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Anaerobic microbial transformation of chlorinated alkanes in cultures derived from Besòs River estuary sediments

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"Anaerobic microbial transformation of chlorinated alkanes in cultures derived from Besòs River estuary sediments"

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CERTIFIQUEM:

Que el titulat en Enginyeria Bioquímica Siti Hatijah Mortan ha realitzat sota la nostra direcció, en els laboratoris del Departament d'Enginyeria Química, Biològica i Ambiental el treball que amb el títol "Anaerobic microbial transformation of chlorinated alkanes in cultures derived from Besòs River estuary sediments", es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I perquè en prengueu coneixement i consti als efectes oportuns, presentem a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat a Bellaterra, Marc 2017.

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ABSTRACT

Halogenated compounds (organohalides) are recalcitrant organic compounds, exhibiting toxic effects on human health and the ecosystem. Among the organohalides, chlorinated alkanes such as chloroethanes and chloropropanes received less attention despite their large use in the industry and have caused serious environmental problems. Physicochemical processes are the most commonly used technology to treat groundwaters contaminated with these compounds, however these techniques imply high operational costs and require a post-treatment step to completely destroy these compounds. Biodegradation has become a more suitable, cost-efficient and environmental friendly technology to address contamination of these compounds.

In this study, we aimed to obtain an enrichment culture containing organohalide-respiring bacteria able to transform halogenated alkanes. A stable nonmethanogenic enriched anaerobic culture that exclusively dehalogenates vicinally chlorinated and brominated alkanes via dihaloelimination was established from Besòs River estuary sediments (Barcelona, Spain). Application of genus specific primers targeting 16S rRNA gene sequences together with the observation of physiological characteristics of the dechlorinating culture indicated that a *Dehalogenimonas* strain was the responsible for chlorinated alkane degradation in the microcosms. The increase of *Dehalogenimonas* 16S rRNA gene copies using quantitative PCR (qPCR) revealed that 1,2-dichloropropane (1,2-DCP) dechlorination was coupled to *Dehalogenimonas* growth in this culture.

Carbon and dual carbon-chlorine (C-Cl) isotope fractionation during anaerobic biodegradation of 1,2-DCP and 1,2-dichloroethane (1,2-DCA), respectively, by *Dehalogenimonas*-containing enrichment culture were determined in this study. Compound specific isotope analysis revealed that the *Dehalogenimonas*-catalyzed carbon isotopic

fractionation (ε^{C}_{bulk}) of the 1,2-DCP-to-propene reaction was −15.0 ± 0.7‰ and differs significantly from other *Dehalococcoides* strains eventhough they harbored the same functional reductive dehalogenase (DcpA) for 1,2-DCP-to-propene dechlorination. The dual element C-Cl isotope correlation obtained (Λ = 1.89 ± 0.02) during 1,2-DCA-to-ethene dichloroelimination by *Dehalogenimonas* was significantly discernible from those reported for *Dehalococcoides* catalyzing a similar dihaloelimination reaction and aerobic oxidation. This illustrates the potential use of dual C-Cl isotope approach to distinguish between different degradation pathways (oxidation, hydrolytic dehalogenation and dihaloelimination).

After overcoming the challenges in developing a stable culture, isolation of *Dehalogenimonas* became the next objective. To date, only three species belonging to *Dehalogenimonas* genus have been isolated and this study constituted the first evidence of a *Dehalogenimonas* culture enriched in Europe. The isolation approach consisted of the dilution to extinction method and the addition of selected antibiotics. After thirteen sequential transfer of this culture fed with 1,2-DCP-to-propene and two consecutive 10^{-7} dilutions followed by the addition of streptomycin for five transfers, a clone library of bacterial amplicons revealed that *Dehalogenimonas sp.* constituted 87 % of the predominant bacteria, followed by *Desulfovibrio sp.* (12 %) and unclassified *Veillonellaceae* (1 %). Further work is currently underway in our lab to isolate the *Dehalogenimonas* sp.

In this study, a preliminary list of reductive dehalogenase (RDase) candidates involved in the transformation of 1,1,2-trichloroethane (1,1,2-TCA) and 1,2-dibromoethane (EDB) by this *Dehalogenimonas* culture was attempted using shotgun proteomics analysis (LTQ-Orbitrap). In addition, blue native polyacrylamide gel electrophoresis (BN-PAGE) approach combined with dechlorination activity assays were performed to identify the RDase responsible for 1,1,2-TCA

dichloroelimination. Eventhough dechlorination activity was detected in a gel slice of the BN-PAGE of the culture growing with 1,1,2-TCA, no RDase was identified by neither liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis nor sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The absence of RDase can be due to several reasons including low protein content and the use of a database constructed from the published genomic annotations of other isolated *Dehalogenimonas* because our strain was not sequenced yet.

Finally, a syntrophic co-culture of *Dehalogenimonas* and *Dehalococcoides mccartyi* strains to achieve complete detoxification of 1,1,2-TCA to ethene was also constructed. In this co-culture, *Dehalogenimonas* transformed 1,1,2-TCA via dihaloelimination to vinyl chloride (VC), whereas *Dehalococcoides* reduced vinyl chloride via hydrogenolysis to ethene. Scale up of the cultivation to a 5-L bioreactor operating in fed-batch mode and the synthetic combination of these bacteria with known complementary metabolic capabilities was demonstrated.

RESUM

Els compostos orgànics halogenats (organohalogenats) són recalcitrants i presenten efectes tòxics per la salut humana i l'ecosistema. Dintre dels organohalogenats, els alcans clorats com els cloroetans i cloropropans reben menys atenció malgrat el seu ampli ús a la indústria i el seu impacte ambiental. Els processos fisicoquímics són la tecnologia més àmpliament utilitzada pel tractament d'aigües subterrànies contaminades amb aquests compostos malgrat que aquestes tècniques tenen un alt cost operacional i requereixen una etapa de post-tractament per destruir completament aquests compostos. En aquest context, la biodegradació es considera més adequada per la seva millor relació cost-eficiència i a que són tecnologies més respectuoses amb el medi ambient.

En aquest estudi es vol obtenir un cultiu enriquit amb bacteris dehalorespiradors capaços de transformar alcans halogenats. A partir d'uns sediments de la desembocadura del riu Besòs (Barcelona, Espanya) es va establir un cultiu anaerobi estable i no metanogènic que exclusivament dehalogenava cloro- i bromoalcans que tenen els àtoms d'halògens en carbonis adjacents via dihaloeliminació.

L'aplicació de cebadors específics derivats a partir del gen 16rRNA de diferents bacteris dehalorespiradors junt amb les observacions fisiològiques del cultiu declorador indicaren que una soca de *Dehalogenimonas* era la responsable de la degradació dels alcans clorats. L'augment de còpies del gen 16S rRNA de *Dehalogenimonas* determinades per qPCR demostraren que la decloració de 1,2-dicloropropà (1,2-DCP) es produïa en paral·lel al creixement de *Dehalogenimonas.*

Durant la biodegradació anaeròbia de 1,2-DCP i 1,2-dicloroetà (1,2-DCA) pel cultiu enriquit amb *Dehalogenimonas* es va determinar el fraccionament isotòpic del C com per la

parella C-Cl, respectivament. L'anàlisi de l'isòtop de carboni durant la degradació de 1,2-DCP per *Dehalogenimonas* presenta un fraccionament isotòpic (ε^{C}_{bulk}) de -15.0 ± 0.7 ‰ que és significativament diferent al d'altres soques de *Dehalococcoides* encara que tenen la mateixa dehalogenasa reductiva funcional (DcpA) implicada en la degradació de 1,2-DCP a propè. La correlació de fraccionament isotòpic obtingut per a la parella C-Cl (Λ = 1.89 ± 0.02) durant la dicloroeliminació de 1,2-DCA per *Dehalogenimonas* era significativament diferent tant dels obtinguts prèviament per *Dehalococcoides* catalitzant una reacció similar de dehaloeliminació com dels de l'oxidació aeròbia de 1,2-DCA. Aquests resultats serveixen per il·lustrar la potencialitat de l'ús del fraccionament isotòpic simultani del C-Cl per a diferenciar entre diferents mecanismes de reacció (oxidació, dehalogenació hidrolítica i dehaloeliminació)

Després d'assolir el repte d'obtenir un cultiu estable, l'aïllament de *Dehalogenimonas* va ser el següent objectiu. Fins avui, només 3 especies del gènere *Dehalogenimonas* han estat aïllades i aquesta és la primera que es cultiva a Europa. Per abordar l'aïllament s'ha fet servir el mètode de dilució fins a l'extinció i l'addició d'antibiòtics selectius. Després de 13 transferències seqüencials d'aquest cultiu de partida alimentat amb 1,2-DCP i dues dilucions consecutives $1{:}10^{7}$ seguides per l'addició d'estreptomicina durant 5 transferències, es va fer una genoteca que va demostrar que el cultiu estava format predominantment per *Dehalogenimonas sp.* (87%), però també hi havia *Desulfovibrio sp.* (12%) i una *Vellonellaceac* no classificada (1%). Actualment s'està continuant el treball en el nostre laboratori per assolir l'aïllament de *Dehalogenimonas sp.*

En aquest estudi s'ha intentat fer una llista preliminar de dehalogenases reductives (RDase) candidates a estar involucrades en la transformació de 1,1,2-tricloroetà (1,1,2-TCA) i 1,2-dibromoetà (EDB) en *Dehalogenimonas* fent servir la tècnica de *shotgun proteomics* (LTQ-

Orbitrap). A més a més, es van fer electroforesis amb gel de poliacrilamida blau natiu (BN-PAGE) combinat amb assajos d'activitat decloradora per identificar la RDase responsable de la dicloroeliminació de 1,1,2-TCA. Encara que es va detectar activitat decloradora en una banda del gel, no es va identificar la RDase ni per cromatografia líquida acoblat amb un espectròmetre de masses (LC-MS/MS) ni per electroforesis en gel de poliacrilamida amb dodecil sulfat de sodi. L'absència de RDAse pot ser degut a vàries raons, entre elles la baixa concentració de proteïna i l'ús d'una base de dades que es va construir a partir d'anotacions dels genomes d'altres *Dehalogenimonas*, perquè la nostra soca no està encara seqüenciada.

Finalment, es va construir un co-cultiu sintròfic de *Dehalogenimonas* i *Dehalococcoides mccartyi* per tal d'assolir la completa detoxificació de 1,1,2-TCA fins a etè. En aquest co-cultiu, *Dehalogenimonas* transforma 1,1,2-TCA via dihaloeliminació a clorur de vinil, alhora que *Dehalococcoides* redueix el clorur de vinil via hidrogenòlisis a etè. S'ha fet un canvi d'escala a un reactor anaerobi de 5 L operant en semicontinu. En aquest estudi es demostra, a partir d'una combinació sintètica de bacteris, la capacitat de detoxificar 1,1,2-TCA quan aquests microorganismes presenten capacitats metabòliques complementàries.

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LIST OF ABBREVIATIONS

ΔG_o'	Gibbs free energy
ε^{C}_{bulk}	Carbon isotope fractionation
ε^{Cl}_{bulk}	Chlorine isotope fractionation
1-CB	Monochlorobenzene
1-CP	1-chloropropane
2-CP	2-chloropropane
1,1-DCA	1,1-dichloroethane
1,2-DBP	1,2-dibromopropane
1,2-DCA	1,2-dichloroethane
1,2-DCP	1,2-dichloropropane
1,6-DBH	1,6-dibromohexane
2,3-D-1-P	2,3-dichloro-1-propene
1,1,1-TCA	1,1,1-trichloroethane
1,1,2-TCA	1,1,2-trichloroethane
1,2,3-TCP	1,2,3-trichloropropane
1,2,4-TCB	1,2,4-trichlorobenzene
1,1,2,2-TeCA	1,1,2,2-tetrachloroethane
2D-CSIA	Two-dimensional compound-specific isotope analysis
ATDSR	Agency for Toxic Substances and Disease Registry
BES	Bromoethanesulfonate
BN-PAGE	Blue native polyacrylamide gel electrophoresis
BvcA	Vinyl chloride reductive dehalogenase strain BAV1
C-Cl	Carbon-chlorine
CA	Chloroethane
CbrA	Chlorobenzene reductive dehalogenase
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CF	Chloroform
CFC	Chlorofluorocarbons

CfrA	Chloroform reductive dehalogenase in *Dehalobacter*
***cis*-DCE**	*cis*-dichloroethylene
CN-PAGE	Clear native polyacrylamide gel electrophoresis
CprA	Chlorophenol reductive dehalogenase
CrdA	Chlorophenol reductive dehalogenase
CSIA	Compound-specific stable isotope analysis
CtrA	Chloroform reductive dehalogenase in *Desulfitobacterium*
D&D	Dig and Dump
DcaA	Dichloroethanes reductive dehalogenase
DCE	Dichloroethylene
DcpA	1,2-DCP reductive dehalogenase
DDT	Dichloro diphenyl trichloroethane
DGGE	Denaturing gradient gel electrophoresis
DI	Dual-inlet
DNAPL	Dense non-aqueous phase liquids
DO	Dissolved oxygen
EDB	1,2-dibromoethane
EPA	Environmental Protection Agency
EU	European Union
EURODEMO	European Co-ordination Action for Demonstration of Efficient Soil and Groundwater Remediation
FID	Flame ionization detector
GC	Gas chromatography
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
HPLC	High performance liquid chromatography
HSSPME	Headspace solid-phase microextraction
IRMS	Isotope ratio mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
ML	Maximum-likelihood
NPL	National Priorities List
OHRB	Organohalide-respiring bacteria

P&T	Pump & Treat
PCE	Tetrachloroethylene or perchloroethylene
PceA	Tetrachloroethylene reductases
PSL	Priority substance list
PDBE	Polybrominated diphenyl ethers
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PVC	Polyvinyl chloride
QM/MM	Quantum mechanical/molecular mechanical
qPCR	Quantitative polymerase chain reaction
RDase	Reductive dehalogenase (characterized)
Rdh	Reductive dehalogenase (uncharacterized)
rdhA	Reductive dehalogenase subunit A
RT-qPCR	Reverse transcription qPCR
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMOC	Standard Mean Ocean Chlorine
SPME	Solid phase micro extraction
TceA	Trichloroethene reductive dehalogenase
TCD	Thermal conductivity detector
TCE	Trichloroethylene
***trans*-DCE**	*trans*-dichloroethylene
TrdA	*trans*-dichloroethylene reductive dehalogenase
VC	Vinyl chloride
VcrA	Vinyl chloride reductive dehalogenase
VPDB	Vienna Pee Dee Belemnite
VSMOW	Vienna Standard Mean Ocean Water

CHAPTER 1

GENERAL INTRODUCTION

1.1. The contamination of the environment by halogenated compounds

Halogenated compounds (organohalides) are a large group of natural and synthetic chemicals, containing one or more halogens combined with carbon and other elements, widely distributed in the environments [1]. Organohalides are considered persistent and bioaccumulative organic compounds, exhibiting toxic effects on animal reproduction, development and immunological function [1], [2].

Many halogenated organic compounds, including aliphatic, aromatic and heterocyclic derivatives, have been produced for close to a century and used for several industrial and agricultural applications. These compounds have been used predominantly as herbicides, insecticides, fungicides, solvents, degreasing agents, pharmaceuticals, plasticizers, hydraulic and heat transfer fluids, flame retardants and intermediates for chemical synthesis; and many other organohalides are by-products of industrial processes [3]. Long-term anthropogenic production of these halogenated compounds coupled to their inappropriate disposal practices have resulted in widespread organohalide contamination into the environment, notably to the soil and groundwater [4]–[7].

Halogenated compounds constitute more than 50% of the top fifty compounds in the 2015 CERCLA Priority List of Hazardous Compounds [8]. This list was prepared by the United States Agency for Toxic Substances and Disease Registry (ATDSR) and the Environmental Protection Agency (EPA) and it indicates a prioritization of substances based on a combination of their frequency at facilities of the National Priorities List (NPL), toxicity, and potential for human exposure at NPL sites. Chlorinated compounds are ranked in the first positions of this list, as illustrated by vinyl chloride (4^{th}), polychlorinated biphenyls (5^{th}), chloroform (11^{th}), Aroclor 1260 (12^{th}), DDT,P,P' (13^{th}), Aroclor 1254 (14^{th}), trichloroethylene (TCE) (16^{th}), dieldrin (18^{th}),

among many others. Although the majority of organohalide contaminants are chlorinated compounds, brominated, fluorinated and iodinated compounds are also used in industrial applications and found in soils and groundwaters [9]. For instance, brominated compounds such as polybrominated diphenyl ethers (PBDEs), used as flame retardants in many types of consumer products and fluorinated compounds (chlorofluorocarbons (CFCs) are ranked 143th on the CERCLA Priority List). Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have been widely used in industrial and consumer applications and they are now widely spread across the Earth and pose considerable risks to the environment and human populations [10]–[12]. In the past few years, iodinated compound iopromide, normally used in pharmaceutical industry, has being included as one of the emerging contaminants and impacted urban surface water [13].

Although organohalides from anthropogenic activities are of major concern in contaminated sites, they can be also produced naturally constituting an important component of the halogen biogeochemical cycles. Around 5000 chlorinated and other halogenated chemicals are discharged into the environment by living organisms such as plants, marine organisms, insects, bacteria, fungi, and mammals, and by other natural processes (volcanic activity, forest fires, and other geothermal processes). Enzymatic, thermal, and other natural processes are constantly occurring in the oceans, in the atmosphere, and in the soil that lead to the formation of chlorinated phenols and myriad other chlorinated chemicals, including dioxins and CFCs, indicating that industry and human actions are not the only source of these xenobiotic compounds. It is clear that these natural processes have been producing chlorinated compounds and have been a vital component of our ecosystem for eons [14].

Once in the environment, it is necessary to consider all aspects of this diverse and biologically challenging group of compounds, including their biodegradation, assimilation, integration (e.g., sorption and coupling to organic matter), and also their persistence in the environment [9], [15]. Most organohalides are volatile, possess varying solubility, and have long half-lives. In many respects, the chemistry of halogenated organic compounds is due to the unique physicochemical properties of their halogen substituent. The physical size and shape of the halogen substituent may also affect reactivity, due to steric constraints and may also hinder uptake into cells and enzymatic attack during biodegradation. In addition, the halogen moiety of an organic compound generally reduces its water solubility and conversely increases lipid solubility. The biological consequence of increased lipophilicity may be reduced biodegradation due to decreased bioavailability, and/or biomagnification in the food chain as the nondegraded haloorganic compounds sequester in the fatty tissues of higher animals [16]. The chemical bonds in these compounds are extraordinarily stable, making them very persistent in most environments. Eventhough organic molecules with few halogens substituents can often be mineralized under aerobic conditions, highly chlorinated compounds are often persistent [17]. According to Nikel et al., the organohalides are recalcitrant to biodegradation for several reasons: (i) they are highly toxic for microbes, (ii) most complex organohalides are unnatural compounds recently introduced into the environment by human activities, and microorganisms are unlikely to possess complete catabolic pathways for their mineralization, and (iii) if degradation is initiated by a dehalogenation reaction catalysed by an existing dehalogenase or oxidase, the lack of enzymes for the rapid conversion of the resulting halogenated alcohols or aldehydes would lead to the accumulation of toxic and/or highly reactive metabolic intermediates [18].

In the past years, degradation of chlorinated ethenes, mainly tetrachloroethylene (PCE) and TCE, has attracted most of the attention of researchers due to their widespread presence in groundwaters. However, chlorinated alkanes such as chloroethanes and chloropropanes received less attention despite their large use in the industry have caused serious environmental problems. Chlorinated alkanes are chlorinated hydrocarbons (n-alkanes) that can have carbon chain lengths ranging from 10 to 38 carbon atoms. Chlorinated alkanes were included on the first priority substance list (PSL) program under the 1999 Canadian Environmental Protection Act and among the top list in United States NPL [19], [20]. Among the most frequently detected chlorinated alkanes in groundwaters are 1,2-DCP, 1,2,3-trichloropropane (1,2,3-TCP), 1,2-DCA, 1,1,2-TCA, and 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA). 1,2-DCP has been used predominantly as a chemical intermediate in the production of carbon tetrachloride and PCE, lead scavenger for antiknock fluids and solvent. According to the Primary Drinking Water Regulations established by the U.S. Environmental Protection Agency (EPA), 1,2-DCP can increase risk of cancer and a maximum contaminant level in drinking water of 5 $\mu g\ L^{-1}$ is legally set for public water. Today, 1,2-DCP is a risk for the environment and drinking water quality especially at historically contaminated sites [21]. 1,2,3-TCP is a non-natural, biodegradation-recalcitrant and toxic compound that occurs in groundwater and soil [22]. 1,2,3-TCP is formed as a by-product during the synthesis of various chemicals, most notably in the classical synthetic route to epichlorohydrin and was present in commercial preparations of the soil fumigant 1,3-dichloropropene (also known under the trade name D-D), which is now abandoned [23]. 1,2-DCA has been found in at least 287 of 932 NPL sites identified by the U.S. EPA. The most common use of 1,2-DCA is to make vinyl chloride, which is used to make a variety of plastic and vinyl products including polyvinyl chloride (PVC) pipes and other important construction

materials, packaging materials, furniture and automobile upholstery, wall coverings, housewares, and automobile parts. Overexposure to 1,2-DCA may cause nervous system disorder and affecting kidney and liver [24], [25]. Meanwhile, polychlorinated ethanes such as 1,1,2-TCA and 1,1,2,2-TeCA have been used for decades as chemical intermediates, solvents, degreasing agents, paint removers and also in pesticides industries [26]. For instance, 1,1,2-TCA and 1,1,2,2-TeCA have been found in at least 157 and 112 out of the 1,774 National Priorities List sites identified by the EPA, respectively [20]. This raises concern due to the potential ability of these compounds to affect the liver, the kidneys, nervous and immune systems [20], [27].

1.2. Remediation of organochlorines

Contamination of groundwaters by organohalides is particularly significant because groundwater represents about 98% of the available fresh water of the planet [28]. More than one third of the Spanish national areas contain aquifers and nearly a quarter of the water consumed can be obtained from groundwater resources [29]. Chlorinated hydrocarbons was found to be contributing 10% of the groundwater contaminants in Europe [30]. In 2007, the European Union (EU) indicated that chlorinated hydrocarbon represents 7.2% among the contaminants affecting soil and groundwater in Spain [29].

Chlorinated solvents were often released to the subsurface environment in wastewater or in the form of dense non-aqueous phase liquids (DNAPLs). When chlorinated solvents are discharged onto soil, their movement and fate are determined by their physical, chemical and biological properties and by site hydrogeological characteristics. The solvent will continue its migration, probably leaving some residual solvent behind as it follows the path of least resistance, which may take many turns through a complex subsurface structure. It is now

recognized that there are thousands of public and private sites with chlorinated solvent related groundwater contamination problems [29], [31].

Remediation methods can be categorized as biological, chemical, or physical treatments and these technologies sometimes were used in conjunction with one another to reduce the contamination to a safe and acceptable level [32]. Physicochemical processes are the most common cleanup methods for groundwater remediation in most part of the world including Europe. An EU-funded project called the European Co-ordination Action for Demonstration of Efficient Soil and Groundwater Remediation (EURODEMO) confirmed that the Dig and Dump (D&D) and Pump & Treat (P&T) remain the most common approaches to soil and groundwater remediation, respectively [29]. P&T methods involve installing one or more wells to extract the contaminated groundwater. Groundwater is pumped from these "extraction wells" to the ground surface, either directly into a treatment system or into a holding tank until treatment can begin (Figure 1.1). The treatment system may consist of a single cleanup method, such as activated carbon or air stripping, to clean the water [33].

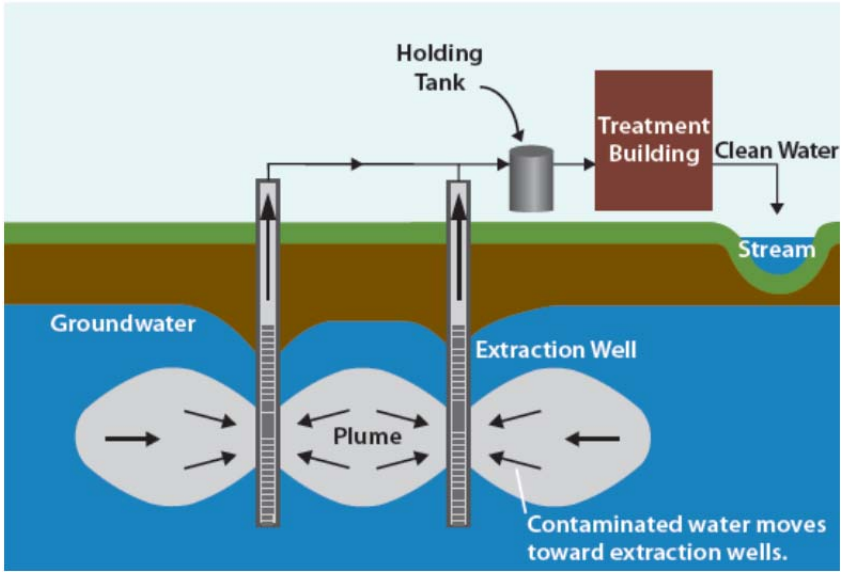

Figure 1.1: Example of a pump and treat system with two extraction wells [33].

