobacter*-like populations is further reinforced by their involvement in the dechlorination of CBs in **Chapter 5**, indicating their potential to be applied in future bioremediation of anoxic contaminated environments.

Remodelling the lower ligand of phenolic corrinoids with 5,6-dimethylbenzimidazole (DMB) to produce the functional corrinoid cofactor, cobalamin, was reported before in *Dehalococcoides mccartyi*, which is also known as corrinoid auxotroph because of the lack of a complete *de novo* corrinoid biosynthetic pathway [93-95, 299]. It would be interesting to

know if remodelling is a shared trait between restricted OHRB i.e. if *Dehalobacter* species also apply such a corrinoid remodelling strategy, however, this has not been investigated.

Our research in **Chapter 4** suggested the importance of the application of functional analyses (e.g. transcriptional analysis and proteomics) in corrinoid metabolism studies of OHRB, as studies at the DNA level such as 16S rRNA genes amplicon sequencing and (meta)genomics can only answer the question ‘who is there?’ and ‘what might they be able to do’, but not ‘what are they doing and how?’. To further expand our knowledge of corrinoid metabolism of OHRB in a complex community, ‘meta’ approaches such as metatranscriptomics [457, 458] and metaproteomics [459, 460] can be considered to gain comprehensive understanding of the functional role of OHRB, the active metabolic interaction between OHRB and co-existing microbes, and the strategies OHRB employ to meet their nutritional need such as corrinoids, especially for corrinoid auxotrophic OHRB. However, these approaches are often technically challenging with respect to extraction of high quality RNA and proteins from environmental samples and complicated bioinformatic analysis that demand further wet-lab optimization and computational analysis simplification.

Syntrophic interactions in OHR consortia and the role of non-dehalogenating community members

Many OHRB can grow in axenic cultures with exogenous supplement of required micro- and macronutrients, whereas in natural habitats they rely on other microbes in the community to fulfil their nutritional needs (see Figure 8.1). Application of synthetic/enriched co-cultures as simplified model consortia has revealed some syntrophic interactions between OHRB and their companion microbes [63, 276, 324, 325, 461, 462]. The non-dechlorinating partners can stimulate the growth and dehalogenation activities of OHRB by i) providing carbon source (e.g. acetate) and electron donor (e.g. hydrogen) produced by fermenters like *Acetobacterium* [324] and *Desulfovibrio* [242, 324, 325]; ii) providing essential growth factors like corrinoids by methanogens and acetogens [299], *Desulfovibrio* [324, 325], *Geobacter* [93], and *Sedimentibacter* [63]. Other goods and services include vitamin K by provided by an *Enterococcus* [166], iii) and removal of carbon monoxide (CO) by CO-oxidizing bacteria such as *Desulfovibrio vulgaris* Hildenborough that *D. mccartyi* produces but cannot tolerate [37]. In return, OHRB may stimulate growth of non-dechlorinators by detoxifying notorious organohalides [63]. Furthermore, it is possible that OHRB provide additional goods/services in return to non-dechlorinators that have not been discovered yet. In addition to those