However, today it is widely recognized in Europe that established conventional remediation technologies like P&T cannot be regarded as sustainable in all cases in the sense of cost-efficiency, contamination reduction, or environmental balance [34]. Owing to specific properties of some contaminants, remediation efforts devoted at macroscale for removing soil and groundwater contamination by applying P&T have had quite limited success during time. With regards to chlorinated compounds, the treatment is basically achieved through activated carbon adsorption, which has very high operating costs and requires a post-treatment step to actually 'destroy' the chlorinated compounds (e.g. during thermal regeneration of activated carbon) [29]. An additional drawback of P&T systems is the rather long operational time needed for proper remediation. The concentration of the contaminants received in the pumping wells decreases only slowly during operation, and residual concentrations can still exceed the clean-up standards [35].

Alternatively, bioremediation offers several advantages when compared to P&T treatments to remove hazardous compounds from contaminated soil or groundwater [36]. Bioremediation is a technique that involves the use of microorganisms to degrade and detoxify environmental contaminants [37]. Similar to many industrial processes, the main motivation to use biotechnology for environmental cleanup is because it is more cost-effective. Unlike P&T treatments that transfer the pollutants from one phase (or location) to another, bioremediation offers a terminal solution by destroying the pollutants [28]. An additional advantage is that bioremediation is commonly applied *in situ*, avoiding the exposure to contaminants for both the workers at the site and the nearby residents and eliminating transportation costs and liabilities [34]. In many instances, manufacturing and industrial use of the site can continue while the bioremediation process is being implemented. Finally, bioremediation can be coupled (i.e.,

integrated) with other treatment technologies into a treatment chain allowing for the treatment of mixed and complex wastes [37].

Enhanced *in situ* bioremediation is an effective strategy to degrade various chlorinated solvents dissolved in groundwater, including chloroethenes, chloroethanes, and chloromethanes [38]. The *in situ* bioremediation processes consist of three fundamental approaches: monitored natural attenuation, biostimulation, and bioaugmentation. Monitored natural attenuation involves passive remediation of polluted sites, without any external force (human intervention). The process relies on both microbial aerobic and anaerobic reactions to biodegrade pollutants but also in other physicochemical processes (e.g. sorption, etc.) that either degrades or binds contaminants to sorbent [39], [40]. Biostimulation involves the modification of the environment to stimulate autochthonous microorganisms capable of bioremediation. In the case of organohalide contamination, organohalide-respiring bacteria (OHRB) are commonly stimulated by delivering a fermentable organic substrate to produce hydrogen (electron donor) and acetate (carbon source) to the subsurface. The successful application of biostimulation requires to know the presence at the site of key bacteria able to detoxify the contaminants. Last, bioaugmentation is the addition of highly concentrated and specialized populations (single strains or consortia) to the site contaminated with recalcitrant toxic compounds [41]. This technique is best suited for sites that do not have microbial populations with the metabolic routes necessary to metabolize the compounds under concern [36]. The last decade has seen the development and commercialization of several new microbial inoculants for the remediation of chlorinated solvents in groundwater, and controlled field demonstrations have shown that these cultures can improve bioremediation performance at many sites [42].

1.3. Biodegradation of chlorinated alkanes in the environment

Regardless of which bioremediation techniques involved, bioremediation is only possible because organisms have developed an impressive enzymatic capability to transform a wide array of organohalides. Numerous mixed and pure culture studies of the past two decades have revealed that predominantly reductive dehalogenation processes, but also oxidative and fermentative mechanisms, are responsible for the initial attack and degradation of a wide variety of aliphatic and aromatic organohalides pollutants.

Figure 1.2 summarizes the main reaction pathways observed during biodegradation of chlorinated alkanes. Under anoxic conditions, the three predominant reactions include dichloroelimination, hydrogenolysis and dehydrochlorination. In dichloroelimination, an electron donor supplies a pair of electrons to the organohalide, which serves as electron acceptor, resulting in the loss of halogens from adjacent (i.e. vicinal) carbon centers and forms a double bound (Figure 1.2, iii). This reaction can only occur when the organohalide contains vicinal chlorine pairs. Dichloroelimination can occur in two reaction pathways, (i) concerted reaction where the chlorines were removed simultaneously (Figure 1.2, iiia) or (ii) stepwise reaction in where the chlorine was removed in at least two consecutive elementary reactions with the formation of reaction intermediates (Figure 1.2, iiib).

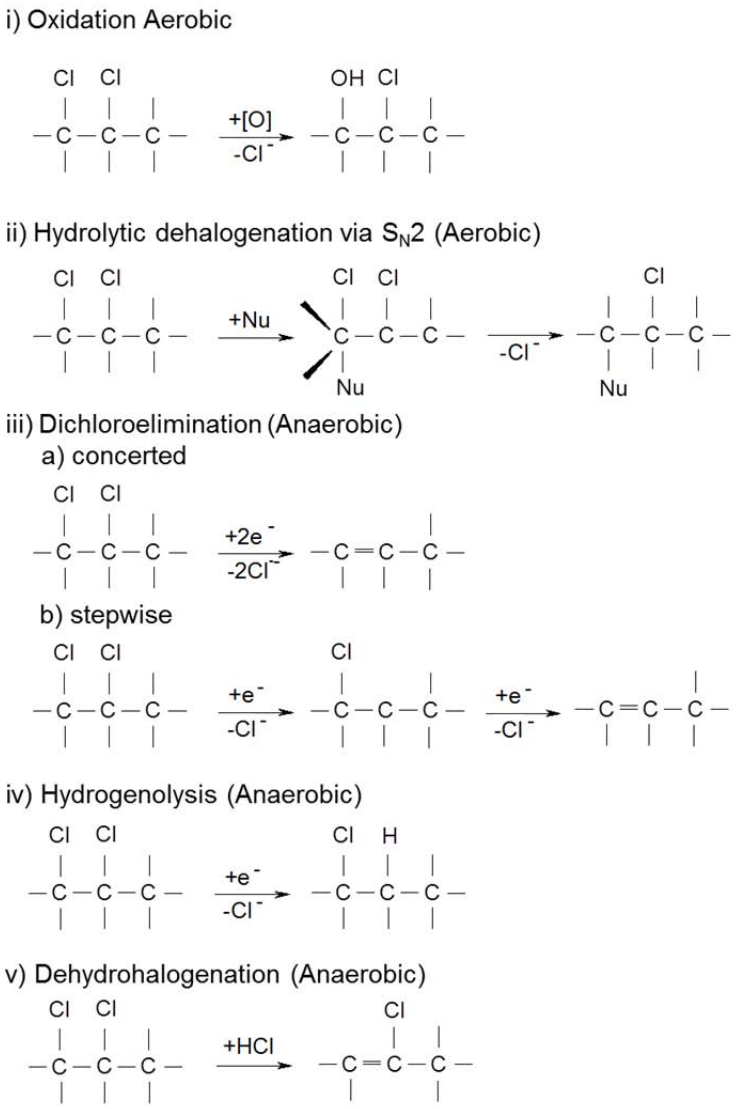

Figure 1.2: Aerobic and anaerobic biodegradation pathways of chlorinated alkanes (1,2-dichloropropane biodegradation pathways were shown as an example).

During hydrogenolysis, a pair of electrons from an external donor drives the reaction but unlike dichloroelimination the departing halogen is replaced by a proton (Figure 1.2, iv). Dehydrohalogenation has been reported as an abiotic reaction under anoxic conditions and implies that a hydrogen and a chlorine are removed simultaneously, resulting in a less chlorinated compound and in a double bond (Figure 1.2, v). Finally, under oxic conditions,

chlorinated alkanes can be degraded by oxidation or hydrolytic dehalogenation (Figure 1.2, i and ii).

As organohalide-respiring bacteria harness energy using halogenated organic compounds as electron acceptors, the thermodynamics can gives us insight into the amount of energy that is available from a dehalogenation reaction, or how far from equilibrium that reaction is [43]. It provides the basis for the assessment of the energetic feasibility of different pathways for the dehalogenation of organohalides at different environmental conditions [15], [43]. Thermodynamics can be used as a tool to gain insight into the amount of energy that is available from a reaction. The change in Gibbs free energy (ΔG_o') for hydrogenolysis is generally -120 to -170 kJ mol^{-1} and is driven by the energy released in the formation of HCl. As in the dihaloelimination two moles of HCl are formed per reaction, ΔG_o' was expected to be twice of the hydrogenolysis. Thus under conditions where organohalides are limited, from a thermodynamical point of view dihaloelimination is a more energetically favorable process as compared to hydrogenolysis [44].

Chlorinated alkanes can be divided in two categories based on the distinct behavior patterns in their biodegradability, the lower chlorinated ethanes or the higher chlorinated ethanes. Lower chlorinated ethanes can be utilized as primary growth substrates by both aerobic and anaerobic bacteria. In addition, lower chlorinated alkanes are also known to be cometabolized. Higher chlorinated alkanes are hardly used as primary growth substrate under aerobic conditions and their biodegradation is almost exclusively under anaerobic conditions.

Lower chlorinated ethanes, which include chloroethane, 1,1-dichloroethane (1,1-DCA), 1,2-DCA or 1,2-DCP can be used either as primary growth substrates by aerobic bacteria [45], [46]. The growth of bacteria linked to the use of 1,2-DCA as carbon and energy source was first

reported by Stucki et al. (1983) for a *Pseudomonas* strain DE2 [47]. Since then, several other bacteria with ability to grow with 1,2-DCA have been isolated, such as *Xanthobacter autotrophicus* GJ10, *Ancylobacter aquaticus* AD20, and *Pseudomonas sp.* strain DCA1 [48]–[50]. Two different degradation mechanisms have been described for aerobic metabolic degradation of 1,2-DCA: removal of a chlorine group and replacement with a hydroxyl group from water yielding 2-chloroethanol as the first product via a haloalkane dehalogenase (i.e. *X. autotrophicus* GJ10, *A. aquaticus* AD20) or dechlorination via a monooxygenase that forms an unstable 1,2-dichloroethanol intermediate that abiotically descomposes to chloroacetaldehyde (i.e. *Pseudomonas sp.* strain DCA1). Aerobic co-metabolism of lower chlorinated ethanes has been also well documented. For instance, 1,2-DCA and 1,1-DCA was completely degraded with stoichiometric release of chloride by methane-and ammonia-oxidizing bacteria [51], [52]. Under anaerobic conditions, pure cultures of methanogens have shown to reductively dechlorinate 1,2-DCA to chloroethane via hydrogenolysis or cause dichloroelimination to ethene. Similarly, the acetogenic bacterium *Acetobacterium* has been also demonstrated to cometabolize 1,2-DCA without coupling to growth [53]. Finally, use of 1,2-DCA, and 1,2-DCP as terminal electron acceptor and hydrogen as electron donor has been observed in several OHRB supporting microbial growth in an energy conservation reaction. In this case, dihaloelimination is the major observed pathway. However, traces of vinyl chloride from 1,2-DCA were formed in some *Dehalococcoides* strains, indicating that hydrogenolysis is also taken place [54].

Several higher chlorinated ethanes can be degraded cometabolically under aerobic conditions via monooxygenases. For instance, 1,1,1-TCA is transformed to 2,2,2-trichloroethanol using pure cultures that uses as primary growth substrate methane, butane, propane or ammonia [55]. Anaerobic cometabolism of higher chlorinated ethanes have been

observed in mixed methanogenic cultures degrading hexachloroethane, pentachloroethane, 1,1,1,2-TeCA, and 1,1,2,2-TeCA, among others. In this case, the results taken as a whole indicate three major types of reactions: dichloroelimination, hydrogenolysis, and dehydrochlorination [55]. The latter is the main abiotic mechanism, but dichloroelimination and hydrogenolysis are biologically catalyzed reactions. To illustrate the three reactions, the methanogen *Methanobacterium thermoautotrophicum* converts 1,1,1-trichloroethane (1,1,1-TCA) to 1,1-DCA via hydrogenolysis [45]; and 1,1,2,2-TeCA can be abiotically transformed to TCE under reduced conditions via dehydrochloroelimination but also to *trans*- and *cis*-dichloroethylene (*trans*-DCE and *cis*-DCE) in an anaerobic sludge via hydrogenolysis [56]. These three pathways are also used during the transformation of higher chlorinated alkanes by OHRB. For instance, 1,1,1-TCA was transformed into chloroethane (CA) with transient formation of 1,1-DCA as intermediate by *Dehalobacter* sp. strain TCA1 (hydrogenolysis) [57]. On the other hand, 1,1,2-TCA can be transformed via dichloroelimination to vinyl chloride by *Dehalogenimonas* strains [58].

1.4. Organohalide-respiring bacteria

Organohalide respiration is an energy-conserving respiratory process wherein a halogen-carbon bond is broken and the halogen atom is liberated as a halide [59]. OHRB can be defined as the anaerobic microorganisms that are capable of deriving energy for its growth from dehalogenation of aliphatic or aromatic halogenated compounds [59]. Organohalide-respiring bacteria (OHRB) metabolic dechlorination rates are several orders of magnitude higher than those observed for co-metabolic conversion, hence become an important contributor for the effective removal of chlorinated pollutants from contaminated environments [60], [61].

Hazardous halogenated organic compounds are highly resistant to degradation by aerobic organisms [62]. Additionally, several OHRB strains can stably coexist in the same niche and thus broadening the potential use of OHRB in contaminated sites with variety of contaminants [63].

OHRB have been identified from diverse bacterial phyla, including the Proteobacteria, Firmicutes and Chloroflexi (Figure 1.3) and comprised both Gram-positive and Gram-negative bacteria [64]. There is little to no correlation between phylogenetic affiliation and chlorinated substrate specificities: aliphatic and aromatic substrates are used by taxonomically diverse organisms. The known OHRB can be grouped as either obligate or facultative organohalide respirers [59]. Obligate OHRB are highly niche-specialized to organohalide respiration, these bacteria only utilized halogenated compound as electron acceptor and in nearly all studied strains of this group are restricted to H_2 as the electron donor. Meanwhile, facultative OHRB are metabolically-versatile and they can either use halogenated compound or other compound such as NO_3^-, Fe(III), Mn(IV), or oxidized sulfur compounds as electron acceptor. Facultative OHRB also can use diverse electron donors, typically the organic end products of primary fermenters (lactate, pyruvate, butyrate), as well as H_2 and formate [65].

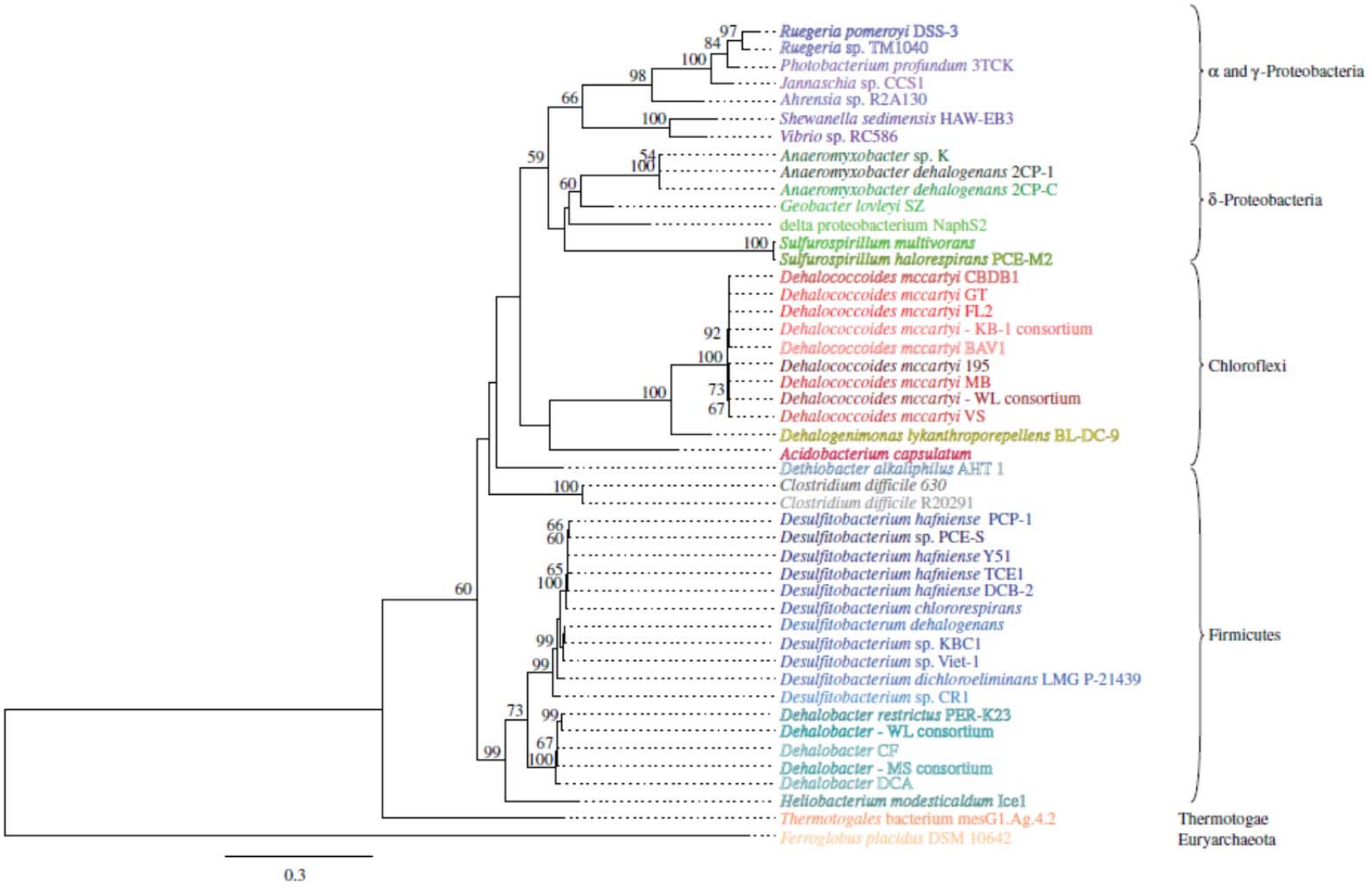

Figure 1.3: Maximum-likelihood (ML) tree of 16S rRNA gene sequences from all known organisms containing a reductive dehalogenase homologous (rdhA) gene [59].

The Firmicutes contain non-obligate organohalide-respiring *Desulfitobacterium* spp. as well as metabolically restricted *Dehalobacter* OHRB [59], [66]. *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 H_2 as sole electron donor except for two reports on fermentative growth of *Dehalobacter* spp. with dichloromethane [64]. *Dehalobacter* spp. were found to be responsible for transforming chloroethenes (PCE and TCE), chlorinated ethanes, trichlorophenol, tribromophenol, chloroform, chlorinated benzenes and chlorotoluenes [64], [67]. *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 [64]. Most of the isolated *Desulfitobacterium* strains show versatile dehalogenation of both chlorinated aliphatic and aromatic compounds such as chloroethenes and chlorophenols. In addition to the ability to respire with organohalides, most *Desulfitobacterium* isolates can grow fermentatively on pyruvate and can utilize a variety of alternative electron acceptors, including sulfite, thiosulfate, fumarate, Fe(III), and Mn(IV) [68].

The members of Proteobacteria OHRB such as *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter, Desulfomonile*, *Desulfovibrio* and *Sulfurospirillum* are metabolically versatile with respect to their spectrum of electron donors and acceptors (e.g. sulfate, iron, nitrate, dimethylsulfoxide), and can dehalogenate a wide range of halogenated aromatic and aliphatic compounds [63], [66]. *Anaeromyxobacter* spp. strains were shown to dehalogenate various chlorophenols and bromophenols with acetate, H_2, succinate, pyruvate, formate and lactate as electron donors [64]. *Geobacter lovleyi* strains dechlorinate the priority pollutants PCE and TCE in freshwater aquifers whereas the sponge-associated species *Desulfoluna spongiiphila*

uses bromo- and iodophenols as electron acceptors in marine environments [69]. Meanwhile, isolates of *Desulfovibrio* spp. isolated from marine sediments use halophenols as electron acceptors [64]. A highly enriched culture of *Sulfurospirillum* strains dechlorinate PCE to TCE and *cis*-DCE exclusively [70].

The isolates in the Chloroflexi phylum are obligate organohalide respirers and share similar limited metabolic capabilities with respect to nutrients and electron donors [71]. These isolates include the genus *Dehalococcoides, Dehalobium* and *Dehalogenimonas*. These Chloroflexi OHRB appear as highly specialized bacteria that strictly depend on orgahohalide respiration for growth and in most cases coupled to H_2 as the sole electron donor. Members of the genus *Dehalococcoides* comprise the biggest groups of isolates to date (19 isolates) and have been found to dechlorinate a wide range of persistent organic contaminants [64]. *Dehalococcoides* strains were implicated in the dehalogenation of chloroethenes, chloroethanes, chlorophenols, chlorobenzenes and several brominated contaminants [72]. Phylogenetically most similar to the *Dehalococcoides, Dehalogenimonas* strains are able to couple growth with the reductive dehalogenation of a variety of polychlorinated alkanes [73]. The other OHRB Chloroflexi, genus *Dehalobium* selectively dechlorinates PCBs with formate or H_2 as the electron donor and carbon source [64].

Organohalide respiration reactions are catalyzed by enzymes known as reductive dehalogenases (RDase). RDase consists of RdhA which encode the catalytic subunit of the enzyme, and RdhB, encoding a putative membrane-anchoring protein for RdhA [15], [66], [74]. RdhA of the enzyme harbors a cobalamin (vitamin B12) cofactor and two Fe-S clusters [59]. Reductive dehalogenation occurs at the catalytic site where reduced cobalamin cofactor ($Co^{I,II}$) attacks the halogen atom of an organohalide to cleave a carbon-halogen bond, leading to

sequential removal of the halogen substituents from the organic backbone [75]. Since corrinoids (e.g. cobalamin) are key cofactors in RDases, they need to be added exogenously as vitamin B12 (cyanocobalamin, CN-Cbl) in the medium in case the OHRB lacks the ability to synthetize it *de novo* [76]–[78].

Recently, X-ray crystal structures of two RDases of PceA from microaerophilic *S. multivorans* and an NpRdhA from *N. pacificus* pht-3B revealed details of the active site, specific cobalamin cofactors, and led to the proposal of a new mechanism of cobalt–halogen chemistry. Norpseudovitamin-B12 was found to be a cofactor used in the PceA of *S. multivorans* and it was shown to be deeply buried inside the internal substrate-binding pocket [79], [80]. The scarcity of RDase crystal structures with different substrates and corrinoid cofactors have been the main limiting factors for complete biochemical characterization of RDases.

The RDases of several OHRB have been functionally characterized via proteomic (e.g., blue native gel followed by mass spectrometry analysis, liquid chromatographic purification) or transcriptional (e.g., RT-qPCR) analysis [80]. The first RDase that was biochemically characterized was the 3-chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei* strain DCB1 [59], [81]. Eventhough RDase encoding genes have been identified in wide variety of strictly anaerobic bacteria such as *Sulfurospirillum* [82] and microaerophilic bacteria *Anaeromyxobacter* [83], RDases from *Dehalococcoides, Dehalobacter* and *Desulfitobacterium* strains are amongst the most extensively studied of these enzymes [75], [84]. Analysis of genome sequences divides the OHRB into those whose genomes contain one or two *rdhA* genes and those containing several (more than 2) different *rdhA* [59], [85]. Genomes of *Dehalococcoides, Dehalogenimonas, Dehalobacter* and *Desulfitobacterium* strains contain multiple *rdhA* genes.

Phylogenetic analysis shows various clusters of RDase with similar functions (Figure 1.4), such as chlorophenol reductases (CprA), trichloroethene reductases (TceA) and vinyl chloride reductases (VcrA, BvcA). However, RdhAs with an identical function (e.g. tetrachloroethene reductases, PceA) are also present in different phylogenetic branches, suggesting a convergent evolution in different phylogenetic lineages of OHRBs [66]. In the last few years, several RDases have been purified and characterized: PCE RDase (PceA) in *Dehalococcoides*, *Dehalobacter, Desulfitobacterium*, and *Sulfurospirillum* [86]–[89]; TCE RDase (TceA) from *Dehalococcoides* strain 195 [89]; VC RDase (VcrA and BvcA) from *Dehalococcoides* strains VS and BAV1 [90], [91], 1,2-DCP RDase (DcpA) from *Dehalococcoides* strains KS, RC and *Dehalogenimonas lykanthroporepellens* [92], chlorobenzene RDase (CbrA) from *Dehalococcoides* strain CBDB1 [93], chlorophenol RDase (CprA and CrdA) in *Desulfitobacterium* [94], chloroform RDase (CfrA and CtrA) in *Dehalobacter* and *Desulfitobacterium* [57], [95], dichloroethanes RDase (DcaA and DcrA) from *Desulfitobacterium* and *Dehalobacter* [57], [96] and *trans*-dichloroethene RDase (TdrA) from *Dehalogenimonas* WBC-2 [97].

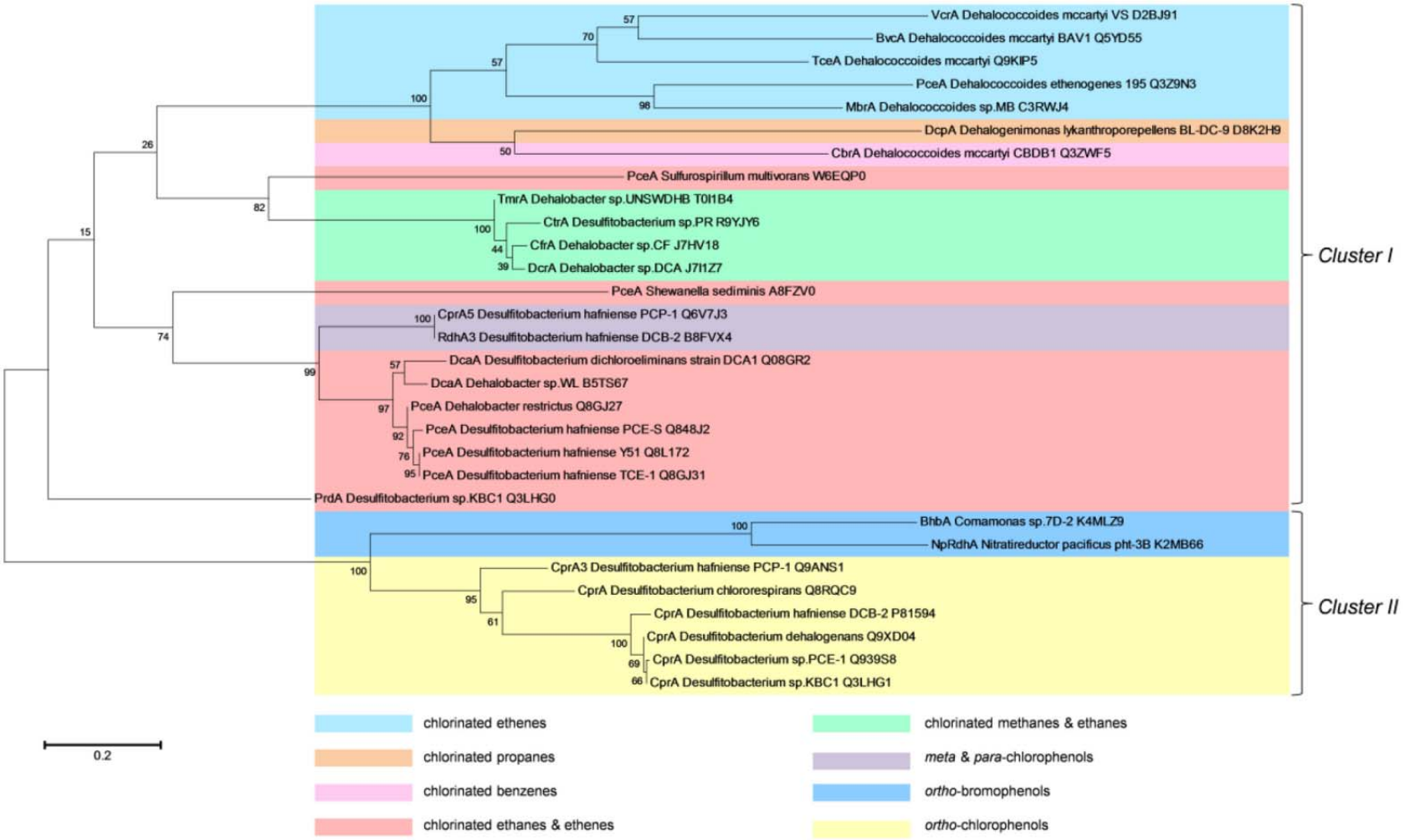

Figure 1.4: Maximum Likelihood phylogenetic analysis of reductive dehalogenases characterized to date [75].

Since many of the OHRB have multiple reductive dehalogenase genes, it is difficult to assess the substrate specificity based on genome information [59], [75]. In the case of *Dehalococcoides, Dehalogenimonas* and some *Dehalobacter*, detailed studies on the biochemistry of RDase were hindered by difficulties in obtaining sufficient biomass [59]. A broad range of substrate specificity has been reported among OHRB. The PceAs identified from *Dehalobacter, Desulfitobacterium*, and *Sulfurospirillum* dechlorinated chloroethenes such as PCE and TCE [86]–[88]. The PceA of *Desulfitobacterium* sp. Y51 was found to dechlorinate not only PCE and TCE but also various chloroethanes, especially those with a high number of chloride substituents [98]. A broad substrate ranges (including haloalkanes and haloalkenes with three to five carbon atoms) have been described for *Dehalococcoides ethenogenes* strain 195's TceA [89]. TceA from *Dehalospirillum multivorans* also catalyzes various chlorinated ethanes and propanes [86]. BvcA and VcrA from *Dehalococcoides* strain BAV1 and VS which dechlorinate VC to ethene were found in *Dehalococcoides ethenogenes* [99]. Chlorobenzene Rdh-encoding gene (cbrA) was identified in *Dehalococcoides* sp. strain CBDB1 [93]. CprA and CdrA dehalogenases from *Desulfitobacterium dehalogenans* and *Desulfitobacterium hafniense* dechlorinate a variety of chlorinated phenols [94], [100]. Recently, the function of the RDase TdrA in *Dehalogenimonas* sp. strain WBC-2 was known and it catalyzes the dechlorination of *trans*-DCE to VC [97].

1.5. References

[1] A. Cincinelli, F. Pieri, Y. Zhang, M. Seed, and K. C. Jones, "Compound specific isotope analysis (CSIA) for chlorine and bromine: A review of techniques and applications to elucidate environmental sources and processes," *Environ. Pollut.*, vol. 169, pp. 112–127, 2012.

[2] D. Leys, L. Adrian, and H. Smidt, "Organohalide respiration: microbes breathing chlorinated molecules," *Philos. Trans. R. Soc. B.*, vol. 368, p. 20120316, 2013.

[3] G. Zanaroli, A. Negroni, M. M. Häggblom, and F. Fava, "Microbial dehalogenation of organohalides in marine and estuarine environments," *Curr. Opin. Biotechnol.*, vol. 33, pp. 287–295, 2015.

[4] P. Bhatt, M. S. Kumar, S. Mudliar, and T. Chakrabarti, "Biodegradation of chlorinated compounds-A review," *Crit. Rev. Environ. Sci. Technol.*, vol. 37, pp. 165–198, 2007.

[5] W. Reineke and B. Universita, "Chlorinated hydrocarbon metabolism," *Life Sci.*, pp. 1–11, 2002.

[6] R. Alfan-Guzman, M. Lee, and M. Manefield, "Anaerobic bioreactors for the treatment of chlorinated hydrocarbons," in *Industrial Biotechnology: Sustainable Production and Bioresource Utilization*, D. Thangadurai and J. Sangeetha eds. CRC Press, Sydney, 2016, pp. 421–451.

[7] J. F. Ferguson and J. M. H. Pietari, "Anaerobic transformations and bioremediation of chlorinated solvents," *Environ. Pollut.*, vol. 107, no. 2, pp. 209–215, 2000.

[8] Agency for Toxic Substances and Disease Registry, "2015 Priority list of hazardous substances | ATSDR," 2015. [Online]. Available: https://www.atsdr.cdc.gov/spl/. [Accessed: 21-Nov-2016].

[9] M. M. Haggblom, D. E. Fennell, Y. B. Ahn, B. Ravit, and L. J. Kerkhof, "Anaerobic dehalogenation of halogenated organic compounds: Novel strategies for bioremediation of contaminated sediments," *Soil Water Pollut. Monit. Prot. Remediat.*, pp. 505–521, 2006.

[10] L. P. Padhye and U. Tezel, "Fate of Environmental Pollutants," *Water Environ. Res.*, vol. 85, no. 10, pp. 1734–1785, 2013.

[11] R. a Hites, "Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations," *Environ. Sci. Technol.*, vol. 38, no. 4, pp. 945–956, 2004.

[12] D. E. C. Adams and R. U. Halden, "Fluorinated chemicals and the impacts of anthropogenic use," in *ACS Symposium Series* vol. 1048, R. U. Halden. ACS Publications. 2010, pp. 539–560.

[13] A. Pal, Y. He, M. Jekel, M. Reinhard, and K. Y. H. Gin, "Emerging contaminants of public health significance as water quality indicator compounds in the urban water cycle," *Environ. Int.*, vol. 71, pp. 46–62, 2014.

[14] G. W. Gribble, "The natural production of chlorinated compounds," *Environ. Sci. Technol.*, vol. 28, no. 7, p. 310A, 1994.

[15] H. Smidt and W. M. de Vos, "Anaerobic microbial dehalogenation," *Annu. Rev. Microbiol.*, vol. 58, pp. 43–73, 2004.

[16] M. M. Häggblom and I. D. Bossert, "Halogenated organic compounds - A global perspective," in *Dehalogenation Microbial Processes and Environmental Applications*, M. M. Häggblom and I. D. Bossert, Eds. Kluwer Academic Publishers, Boston. 2004, pp. 3–29.

[17] L. Adrian and F. E. Löffler, "Outlook-the next frontiers for research on organohalide-respiring bacteria," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016, pp. 621–627.

[18] P. I. Nikel, D. Pérez-Pantoja, and V. de Lorenzo, "Why are chlorinated pollutants so difficult to degrade aerobically? Redox stress limits 1,3-dichloroprop-1-ene metabolism by *Pseudomonas pavonaceae*," *Philos. Trans. R. Soc. B*, vol. 368, no. 1616, p. 20120377, 2013.

[19] Environment and Climate Change Canada - Acts & Regulations, "Toxic substances list - schedule 1," 2016. [Online]. Available: http://www.ec.gc.ca/lcpe-cepa/default.asp [Accessed: 21-Nov-2016].

[20] Agency for Toxic Substances and Disease Registry, "Summary data for 2015 priority list of hazardous substances," *ATSDR, Div. Toxicol. Hum. Heal. Sci.*, pp. 1–6, 2015.

[21] L. Martín-González, S. H. Mortan, M. Rosell, E. Parladé, M. Martínez-Alonso, N. Gaju, G. Caminal, L. Adrian, and E. Marco-Urrea, "Stable carbon isotope fractionation during 1,2-dichloropropane-to-propene transformation by an enrichment culture containing *Dehalogenimonas* strains and a dcpA gene.," *Environ. Sci. Technol.*, vol. 49, no. 14, pp. 8666–74, 2015.

[22] G. Samin and D. B. Janssen, "Transformation and biodegradation of 1,2,3-trichloropropane (TCP).," *Environ. Sci. Pollut. Res. Int.*, vol. 19, no. 8, pp. 3067–78, 2012.

[23] A. J. Tesoriero, F. E. Löffler, and H. Liebscher, "Fate and origin of 1,2-dichloropropane in an unconfined shallow aquifer," *Environ. Sci. Technol.*, vol. 35, no. 3, pp. 455–461, 2001.

[24] Agency for Toxic Substances and Disease Registry, "Toxicological profile for 1,2-dichloroethane," 2001. [Online]. Available: https://www.atsdr.cdc.gov/toxprofiles/tp38.pdf. [Accessed: 18-Nov-2016].

[25] A. Grostern and E. A. Edwards, "Characterization of a *Dehalobacter* coculture that dechlorinates 1,2-dichloroethane to ethene and identification of the putative reductive dehalogenase gene," *Appl. Environ. Microbiol.*, vol. 75, no. 9, pp. 2684–2693, 2009.

[26] Agency for Toxic Substances and Disease Registry, "Toxicological profile on 1,1,2-Trichloroethane," 1999. [Online]. Available: http://www.atsdr.cdc.gov/toxfaqs/tfacts148.pdf. [Accessed: 30-Jul-2016].

[27] Agency for Toxic Substances and Disease Registry, "ToxGuide for 1,1,2,2-tetrachloroethane," 2012. [Online]. Available: https://www.atsdr.cdc.gov/toxguides/toxguide-93.pdf. [Accessed: 30-Jul-2016]

[28] P. J. J. Alvarez and W. A. Illman, "Introduction to bioremediation," in *Bioremediation and Natural Attenuation*, J.L. Shnoor and A. Zehnder Eds. John Wiley & Sons, Inc., Hoboken, NJ, USA, 2005, pp. 1–23.

[29] M. Majone, R. Verdini, F. Aulenta, S. Rossetti, V. Tandoi, N. Kalogerakis, S. Agathos, S. Puig, G. Zanaroli, and F. Fava, "In situ groundwater and sediment bioremediation: Barriers and perspectives at European contaminated sites," *N. Biotechnol.*, vol. 32, no. 1, pp. 133–146, 2015.

[30] P. Panagos, R. Hiederer, M. Van Liedekerke, and F. Bampa, "Review article contaminated sites in europe: review of the current situation based on data collected through a European network," *J. Environ. Public Health*, pp. 1–11, 2013.

[31] P. L. McCarty, "Groundwater contamination by chlorinated solvents: History, remediation technologies and strategies," in *in-Situ of Chlorinated Solvent Plumes, H.Stroo, H.F. Ward and C. Herb Eds.* Springer, New York, 2010, pp. 1–28.

[32] F. I. Khan, T. Husain, and R. Hejazi, "An overview and analysis of site remediation technologies," *J. Environ. Manage.*, vol. 71, no. 2, pp. 95–122, 2004.

[33] U.S. Environmental Protection Agency, "A citizen's guide to pump and treat." [Online]. Available:https://clu-in.org/download/Citizens/a_citizens_guide_to_pump_and_treat.pdf. [Accessed: 31-Jan-2017].

[34] Y. Spira, J. Henstock, P. Nathanail, D. Müller, and D. Edwards, "A European approach to increase innovative soil and groundwater remediation technology applications," *Remediat. J.*, vol. 16, no. 4, pp. 81–96, 2006.

[35] F.-G. Simon, T. Meggyes, and T. Tünnermeier, "Groundwater remediation using active and passive processes," in *Advanced groundwater remediation: Active and passive technologies*, F. G.Simon, T. Meggyes and C. McDonald Eds, Thomas Telford Publishing, London, 2002, pp. 3–34.

[36] M. Tyagi, M. M. R. da Fonseca, and C. C. C. R. de Carvalho, "Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes," *Biodegradation*, vol. 22, pp. 231–241, 2011.

[37] A. A. Juwarkar, S. K. Singh, and A. Mudhoo, "A comprehensive overview of elements in bioremediation," *Rev. Environ. Sci. Biotechnol.*, vol. 9, pp. 215–288, 2010.

[38] A. Leeson, E. Beevar, B. Henry, J. Fortenberry, and C. Coyle, "Principles and practices of enhanced anaerobic bioremediation of chlorinated solvents," for Environmental Security Technology Certification Program California, 2004.

[39] C. C. Azubuike, C. B. Chikere, and G. C. Okpokwasili, "Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects," *World J. Microbiol. Biotechnol.*, vol. 32, no. 11, pp. 1–18, 2016.

[40] R. Naidu, "Recent advances in contaminated site remediation," *Water. Air. Soil Pollut.*, vol. 224, no. 12, pp. 1–11, 2013.

[41] T. Gentry, C. Rensing, and I. Pepper, "New approaches for bioaugmentation as a remediation technology," *Crit. Rev. Environ. Sci. Technol.*, vol. 34, no. 5, pp. 447–494, 2004.

[42] H. F. Stroo, D. W. Major, and J. M. Gossett, "Bioaugmentation for anaerobic bioremediation of chlorinated solvents," in *In Situ Remediation of Chlorinated Solvent Plumes*, H. F. Stroo and C. H. Ward, Eds.Springer Science Business Media, New York, 2010, pp. 425–454.

[43] D. Jan, "Thermodynamic considerations for dehalogenation," in *Dehalogenation*, M.M. Häggblom and I.D. Bossert Eds., Kluwer Academic Publishers, Boston, 2004, pp. 89–114.

[44] J. Dolfing, "Energetic considerations in organohalide respiration," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016, pp. 31–48.

[45] C. Egli, R. Scholtz, A. M. Cook, and T. Leisinger, "Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of

Desulfobacterium sp. and *Methanobacterium* sp.," *FEMS Microbiol. Lett.*, vol. 43, no. 3, pp. 257–261, 1987.

[46] C. Holliger, G. Schraa, A. J. M. Stams, and A. J. B. Zehnder, "Reductive dechlorination of 1,2-dichloroethane and chloroethane by cell suspensions of methanogenic bacteria," *Biodegradation*, vol. 1, no. 4, pp. 253–261, 1990.

[47] G. Stucki, U. Krebser, and T. Leisinger, "Bacterial growth on 1,2-dichloroethane.," *Experientia*, vol. 39, no. 11, pp. 1271–3, 1983.

[48] D. B. Janssen, A. Scheper, L. Dijkhuizen, and B. Witholt, "Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10," *Appl. Environ. Microbiol.*, vol. 49, no. 3, pp. 673–677, 1985.

[49] A. J. Van den Wijngaard, K. W. H. J. Van der Kamp, J. Van der Ploeg, F. Pries, B. Kazemier, and D. B. Janssen, "Degradation of 1,2-dichloroethane by A*ncylobacter aquaticus* and other facultative methylotrophs," *Appl. Environ. Microbiol.*, vol. 58, no. 3, pp. 976–983, 1992.

[50] J. C. Hage and S. Hartmans, "Monooxygenase-mediated 1,2-dichloroethane degradation by *Pseudomonas* sp. strain DCA1," *Appl. Environ. Microbiol.*, vol. 65, no. 6, pp. 2466–2470, 1999.

[51] R. Oldenhuis, R. L. J. M. Vink, D. B. Janssen, and B. Witholt, "Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase," *Appl. Environ. Microbiol.*, vol. 55, no. 11, pp. 2819–2826, 1989.

[52] H. L. Chang and L. Alvarez-Cohen, "Biodegradation of individual and multiple chlorinated aliphatic hydrocarbons by methane-oxidizing cultures," *Appl. Environ. Microbiol.*, vol. 62, no. 9, pp. 3371–3377, 1996.

[53] S. De Wildeman and W. Verstraete, "The quest for microbial reductive dechlorination of C(2) to C(4) chloroalkanes is warranted," *Appl. Microbiol. Biotechnol.*, vol. 61, no. 2, pp. 94–102, 2003.

[54] J. Palau, R. Yu, S. H. Mortan, O. Shouakar-Stash, M. Rosell, D. L. Freedman, C. Sbarbati, S. Fiorenza, R. Aravena, E. Marco-Urrea, M. Elsner, A. Soler, and D. Hunkeler, "Distinct dual C-Cl isotope fractionation patterns during anaerobic biodegradation of 1,2-dichloroethane: potential to characterize microbial degradation in the field," *Environ. Sci. Technol.*, vol. 51, pp. 2685-2694, 2017.

[55] J. A. Field and R. Sierra-Alvarez, "Biodegradability of chlorinated solvents and related chlorinated aliphatic compounds " *Rev. Environ. Sci. Bio/Technology*, vol. 3, pp. 185–254, 2004.

[56] J. Yan, B. A. Rash, F. A. Rainey, and W. M. Moe, "Isolation of novel bacteria within the Chloroflexi capable of reductive dechlorination of 1,2,3-trichloropropane," *Environ. Microbiol.*, vol. 11, no. 4, pp. 833–843, 2009.

[57] S. Tang and E. Edwards, "Identification of *Dehalobacter* reductive dehalogenases that catalyse dechlorination of chloroform, 1,1,1-trichloroethane and 1,1-dichloroethane," *Philos. Trans. R. Soc. B,* vol. 368, no. 1616, p. 20120318, 2013.

[58] A. D. Maness, K. S. Bowman, J. Yan, F. A. Rainey, and W. M. Moe, "*Dehalogenimonas* spp. can reductively dehalogenate high concentrations of 1,2-dichloroethane, 1,2-dichloropropane, and 1,1,2-trichloroethane," *AMB Express*, vol. 2, no. 1, p. 54, 2012.

[59] L. A. Hug, F. Maphosa, D. Leys, F. E. Löffler, H. Smidt, E. a Edwards, and L. Adrian, "Overview

of organohalide-respiring bacteria and a proposal for a classification system for reductive dehalogenases.," *Philos. Trans. R. Soc. B,* vol. 368, no. 1616, pp. 1–10, 2013.

[60] H. Smidt, A. D. L. Akkermans, J. Van Der Oost, and W. M. De Vos, "Halorespiring bacteria – molecular characterization and detection," *Enzyme Microb. Technol.*, vol. 27, pp. 812–820, 2000.

[61] S. Fetzner, "Bacterial dehalogenation," *Appl. Microbiol. Biotechnol.*, vol. 50, no. 6, pp. 633–657, 1998.

[62] P. L. McCarty, "Discovery of organohalide-respiring processes and the bacteria involved," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016,pp. 51–62.

[63] R. E. Richardson, "Genomic insights into organohalide respiration," *Curr. Opin. Biotechnol.*, vol. 24, no. 3, pp. 498–505, 2013.

[64] S. Atashgahi, Y. Lu, and H. Smidt, "Overview of known organohalide-respiring bacteria—phylogenetic diversity and environmental distribution," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016, pp. 63–105.

[65] K. Mayer-Blackwell, H. Sewell, M. Fincker, and A. M. Spormann, "Comparative physiology of organohalide-respiring bacteria," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016, pp. 259–280.

[66] F. Maphosa, W. M. de Vos, and H. Smidt, "Exploiting the ecogenomics toolbox for environmental diagnostics of organohalide-respiring bacteria," *Trends Biotechnol.*, vol. 28, no. 6, pp. 308–316, 2010.

[67] I. Nijenhuis and K. Kuntze, "Anaerobic microbial dehalogenation of organohalides-state of the art and remediation strategies," *Curr. Opin. Biotechnol.*, vol. 38, pp. 33–38, 2016.

[68] T. Futagami and K. Furukawa, "The genus *Desulfitobacterium*" in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016, pp. 173–207.

[69] R. A. Sanford, J. Chowdhary, and F. E. Löffler, "Organohalide-respiring Deltaproteobacteria," in *Organohalide-Respiring Bacteria*, L. Adrian and F. E. Loffler, Eds. Springer Berlin. Heidelberg, 2016, pp. 235–258.

[70] T. Goris and G. Diekert, "The genus *Sulfurospirillum*," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016,pp. 209–234.

[71] M. J. Krzmarzick, B. B. Crary, J. J. Harding, O. O. Oyerinde, A. C. Leri, S. C. B. Myneni, and P. J. Novak, "Natural niche for organohalide-respiring chloroflexi," *Appl. Environ. Microbiol.*, vol. 78, no. 2, pp. 393–401, 2012.

[72] S. H. Zinder, "The genus *Dehalococcoides*," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016, pp. 107–136.

[73] W. M. Moe, F. A. Rainey, and J. Yan, "The Genus *Dehalogenimonas*," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016, pp. 137–151.

[74] T. Futagami, M. Goto, and K. Furukawa, "Biochemical and genetic bases of dehalorespiration," *Chem. Rec.*, vol. 8, no. 1, pp. 1–12, 2008.

[75] B. E. Jugder, H. Ertan, S. Bohl, M. Lee, C. P. Marquis, and M. Manefield, "Organohalide respiring bacteria and reductive dehalogenases: Key tools in organohalide bioremediation," *Front. Microbiol.*, vol. 7, pp. 1–12, 2016.

[76] Y. Men, H. Feil, N. C. Verberkmoes, M. B. Shah, D. R. Johnson, P. K. H. Lee, K. A. West, S. H. Zinder, G. L. Andersen, and L. Alvarez-Cohen, "Sustainable syntrophic growth of *Dehalococcoides ethenogenes* strain 195 with *Desulfovibrio vulgaris* and *Methanobacterium congolense*: global transcriptomic and proteomic analyses.," *ISME J.*, vol. 6, no. 2, pp. 410–21, 2012.

[77] R. Banerjee and S. W. Ragsdale, "The many faces of vitamin B12: catalysis by cobalamin-dependent enzymes1," *Annu. Rev. Biochem.*, vol. 72, no. 1, pp. 209–247, 2003.

[78] J. Yan, J. Im, Y. Yang, and F. E. Loffler, "Guided cobalamin biosynthesis supports *Dehalococcoides mccartyi* reductive dechlorination activity.," *Philos. Trans. R. Soc. B,*vol. 368, no. 1616, p. 20120320, 2013.

[79] M. Bommer, C. Kunze, J. Fesseler, T. Schubert, and G. Diekert, "Structural basis for organohalide respiration," *Science*, vol. 346, no. 6208, pp. 455–458, 2014.