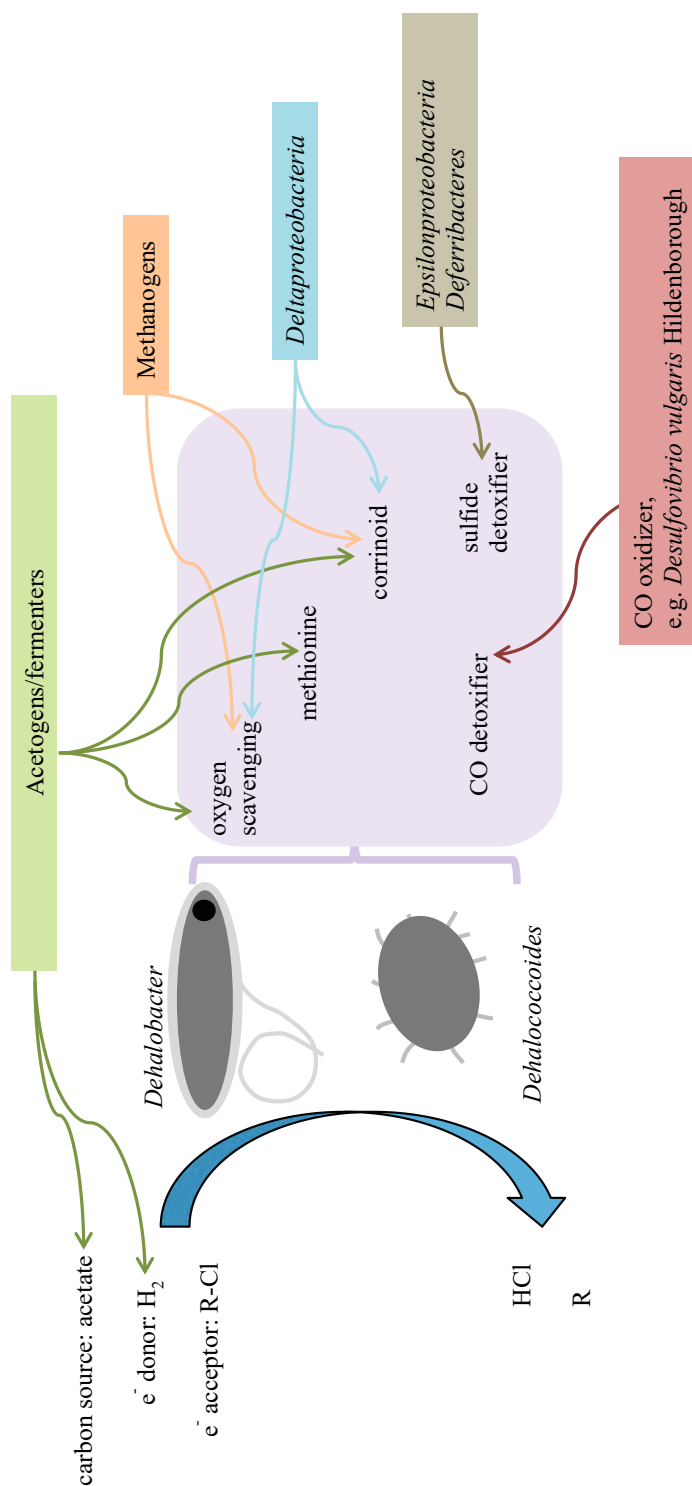


Figure 8.1 Conceptual schematic of metabolic interactions between obligate OHRB (*Dehalobacter* and *Dehalococcoides*) and co-existing, non-dechlorinating microbes in the community. Modified from Hug [237].

syntrophic interactions between OHRB and non-dechlorinators mentioned above, more potential interactions were discovered based on (meta)genomics studies of the metabolism of OHRB. For instance, the comparative metagenomics of three *Dehalococcoides*-containing enrichment consortia suggested *Deltaproteobacteria* and/or acetogens/fermenters as oxygen scavengers and methionine providers for *Dehalococcoides* [237].

Perhaps one of the most frequently encountered guilds in organohalide-respiring consortia at lab and field scales comprise the microbes driving the sulfur-cycle (**Chapter 5** and **6** of this thesis [227, 352]. Sulfide as a product of sulfate reducers is a known inhibitor of OHRB in the laboratory [41, 97, 161] and *in situ* [415]. Similarly, the abundance of *Dehalococcoidia* and other organohalide-respiring *Chloroflexi* was reported to be negatively impacted by reduced sulfur species in lake sediments [463]. In our study described in **Chapter 6**, we showed a yet unrecognised potential role for putative sulfur-oxidizing bacteria such as *Deferribacteres*, *Epsilonproteobacteria* (*Sulfuricurvum* and *Sulfurospirillum*) and candidate phylum OD1 under field conditions. These guilds seem to support *D. mccartyi* growth by oxidising sulfide and reducing its toxicity. However, these putative sulfur oxidisers seem to be competitive successors of the more established companions of OHRB, i.e. sulfate reducers and fermenters, and hence might impact OHR negatively. The net impact is not known and its verification remains challenging. Finally, sulfite and thiosulfate were reported before to inhibit OHRB strains like *Desulfomonile tiedjei* DCB-1^T [11, 136], which suggest microbes in the community that are capable of removing these compounds may stimulate OHR activity.

Members of the *Bacteroidetes* are reported in many environments such as freshwater, soil, the ocean, and the mammalian gut, all of which are characterized by the presence of a large spectrum of complex organic matter (e.g. proteins and carbohydrates) [464]. We found an exceptionally high relative abundance of members of the *Bacteroidetes* in CBs-dechlorinating microbial communities (with average relative abundance of 79.7 %, **Chapter 5**). To test the potential link between predominant *Bacteroidetes* and CBs dechlorinators, functional metagenomics studies encompassing metatranscriptomics and metaproteomics can be applied.

Concluding remarks and perspectives

Bioremediation is considered a sustainable, relatively efficient and cost-effective technology to treat contaminated sites [465]. OHRB continue to draw great scientific interest due to their potential application in bioremediation of organohalide contaminated subsurface environments [12, 13]. Their presence and activity is pivotal for effective bioremediation, but

lack of comprehensive information about their metabolism, nutritional requirements and interactions with geochemical geophysical parameters limits *in situ* implementation. The knowledge generated in this thesis that can be used to guide design and optimization of bioremediation strategies is summarized in Figure 8.2.

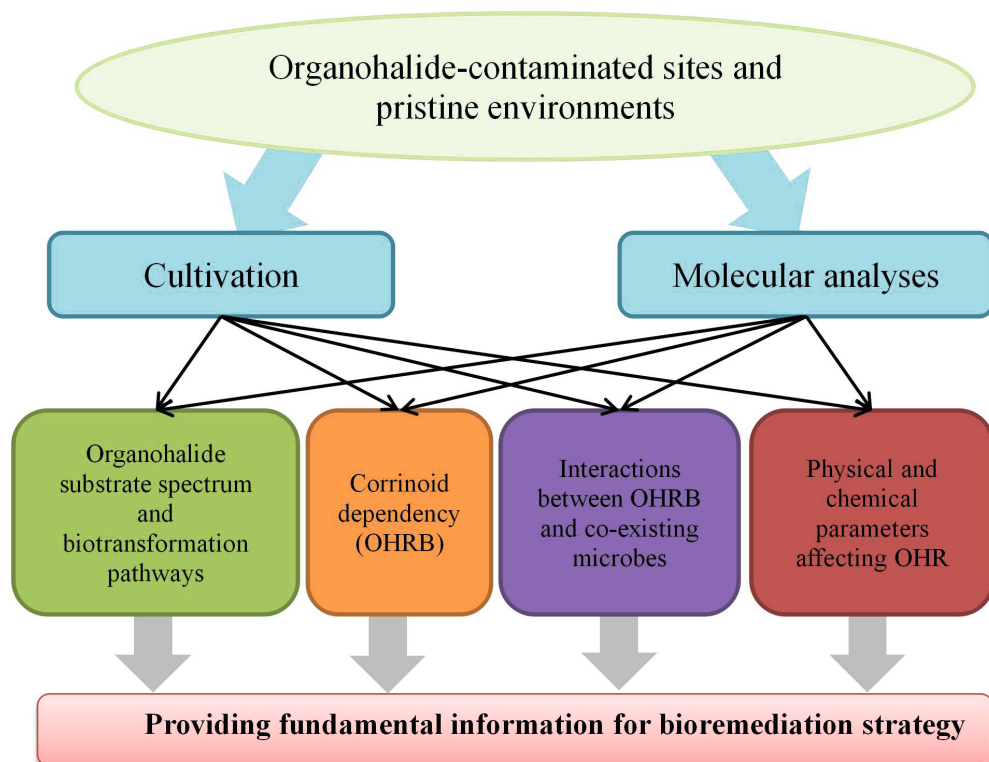


Figure 8.2 Overview of this study contributes to the bioremediation strategy.

As reviewed in **Chapter 2**, many environmental factors can affect the distribution and activity of OHRB, such as redox conditions, micronutrients, electron acceptors and interaction with chemical species, which further impact the outcome of bioremediation. An intriguing factor is DMB as essential lower ligand of a functional corrinoid cofactor (cobalamin) for RDases in the obligate OHRB *D. mccartyi* and *Dehalobacter*. It is likely that this compound will be used in attempts to biostimulate *in situ* reductive dehalogenation. However, DMB is known to enforce some community members to produce cobalamin that is non-functional for them. A recent example is the case of *S. multivorans* [145] which requires norpseudo-B12 as corrinoid cofactor [144]. Hence stimulating *D. mccartyi* guilds can on the other hand prohibit *S.*

multivorans which as discussed in **Chapter 2** can play multiple beneficiary roles in bioremediation of chlorinated ethene.