[80] B. E. Jugder, H. Ertan, M. Lee, M. Manefield, and C. P. Marquis, "Reductive dehalogenases come of age in biological destruction of organohalides," *Trends Biotechnol.*, vol. 33, no. 10, pp. 595–610, 2015.

[81] S. Ni, J. K. Fredrickson, and L. Xun, "Purification and characterization of a novel 3-chlorobenzoate-reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile tiedjei* DCB-1.," *J. Bacteriol.*, vol. 177, no. 17, pp. 5135–9, 1995.

[82] A. Neumann, H. Scholz-Muramatsu, and G. Diekert, "Tetrachloroethene metabolism of *Dehalospirillum multivorans*," *Arch. Microbiol.*, vol. 162, no. 4, pp. 295–301, 1994.

[83] S. H. Thomas, R. D. Wagner, A. K. Arakaki, J. Skolnick, J. R. Kirby, L. J. Shimkets, R. A. Sanford, and F. E. Löffler, "The mosaic genome of *Anaeromyxobacter dehalogenans* strain 2CP-C suggests an aerobic common ancestor to the delta-proteobacteria," *PLoS One*, vol. 3, no. 5, 2008.

[84] L. A. Hug, "Diversity, evolution, and environmental distribution of reductive dehalogenase genes," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin. Heidelberg, 2016,pp. 377–393.

[85] Y. Sung, K. E. Fletcher, K. M. Ritalahti, R. P. Apkarian, N. Ramos-Hernández, R. A. Sanford, N. M. Mesbah, and F. E. Löffler, "*Geobacter lovleyi* sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium," *Appl. Environ. Microbiol.*, vol. 72, no. 4, pp. 2775–2782, 2006.

[86] A. Neumann, A. Siebert, T. Trescher, S. Reinhardt, G. Wohlfarth, and G. Diekert, "Tetrachloroethene reductive dehalogenase of *Dehalospirillum multivorans*: substrate specificity of the native enzyme and its corrinoid cofactor.," *Arch. Microbiol.*, vol. 177, no. 5, pp. 420–6, 2002.

[87] W. Schumacher, C. Holliger, A. J. . Zehnder, and W. R. Hagen, "Redox chemistry of cobalamin and iron-sulfur cofactors in the tetrachloroethene reductase of *Dehalobacter restrictus*," *FEBS Lett.*, vol. 409, no. 3, pp. 421–425, 1997.

[88] J. Maillard, W. Schumacher, F. Vazquez, C. Regeard, W. R. Hagen, and C. Holliger, "Characterization of the corrinoid iron-sulfur protein tetrachloroethene reductive dehalogenase of *Dehalobacter restrictus*," *Appl. Environ. Microbiol.*, vol. 69, no. 8, pp. 4628–38, 2003.

[89] J. K. Magnuson, M. F. Romine, D. R. Burris, and M. T. Kingsley, "Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: sequence of *tceA* and substrate range characterization.," *Appl. Environ. Microbiol.*, vol. 66, no. 12, pp. 5141–7, 2000.

[90] J. A. Müller, B. M. Rosner, G. Von Abendroth, G. Meshulam-Simon, P. L. McCarty, and A. M. Spormann, "Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution," *Appl. Environ. Microbiol.*, vol. 70, no. 8, pp. 4880–4888, 2004.

[91] S. Tang, W. W. M. Chan, K. E. Fletcher, J. Seifert, X. Liang, F. E. Löffler, E. A. Edwards, and L. Adrian, "Functional characterization of reductive dehalogenases by using blue native polyacrylamide gel electrophoresis," *Appl. Environ. Microbiol.*, vol. 79, no. 3, pp. 974–981, 2013.

[92] E. Padilla-Crespo, J. Yan, C. Swift, D. D. Wagner, K. Chourey, R. L. Hettich, K. M. Ritalahti, and F. E. Löffler, "Identification and environmental distribution of *dcpA*, which encodes the reductive dehalogenase catalyzing the dichloroelimination of 1,2-dichloropropane to propene in organohalide-respiring Chloroflexi," *Appl. Environ. Microbiol.*, vol. 80, no. 3, pp. 808–818, 2014.

[93] L. Adrian, J. Rahnenführer, J. Gobom, and T. Hölscher, "Identification of a chlorobenzene reductive dehalogenase in *Dehalococcoides* sp. strain CBDB1," *Appl. Environ. Microbiol.*, vol. 73, no. 23, pp. 7717–7724, 2007.

[94] A. Bisaillon, R. Beaudet, F. Lépine, E. Déziel, and R. Villemur, "Identification and characterization of a novel CprA reductive dehalogenase specific to highly chlorinated phenols from *Desulfitobacterium hafniense* strain PCP-1," *Appl. Environ. Microbiol.*, vol. 76, no. 22, pp. 7536–7540, 2010.

[95] C. Ding, S. Zhao, and J. He, "A *Desulfitobacterium* sp. strain PR reductively dechlorinates both 1,1,1-trichloroethane and chloroform," *Environ. Microbiol.*, vol. 16, no. 11, pp. 3387–3397, 2014.

[96] M. Marzorati, F. De Ferra, H. Van Raemdonck, S. Borin, E. Allifranchini, G. Carpani, L. Serbolisca, W. Verstraete, N. Boon, and D. Daffonchio, "A novel reductive dehalogenase, identified in a contaminated groundwater enrichment culture and in *Desulfitobacterium dichloroeliminans* strain DCA1, is linked to dehalogenation of 1,2-dichloroethane," *Appl. Environ. Microbiol.*, vol. 73, no. 9, pp. 2990–2999, 2007.

[97] O. Molenda, A. T. Quaile, and E. A. Edwards, "*Dehalogenimonas* sp. strain WBC-2 genome and identification of its trans-dichloroethene reductive dehalogenase, TdrA," *Appl. Environ. Microbiol.*, vol. 82, no. 1, pp. 40–50, 2016.

[98] A. Suyama, M. Yamashita, S. Yoshino, and K. Furukawa, "Molecular characterization of the PceA reductive dehalogenase of *Desulfitobacterium* sp. strain Y51," *J. Bacteriol.*, vol. 184, no. 13, pp. 3419–25, 2002.

[99] K. M. Ritalahti, B. K. Amos, Y. Sung, Q. Wu, S. S. Koenigsberg, and F. E. Loffler, "Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains," *Appl. Environ. Microbiol.*, vol. 72, no. 4, pp. 2765–2774, 2006.

[100] B. A. Van De Pas, H. Smidt, W. R. Hagen, J. Van Der Oost, G. Schraa, A. J. M. Stams, and W. M.

De Vos, “Purification and molecular characterization of ortho-chlorophenol reductive dehalogenase, a key enzyme of halorespiration in *Desulfitobacterium* dehalogenans,” *J. Biol. Chem.*, vol. 274, no. 29, pp. 20287–20292, 1999.

CHAPTER 2

OBJECTIVES OF THE THESIS

The main objective of this study is to isolate and characterize organohalide respiring bacteria (OHRB) from a microbial consortium obtained from Besòs River estuary sediments that selectively converts vicinal halogenated alkanes via dihaloelimination.

The specific objectives of the current study are to:

1) Enrich, identify and to study the physiological and molecular characterization of the OHR responsible for this dihaloelimination reaction.
2) To determine the stable isotope fractionation during the dichloroelimination of selected chloroalkanes by a mixed culture containing *Dehalogenimonas* spp.
3) To isolate the *Dehalogenimonas* strain.
4) To identify the reductive dehalogenases involved in the dichloroelimination of selected chloroalkanes by *Dehalogenimonas* spp.
5) To construct a co-culture of OHRB consisting of *Dehalogenimonas* spp and *Dehalococcoides mccartyi* strain BTF08 to achieve complete dechlorination of highly chlorinated ethanes in a bioreactor.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1. Chemicals

All chemicals were purchased at the highest purity available. The companies, purities and state of the matter of the compounds are outlined in Table 3.1.

Table 3.1: List of chemicals

Chemicals	Purity (%)	Supplier	State of matter
1,1,2-Trichloroethane	≥98%	J.T Baker	Liquid
1,1,2,2-Tetrachloroethane	≥99%	Sigma	Liquid
1,2,4-Trichlorobenzene	≥99%	Sigma	Liquid
1,2-Dibromopropane	≥99%	Sigma	Liquid
1,2-Dichloroethane	≥99%	Sigma	Liquid
1,2-Dichloropropane	≥99%	Sigma	Liquid
1,6-Dibromohexane	≥99%	Sigma	Liquid
1-Chloropropane	≥99%	Sigma	Liquid
2-Chloropropane	≥99%	Sigma	Liquid
2,3-Dichloro-1-propene	≥99%	Sigma	Liquid
Allyl chloride	≥99%	Sigma	Liquid
Allyl disulfide	≥99%	Sigma	Liquid
Allyl sulfide	≥99%	Sigma	Liquid
Chlorobenzene	≥99%	Sigma	Liquid
Chloroform	≥98%	Sigma	Liquid
cis-1,2-Dichlorethene	≥99%	Chem Service	Liquid
4-Aminobenzoic acid		Sigma	Solid
Acetic acid		Sigma	Liquid
Acetone		Sigma	Liquid
Ammonium chloride		Panreac	Liquid
Ampicillin		Sigma	Solid
Biotin		Sigma	Solid
Boric acid		Panreac	Solid
Calcium chloride		Panreac	Solid
Calcium pantothenate		Sigma	Solid
Chloramphenicol		Sigma	Solid
Cobalt chloride 6-hydrate		VWR	Solid
Copper(II) chloride dihydrate		Sigma	Solid
Cyanocobalamin		Sigma	Solid
Diclofenac sodium salt	≥99%	Sigma	Solid
Ethanol	≥96%	Fisher	Liquid

Table 3.1 (*continued*)

Chemicals	Purity (%)	Supplier	State of matter
Ethylene	≥99%	Sigma	Gas
Formic acid		Merck	Liquid
HEPES sodium salt		Merck	Solid
Iron(II) chloride tetrahydrate		Sigma	Solid
Kanamycin sulfate		Sigma	Solid
L-Cysteine		Sigma	Liquid
Manganese (II) chloride 4-hydrate		Pancreac	Solid
Magnesium chloride hexahydrate		Sigma	Solid
Methyl viologen dichloride hydrate		Sigma	Liquid
Nalidixic acid		Sigma	Solid
Nickel (II) chloride		Sigma	Solid
Nicotinic acid		Sigma	Solid
Nitrilotriacetic acid		Sigma	Liquid
Potassium chloride		Panreac	Solid
Potassium dihydrogen phosphate		VWR	Solid
Propene		Chem Service	Gas
Propionic acid		Panreac	Liquid
Pyridoxine hydrochloride		Sigma	Solid
Resazurin sodium salt		Sigma	Solid
Sodium bicarbonate			Solid
Sodium 2-bromoethanesulfonate	≥99%	Sigma	Solid
Sodium chloride		Panreac	Solid
Sodium DL-lactate	≥99%	Sigma	Soild
Sodium hydroxide		Panreac	Solid
Sodium molybdenum oxide dihydrate	98%	VWR	Solid
Sodium pyruvate		VWR	Solid
Streptomycin sulfate salt		Sigma	Solid
Sodium sulfide nonahydrate	98%	Sigma	Solid
Thiaminchloride-hydrochloride		Merck	Solid
trans-1,2-Dichloroethylene		Sigma	Liquid
Triclosan		Sigma	Solid
Vancomycin hydrochloride hydrate		Sigma	Solid

Table 3.1 (*continued*)

Chemicals	Purity (%)	Supplier	State of matter
Vinyl chloride	≥99%	Chem Service	Liquid
Vitamin B12	≥99%	Sigma	Solid
Zinc chloride		VWR	Solid

3.2. Microcosms setup

Anaerobic basal media was prepared as described by Adrian et. al (2000) [1]. The anaerobic medium contained the following constituents (per liter): KH_2PO_4, 0.2 g; NH_4Cl, 0.27 g; NaCl, 1 g; $MgCl_2.6H_2O$, 0.41 g; KCl, 0.52 g; $CaCl_2.2H_2O$, 0.15 g; $ZnCl_2$, 0.7 mg; $MnCl_2.4H_2O$, 0.8 mg; H_3BO_3, 0.06 mg; $CoCl_2.6H_2O$, 0.19 mg; $CuCl_2.2H_2O$, 0.02 mg; $NiCl_2.6H_2O$, 0.24 mg; $Na_2MoO_4.2H_2O$, 0.36 mg; $FeCl_2.4H_2O$, 20 mg; nitriloacetic acid, 128 mg.

To remove oxygen from the medium, it was bubbled with nitrogen gas for at least 20 minutes. The serum bottles were sealed with Teflon coated stoppers and crimped inside the anaerobic tent. All bottles were autoclaved at 121 °C for 40 min.

After the medium was autoclaved and cooled, the following components were added aseptically from sterile and anoxic stock solutions; $NaHCO_3$ solution (1 g L^{-1}), $Na_2S.9H_2O$ + L-cysteine solution (25 mg L^{-1}), filter sterilized vitamin solution containing (40 mg L^{-1} 4-aminobenzoic acid, 10 mg L^{-1} D(+)-biotin, 100 mg L^{-1} nicotinic acid, 50 mg L^{-1} calcium D (+)-pantothenate, 150 mg L^{-1} pyridoxine dihydrochloride, 0.1 mL thiamin thiamine chloride).

Each microcosm was inoculated with 10 % v/v of inoculum in 120 mL glass serum bottles containing 65 mL of a medium. The serum bottles were then gassed with N_2/CO_2 (4:1, v/v, 0.2 bar overpressure) and H_2 (added to an overpressure of 0.4 bar). Halogenated compound was added with a syringe from a stock solution in acetone to a desired nominal concentration. Microcosms were incubated at 25 °C in the dark in static conditions.

For the maintenance of the culture, microcosms that depleted the initial dose of halogenated compound were reamended with the same amount of the electron acceptor and transferred to fresh medium (10% v/v) when approximately 80% of the initial dose was consumed.

3.3. Analytical methods

Chlorinated compounds, propene, ethene, hydrogen and methane, were detected and quantified by analyzing 0.5 mL headspace samples respectively taken with a 1.0 mL pressurelock precision analytical syringe (Vici, USA) from the serum bottles. All compounds were identified using retention times of chemical standards.

3.3.1. Chlorinated compounds

A gas chromatograph (GC) model 6890N (Agilent Technologies; Santa Clara, USA) equipped with a DB-624 column (30 m × 0.32 mm with 0.25 μm film thickness; Agilent Technologies) and a flame ionization detector (FID) was used to analyze all volatile organic compounds. Helium was used as the carrier gas (0.9 mL min^{-1}). The injector and detector temperatures were set at 250 and 300 °C respectively. After the injection of the sample (split ratio = 2), the initial oven temperature (35 °C) was held for 3 min and then ramped at 10 °C min^{-1} to 240 °C, which was held for 4 min. Peak areas were calculated using Millennium/Empower software (Waters, Milford, USA).

Calibration was based on aqueous standards, with the same liquid and headspace volumes as in the microcosms. The solutions were allowed to equilibrate overnight, and afterwards 0.5 mL aliquots of the headspace were analyzed in the GC as described above. Results are presented as either nominal concentrations (μmol L^{-1} of liquid volume) or total μmoles in the serum bottles.

3.3.2. Ethene and propene measurements

Propene and ethene were analyzed using the GC-FID described in section 3.3.1 but equipped with a HP Plot Q column (30 m × 0.53 mm with 40 μm film thickness, Agilent Technologies). The oven temperature was fixed at 150 °C, the injector temperature at 250 °C and the detector temperature at 260 °C. Run time lasted 7 min.

Calibration was done by injecting different known volumes of gas standards into 120 mL serum bottles sealed with Teflon-coated butyl rubber septa and aluminum crimp caps. The gas stocks were prepared diluting 1mL of the pure gas (ethene or propene) in 1 L nitrogen in a Tedlar gas sampling bag. Calibration curves were prepared by plotting peak area versus known concentrations.

3.3.3. Hydrogen measurements

Hydrogen concentration in the gas headspace was measured using an Agilent 7820A GC fitted with MolSieve 5A 60/80 SS (Agilent Technologies; Santa Clara, USA) and Porapak Q 60/80 UM columns (Agilent Technologies; Santa Clara, USA) and a thermal conductivity detector (TCD). Oven temperature was held isothermal at 40 °C, the injector temperature at 200 °C and the detector temperature at 250 °C. Run time lasted 5 min. The calibration was done as described in section 3.3.2.

3.3.4. Methane measurements

Methane concentration was analyzed using a GC HP 5890 ((Agilent Technologies; Santa Clara, USA) with a thermal conductivity detector (TCD) equipped with a Porapack Q column (3 m x 3.2 mm, Sigma-Aldrich, Barcelona, Spain) using helium at 338 kPa as the carrier gas. The oven temperature was fixed at 70 °C, the injector temperature at 150 °C and the detector

temperature at 180 °C. Run time was 3 min. The calibration was done as described in section 3.3.2.

3.3.5. Volatile fatty acids

Organic acids (acetate, pyruvate, formate, and propionate) were analyzed using a Dionex 3000 Ultimate high-pressure liquid chromatography (ThermoFisher Scientific, Waltham, USA) equipped with a UVD 170S UV detector set at 210 nm and an autosampler (injection volume, 20 µL) after filtering 1 mL liquid sample from the medium through a 0.22 µm filter. The eluent was 6 mM aqueous H_2SO_4, which was pumped at a flow rate of 0.5 ml min^{-1} through a Transgenomic ICSep ICE-COREGEL 87H3 column (300 × 4.6 mm) (Chrom Tech, Apple Valley, USA).

3.4. Reference

[1] L. Adrian, U. Szewzyk, J. Wecke, and H. Görisch, "Bacterial dehalorespiration with chlorinated benzenes.," *Nature*, vol. 408, no. 6812, pp. 580–3, 2000.

CHAPTER 4

ENRICHMENT, PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF A DEHALOGENATING BACTERIAL CONSORTIUM OBTAINED FROM BESÒS RIVER ESTUARY SEDIMENTS

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ABSTRACT

A stable enrichment culture derived from Besòs river estuary sediments stoichiometrically dechlorinated 1,2-DCP to propene. Sequential transfers in defined anaerobic medium with the methanogenic inhibitor bromoethanesulfonate produced a sediment-free culture dechlorinating 1,2-DCP in the absence of methanogenesis. Application of previously published genus specific primers targeting 16S rRNA gene sequences revealed the presence of a *Dehalogenimonas* strain, and no amplification was obtained with *Dehalococcoides* specific primers. The partial sequence of the 16S rRNA amplicon was 100% identical with *Dehalogenimonas alkenigignens* strain IP3-3. Also, *dcpA*, a gene described to encode a corrinoid-containing 1,2-DCP reductive dehalogenase was detected. Resistance of the dehalogenating activity to vancomycin, exclusive conversion of vicinally chlorinated alkanes, and tolerance to short-term oxygen exposure is consistent with the hypothesis that a *Dehalogenimonas* strain is responsible for 1,2-DCP conversion in the culture. Quantitative PCR showed a positive correlation between the number of *Dehalogenimonas* 16S rRNA genes copies in the culture and consumption of 1,2-DCP. The monitoring of bacterial composition by 16S rDNA-based methods employing DGGE revealed that the microbial composition of the culture was maintained for over one and a half year, suggesting that a stable consortia containing *Dehalogenimonas* as the dechlorinating bacteria was established.

4.1. Introduction

Biological transformation of chlorinated alkanes under anaerobic conditions has not been studied in detail despite they are frequently detected in groundwaters contaminated with chlorinated solvents. In this work, our purpose was establishing a microbial consortium with dechlorinating activity towards chlorinated alkanes and sediments collected from the Besòs River estuary (Barcelona, Spain) were used as inoculum for this purpose. This location was chosen because sediments of this coastal area have been historically contaminated with short-chain chlorinated paraffins [1], providing a potential niche for OHRB.

1,2-DCP was selected as chlorinated alkane model in the first generation of these cultures. This compound has been used predominantly as a chemical intermediate in the production of tetrachloromethane (carbon tetrachloride) and tetrachloroethylene, lead scavenger for antiknock fluids and solvent. According to the National Primary Drinking Water Regulations established by the U.S. Environmental Protection Agency [2], 1,2-DCP can increase risk of cancer so a maximum contaminant level in drinking water of 5 μg L^{-1} is legally set for public water systems. Today, 1,2-DCP is a risk for the environment and drinking water quality especially at historically contaminated sites.

Under aerobic conditions, 1,2-DCP can be partially cometabolized to less-chlorinated alkanes. For instance, *Pseudomonas* sp. strain DCA1 oxidized cometabolically 1,2-DCP to 2,3-dichloro-1-propanol and 2-chloroethanol during growth on 1,2-DCA [3]. However, 1,2-DCP exerted a strong inhibitory effect on the growth of this *Pseudomonas* strain, probably due to a transient toxic intermediate [3]. Similarly, resting cells of the methanotroph *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase catalyzed the transformation of 1,2-DCP to 2,3-dichloro-1-propanol, 1-chloro-2-propanol, and 2-chloro-1-propanol [4]. So

far, complete dechlorination of 1,2-DCP to propene or propane has only been demonstrated for OHRB, which use halogenated compounds as terminal electron acceptors during electron transport-based energy conservation [5]. To date, few isolated OHRB have been described to derive energy for growth from dechlorination of 1,2-DCP, including *Dehalogenimonas alkenigignens* strain IP3-3[6], *Dehalogenimonas lykanthroporepellens* strain BL-DC-8 and strain BL-DC-9 [7], *Dehalogenimonas formicexedens* [8], *Dehalococcoides mccartyi* strain RC and strain KS[9] , and *Desulfitobacterium dichloroeliminans* strain DCA1 [10]. A *Dehalobacter* strain was involved in the reductive dechlorination of 1,2-DCP in a consortium [11]. Although these bacteria share several common phenotypic features including growth under anaerobic conditions, the use of halogenated compounds as respiratory electron acceptors and hydrogen as electron donor, isolated *Dehalogenimonas* strains differ from other 1,2-DCP transforming isolates in their exclusive utilization of polychlorinated alkanes as halogenated electron acceptors. Recently, a gene designated *dcpA* was identified as encoding the reductive dehalogenase that catalyzes the dechlorination of 1,2-DCP to propene in *Dehalococcoides* [12].

4.2. Materials and methods

4.2.1. Chemicals

All the chemicals used were reagent grade and described in Chapter 3 unless otherwise specified.

4.2.2. Sampling and cultivation

Inocula derived from Besòs river estuary sediments (Spain). The samples were collected from layers 15 cm below the surface. Sediments were transported to the lab, transferred to an anaerobic glovebox and used to set up microcosms on the same day. Each microcosm consisted

of 6 g of sediment (wet weight) in 120 mL glass serum bottles containing 65 mL of a sterilized anaerobic synthetic medium previously used to grow *Dehalococcoides mccartyi* strain CBDB1 [13]. The microcosm setup was described in Chapter 3. 1,2-DCP was added with a syringe from a 3.2 mM stock solution in acetone to a nominal concentration of 50 μM. Microcosms were prepared at least in triplicate and incubated at 25 °C in the dark in static conditions. Microcosms that depleted the initial dose of 1,2-DCP were reamended with the same amount of the electron acceptor and transferred to fresh medium (10% v/v) when approximately 80% of the initial dose of 1,2-DCP was consumed.

4.2.3. Analytical methods

Chlorinated compounds, propene and methane were analyzed in the headspace by gas chromatography as described in Chapter 3.

Diclofenac and triclosan concentrations were measured using high performance liquid chromatography (HPLC). Liquid samples (1 mL) from experimental bottles were diluted 1:1 (v:v) with acetone as a solvent before mixing on a vortex (Zx, Velp Scientifica, Italy). Next, the samples were filtered (Millex-GV, PVDF, 0.22 μm, Millipore) and analyzed using a Dionex 3000 Ultimate HPLC (Barcelona, Spain) that was equipped with a UV detector at 277 nm. The column temperature was 30 °C, and a sample volume of 10 μL was injected from a Dionex autosampler. Chromatographic separation was achieved using a GraceSmart RP-18 column (250 × 4.6 mm, particle size of 5 μm) (Grace, Columbia, USA). The mobile phase consisted of a 0.1% formic acid solution (A) and acetonitrile (B). The analysis was performed isocratically (30% A) at 1 mL min^{-1}. The retention times for diclofenac and triclosan were 4.3 and 5.4 min, respectively. The quantification limit for both compounds was 2 mg L^{-1}.

The presence of bromide ions was evaluated on a Dionex ICS-2000 ion chromatography system (Dionex, Sunnyvale, USA) equipped with an IonPac AS18 anion-exchange column (Dionex, Sunnyvale, USA). The column was operated at a temperature of 30 °C and a flow rate of 1 mL min^{-1}. The injection volume was 25 µL. The potassium hydroxide concentration of the eluent varied from 25 mM to 50 mM along the 10 min analysis.