The enrichment cultures we obtained in **Chapter 7** seem to be well adapted to alkaline pH and high salinity in hypersaline sediments. Besides serving as a source for isolating novel OHRB, the enriched consortia could be a good option for bioremediation of contaminated sites with harsh pH and/or salinity, such as hypersaline wastewater [466] and/or alkaline wastewater if they are contaminated with target organohalides compounds.

Studies described in this thesis contribute to expanding our understanding of the ecophysiology and environmental distribution of OHRB from different angles, such as the presence of OHRB in pristine extreme environments, the corrinoid auxotrophy of OHRB, and their syntrophic interactions with co-existing, non-dechlorinating microbes. Our research provides knowledge for evaluating and optimizing bioremediation strategies and contributes to building a scientific foundation for regulatory bodies and companies to improve the efficacy of the bioremediation of organohalide pollutants.

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Summary

Many halogenated organic compounds (organohalides) are known environmental pollutants, threatening the health of humans and other animals. To address the environmental problems caused by organohalide pollution, successful application of bioremediation with efficient microbes is essential. Organohalide-respiring bacteria (OHRB) are able to breathe organohalides persistent in a broad range of oxygen-depleted environments and therefore present considerable potential for organohalide bioremediation. Nevertheless, to assess and adjust *in situ* bioremediation strategies, thorough understanding of ecophysiology of OHRB and their interaction with surrounding biotic and abiotic forces is necessary. To this end, this thesis focused on exploring ecophysiology and environmental distribution of OHRB and unraveling their interactions with the co-existing microbial guilds in the community and geochemical parameters by application of a suite of physiological, molecular and geochemical analyses.

Firstly, we provided a comprehensive overview of currently known OHRB isolates and highlighted their environmental distribution and factors impacting it (**Chapter 2**). In contrast to the limited/distinct locations sampled for isolation, no restriction for the geographical distribution of OHRB was observed in molecular surveys. The important environmental factors that affect the distribution and activity of OHRB were discussed, and chasing novel OHRB from extreme environments was proposed as the known OHRB isolates survive/thrive in moderate pH and salinity range in laboratory culture. Therefore, in **Chapter 7**, with the organohalide respiration (OHR) potential was surveyed in hypersaline sediments collected from Lake Strawbridge, Western Australia, to assess the presence of OHRB. As a result, for the first time, the dechlorination of chloroform and perchloroethene (PCE) to dichloromethane and trichloroethene was documented from an alkaline hypersaline pristine environment. Combined with former report on biological production of organohalides at that site, **Chapter 7** provided intriguing evidence of potentially interdependent biological chlorination and dechlorination in local halogen cycles occurring in hypersaline lakes.

Most molecular surveys for the presence and functionality of OHRB employ primer-based detection of their 16S rRNA and reductive dehalogenase (RDase) encoding genes. A comprehensive overview of degenerate/specific primers designed to target RDase encoding genes and primer-dependent techniques applied to date was provided **Chapter 3**. Further, the current trend in application of primer-independent, high-throughput approaches to expand our

knowledge of the OHRB and their RDase genes pool was discussed. Due to pros and cons associated with primer-(in)dependent methods, a combined application is more advantages.

Dehalobacter restrictus PER-K23^T was applied as a model strain to study corrinoid auxotrophy in **Chapter 4**. Corrinoid at fifty µg/L was found essential for sufficient PCE dechlorination. Transcriptional analysis and shotgun proteomics confirmed that the cobalamin biosynthesis pathway in *D. restrictus* is non-functional due to a 101-bp truncation in the *cbiH* gene, and suggested up-regulation of relevant cobalamin salvaging and transport pathways to ensure sufficient corrinoid supply under partial corrinoid starvation.

The importance of syntrophic interactions to support robust growth of OHRB was described in **Chapters 5&6**. In **Chapter 5**, bacteria belonging to the *Bacteroidetes* phylum and *Clostridiales* order as well as sulfate-reducing members of *Deltaproteobacteria* class were suggested as putative stimulating guilds that provide electron donor and/or organic cofactors to chlorinated benzenes dechlorinators, i.e. *D. mccartyi* and *Dehalobacter*, in sediment microcosms and transferred cultures derived from contaminated harbor sludge. In **Chapter 6**, a similar syntrophic interaction between *D. mccartyi* and fermenting and/or sulfate reducing bacteria were noticed for robust dechlorination of chlorinated ethenes after *in situ* biostimulation by glycerol injection. Furthermore, the appearance of *Epsilonproteobacteria* and *Deferribacteres* as putative oxidizers of reduced sulfur compounds in the late stage of the filed study suggested that on one hand they might serve as detoxifiers of sulfide stimulating *D. mccartyi*, but on the other hand could be inhibitory as successors of the more important syntrophs belonging to fermenters and sulfate reducers.

In conclusion, this thesis expanded our understanding of ecophysiology and environmental distribution of OHRB, addressing their presence in pristine environments as well as providing further evidence for their dependencies on other microbial community members in order to meet their nutritional requirements. Hence, research described here contributes to increasing our knowledge as a foundation for evaluating and optimizing strategies for the bioremediation of organohalide contaminated sites.

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Nederlandse samenvatting

Veel organische halogeenverbindingen zijn vervuילend voor het milieu en gevaarlijk voor de gezondheid van mensen en dieren. Om de problemen van vervuiling met organische halogeenverbindingen te overkomen, is het essentieel dat er succesvol bioremediatie met efficiënte microben wordt toegepast. Organische halogeenverbinding-gebruikende bacteriën (OHGB) kunnen deze verbindingen gebruiken als elektronen acceptor in een groot scala aan zuurstof arme milieus en kunnen daardoor worden ingezet voor bioremediatie. Echter, voor een optimalisatie van bioremediatie strategieën moet er nog beter begrip komen van de ecofysiologie van OHGB en de interactie met biotische en a-biotische factoren. Om dit te bereiken wordt in dit proefschrift de ecofysiologie en de verspreiding van OHGB onderzocht en hun interactie en samenleving met de lokale microbiële gemeenschap en geochemische parameters. Dit wordt onderzocht met behulp van een scala aan fysiologische, moleculaire en geochemische benaderingen.

Om te beginnen, hebben wij een uitgebreid overzicht gemaakt van de huidige bekende OHGB isolaten en hebben daarbij de nadruk gelegd op hun verspreiding in het milieu en de effecten die dat heeft (**Hoofdstuk 2**). Ook al zijn OHRB tot nu toe nog maar van een gelimiteerd aantal locaties geïsoleerd, blijkt er volgens kweek-onafhankelijke moleculaire analyses geen beperking te zijn voor hun geografische verspreiding. De belangrijkste omgevingsfactoren die de verspreiding en activiteit van de OHGB beïnvloeden worden besproken. Daarnaast worden de mogelijkheden om nieuwe OHGB te vinden in extreme milieus voorgesteld, aangezien de huidige OHGB isolaten bij neutrale pH's en lage zout concentraties overleven en groeien. Hieruit voortvloeiend is er in **Hoofdstuk 7** de potentie voor het gebruik van organische halogeenverbindingen bekeken in de extreem zoute sedimenten van Lake Strawbridge, Australië. Dit resulteerde in de eerste beschrijving van de dechlorinering van chloroform en perchloroetheen (PCE) naar dichloromethaan en trichloroetheen uit een ongerepte alkalische en extreem zoute omgeving. In combinatie met de eerder door anderen beschreven productie van organische halogeenverbindingen op deze plek geeft **Hoofdstuk 7** een interessant bewijs van de mogelijkheid van onafhankelijke biologische chlorinering en dechlorinering in halogeen- cycli die zich in extreem zoute meren afspelen.

De meeste moleculaire onderzoeken om de aanwezigheid en functionaliteit van OHGB aan te tonen, gebruiken een primer gebaseerde detectie van 16S ribosomaal RNA- en reductief dehalogenase (RDase) coderende genen. Een uitgebreid overzicht van de specifieke primers

voor RDase coderende genen en de toepassing van primer-afhankelijke technieken wordt gegeven in **Hoofdstuk 3**. Daarnaast bespreken we de huidige trend in het gebruik van primer-onafhankelijke technieken om onze kennis over OHGB en hun RDase genen te vergroten. Door de voor- en nadelen van primer-afhankelijke en –onafhankelijke methodes is het beter om een combinatie van beide te gebruiken.

Dehalobacter restrictus PER-K23^T is gebruikt in **Hoofdstuk 4** als model organisme om corrinoïde auxotrofie te bestuderen. Corrinoïdes zijn essentiële co-factoren van RDase enzymen. Vijftig µg/L corrinoïde is de essentiële hoeveelheid voor het dechloreren van PCE. Dit kon worden bevestigd door transcriptie en proteoom analyses. Genoom analyse heeft verder uitgewezen dat de niet functionele cobalamine biosynthese in *D. restrictus* wordt veroorzaakt door een verkorting van 101 bp in het *chiH* gen. Dit suggereert een verhoogde regulatie van cobalamine opname en transport systemen, om aan de corrinoïde behoefte te kunnen voldoen.