4.2.4. DNA extraction and PCR

Genomic DNA was extracted from 50 mL of the consortium using an UltraClean water DNA isolation kit (MoBio, Carlsbad, USA). Amplification of bacterial 16S rRNA genes was conducted with two sets of primers. The first primer combination (BL-DC-142f and BLDC-1351r) was specific for the genus *Dehalogenimonas* [14]. The second primer set specifically targeted the genus *Dehalococcoides* (Dch1F and Dch264R) [15]. Genomic DNA was also analyzed for the presence of the *dcpA* gene. Primers dcpA-360F and dcpA-1449R10 designed for conventional PCR were used. Genomic DNA from *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 (=ATCC BAA-1523 = JCM 15061) and *Dehalococcoides mccartyi* strain CBDB1 were used as positive controls. Each 50 µL reaction mixture contained 50 ng of template DNA, 1× PCR buffer (20 mM Tris/HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 0.5 µM of each primer and 2.5 U of Taq DNA polymerase (Invitrogen, Carlsberg, USA). The thermal programs used for PCR amplification of *Dehalogenimonas* and *dcpA* gene were previously described [12], [14]. For primer set Dch1F and Dch264R, the program used an initial denaturation at 95 °C for 5 min and then 35 cycles at 95 °C for 30 s, the desired annealing temperature (59 °C) for 30 s, and extension at 72 °C for 60 s, followed by a final extension step at 72 °C for 7 min. Amplicons were analyzed by electrophoresis in a 2% (wt/v) agarose gel at 75 V for 40 min. Primers used in the second

amplification in the nested PCR approach were 341f-GC and 907r. Temperature cycling was done as described previously [16]. Sequences of the different oligonucleotide primer sets used in this study are given in Table 4.1.

Table 4.1: Designations and sequences of primers used in this study.

Primer set	Primer sequence (5'-3')	Target	Reference
341f	CCTACGGGAGGCAGCAG	Bacterial 16S rRNA gene	[17]
907r	CCGTCAATTCMTTTGAGTTT	Bacterial 16S rRNA gene	[18]
BL-DC-142f	GTGGGGGATAACACTTCGAAAGAAGTGC	*Dehalogenimonas* 16S rRNA gene	[14]
BL-DC-1351r	AACGCGCTATGCTGACACGCGT	*Dehalogenimonas* 16S rRNA gene	[14]
mod-BL-DC-1243f	GGYACAATGGGTTGCCACCGG	*Dehalogenimonas* 16S rRNA gene	[14]
BL-DC-1351r	AACGCGCTATGCTGACACGCGT	*Dehalogenimonas* 16S rRNA gene	[14]
Dch1F	GATGAACGCTAGCGGCG	*Dehalococcoides*16S rRNA gene	[15]
Dch264R	CCTCTCAGACCAGCTACCGATCGAA	*Dehalococcoides*16S rRNA gene	[15]
dcpA-360F	TTGCGTGATCAAATTGGAGCCTGG	*dcpA* gene	[12]
dcpA-1449R	TTTAAACAGCGGGCAGGTACTGGT	*dcpA* gene	[12]

4.2.5. Denaturing gradient gel electrophoresis (DGGE) analysis and sequencing

Five-hundred ng of PCR product from nested PCR using the primer sets BL-DC-142f/BL-DC1351r and 341f-GC/907r were loaded onto a denaturing gradient gel. DGGE was carried out using a Bio-Rad DCode system, as described elsewhere [16], in a 6% polyacrylamide gel with 30 − 70% denaturant gradient (100% denaturant contained 7 M urea and 40% v/v

deionized formamide). Electrophoresis was performed at 60 °C with a constant voltage of 75 V for 16 h. The gels were stained with ethidium bromide (0.5 μg mL^{-1}), then inspected under UV illumination and photographed. Prominent bands were excised from the gels, reamplified, and then purified using the MoBio PCR Clean up Kit (Carlsbad, USA) for subsequent sequencing.

Sequencing reactions were performed by Macrogen (South Korea) using the Big Dye Terminator v3.1 sequencing kit; reactions were run in an automatic capillary type ABI 3730XL analyzer-96. Sequences were first screened to detect potential chimeric artifacts using the Chimera.uchime program in Mothur 1.33.3 (http://www.mothur.org/wiki/Download_ mothur) [19] and then compared to those deposited in the GenBank nucleotide database using the BLASTN program [20]. The 16S rRNA gene sequences determined in this study are available at the GenBank database under accession numbers KP780280 through KP780282. Each band designation includes a code specifying its origin (BREd, Besòs River Estuary *Dehalogenimonas*) followed by a number indicating the order in which the sequence was isolated from the gel.

4.2.6. Quantitative PCR (qPCR)

The qPCR assays were performed with DNA extracted from cultures growing in parallel consuming different amounts of 1,2-DCP. Primer set mod-BL-DC-1243f and BL-DC-1351r21 was used in qPCR to quantify *Dehalogenimonas* 16S rRNA gene copies in the consortia (Table 4.1). qPCR was performed using a CFX96 Real-Time System (Bio-Rad) in 20 μL total reaction volumes containing 1× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.5 μM of each primer and 36 ng of sample DNA. The amplification program used was reported previously [14]. A melting curve analysis to assess product specificity followed each PCR reaction. Melting curves were generated from 65 to 95 °C with increments of 0.5 °C each cycle and a dwell time at each temperature of 5 s. Samples and nontemplate controls were analyzed in triplicates and the

latter were included in each assay. Each calibration curve was prepared using purified PCR product of a partial 16S rRNA gene (1199 bp) from *Dehalogenimonas lykanthroporepellens* BL-DC-9T. Six serial dilutions were prepared independently in triplicates and concentrations were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific). Gene copies per qPCR reaction and PCR amplification efficiency were calculated as described previously [21]. PCR amplification efficiency ranged from 93.2% to 102.3%.

4.3. Results and discussion

4.3.1. Enrichment of dehalogenating bacteria

Sediments collected from the Besòs river estuary were incubated in reduced medium containing 50 μM 1,2-DCP. In the first transfer, hydrogenolysis of 1,2-DCP to 1-chloropropane (1-CP) and minor amounts of 2-chloropropane (2-CP) was the predominant reaction in most of the cultures (Figure 4.1A). Hydrogenolysis of chloroalkanes was an unexpected reaction which has been reported only once before [22], and therefore it was specifically followed. A decrease in the production of chloropropanes in the subsequent transfers was observed, and titanium(III) citrate was replaced by Na_2S and L-cysteine (0.2 mM each) in parallel cultures to test whether titanium(III) citrate exerted inhibition to the dechlorinating population catalyzing hydrogenolysis. Production of chloropropanes was favored in the cysteine-sulfide medium but after the fourth transfer hydrogenolytic activity was lost and propene became the unique identified transformation product (Figure 4.1B). Although we corroborated that hydrogenolysis of chloropropanes is possible, the identity and characteristics of the bacteria catalyzing this process remained unknown. Similarly to the results showed by Löffler et al. [22], hydrogenolysis

was not observed in sediment-free cultures, which is consistent with the hypothesis that microorganisms catalyzing this reaction may be favored in sedimentary environments.

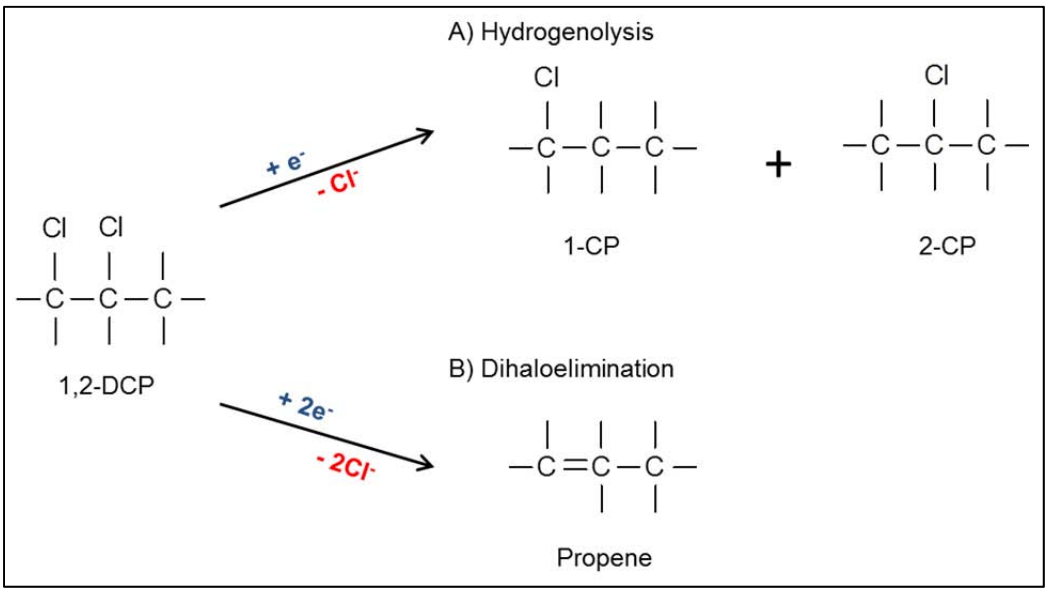


Figure 4.1: Degradation pathways of 1,2-DCP observed in microcosms containing sediments from the Besòs River Estuary.

Dihaloelimination of 1,2-DCP to propene (Figure 4.1B) was sustained in subsequent transfers inoculating 10% (v/v) from the original microcosms using the L-cysteine-sulfide reducing agent. A sediment-free culture was obtained after five consecutive transfers of the supernatant to fresh medium.

Methane was produced concomitantly to the dechlorination of 1,2-DCP. To investigate the role of methanogens in dichloroelimination of 1,2-DCP, we tested the effect of bromoethanesulfonate (BES) at two different concentrations (5 and 25 mM) and of 1,2-dibromopropane (10 µM) on dechlorination activity (Figure 4.2A and B). The latter was initially tested as a possible electron acceptor (see below) but instead of that, we observed that it completely inhibited methane production in our cultures. Methane production was not inhibited

at 5 mM BES, but it completely ceased at a concentration of 25 mM with no negative effect on dechlorinating activity (Figure 4.2B). Addition of 10 µM of dibromopropane completely inhibited both methanogenic activity and 1,2-DCP dechlorination.

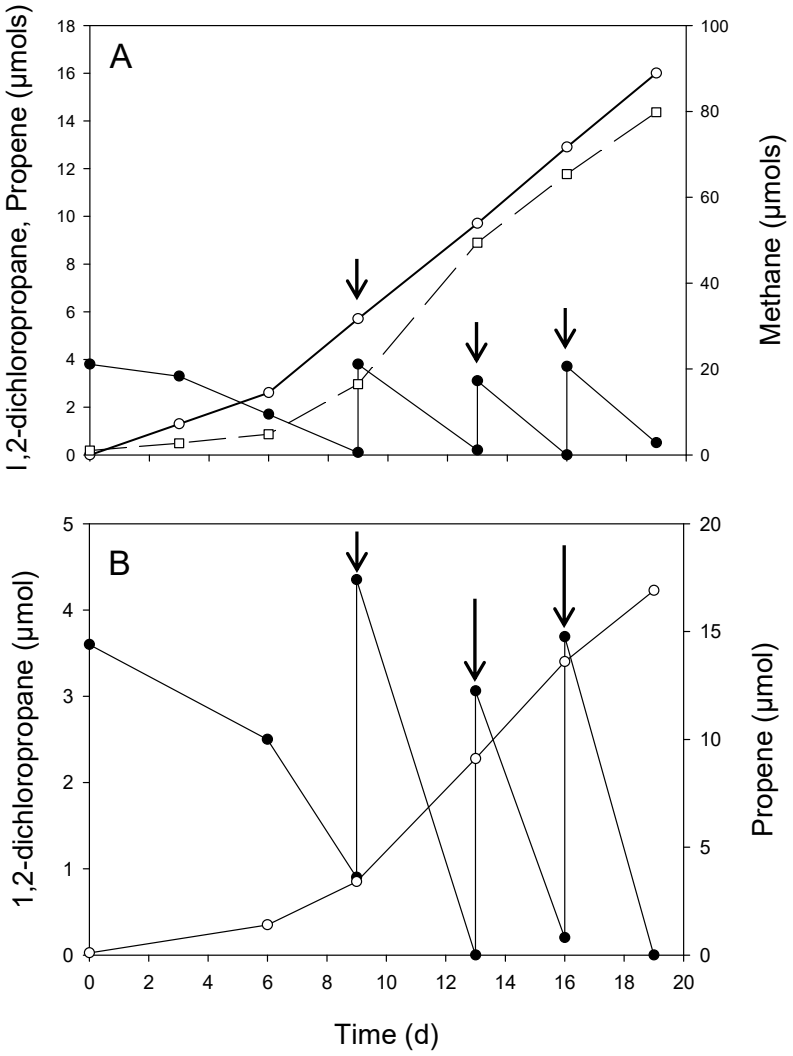


Figure 4.2: Dichloroelimination of 1,2-DCP to propene in enriched methanogenic microcosms containing 5 mM BES which did not inhibit methanogenesis at this concentration (Panel A) and in non-methanogenic enrichment cultures containing 25 mM BES (Panel B). Arrows indicate additional amendments of 1,2-DCP. Concentrations are presented as total µmoles in the serum bottle to facilitate the molar balance calculations. Results presented are from one individual microcosm (8^{th} transfer, 10% (v/v)) but the experiments were done in triplicate with similar results in replicates. (Symbols: ●: 1,2-DCP, ○: propene, □: methane).

The repeated addition of 1,2-DCP led to faster dechlorination rates suggesting that dichloroelimination was supporting growth of the dechlorinating bacteria (Figure 4.2A and 4.2B). The stoichiometric relationship between 1,2-DCP consumed and propene produced revealed a closed molar balance and excluded the production of alternative metabolites (Table 4.2). Neither dechlorination of 1,2-DCP nor propene production were detected in the abiotic and heat-killed controls indicating that the reaction was biotically mediated.

Table 4.2: Molar balance between 1,2-DCP dechlorinated and propene produced in the microcosm from Figure 4.1A.

Time (d)	1,2-DCP consumed (μmol)	Propene produced (μmol)	Ratio between 1,2-DCP consumed/Propene produced
6	2.3	2.6	0.9
9	6.2	5.7	1.1
13	9.9	9.7	1.0
16	13.0	12.9	1.0
19	16.2	16.0	1.0

The addition of vancomycin (5 mg L^{-1}), an inhibitor of peptidoglycan cell-wall biosynthesis, provoked an increase in the dichloroelimination rate of 1,2-DCP. The consortia containing vancomycin were transferred for more than 11 subsequent transfers (10% v/v) without losing this dechlorinating activity.

4.3.2. Dehalogenation of alternative electron acceptors

Besides 1,2-DCP, the potential of the consortium to reductively dehalogenate various chlorinated alkanes, chlorinated alkenes, chloroaromatics and bromoalkanes were tested as listed in Table 4.3 by adding the compounds at concentrations between 10 and 50 μM. After one month of incubation, dehalogenation was only observed in cultures containing chlorinated alkanes (including 1,2-DCA, 1,1,2-TCA, 1,1,2,2-TeCA, 1,2,3-TCP) and the brominated compound 1,2-

dibromoethane (EDB) with halogens on vicinal carbons. All the halogenated alkanes degraded were transformed to the corresponding alkene (Table 4.3) indicating that dihaloelimination was the unique degradation pathway observed. These results illustrate that the species responsible for the dechlorination of these substrates is highly specialized.

Table 4.3: List of alternative electron acceptors tested in the microcosms established with sediments from the Besòs River estuary

Electron acceptors	Initial concentrations (μM)	Dechlorinating activity	Dechlorination end product
1,2-dichloroethane (1,2-DCA)	10, 50	Yes	Ethene
1,1,2-trichloroethane (1,1,2-TCA)	20, 50	Yes	Vinyl chloride (VC)
1,2,3-trichlopropane (1,2,3-TCP)	20, 50	Yes	Allyl chloride, allyl alcohol
1,1,2,2-tetrachloroethane (1,1,2,2-TeCA)	20, 50	Yes	*trans*-DCE, *cis*-DCE
1-chloropropane (1-CP)	10	No	none
2-chloropropane (2-CP)	10	No	none
2,3-dichloro-1-propene (2,3-D-1-P)	10	No	none
Tetrachloroethylene (PCE)	10, 50	No	none
Trichloroethylene (TCE)	10, 50	No	none
trans-dichloroethene (*trans*-DCE)	10	No	none
Chloroform (CF)	10	No	none
Monochlorobenzene (1-CB)	10	No	none
1,2,4-trichlorobenzene (1,2,4-TCB)	10	No	none
1,6-dibromohexane (1,6-DBH)	10	No	none
1,2-dibromopropane (1,2-DBP)	20, 50	Yes	Propene
1,2-dibromoethane (EDB)	20, 50	Yes	Ethene
Diclofenac	10	No	none
Triclosan	10	No	none

4.3.3. Identification of Dehalogenimonas spp. and dcpA gene

To date, four OHRB populations (*Dehalogenimonas, Dehalococcoides, Desulfitobacterium,* and *Dehalobacter*) have been implicated in the dechlorination of 1,2-DCP to propene. The presence of OHRB belonging to the genus *Dehalobacter* and *Desulfitobacterium* in our cultures was ruled out because they cannot grow in the presence of vancomycin. In addition, morphological evidence supported the involvement of either *Dehalogenimonas* or *Dehalococcoides* since microscopic observations of cultures fed with several 1,2-DCP additions showed predominantly irregular cocci, but not rod-shaped cells as those described for *Desulfitobacterium* and *Dehalobacter* spp. (data not shown). In order to determine if known OHRB were present in the dehalogenating consortium we performed PCR reactions with genus-specific primers for *Dehalococcoides* and *Dehalogenimonas* 16S rRNA. The tested PCR primers targeting for *Dehalococcoides* spp. failed to produce an amplicon, but confirmed the presence of *Dehalogenimonas* spp. in our culture (Figure 4.3A and 4.3B). Furthermore, nested PCR followed by DGGE allowed the visualization of a prominent band (Figure 4.4). Several band replicates were excised from the gel, sequenced, and partial 16S rRNA gene sequences obtained (accession numbers from KP780280 to KP780282) showed 100% identity with *Dehalogenimonas alkenigignens* strain IP3−3.

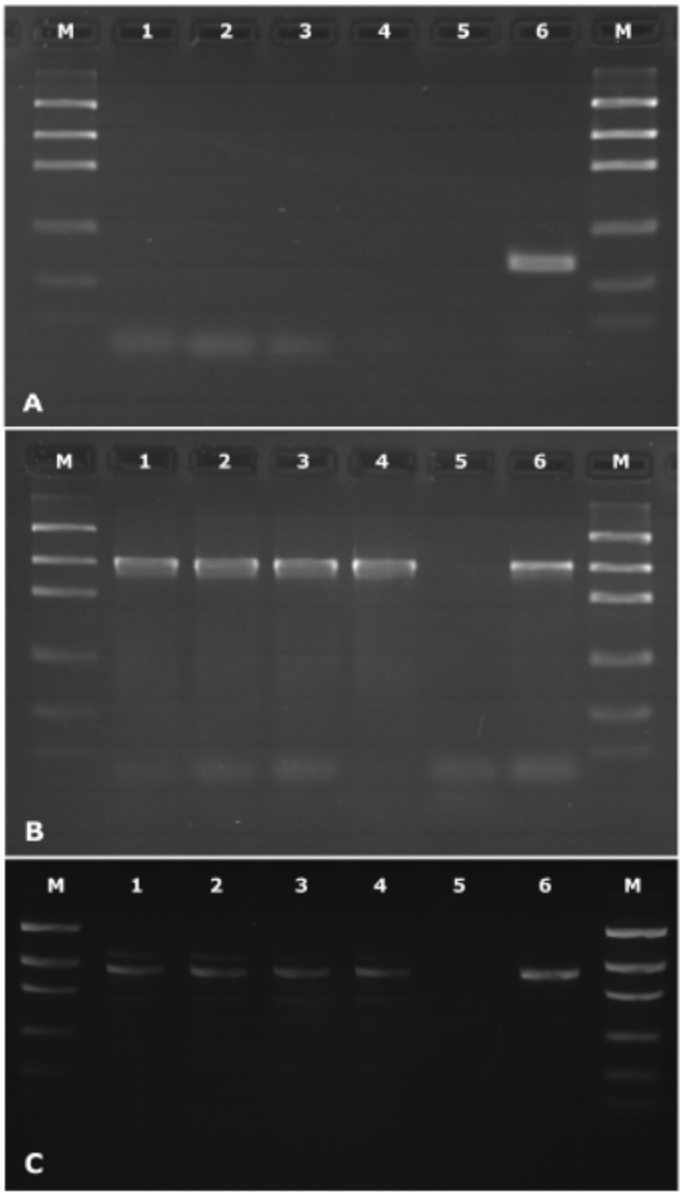


Figure 4.3: Detection of *Dehalococcoides* (A) and *Dehalogenimonas* (B) 16S rRNA genes in the consortia, and of the *dcpA* gene (C). Lane M: DNA size and mass marker (Low DNA mass Ladder, Invitrogen), lanes 1-4: PCR products generated using genomic DNA from the consortia, lane 5: negative control and lane 6: positive control (DNA from *Dehalococcoides mccartyi* CBDB1 [upper panel] and DNA from *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 [middle and lower panel] as template.

Figure 4.4: DGGE from OHRB enrichment cultures obtained from Besos River estuary. Lane M: sequence marker, and lines 1-6: PCR products generated using genomic DNA from the consortia. Bands retrieved and sequenced: A, BREd1; B, BREd2 and C, BREd3.

The hypothesis that *Dehalogenimonas* strains are involved in the dichloroelimination of 1,2-DCP in these enrichment cultures was consistent with three complementary lines of evidence. First, we tested the oxygen tolerance of the OHRB contained in our culture by exposing the inoculum to air until the redox indicator resazurin turned pink, and afterward it was injected into fresh reduced medium. These cultures grew at the same dechlorination rate than positive controls not exposed to oxygen, which is consistent with the oxygen tolerance observed for *Dehalogenimonas* [23] but disfavors the involvement of *Dehalococcoides* species due to their strong sensitivity to short-term oxygen exposure [24]. We repeated the short-time exposure of the inoculum to oxygen for three serial transfers with identical results. Second, the OHRB present in our culture solely dechlorinate vicinally chlorinated alkanes. This strong substrate

specialization is one of the remarkable characteristics observed for *Dehalogenimonas* strains [6], [7]. Although it has been described by others that growth of a *Dehalogenimonas* population in a consortium was coupled to reductive dechlorination of *trans*-DCE to VC [15], our culture could not transform chlorinated ethenes within one month of incubation. Third, we monitored the abundance of *Dehalogenimonas* 16S rRNA gene copies using quantitative PCR (qPCR) to determine whether 1,2-DCP dechlorination was coupled to *Dehalogenimonas* growth in the Besòs river cultures. The addition of several successive doses of 1,2-DCP when all the electron acceptor was consumed (Figure 4.5A) resulted in a concomitant increase in the number of *Dehalogenimonas* 16S rRNA gene copies detected in the cultures (Figure 4.5B). No growth was observed in control cultures that received no 1,2-DCP, showing that 1,2-DCP dechlorination was a growth linked respiratory process. These findings were consistent with the detection of the *dcpA* gene encoding 1,2-DCP reductive dehalogenase in *Dehalogenimonas* strain BL-DC-9, *Dehalococcoides mccartyi* strain RC and strain KS [12]. As shown in Figure 4.3C, a conventional PCR using dcpA-360F and dcpA-1449R primers confirms the presence of a unique amplicon of the expected size (1089 bp) when applied to genomic DNA from the consortium.