Het belang van syntrofe interacties voor goede groei van OHGB staat in de **Hoofdstukken 5 en 6** beschreven. In **Hoofdstuk 5** zijn dit bacteriën die behoren tot het *Bacteroidetes* phylum, de *Clostridiales* orde en sulfaat reduceerders uit de *Deltaproteobacteria* klasse. Deze bacteriën zouden elektronen en/of cofactoren kunnen bieden aan OHGB die chloor-benzeen kunnen gebruiken, zoals *Dehalococcoides mccartyi* en *Dehalobacter*. De bacteriën werden gevonden in ophopingen die werden verkregen van verontreinigd rivier- en havensedimenten. In **Hoofdstuk 6** is een vergelijkbare syntrofe interactie geobserveerd tussen *D. mccartyi* en fermenterende en/of sulfaat reducerende bacteriën. De syntrofie tussen deze bacteriën bestond uit een robuuste dechlorering van gechloreerde ethenen welke ontstond na *in situ* biostimulatie door middel van glycerol injectie. Daarnaast dienen bacteriën behorend bij de *Epsilonproteobacteria* en *Deferribacteres*, welke in een laat stadium van de studie zijn gaan groeien, mogelijk als detoxificeerders van sulfide en stimuleren daarmee *D. mccartyi*. Echter deze bacteriën zouden mogelijk ook inhibeerders kunnen zijn en verschijnen na de belangrijkere syntrofen die de feitelijke fermentatie en sulfaat reductie hebben gedaan.

Concluderend, verbreedt deze thesis ons begrip binnen de ecofysiologie en verspreiding van OHGB, waarbij hun aanwezigheid is gevonden in de meeste milieus. Daarnaast geven we meer bewijs voor hun afhankelijkheid van nutriënten van andere microben in het ecosysteem. Daarmee vergroot dit onderzoek onze kennis over de anaërobe microbiële afbraak van

organische halogeneenvbindingen, en kunnen zo strategieën voor bioremediatie worden geëvalueerd en verder worden verbeterd.

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About the author

Yue Lu was born on April 9th, 1989, in Shaoshan, Hunan province, China. In 2006, she started her bachelor study at the Department of Resources and Environmental Science, China Agricultural University (CAU), Beijing. In 2009, she started her BSc thesis entitled “Transcriptional response of methanogenic community to oxygen exposure in rice field soil”, under the supervision of Prof. Dr. Yahai Lu in the Lab of Molecular Ecology-Max Planck Partner Group. In 2010, she obtained her BSc degree of Science and received the award of Outstanding Undergraduate in Beijing. Inspired by the great experience of her stay in the lab of Prof. Dr. Yahai Lu, she continued with her MSc studies in the same year and completed her MSc thesis entitled “Effect of temperature on the structure and activity of a methanogenic archaeal community during rice straw decomposition”, which resulted in a first-author publication in the journal of Soil Biology and Biochemistry. After obtaining her MSc degree in 2012, she moved to the Netherlands and started a PhD project with the scholarship from China Scholarship Council at the Laboratory of Microbiology, Wageningen University, under the supervision of Prof. Dr. Hauke Smidt and Dr. Siavash Atashgahi.

During her study time, Yue Lu has been actively involved in diverse social activities. She joined the Student Union in Department of Resources and Environmental Sciences in CAU for two years, and she served as a volunteer in 29th Olympic Games, Beijing and in China Environmental Culture Promotion Association (member of the Ministry of Environmental Protection of China).

List of Publications

Yue Lu, Li Fu, Yahai Lu, Floor Hugenholtz, Ke Ma (2015). Effect of temperature on the structure and activity of a methanogenic archaeal community during rice straw decomposition. *Soil Biology and Biochemistry* **81**: 17-27. doi: 10.1016/j.soilbio.2014.10.031

Aamani Rupakula*, **Yue Lu***, Thomas Kruse, Sjeff Boeren, Christof Holliger, Hauke Smidt, Julien Maillard (2015). Functional genomics of corrinoid starvation in the organohalide-respiring bacterium *Dehalobacter restrictus* strain PER-K23. *Frontiers in Microbiology* **5**:751. doi: 10.3389/fmicb.2014.00751.

Yue Lu*, Siavash Atashgahi*, Laura A. Hug, Hauke Smidt (2015). Primer that target functional genes of organohalide-respiring bacteria. In: T.J. McGenity et al. (eds.) *Hydrocarbon and Lipid Microbiology Protocols*, Springer Protocols Handbooks. Springer. doi: 10.1007/8623_2015_75

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Yue Lu, Javier Ramiro-Garcia, Pieter Vandermeeren, Steffi Herrmann, Danuta Cichocka, Dirk Springael, Siavash Atashgahi, Hauke Smidt (2016). Dehalogenation of three tetrachlorobenzenes isomers by contaminated harbor sludge-derived enrichment cultures follows thermodynamically favorable reactions. Under review.

Siavash Atashgahi, **Yue Lu**, Ying Zheng, Edoardo Saccenti, Maria Suarez Diaz, Javier Ramiro-Garcia, Heinrich Eisenmann, Martin Elsner, Alfons JM Stams, Dirk Springael, Winnie Dejonghe, Hauke Smidt (2016). Geochemical and microbial analysis of groundwater at a site with biostimulated reductive dechlorination. Under review.

Siavash Atashgahi, **Yue Lu***, Javier Ramiro-Garcia*, Peng Peng, Farai Maphosa, Detmer Sipkema, Winnie Dejonghe, Hauke Smidt, Dirk Springael (2016). Geochemical parameters and reductive dechlorination determine cometabolic vs metabolic aerobic VC biodegradation at oxic/anoxic interface of hyporheic zones. Submitted for publication.

Yue Lu*, Peng Peng*, Alexander Martin Ruecker, Javier Ramiro-Garcia, Alexandr Umanetc, Andreas Kappler, Detmer Sipkema, Siavash Atashgahi, Hauke Smidt (2016). Organohalide respiration in pristine hypersaline lakes. Manuscript in preparation.

Ying Zheng, **Yue Lu**, Maria Suarez Diaz, Alfons JM Stams, Siavash Atashgahi, Hauke Smidt (2016). Anaerobic biodegradation of long-chain fatty acid methyl esters by an oleate-degrading enrichment culture. Manuscript in preparation.

Peng Peng, Jasper Koehorst, **Yue Lu**, Detmer Sipkema, Jaap S. Sinninghe Damste, Alfons JM Stams, Siavash Atashgahi, Hauke Smidt (2016). Isolation, identification and genomic characterization of an organohalide-respiring *Desulfoluna* bacterium. Manuscript in preparation.

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The SENSE Research School declares that **Ms Yue Lu** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 37.7 EC, including the following activities:

SENSE PhD Courses

- o Environmental research in context (2013)
- o Research in context activity: 'Co-organising PhD trip 2015 to Californian research institutes', Laboratory of Microbiology and Laboratory of Systems and Synthetic Biology of Wageningen University (2015)

Other PhD and Advanced MSc Courses

- o Information literacy including EndNote, Wageningen University (2012)
- o Systems biology: statistical analysis of ~omics data, Wageningen University (2012)
- o Hydrogen sulphide gas (H₂S) course, Netherlands Safety Education BV (2013)
- o Techniques for writing and presenting scientific papers, Wageningen University (2013)
- o Interpersonal communication for PhD students, Wageningen University (2013)
- o Metagenomics approaches and data analysis, Netherlands Bioinformatics Centre (NBIC) (2013)
- o Basic training in software packages ARB and SILVA, Wageningen University (2014)

Management and Didactic Skills Training

- o Teaching practical course 'Microbial Physiology', Wageningen University (2013)
- o Supervising MSc student with thesis entitled 'Enrichment of chlorobenzene degrading cultures', Wageningen University (2014)
- o Teaching practical course 'Research Methods Microbiology', Wageningen University (2015-2016)

Oral Presentations

- o *Functional genomics of Dehalobacter sp. E1 in syntrophic interaction with Sedimentibacter sp. B4.* FOR 1530 Research Unit Conference - Friedrich Schiller University Jena, 19-20 July 2013, Leipzig, Germany
- o *Chasing organohalide respiring bacteria (OHRB) from pristine hypersaline lakes.* FOR 1530 Research Unit Meeting - Friedrich Schiller University Jena, 14-15 April 2016, Weimar, Germany

SENSE Coordinator PhD Education


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