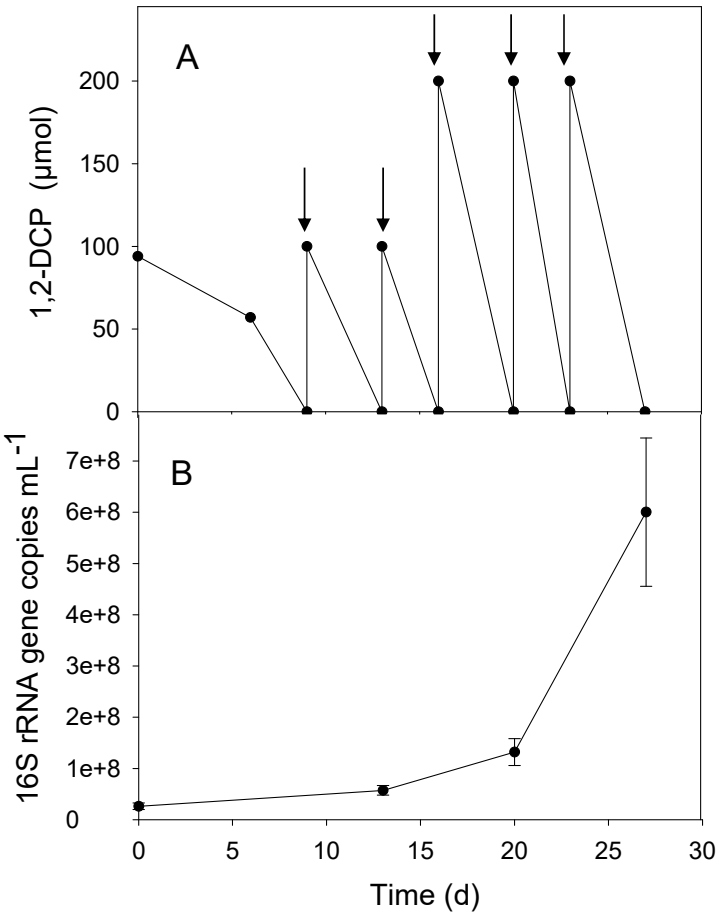


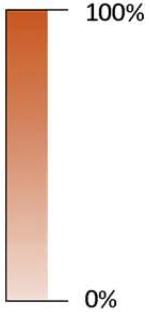
Figure 4.5: Increase of *Dehalogenimonas* 16S rRNA gene copy numbers in Besòs River cultures after consuming different doses of 1,2-DCP. Panel A: Consumption of 1,2-DCP over time. Concentrations are presented as total µmoles in the serum bottle. The culture received several additions of 1,2-DCP, as indicated by the arrows. Panel B: *Dehalogenimonas* 16S rRNA gene copies per mL of DNA in the 1,2-DCP amended cultures. Error bars represent the standard deviation of copy numbers measured in duplicate experiments for each point injected in triplicate.

4.3.4. Monitoring the stability of the dechlorinating consortium by DGGE

The use of 16S rDNA-based methods employing DGGE provides an accurate estimate of the microbial composition and diversity in a complex microbial community. Here, we monitored the stability of the consortia analyzing the predominant microbes present in the microcosms maintained by routine transfers in fresh medium through more than one year and a half. A total of six bacteria genus were detected throughout this period: *Acholeplasma, Azonexus, Dehalogenimonas, Desulfovibrio, Geobacter* and *Sphaerochaeta* (Table 4.4). In the beginning (May, 2014), *Azonexus* showed the highest abundance (>30%) followed by *Dehalogenimonas*. Low abundance of *Acholeplasma, Desulfovibrio,* and *Sphaerochaeta* were detected. After more than a year (July, 2015), *Azonexus* and *Dehalogenimonas* were the predominant bacteria in the consortia whereas *Acholeplasma, Desulfovibrio, Sphaerochaeta, Geobacter* and uncultured *Synergistetes* were present in minor abundance (≤10%). Comparison of DGGE band-patterns revealed that this microbial composition was maintained for five additional months (December 2015), suggesting that a stable consortia containing *Dehalogenimonas* as the dechlorinating bacteria was established.

Table 4.4: Bacterial genera detected during the consortium stabilization presented along with their abundance in DGGE profiles. Relative abundance is reflected in color intensity and categorized in absent (0%; -), minority (≤10%; +), moderate (≤30%; ++) and abundant (>30%; +++). The numbers of sequential transfer of the consortia corresponding to each sample are as follows: May-14(8th Transfer), Jun-14(10th Transfer), Jan-15(13th Transfer), Feb-15(16th Transfer), Jul-15(21st Transfer), Nov-15(27th Transfer), Dec-15(31st Transfer).Table courtesy of E. Parladé.

	May-14	Jun-14	Jan-15	Feb-15	Jul-15	Nov-15	Dec-15
Acholeplasma	+	-	-	+	+	+	+
Azonexus	+++	++	+	+++	+++	+++	+++
Dehalogenimonas	++	+++	+++	++	+++	++	++
Desulfovibrio	+	-	+	+	+	+	+
Geobacter	-	+	+	+	+	+	+
Sphaerochaeta	+	-	-	-	+	+	+
Uncultured Synergistetes	-	-	-	-	+	+	+



4.4. Conclusions

In summary, a stable *Dehalogenimonas* containing culture that exclusively dehalogenates vicinally chlorinated and brominated alkanes via dihaloelimination was established. Production of propene from 1,2-DCP was stoichiometric and the *dcpA* gene encoding 1,2-DCP reductive dehalogenase was also identified in our culture. All the molecular and physiological evidences pointed out that *Dehalogenimonas* sp. is indeed the bacteria responsible for the dechlorination of halogenated alkanes in the consortia. The stable culture was predominantly constituted by bacteria belonging to seven different genera, with *Dehalogenimonas* and *Azonexus* being the more abundant bacteria. Once stable climax consortium was obtained, and no further enrichment can be achieved under the incubation conditions applied, the isolation of *Dehalogenimonas* sp. present in the consortium was further attempted in Chapter 5.

4.5. References

[1] P. Castells, J. Parera, F. J. Santos, and M. T. Galceran, "Occurrence of polychlorinated naphthalenes, polychlorinated biphenyls and short-chain chlorinated paraffins in marine sediments from Barcelona (Spain)," *Chemosphere*, vol. 70, no. 9, pp. 1552–1562, 2008.

[2] Agency for Toxic Substances and Disease Registry (ATSDR), "Toxicological profile for 1,2-dichloropropane," 1989. [Online]. Available: https://www.atsdr.cdc.gov/toxprofiles/tp134.pdf. [Accessed: 21-Nov-2016].

[3] J. C. Hage, F. D. G. Kiestra, and S. Hartmans, "Co-metabolic degradation of chlorinated hydrocarbons by *Pseudomonas* sp. strain DCA1," *Appl. Microbiol. Biotechnol.*, vol. 57, no. 4, pp. 548–554, 2001.

[4] T. Bosma and D. B. Janssen, "Conversion of chlorinated propanes by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase," *Appl. Microbiol. Biotechnol.*, vol. 50, no. 1, pp. 105–112, 1998.

[5] L. A. Hug, "Diversity, evolution, and environmental distribution of reductive dehalogenase genes," in *Organohalide-Respiring Bacteria*, L. Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 377–393.

[6] K. S. Bowman, M. F. Nobre, M. S. da Costa, F. A. Rainey, and W. M. Moe, "*Dehalogenimonas alkenigignens* sp. nov., a chlorinated-alkane-dehalogenating bacterium isolated from groundwater," *Int. J. Syst. Evol. Microbiol.*, vol. 63, no. PART4, pp. 1492–1498, 2013.

[7] W. M. Moe, J. Yan, M. F. Nobre, M. S. da Costa, and F. A. Rainey, "*Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater," *Int. J. Syst. Evol. Microbiol.*, vol. 59, no. 11, pp. 2692–2697, 2009.

[8] T. A. Key, K. S. Bowman, I. Lee, J. Chun, M. da Costa, L. Albuquerque, F. A. Rainey, and W. M. Moe, "*Dehalogenimonas formicexedens* sp. nov., a chlorinated alkane respiring bacterium isolated from contaminated groundwater," *Int. J. Syst. Evol. Microbiol.*, In press, doi: 10.1099/ijsem.0.001819, 2017.

[9] K. M. Ritalahti and F. E. Löffler, "Populations implicated in anaerobic reductive dechlorination of 1,2-dichloropropane in highly enriched bacterial communities," *Appl. Environ. Microbiol.*, vol. 70, no. 7, pp. 4088–4095, 2004.

[10] S. De Wildeman, G. Diekert, H. Van Langenhove, and W. Verstraete, "Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes," *Appl. Environ. Microbiol.*, vol. 69, no. 9, pp. 5643–5647, 2003.

[11] C. Schlötelburg, C. Von Wintzingerode, R. Hauck, F. Von Wintzingerode, W. Hegemann, and U. B. Göbel, "Microbial structure of an anaerobic bioreactor population that continuously dechlorinates 1,2-dichloropropane," *FEMS Microbiol. Ecol.*, vol. 39, no. 3, pp. 229–237, 2002.

[12] E. Padilla-Crespo, J. Yan, C. Swift, D. D. Wagner, K. Chourey, R. L. Hettich, K. M. Ritalahti, and F. E. Löffler, "Identification and environmental distribution of dcpA, which encodes the reductive dehalogenase catalyzing the dichloroelimination of 1,2-dichloropropane to propene in organohalide-respiring Chloroflexi," *Appl. Environ. Microbiol.*, vol. 80, no. 3, pp. 808–818, 2014.

[13] L. Adrian, U. Szewzyk, J. Wecke, and H. Görisch, "Bacterial dehalorespiration with chlorinated

benzenes.," *Nature*, vol. 408, no. 6812, pp. 580–3, 2000.

[14] J. Chen, K. S. Bowman, F. A. Rainey, and W. M. Moe, "Reassessment of PCR primers targeting 16S rRNA genes of the organohalide-respiring genus *Dehalogenimonas*," *Biodegradation*, vol. 25, no. 5, pp. 747–756, 2014.

[15] M. J. Manchester, L. A. Hug, M. Zarek, A. Zila, and E. A. Edwards, "Discovery of a trans-dichloroethene-respiring *Dehalogenimonas* species in the 1,1,2,2-tetrachloroethane-dechlorinating WBC-2 consortium," *Appl. Environ. Microbiol.*, vol. 78, no. 15, pp. 5280–5287, 2012.

[16] H. Schäfer and G. Muyzer, "Denaturing gradient gel electrophoresis in marine microbial ecology," *Methods Microbiol.*, vol. 30, pp. 425–468, 2001.

[17] G. Muyzer, E. C. De Waal, and A. G. Uitterlinden, "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA," *Appl. Environ. Microbiol.*, vol. 59, no. 3, pp. 695–700, 1993.

[18] G. Muyzer, A. Teske, C. O. Wirsen, and H. W. Jannasch, "Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments," *Arch. Microbiol.*, vol. 164, no. 3, pp. 165–172, 1995.

[19] R. C. Edgar, B. J. Haas, J. C. Clemente, C. Quince, and R. Knight, "UCHIME improves sensitivity and speed of chimera detection," *Bioinformatics*, vol. 27, no. 16, pp. 2194–2200, 2011.

[20] T. A. Tatusova and T. L. Madden, "BLAST 2 SEQUENCES, a new tool for comparing protein and nucleotide sequences," *FEMS Microbiol. Lett.*, vol. 174, no. 2, pp. 247–250, 1999.

[21] K. M. Ritalahti, B. K. Amos, Y. Sung, Q. Wu, S. S. Koenigsberg, and F. E. Loffler, "Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains," *Appl. Environ. Microbiol.*, vol. 72, no. 4, pp. 2765–2774, 2006.

[22] F. E. Löffler, J. E. Champine, K. M. Ritalahti, S. J. Sprague, and J. M. Tiedje, "Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria," *Appl. Environ. Microbiol.*, vol. 63, no. 7, pp. 2870–2875, 1997.

[23] J. Yan, B. A. Rash, F. A. Rainey, and W. M. Moe, "Isolation of novel bacteria within the Chloroflexi capable of reductive dechlorination of 1,2,3-trichloropropane," *Environ. Microbiol.*, vol. 11, no. 4, pp. 833–843, 2009.

[24] F. E. Löffler, J. Yan, K. M. Ritalahti, L. Adrian, E. A. Edwards, K. T. Konstantinidis, J. A. Müller, H. Fullerton, S. H. Zinder, and A. M. Spormann, "*Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and famil," *Int. J. Syst. Evol. Microbiol.*, vol. 63, pp. 625–635, 2013.

CHAPTER 5

STABLE ISOTOPE FRACTIONATION DURING DICHLOROELIMINATION OF CHLOROALKANES BY A MIXED CULTURE CONTAINING *Dehalogenimonas* sp.

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ABSTRACT

This study investigates the carbon and dual C-Cl isotope fractionation during anaerobic biodegradation of 1,2-dichloropropane (1,2-DCP) and 1,2-dichloroethane (1,2-DCA), respectively, by a *Dehalogenimonas*-containing culture derived from Besòs river estuary sediments. Compound specific isotope analysis revealed that the carbon isotopic fractionation (ε^{C}_{bulk}) of the 1,2-DCP-to-propene reaction was −15.0 ± 0.7‰ under both methanogenic and nonmethanogenic conditions. The calculated ε^{C}_{bulk} was in the same order of magnitude but differs from that reported for *Dehalococcoides mccartyi* strain RS and strain KC, although the *dcpA* gene encoding 1,2-DCP reductive dehalogenase was present in both *Dehalococcoides* and *Dehalogenimonas* cultures. Carbon and chlorine isotopic fractionation of 1,2-DCA-to-ethene (ε^{C}_{bulk} = -23±2‰ and ε^{Cl}_{bulk} = -12.0±0.8‰) resulted in a dual element C-Cl isotope correlation ($\Lambda = \Delta\delta^{13}C/\Delta\delta^{37}Cl \approx \varepsilon^{C}_{bulk}/\varepsilon^{Cl}_{bulk}$) of 1.89±0.02. Interestingly, Λ values of 1,2-DCA determined in two contaminated sites under reducing conditions (2.1 ± 0.1 and 2.2 ± 2.9) were similar to the one obtained for the *Dehalogenimonas*-containing microcosms and very different from those reported for aerobic degradation pathways. This study demonstrates the potential of using dual isotope analysis to distinguish between aerobic and anaerobic biodegradation pathways of 1,2-DCA in the field and suggests that this approach might also be used to characterize dihaloelimination of 1,2-DCA by different bacteria.

5.1. Introduction

The isotope techniques are becoming attractive tools to study a wide array of environmental processes such as the global biogeochemical cycles, water resources development and management, forensic investigations, or the bioremediation of soils and aquifers [1].

For monitoring groundwater contamination, some approaches rely exclusively on measuring the contaminant concentration through the time. However, the disappearance of the contaminants along the groundwater flow path can also be caused by several physicochemical processes such as adsorption, dilution, volatilization and chemical transformation [2], [3]. Furthermore, if the monitoring is not oriented to the direction of the groundwater flow and samples are taken only at the edge of the plume, the decreasing concentrations will give an incorrect impression that biodegradation occurs. Compound-specific stable isotope analysis (CSIA) can potentially bridge this gap as this technique can provide convincing evidence of *in situ* biodegradation, quantify the extent of transformation, establish links between parent and daughter compounds and distinguish between different sources of the same contaminant [4].

Chlorinated alkanes are made of atoms of various elements, but most importantly carbon, chlorine, and hydrogen. Isotopes of the same element have an identical number of protons but differ in their number of neutrons leading to different atomic mass [2], [5]. These elements have at least two stable (nonradioactive) isotopes which can be distinguished by mass spectrometry. Each element has lighter isotopes (for example, ^{12}C, “carbon twelve” for carbon) and heavier isotopes (^{13}C, “carbon thirteen”). In most elements, the lightest stable isotope is the most abundant (Table 5.1) [5]. Due to their larger masses, heavier isotopes tend to form shorter and more stable chemical bonds. Isotope fractionation occurs because it takes less energy to break a bond between light isotope than it takes to break the heavier isotope. As the rates of reaction

involving the heavier isotopes are slower, the percentage of heavy isotopes increases and accumulates in the residual part of the contaminant [1]. Hence, the enrichment of the heavier isotopes over the lighter isotopes (isotope fractionation) can serve as a qualitative indicator for biodegradation in the field even when mass balances cannot be closed and metabolites are not detected. In this context, it is assumed that physical processes occurring in natural systems (e.g. sorption, volatilization, diffusion of the contaminants) are associated with a negligible isotope fractionation.

Table 5.1: Relative abundance of hydrogen, carbon, and chlorine isotopes and the corresponding (international) reference scales: VSMOW (Vienna Standard Mean Ocean Water), VPDB (Vienna Pee Dee Belemnite), and SMOC (Standard Mean Ocean Chlorine). E means element [5].

Element	Stable isotopes	Natural abundance (%)		Relative mass difference (%)	International reference standard
		$^{heavy}E$	$^{light}E$		
Hydrogen	$^{2}H/^{1}H$	0.0155	99.98	100	VSMOW
Carbon	$^{13}C/^{12}C$	1.1060	98.89	8.3	VPDB
Chlorine	$^{37}Cl/^{35}Cl$	24.220	75.78	5.7	SMOC

The abundance of different stable isotopes in a system are reported as isotope ratio, R [6] (Eq. 1):

$$R_E = \frac{^{H}E}{^{L}E} \quad (1)$$

where ^{H}E is the abundance of heavy isotope in the system and ^{L}E is the abundance of the light isotopes.

Isotope ratios of individual compounds are reported using delta notation (δ) and expressed in parts per mil (‰) (Eq. 2) [7]:

$$\delta(‰) = \left(\frac{R_{sample-R_{std}}}{R_{std}}\right) \times 1000 = \left(\frac{R_{sample}}{R_{std}}\right) \times 1000 \quad (2)$$

where R_{sample} is the isotope ratios of heavy and light isotopes of the element (e.g., $^{13}C/^{12}C$ and $^{37}Cl/^{35}Cl$) and R_{std} is the stable isotope ratio of the international reference standard (see Table 5.1 for the C, H, and Cl standards). These international reference standards ensure the comparability of isotope analysis among different laboratories.

The changes in isotope composition of single compound during a reaction can be described using the Rayleigh model, relating changes in isotope composition to changes in concentration under closed-system conditions and the extent of isotope fractionation is usually expressed as isotopic fractionation value, ε_{bulk} (Eq. 3) [5]. While the isotope ratios of the contaminant provide qualitative indication for biodegradation, the quantitative evaluation of field isotope data can be based on the Rayleigh equation [2], [8], [9] (Eq. 3):

$$\ln\left(\frac{R_t}{R_0}\right) = \left(\frac{\varepsilon_{bulk}}{1000}\right) \times \ln\left(\frac{C_t}{C_0}\right) = \varepsilon_{bulk} \bullet \ln f \quad (3)$$

where R and C represents the compound stable isotope ratios and corresponding concentration, respectively, of the substrate at time 0 and t.

Currently, CSIA of carbon is the most common routine application. The value of carbon isotopic fractionation (ε^{C}_{bulk}) calculated from Eq. 3 often reflect the reaction mechanism involved in the biodegradation of certain contaminants and it can be used to identify degradation pathways [10]. However, one of the drawbacks associated with the isotope fractionation of one element alone is the establishment of the reaction mechanism in the field, because in this case the extent of degradation needs to be linked to shifts in isotope ratios. In this case, it is difficult to

distinguish between biodegradation of the reactant and other processes such as sorption, volatilization or mixing through dispersion under natural conditions. This limitation can be overcome by combining CSIA of two (or more) elements, called two-dimensional compound-specific isotope analysis (2D-CSIA) [11]. The natural abundance and analytical techniques to measure C, Cl, and H stable isotopes are summarized in Table 5.1 and 5.2 [4]. The dual isotope analysis are presented as a dual-element slope m (or Λ), as shown below for carbon and chlorine (Eq. 4) [3] which represents the relative change in the isotopic fractionation of carbon (ε^{C}_{bulk}) and chlorine (ε^{Cl}_{bulk}):

$$\Lambda = \frac{\Delta^{13}C}{\Delta^{37}Cl} \approx \frac{\varepsilon^{C}_{bulk}}{\varepsilon^{Cl}_{bulk}} \quad (4)$$

The combined change in isotope fractionation of two elements for a given reactant is often pathway-specific and it is reflected in the dual-element slope. Thus, the Λ value can act as direct indicator for different initial reaction mechanisms independently of the knowledge on the extent of degradation in the field.

Table 5.2: Analytical methods for carbon, chlorine and hydrogen isotope analysis in chlorinated hydrocarbons and typical detection limits and precisions [4].

Element	Measurement technique	Sample extraction/injection	Typical detection limits	Typical uncertainty	Reference
C	GC-C-IRMS	Purge and Trap	2-10 µg L^{-1}	0.3-0.5 ‰	[12]
Cl	GC-IRMS	SPME	5-20 µg L^{-1}	0.1-0.3 ‰	[13]
	GC-qMS	Headspace injection	50-100 µg L^{-1}	0.3-0.8 ‰	[14]
	GC-HTC-IRMS	Headspace/liquid injection	n.d	<0.3 ‰	[15]
H	GC-IRMS	SPME	200-400 µg L^{-1}	2-7 ‰	[16]

Measurements of 2D-CSIA can provide key information to investigate contaminant degradation pathways in the field, which has not been applied to biodegradation field studies of chlorinated ethanes such as 1,2-DCA and 1,2-DCP [17].

Previous studies showed that the biodegradation of 1,2-DCA and 1,2-DCP can occur via aerobic and anaerobic pathways. 1,2-DCP are partially cometabolized to less chlorinated alkanes under aerobic condition while complete dechlorination of 1,2-DCP have been only demonstrated under anaerobic condition by OHRB belonging to the genus *Dehalococcoides, Dehalogenimonas, Desulfitobacterium* and *Dehalobacter*, mostly by dichloroelimination [18]. This reaction involves the transformation of 1,2-DCP to propene via either a stepwise mode (involving a transition state with one C-Cl bond) or a concerted mode (involving simultaneous cleavage of both C-Cl bonds). To date, only stable carbon isotope fractionation by *Dehalococcoides mccartyi* strain KS and RC has been studied. The ε^{C}_{bulk} values obtained for the two *Dehalococcoides mccartyi* strains in their respective 1,2-DCP-to-propene consortia were statistically identical (−10.8 ± 0.9‰ and −11.3 ± 0.8‰, respectively) [19].

Several field and laboratory studies have shown that 1,2-DCA can be either aerobically or anaerobically biodegraded via different reaction pathways (Figure 5.1). Under aerobic conditions, 1,2-DCA can be degraded either via oxidative cleavage of a C-H bond catalyzed by hydrolytic dehalogenase or monooxygenase enzyme (Figure 5.1a) or via nucleophilic substitution (S_N2) (Figure 5.1b) [20]. Only few bacterial strains have been described to anaerobically dehalogenate 1,2-DCA, which includes *Dehalococcoides mccartyi* strains [9], *Desulfitobacterium dichloroeliminans* strain DCA1[21] and *Dehalogenimonas* strains [22]. The carbon isotopic fractionation (ε^{C}_{bulk}) determined for anaerobic 1,2-DCA dichloroelimination to ethene (Figure 5.1c,d) were -30.8 ± 1.3‰ and -29.0 ± 3.0‰ for *Dehalococcoides mccartyi*

strains 195 and BTF08, respectively [9]. The aerobic mineralization of 1,2-DCA (S_N2 reaction, Figure 5.1b) by *Xanthobacter autotrophicus* GJ10 was ε^{C}_{bulk}= -32 ‰ and it resulted similar with the fractionation obtained for *Dehalococcoides* strains, showing that carbon stable isotope alone cannot be used to distinguish between aerobic and anaerobic pathways of 1,2-DCA [23]. Recently, Palau et al. applied 2D-CSIA of carbon and chlorine to study the aerobic transformation of 1,2-DCA by *Xanthobacter autotrophicus* GJ10 and *Ancylobacter aquaticus* AD20 (via aerobic cleavage of C-Cl bond, S_N2 reaction) that exhibited a similar Λ value of 7.7 ± 0.2, while transformation by *Pseudomonas* sp. strain DCA1 (via aerobic cleavage of a C-H bond) was associated with a much smaller value 0.78 ± 0.03‰ [7]. To date, application of 2D-CSIA to study the anaerobic reductive dechlorination of 1,2-DCA remains unknown.

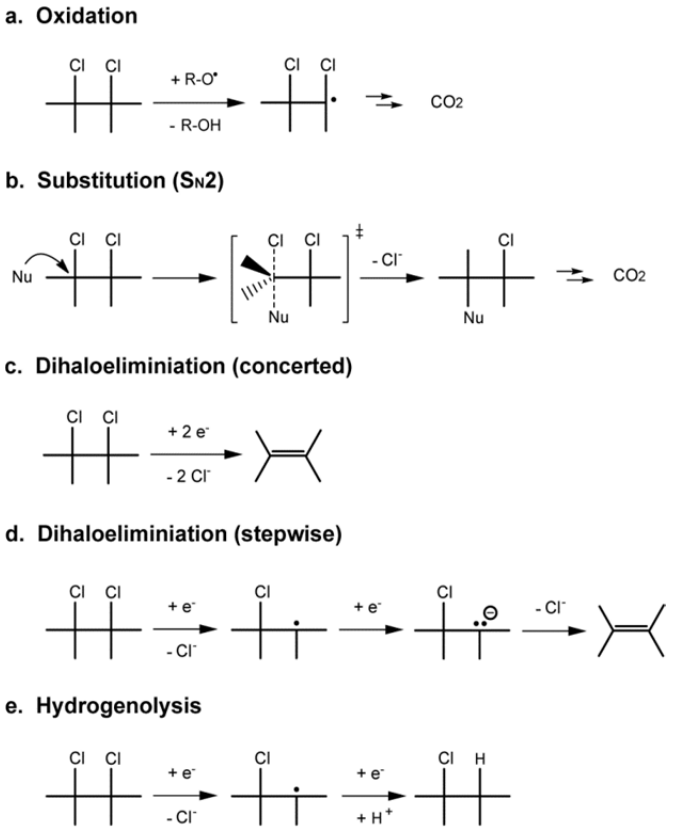


Figure 5.1: Aerobic and anaerobic biodegradation pathways of 1,2-DCA in aqueous systems [17].

In this chapter, we aimed to determine the carbon stable isotope fractionation during dechlorination of 1,2-DCP by *Dehalogenimonas* and compared it to the isotopic fractionation values reported for *Dehalococcoides mccartyi* strains KS and RC in a previous study. In addition, dual isotope enrichment factor of carbon and chlorine for the 1,2-DCA-to-ethene transformation by *Dehalogenimonas*-containing enrichment culture was determined to overcome limitations observed when using ε^{C}_{bulk} to distinguish between anaerobic dichloroelimination and aerobic oxidation of 1,2-DCA. Therefore, the value of Λ obtained in this study was compared to data reported previously to determine whether Λ value is sufficiently different to distinguish among different degradation pathways. In addition, the Λ value from two groundwaters contaminated with 1,2-DCA in Italy and USA were also determined and compared with the dual element isotope patterns obtained in our laboratory experiments.

5.2. Materials and methods

5.2.1. Biodegradation experiments

Two biodegradation experiments using the stable *Dehalogenimonas* enrichment culture described in Chapter 4 as inoculum were performed in parallel with the chlorinated compounds 1,2-DCP and 1,2-DCA. The microcosms were set up as described in section 3.2.

For 1,2-DCP experiment, 10 serum bottles were inoculated with 5 mL of a *Dehalogenimonas*-enrichment culture with methanogenic activity (5^{th} transfer) whereas 12 serum bottles were inoculated with 5 mL of a nonmethanogenic *Dehalogenimonas*-enrichment culture (11^{th} transfer) containing 25 mM BES. 1,2-DCP was added with a syringe from a 3.2 mM stock solution in acetone to a nominal concentration of 50 μM. Three types of controls were included at least in duplicate: (i) controls containing heat-killed inoculum and 1,2-DCP, (ii) live controls

without 1,2-DCP, and (iii) abiotic controls containing the growth medium with 1,2-DCP without inoculum, to control losses, abiotic transformations, and the transfer of compounds from previous degradation experiment with the inoculum or potential impurities from the stock solution, respectively. Six mL of NaOH was added to parallel incubations to stop biological transformations at different time points and bottles were preserved at 4 °C until analysis.

In the case of 1,2-DCA, a total of 16 serum bottles were prepared but in half of the samples pyruvate (5 mM) was replaced by acetate (5 mM) as carbon source. The microcosms were inoculated with 3 mL of a *Dehalogenimonas*-enrichment culture (23rd transfer) and 1,2-DCA was added with a syringe from a stock solution in acetone to an initial aqueous phase concentration of ~9.5 mg L^{-1}. Two types of controls were included in this experiment to account for losses, abiotic transformations, and the transfer of compounds from previous degradation experiments with the inoculum or potential impurities from the stock solution: live controls without 1,2-DCA and abiotic controls containing the growth medium with 1,2-DCA but without inoculum. To stop biological transformations at different time points, the cultures were killed by adding 9 mL of a saturated Na_2SO_4 solution (pH 1) and bottles were preserved at 4 ºC until isotopic analysis.

5.2.2. Analytical methods

5.2.2.1.Carbon isotope analysis.

Carbon isotope ratios of 1,2-DCP and 1,2-DCA were analyzed at the laboratories of the Universitat de Barcelona (Grup de Mineralogia Aplicada i Geoquímica de Fluids, Departament de Mineralogia Petrologia i Geologia Aplicada) under the supervision of Dr Mònica Rosell.

Carbon isotope analyses of propene and 1,2-DCP were performed using a Agilent 6890 gas chromatograph (Palo Alto, USA) equipped with a split/splitless injector, coupled to a Delta

Plus isotope ratio mass spectrometer through a GC-Combustion III interface (ThermoFinnigan, Bremen, Germany). A headspace gas sample with a volume between 0.6 and 1 mL, depending on propene concentration measured previously, was injected by a gas syringe. For propene, the GC was equipped with a HPPLOT/Q column (30 m × 0.32 mm, 20 μm film thickness, Agilent Technologies, Palo Alto, USA). The injector was set at 220 °C in split mode (1:10). The oven temperature program was kept at 40 °C for 10 min, heated to 220 °C at a rate of 15 °C min^{-1} and finally held at 220 °C for 10 min. Helium was used as a carrier gas with a gas flow rate of 1.2 mL min^{-1}. The retention time of propene was identified by injecting 300 μL of a standard gas mixture (Supelco Scotty Analyzed Gases, C2 − C6 Olefins, Sigma-Aldrich, St. Louis, USA) containing ethene, propene, 1-butene, 1-pentene, 1-hexene each at 100 mg L^{-1}. Once the $\delta^{13}C$ of propene was analyzed, liquid aliquots were removed from the experimental bottles and placed in 20 mL vials filled with 10 mL aqueous phase (samples were diluted or not in Milli-Q water depending on the 1,2-DCP concentration) and containing a 30 mm PTFE-coated stir bar. This solution was stirred at room temperature and 1,2-DCP was extracted during 20 min by headspace solid-phase microextraction (HSSPME) using a manual sampler holder equipped with a 75 μm Carboxen-PDMS fiber (Supelco, Bellefonte, USA). For 1,2-DCP, the GC was equipped with a Supelco SPB-624 column (60 m × 0.32 mm, 1.8 μm film thickness; Bellefonte, USA). The injector was set at 220 °C in split mode (1:10). The oven temperature program was kept at 60 °C for 2 min, heated to 220 °C at a rate of 8 °C min^{-1} and finally held at 220 °C for 5 min. Helium was used as a carrier gas with a gas flow rate of 1.8 mL min^{-1}. Several 1,2-DCP aqueous control standards were prepared daily from the same pure 1,2-DCP standard which was also used for the cultures (standard stock solutions were prepared first in HPLC grade methanol) and analyzed on the same days as the samples to ensure accuracy of the isotopic measurements. All the controls

injected in different replicas, days and concentrations (from 30 to 300 μg L^{-1}) had an average 1,2-DCP-$\delta^{13}C$ values of −29.3 ± 0.5‰ (n = 34).

In the case of 1,2-DCA, liquid aliquots were diluted to a similar 1,2-DCA concentration in 20 mL vials containing a 30 mm PTFE-coated stir bar. The vials were immediately sealed with PTFE/Silicone septa and aluminum crimp caps. Then, the sample solution was stirred at room temperature and 1,2-DCA was extracted during 20 min by HSSPME using a manual sampler holder equipped with a 75 μm Carboxen-PDMS fiber (Supelco, Bellefonte, USA). An isotopic working standard of 1,2-DCA (99%, Sigma-Aldrich) was used to ensure accuracy of the isotopic measurements during the course of samples analysis. The isotopic signature of the working standard ($\delta^{13}C_{V\text{-}PDB}$ = −28.74 ± 0.04‰, ±1σ, n = 10) was determined beforehand by EA-IRMS. Several 1,2-DCA aqueous standards were prepared daily from the same pure 1,2-DCA (stock solutions were prepared first in HPLC grade methanol) and analyzed on the same days as the samples. Standards and samples were analyzed by duplicate as a quality control.

5.2.2.2.Chlorine isotope analysis

Samples from laboratory batch experiments were measured at Isotope Tracer Technologies Inc., Canada. Pure 1,2-DCA isotopic working standards were used for instrument monitoring and external calibration of sample raw $\delta^{37}Cl$ values to the international Standard Mean Ocean Chloride (SMOC) scale: IT2-3001 and IT2-3002 ($\delta^{37}Cl_{SMOC}$ = +0.83 ± 0.09 and −0.19 ± 0.12‰, respectively) and CHYN1 and CHYN2 ($\delta^{37}Cl_{SMOC}$ = +6.30 ± 0.06 and +0.84 ± 0.14‰, respectively). Chlorine compound-specific isotope analysis (CSIA) was performed using a 6890 GC (Agilent, Santa Clara, CA, USA) coupled to a MAT 253 IRMS (Thermo Finnigan, Bremen, Germany). This IRMS, equipped with nine collectors, is a continuous flow IRMS with a

dual-inlet (DI) mode option. The DI bellows are used as the monitoring gas reservoir and reference peaks were introduced at the beginning of each analysis run.

Samples and standards were prepared in 20 mL vials and sealed with crimped septa caps (PTFE/Silicone). These vials contain 16 mL of solution and 4 mL of HS. The 1,2-DCA in solution was extracted with a solid phase micro extraction (SPME) fiber (75 µm Carboxen-PDMS for Merlin MicrosealTM, 23 gauge needle auto holder from Supelco, Bellefonte, USA) using a CombiPAL SPME autosampler (CTC Analytics, Zwingen, Switzerland). The SPME fiber was desorbed into the GC inlet at 270 °C. A SPME injection sleeve (0.75 mm ID) (Supelco, Bellefonte, PA, USA) was used as an inlet liner. The chromatographic separation was performed with a DB-5 MS column (60 m x 0.32 mm x 1 µm) (Agilent, Santa Clara, USA). The carrier gas (Helium) flow rate of the GC was set at 1.8 mL/min and the oven temperature was programmed as follows: 40 °C (9 min), ramp at 10 °C/min to 100 °C and ramp at 46 °C/min to 250 °C (6.75 min). A 4-way VALCO valve (Valco Intruments, Houston, TX, US) with two positions is installed between the GC and the IRMS. This setup is important as it allows a non-stop constant flow of He into the IRMS. The end part of the GC column is connected to one of the two in-ports of the valve, while the other in-port is connected with ultra-pure He gas. The two out-ports are connected to the IRMS and to a FID detector mounted on the GC. A 100 µm deactivated capillary column is used to connect the valve port with the IRMS.

The analysis scheme for the samples calibration with respect to SMOC scale consists of a sequence of samples bracketed by two sets of standards, one set at the beginning of the run and another set at the end of the analysis. Each set of standards comprise six replicates for each of the two standards in varying concentrations, and each run is composed of 12 to 20 samples. Blanks

are included at the beginning of each run, however, there is no carry-over observed along many years of analyzing these compounds by this method.

5.2.3. Calculation of AKIEs

Position-specific carbon isotopic fractionation ($\varepsilon_{reactive}$) values, which are corrected for the presence of nonreactive positions, were calculated for both stepwise and concerted reactions according to Elsner et al. [8] as done by Fletcher et al. [19], where *n* is the number of carbon atoms in the molecule (in the case of 1,2-DCP, *n* = 3) and *x* is the number of carbon atoms in the reactive position (in the case of a stepwise reaction, *x* = 1 and in the case of a concerted reaction, *x* = 2) by plotting again the modified Rayleigh equation:

$$\ln\left(\frac{1000+\delta^{13}C_0+(n/x)\Delta\delta^{13}C}{1000+\delta^{13}C_0}\right)=\left(\frac{\varepsilon_{reactive}}{1000}\right)\ln\left(\frac{C_t}{C_0}\right) \quad (5)$$

For the calculation and interpretation of AKIEs a hypothesis about the reaction mechanism, or assumed reaction mechanism, is necessary. The effects of non-reacting positions within the molecule, as well as of intramolecular competition, are then taken into account using Eqs. 6 and Eq. 7, respectively,

$$\varepsilon_{rp} \approx \frac{n}{x}\cdot\varepsilon_{bulk} \quad (6)$$

$$AKIE_{C,Cl}=\frac{1}{z\cdot\varepsilon_{rp}+1} \quad (7)$$

where ε_{rp} is the isotopic fractionation at the reactive position, *n* is the number of atoms of the element considered, *x* is the number of reactive sites and *z* the number of identical reactive sites undergoing intramolecular competition.

Carbon isotopic mass balance and the associated concentration-weighted average $\delta^{13}C_{sum}$ is calculated similar to the sequential reductive dechlorination of chlorinated ethenes [24], [25] but only considering 1,2-DCP as parental compound and propene as final product:

$$\delta^{13}C_{sum}(‰) = x_{1,2-DCP}\delta^{13}C_{1,2-DCP} + x_{propene}\delta^{13}C_{propene} \quad (8)$$

where *x* is the molar fraction of each compound relative to the total molar mass (1,2-DCP plus propene) at each time. If propene is not further transformed, the $\delta^{13}C_{sum}$ remains constant over the 1,2-DCP dichloroelimination. As a certain amount of propene was transferred with the inoculum from the previous experiment (initial propene detected and quantified in live controls without 1,2-DCP), the $\delta^{13}C_{propene}$ corresponding to newly generated propene in Eq. 7 was calculated as follows:

$$\delta^{13}C_{propene} = \frac{\delta^{13}C_{measured\ propene} - \left(x_{initial\ propene}\delta^{13}C_{initial\ propene}\right)}{x_{generated\ propene}} \quad (9)$$

5.3. Results and discussion

5.3.1. Carbon isotope fractionation of 1,2-DCP

The carbon isotope fractionation of 1,2-DCP was studied in *Dehalogenimonas*-containing enrichment cultures under methanogenic and nonmethanogenic conditions. Methanogenic archaea are often present in dehalogenating communities and compete with OHRB for hydrogen and acetate, but some populations can also synthesize corrinoids *de novo* that can be used by corrinoid auxotrophs such as *Dehalogenimonas* spp. Previous studies have shown that the metabolism of OHRB can be altered by supplying different forms of corrinoids, that is, cyanocobalamin added exogenously to the medium or corrinoids supplied by a methanogenic enrichment culture [26]. Therefore, we aimed to investigate whether the presence of

methanogens may exert an effect on the isotopic fractionation of 1,2-DCP due to the corrinoid source or due to corrinoid concentration. In addition, the results may help to identify a yet unperceived concomitant cometabolic transformation of 1,2-DCP. This information is of importance to interpret and increase confidence in the isotopic fractionation data collected from the field since growth of OHRB is mostly favored in methanogenic zones.

In methanogenic cultures, 94% of 1,2-DCP was transformed to propene within 13 days, whereas nonmethanogenic cultures were much slower and only 77% transformation was reached after 21 days. In both methanogenic and nonmethanogenic cultures, 1,2-DCP was significantly enriched in ^{13}C during the transformation to propene (Figure 5.2).

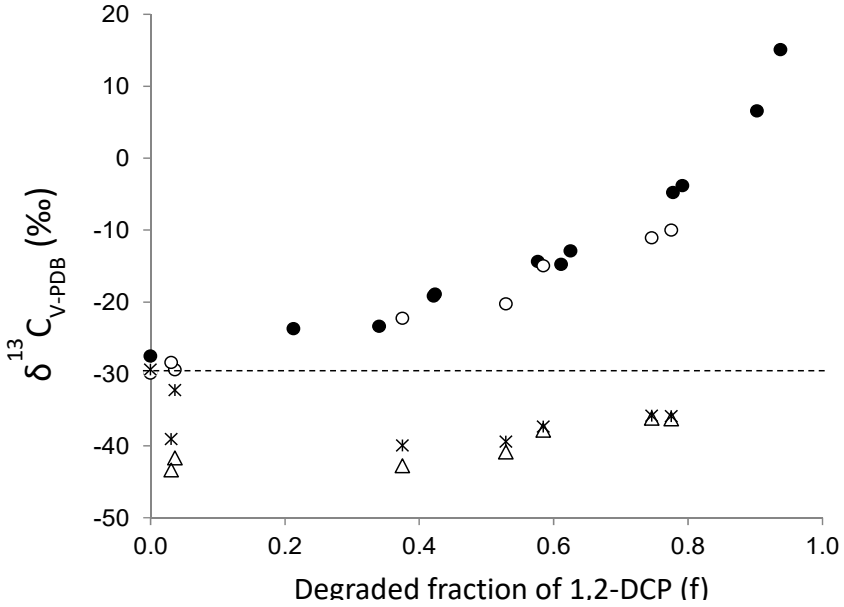


Figure 5.2: Carbon isotopic composition of 1,2-DCP (circles) in methanogenic (●) and non-methanogenic enrichment cultures containing 25 mM BES (○) during 1,2-DCP dechlorination. In non-methanogenic enrichment cultures, propene was measured (stars) and calculated as propene generated (Δ) following Eq. 9 due to certain initial transfer with the inoculum. Dashed line indicates the expected carbon isotopic mass balance which corresponds to the initial 1,2-DCP composition (average standard value of -29.8 ± 0.3‰, n =11). The error bars showing the standard deviation for duplicate measurements are smaller than the symbols.

The considered carbon isotopic compositions of spiked 1,2-DCP was −29.1 ± 0.5 ‰ and −29.8 ± 0.3 ‰, respectively, for the two experiments, and were calculated from the average delta value obtained from aqueous control standards injected together with the samples. These values did not differ significantly from each other (within their 1σ) nor to the ones obtained in abiotic controls after the incubation period (−29.2 ± 0.2 ‰ and −29.9 ± 0.1 ‰, respectively for each series). Therefore, the equilibrium of 1,2-DCP between headspace and liquid in the serum bottles did not affect substantially the measured 1,2-DCP delta value. However, in methanogenic cultures, the inoculum contained a low concentration of residual ^{13}C-enriched 1,2-DCP. This resulted in a more ^{13}C-enriched 1,2-DCP initial value ($\delta^{13}C_0$) of −27.5 ± 0.1 ‰ in this experiment (Figure 5.2). In nonmethanogenic cultures, no 1,2-DCP was detected in live controls without 1,2-DCP, demonstrating that no enriched 1,2-DCP was transferred with the inoculum and the −29.9 ± 0.1‰ value from abiotic controls was used as initial ($\delta^{13}C_0$). Taking this into account, the isotopic fractionation value of 1,2-DCP carbon isotope fractionation was calculated with the Rayleigh (Eq. 3) for methanogenic (ε^{C}_{bulk} = −15.3 ± 0.7‰) and nonmethanogenic (ε^{C}_{bulk}= −13.7 ± 2.0‰) enrichment cultures (Table 5.3). These values were statistically identical according to their 95% confidence intervals indicating that the reaction mechanism was not detectably affected by the presence/absence of methanogens in our consortium. Our results are in agreement with those reported recently in which trichloroethylene fractionation was not significantly different in two different *Dehalococcoides*-containing enrichments cultures when methanogenic activity was either inhibited or promoted [27].

Table 5.3: Isotopic fractionation value (ε^{C}_{bulk}) with 95% confidence intervals (95% CI) and calculated AKIE values assuming both stepwise and concerted reductive dechlorination of 1,2-DCP

						AKIE value[c]		
Experiment	**Suspected degrader**	**ε^{C}_{bulk} (‰)**	**N[a]**	**R^2**	**D[b] %**	**Stepwise**	**Concerted**	**Ref**
Non-methanogenic culture RC	*Dehalococcoides*	-10.8 ± 0.9	7	>0.96	>90	1.033 ± 0.003	1.016 ± 0.001	[28]
Non-methanogenic culture KS	*Dehalococcoides*	-11.3 ± 0.8	7	>0.96	>90	1.033 ± 0.003	1.017 ± 0.001	[28]
Methanogenic culture	*Dehalogenimonas*	-15.3 ± 0.7	12	0.996	94	1.046 ± 0.002	1.023 ± 0.001	This study
Non-methanogenic culture	*Dehalogenimonas*	-13.7 ± 2.0	8	0.98	77	1.042 ± 0.007	1.021 ± 0.003	This study
Total combined data	*Dehalogenimonas*	-15.0 ± 0.7	20	0.991	94	1.045 ± 0.002	1.023 ± 0.001	This study

[a]N: number of data points analyzed in duplicate.

[b]D%: maximum percentage of 1,2-DCP degradation which could be analyzed by GC-IRMS.

[c]AKIE values were calculated according to reference [10] being *n* the number of carbon atoms in the molecule of which *x* is the number of carbon atoms located at the reactive site and *z* is the number of indistinguishable reactive sites, correcting the effects of intramolecular isotopic competition. For 1,2-DCP, *n* =3 and the (*x*,*z*) considered for stepwise (1,1) and for concerted (2,1).

Combined ε^{C}_{bulk} (−15.0 ± 0.7‰) was calculated by plotting methanogenic and nonmethanogenic data together (Figure 5.3) giving a correlation factor of 0.991 indicating that dichloroelimation of 1,2-DCP to propene is well described by the Rayleigh model.

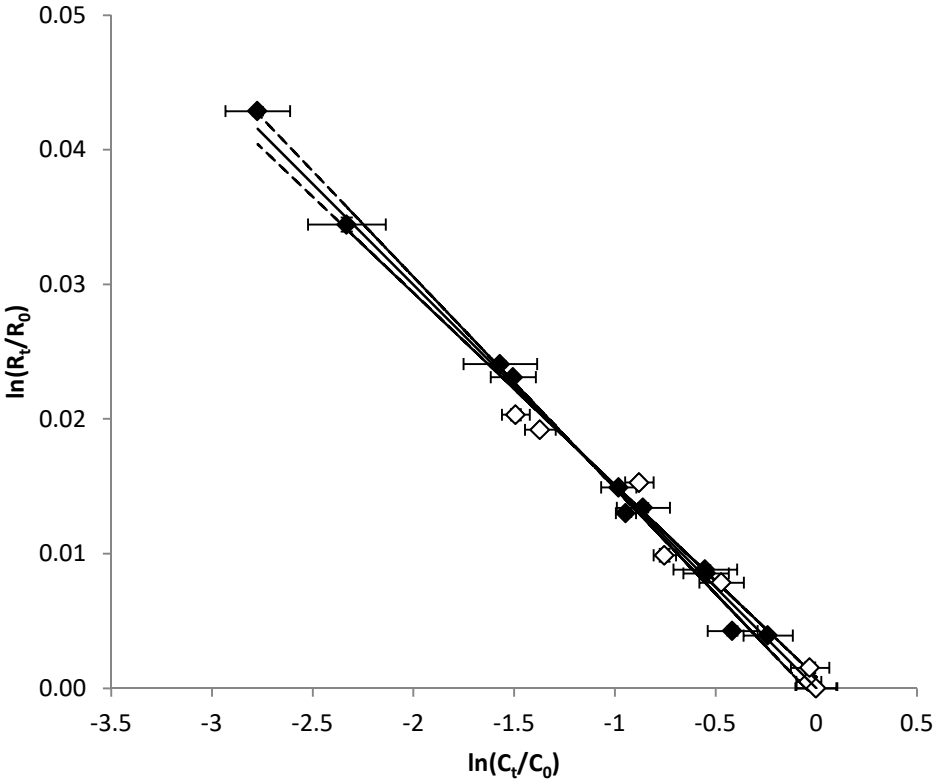


Figure 5.3: Double logarithmic plot according to the Rayleigh equation of the carbon isotope ratio versus the residual concentration of 1,2-DCP during dechlorination by methanogenic (solid symbols) and non-methanogenic enrichment cultures containing 25 mM BES (open symbols). Solid line corresponds to a linear regression model for total combined data (check Table 5.3 for more details) and gray dashed lines to its associated 95% confidential intervals. Data points show their related error bars.

It is expected that species containing the same reductive dehalogenase produce similar fractionation of 1,2-DCP, as previously observed for *Dehalococcoides* strains RC and KS, which

shared the DcpA reductive dehalogenase involved in the dichloroelimination of 1,2-DCP and produced a carbon isotopic fractionation of −10.8 ± 0.9‰ and −11.3 ± 0.8‰, respectively [19]. As previously mentioned in Chapter 4, the *dcpA* gene was also present in our *Dehalogenimonas* enrichment culture, however, the ε^{C}_{bulk} obtained differs significantly from *Dehalococcoides mccartyi* strains RC and KS according to 95% confidence intervals. Although this difference is intriguing, it is known that species belonging to the same genus and harboring the same functional reductive dehalogenase can produce significantly different isotopic fractionation of chlorinated compounds [29]. This phenomenon is commonly attributed to physiological processes such as transport of substrate across the cell membrane or differences in the active site of the enzyme.

To distinguish whether dichloroelimination proceeds via a stepwise or concerted mode AKIE values were calculated (Eq. 8) using the combined carbon isotopic fractionation obtained (Table 5.3). Assuming that the 1,2-DCP dichloroelimination reaction was stepwise, involving the cleavage of one C−Cl bond in the transition state, the combined $\varepsilon_{reactive}$ value was −43 ± 2‰ and the corresponding AKIE 1.045 ± 0.002. Assuming that the reaction was concerted, involving the simultaneous cleavage of both C−Cl bonds, the combined $\varepsilon_{reactive}$ value was −22 ± 1‰ corresponding to an AKIE value of 1.023 ± 0.001. Therefore, both AKIE values were lower than the theoretical maximum carbon primary KIE ("semiclassical Streitwieser Limits") for the complete cleavage of a C−Cl bond (1.057) [30]. However, considering the indication of Elsner et al. 2005 that realistic values with transition states at about 50% bond cleavage can be expected to be half as pronounced (AKIE = 1.03) [8], the concerted reaction with an obtained AKIE value lower than that (1.023) might be more probable in our case than the stepwise (1.045), whereas for Fletcher et al. 2009 both values were still lower enough (1.017 and 1.033, respectively) [19].

In addition, carbon isotopic composition of propene was measured in nonmethanogenic enrichment cultures to confirm the stoichiometric transformation from 1,2-DCP by isotopic mass balance. Eq. 9 was used to calculate the newly generated propene $\delta^{13}C$ value. As shown in Figure 5.2, ^{13}C-depleted propene was generated by 1,2-DCP dechlorination confirming isotope fractionation and it was getting significantly enriched along the 1,2-DCP dichloroelimination ($\delta^{13}C_{propene}$ values from −43.4‰ to −36.3‰). Moreover, by applying Eq. 8, the concentration-weighted average $\delta^{13}C_{sum}$ was nearly constant at −31 ± 1‰ which is not statistically different from the considered initial 1,2-DCP composition in this experiment (−29.8 ± 0.3‰), suggesting a close carbon isotopic mass balance during dichloroelimination.

5.3.2. Carbon and chlorine isotope fractionation of 1,2-DCA

5.3.2.1. Carbon and chlorine isotope fractionation in microcosm experiments

The anaerobic biodegradation batch experiments with *Dehalogenimonas*-containing microcosms lasted approximately 40 days to degrade 100 μM of 1,2-DCA, at which point most all of the initial 1,2-DCA was transformed to ethene (Figure 5.4).

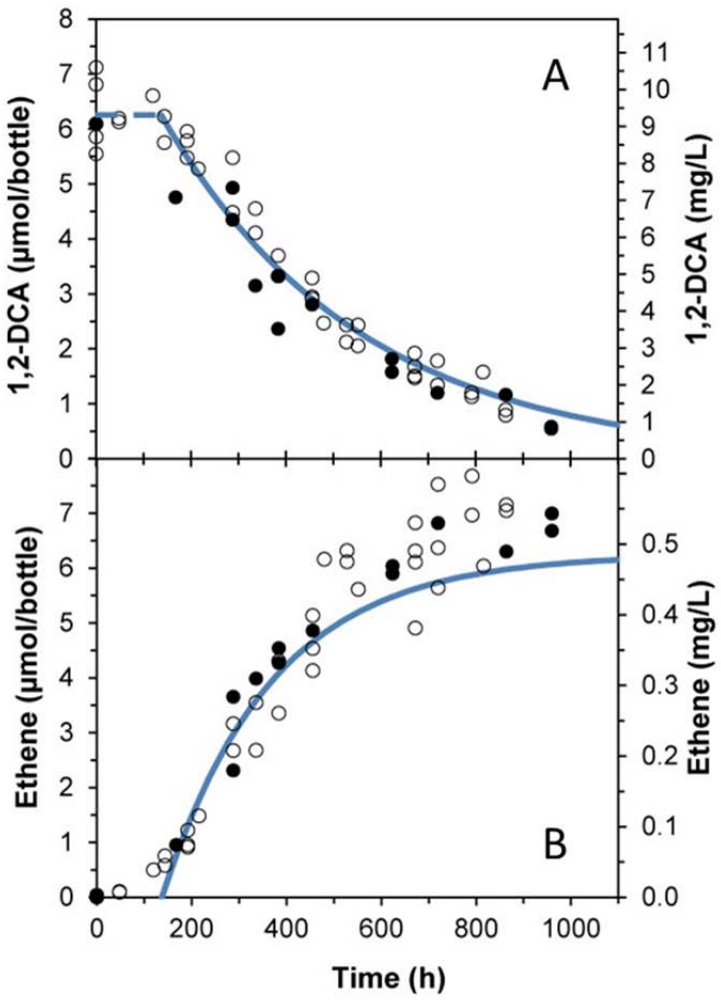


Figure 5.4: Biodegradation of 1,2-DCA (A) and accumulation of ethene (B) in batch experiments with *Dehalogenimonas* containing microcosms. The data from the microcosms with acetate or pyruvate are combined. Open circles (○) represent data points collected by GC headspace measurement on a single bottle that continued to be incubated (headspace monitoring); closed circles (●) indicate those bottles that were sacrificed for isotopic analysis immediately after the GC measurement. The left ordinate shows the total amounts per bottle and directly reveals the stoichiometry of 1,2-DCA conversion to ethene; aqueous phase concentrations that take into account partitioning to the headspace are shown on the right ordinate.

The $\delta^{13}C$ values during dihaloelimination of 1,2-DCA (Figure 5.5B) showed an enrichment in ^{13}C over ^{12}C which followed a Rayleigh trend ($R^2 > 0.98$, Eq. 3, Figure 5.6B). The ε^{C}_{bulk} value for the *Dehalogenimonas*-containing microcosms was -23 ± 2‰ (Figure 5.6B). No difference on concentration and isotope data was observed for the experiments with *Dehalogenimonas*-containing culture prepared with either acetate or pyruvate as carbon source.

The chlorine isotope values of 1,2-DCA showed a trend to more positive values (Figure 5.5A) reflecting an enrichment in ^{37}Cl over ^{35}Cl during dihaloelimination (Eq. 1). As shown in Figure 5.6A, the $\delta^{37}Cl$ values were well-described by a Rayleigh trend ($R^2 > 0.98$). The determined chlorine isotope fractionation (ε^{Cl}_{bulk}) for the *Dehalogenimonas*-containing microcosms is -12.0 ± 0.8‰.

The $\delta^{37}Cl$ and $\delta^{13}C$ values of 1,2-DCA in the controls of the experiment with *Dehalogenimonas*-containing cultures were -0.5 ± 0.1‰ and -27.2 ± 0.2‰, n=5, respectively did not change significantly during the experiments.

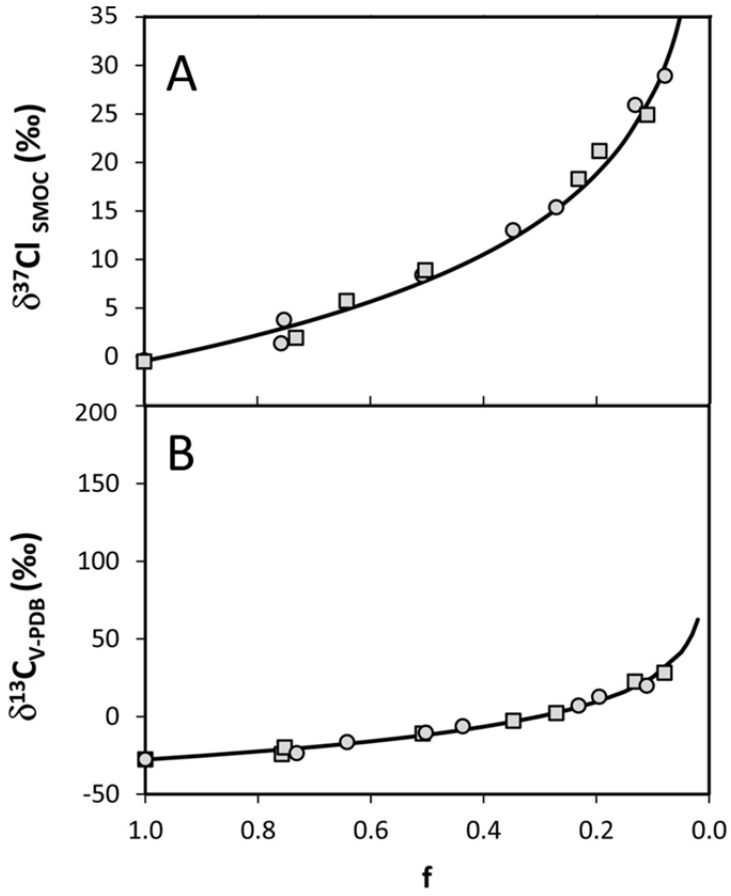


Figure 5.5: Chlorine (A) and carbon (B) isotopic composition of 1,2-DCA during biodegradation by *Dehalogenimonas*-containing cultures; f is the fraction of 1,2-DCA remaining. The error bars for isotope values are smaller than the symbols. Data from the microcosms with acetate (circles) or pyruvate (squares) are combined. The lines are models fit to isotope data according to Eq. 3.

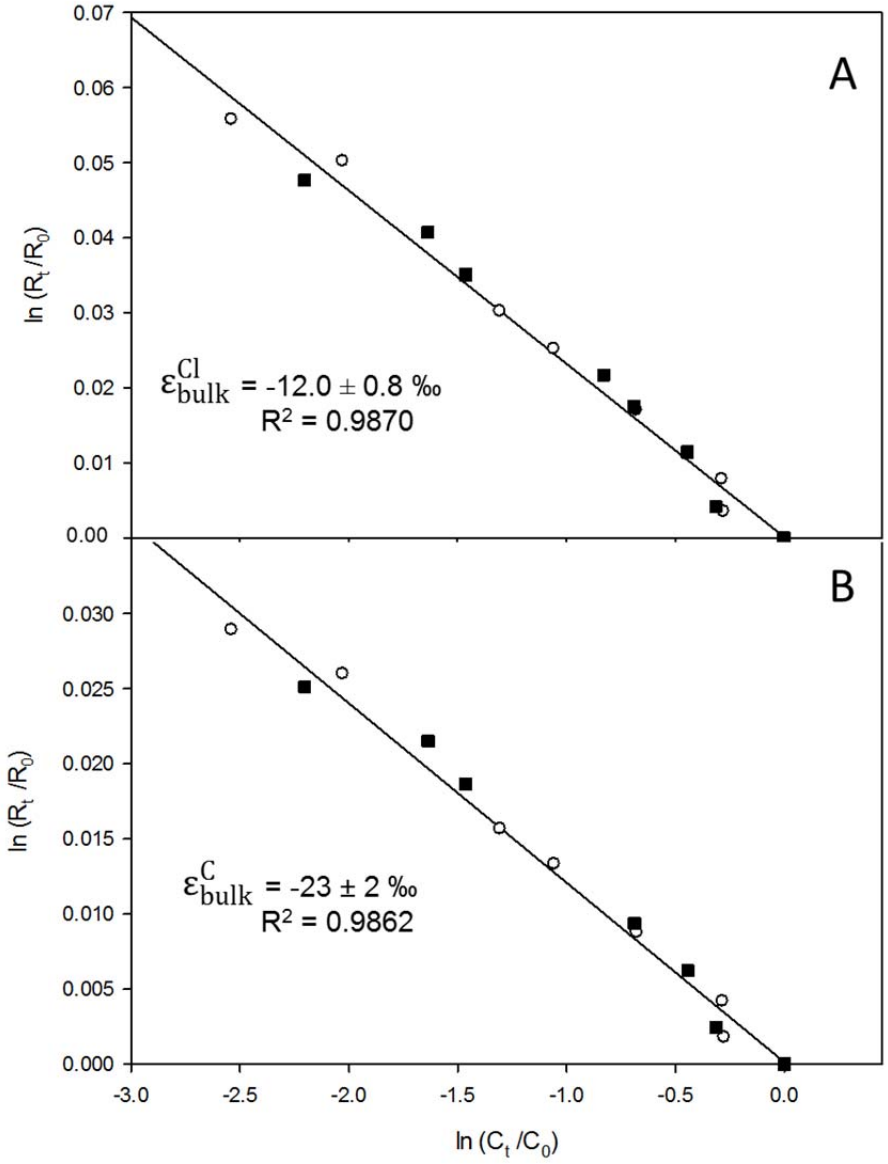


Figure 5.6: Chlorine (A) and carbon (B) isotopes regression during 1,2-DCA transformation by and *Dehalogenimonas*-containing cultures. Data from the microcosms with acetate (circles) or pyruvate (squares) are combined.

This study was made in collaboration with the Clemson University (USA) that possess a mixed culture containing a *Dehalococcoides mccartyi* strain [31]. During the development of this project, the dual isotope analysis of carbon and chlorine during 1,2-DCA dichloroelimination by

this *Dehalococcoides mccartyi* strain was also determined with the aim to compare the isotopic patterns of both OHRB strains. The *Dehalococcoides*-containing microcosms showed a strongest carbon isotope fractionation (ε^{C}_{bulk} = -33.0 ± 0.4‰) and a weaker chlorine isotope fractionation (ε^{Cl}_{bulk} = -5.1 ± 0.1‰) as compared to our *Dehalogenimonas*-containing microcosms [17]. The ε^{Cl}_{bulk} obtained for the *Dehalococcoides*-containing culture from Clemson University was similar to those reported for microbial dihaloelimination of 1,2-DCA in different previous laboratory studies. For instance, Hunkeler et al. obtained a ε^{C}_{bulk} of -32 ± 1‰ in a laboratory experiment with *Dehaloccoides*-containing microcosm prepared with soil and groundwater from a contaminated site [32]. Similar values were also obtained by Schmidt et al. [9] using two *Dehalococcoides* strains (-31 ± 1‰ and -29 ± 3‰ for *D. maccartyi* strains BTF08 and 195, respectively). In contrast, the ε^{C}_{bulk} determined for our *Dehalogenimonas*-containing culture showed a weaker value of -23 ± 2‰. Much lower ε^{C}_{bulk} values (-16.7 ± 0.5‰ and -7.3 ± 0.2‰) were measured by Hirschorn et al. [33] in different anaerobic enrichment cultures originating from contaminated sites. These authors suggested that different enzymes or enzymatic reactions (*stepwise* versus *concerted* dihaloelimination) may control isotopic fractionation during 1,2-DCA dihaloelimination. In addition, if rate-limiting steps preceding dehalogenation occur, the observable isotope effect will be smaller (i.e., masked) compared to the intrinsic isotope effect [34].

With the exception of the two relatively low values determined by Hirschorn et al. [33], the ε^{C}_{bulk} values for dihaloelimination of 1,2-DCA are within a similar range compared to those reported for hydrolytic dehalogenation in previous aerobic biodegradation studies, from -21.5 to -33.0‰ (average of -29 ± 3‰, ± 1σ, n = 24) [7], [23], [35], [36]. Also for anaerobic oxidation of 1,2-DCA under nitrate reducing conditions, Hirschorn et al. [37] measured an ε^{C}_{bulk} of -26 ± 4‰.

Therefore, based on carbon isotope fractionation alone it would be difficult to distinguish between aerobic hydrolytic and anaerobic reductive degradation. In addition, in the field it is not possible to determine the extent of mass removal that is uniquely caused by biodegradation and, consequently, ε^{C}_{bulk} values cannot be evaluated. Hence, a certain extent of observable carbon isotope fractionation in the field (i.e., $\Delta\delta^{13}C$) could have been caused by a strongly isotope fractionating reaction that has proceeded little, or a weakly isotope fractionating reaction that has proceeded further. Analysis of a second element is thus necessary to resolve this issue.

The ε^{Cl}_{bulk} values obtained for *Dehalococcoides mccartyi* and *Dehalogenimonas* are stronger and much variable compared to those reported for hydrolytic dehalogenation of 1,2-DCA (C-Cl bond cleavage) by *A. aquaticus* and *X. autotrophicus* in aerobic biodegradation experiments, -4.4 ± 0.2‰ and -4.2 ± 0.1‰, respectively [7]. In this previous study, a slightly lower value of -3.8 ± 0.2‰ was obtained for aerobic oxidation of 1,2-DCA (C-H bond cleavage) by *Pseudomonas* sp., which was indicative of a large secondary chlorine isotope effect.

Carbon and chlorine δ isotope values of 1,2-DCA from the experiments with *Dehalogenimonas*-containing cultures were combined in a dual isotope plot resulting in linear trends ($r^2 \geq 0.997$) with strongly distinct slopes ($\Lambda = \Delta\delta^{13}C / \Delta\delta^{37}Cl \approx \varepsilon^{C}_{bulk} / \varepsilon^{Cl}_{bulk}$, ± 95% C.I., Figure 5.7). A dual isotope enrichment factor (Λ) of 1.89 ± 0.02‰ was observed for the *Dehalogenimonas*-containing culture and much larger value of 6.8 ± 0.2‰ for *Dehalococcoides*-containing microcosm [17]. This result suggests differences in 1,2-DCA enzymatic dehalogenation by *Dehalococcoides* and *Dehalogenimonas* containing cultures. The interpretation is reinforced by the production of different daughter compounds during 1,2-DCA transformation by the distinct microbial cultures, i.e. formation of ethene and VC in the

experiments with *Dehalococcoides*-containing culture, but only ethene with *Dehalogenimonas*-containing culture.

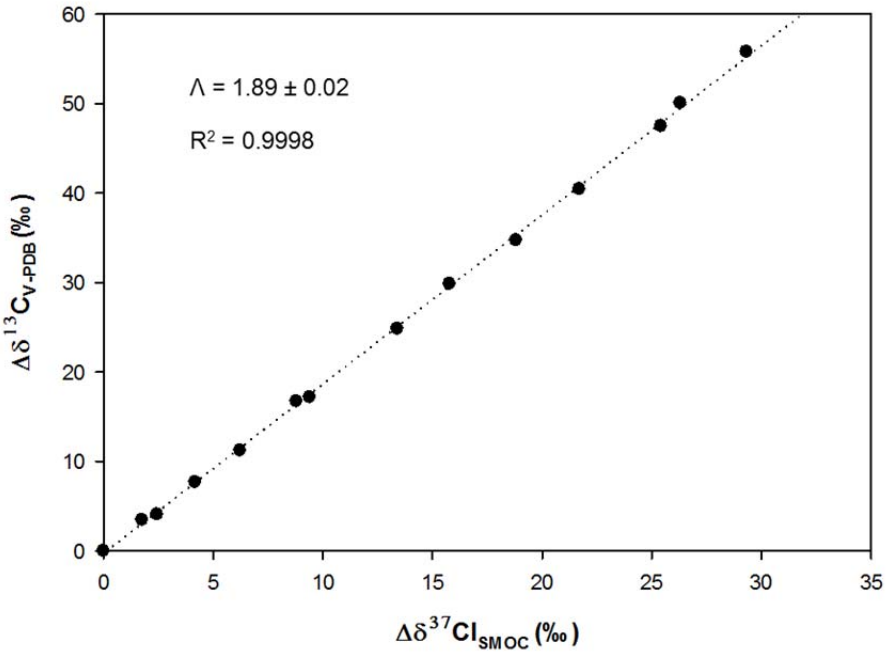


Figure 5.7: Dual C−Cl isotope patterns during biodegradation of 1,2-DCA by *Dehalogenimonas*-containing culture. Data from the microcosms with acetate or pyruvate are combined.

The isotope patterns observed during anaerobic dihaloelimination of 1,2-DCA were compared with those determined for aerobic biodegradation pathways of 1,2-DCA by Palau et al. [7] (Figure 5.8). For the experiments with the *Dehalogenimonas*-containing culture, the Λ value (1.89 ± 0.02‰) is much weaker than the one of hydrolytic dehalogenation (7.6 ± 0.2 ‰, via S_N2) but significantly stronger than for aerobic oxidation (0.78 ± 0.03 ‰). Compared to *Dehalogenimonas*-containing microcosms, the Λ value obtained for those containing *Dehalococcoides mccartyi* strain (6.8 ± 0.2‰) is also stronger than for aerobic oxidation and

closer to, but significantly different (ANCOVA, $p < 0.0001$) from hydrolytic dehalogenation (7.6 ± 0.2‰).

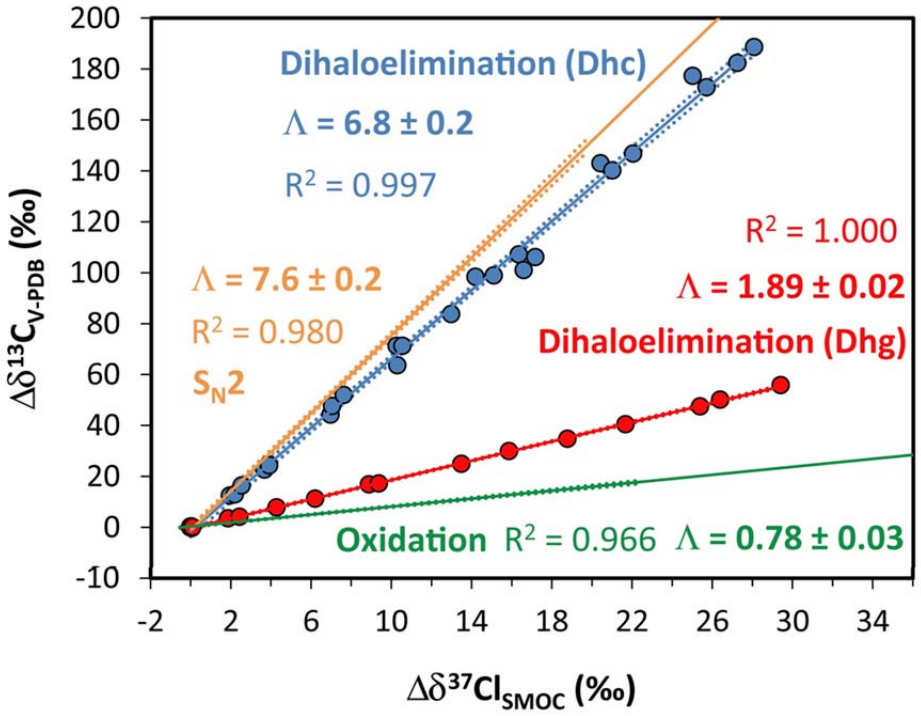


Figure 5.8: Dual C−Cl isotope patterns during biodegradation of 1,2-DCA in the laboratory. Circles are data points for anaerobic dihaloelimination obtained in this study. For the experiments with *Dehalogenimonas*-containing culture, data from the microcosms with acetate or pyruvate are combined. Trend lines determined for aerobic degradation pathways (oxidation and hydrolytic dehalogenation via S_N2) in a previous study are also indicated [20]; these trend lines were slightly extrapolated for better comparison.

5.3.2.2.Calculation of AKIE values

The different Λ values observed for dihaloelimination by cultures harboring different bacteria might be interpreted as a result of different reaction pathways (i.e. stepwise vs concerted) (Figure 1.1). A closer look at the underlying isotope effects, however, allows addressing this question in more detail.

Isotopic fractionation values and derived AKIEs from abiotic reactions are often considered closest to the intrinsic isotope effects [38]. Therefore, AKIEs reported in previous abiotic degradation studies of chlorinated ethanes were used for comparison. Estimated $AKIE^{C}_{stepwise}$ values for dihaloelimination of 1,2-DCA by *Dehalococcoides* (1.0707 ± 0.0009) and *Dehalogenimonas* (1.048 ± 0.004) containing cultures in this study were clearly above the range reported for abiotic *stepwise* dihaloelimination (from 1.0212 ± 0.0005 to 1.037 ± 0.001) [39], [40] suggesting that a *concerted* mechanism involving both C-Cl bonds in the initial transformation step is more likely ($AKIE^{C}_{concerted}$ of 1.0341 ± 0.0004 and 1.024 ± 0.003 for *Dehalococcoides* and *Dehalogenimonas*-containing cultures, respectively). These values agreed well with reported $AKIE^{C}_{concerted}$ of 1.03 and 1.023 for abiotic dihaloelimination of 1,2-DCA by Zn(0) [41] and microbial dihaloelimination of 1,2-DCP by *Dehalogenimonas* [18], respectively, whereas they would be consistent with both, a stepwise ($AKIE^{C}_{stepwise}$ = 1.033) or a concerted ($AKIE^{C}_{concerted}$ = 1.017) mechanism considered to interpret observations by Fletcher et al. [19] during microbial dihaloelimination of 1,2-DCP by *Dehalococcoides* populations.

However, similar Λ values would be expected for the same reaction mechanism and, therefore, the observed difference suggests that despite the evidence of a *concerted* dihaloelimination of 1,2-DCA for both experiments with different microbial cultures, this concerted mechanism must be realized in different ways in both cultures. Recent studies on tetrachloroethylene enzymatic reductive dechlorination [42], [43] postulated that similar dehalogenation reactions could result in different Λ values due to a shift of the rate-limiting step within a reaction sequence. This step, prior to the dehalogenation step, would become rate-limiting and mask the intrinsic primary isotope effect during C-Cl bond cleavage. In addition, if this step produce a small but non-negligible isotope fractionation (e.g., during binding of

substrate to the enzyme), it could lead to a different Λ value. However, this explanation is not consistent with the results of this study: (i) large C and Cl isotope effects reflecting C-Cl bond transformation in both experiments; (ii) large difference of isotope effects between the experiments with *Dehalococcoides* and *Dehalogenimonas*-containing cultures (i.e., ε^{C}_{bulk} values 7 and 10‰ apart for Cl and C, respectively); (iii) countertrends in these isotope effects (i.e., ε^{C}_{bulk} was greater with the *Dehalococcoides*, but ε^{Cl}_{bulk} greater with the *Dehalogenimonas*-containing culture) and (iv) detection of different daughter products in the microcosms with different cultures. These lines of evidence suggest that the observed differences on ε_{bulk} and Λ values between the experiments with cultures harboring different bacteria were associated with a different manner and order of bond breakage (e.g., synchronous / asynchronous or the way how leaving groups were stabilized in different enzyme environments) rather than other reasons invoked previously (i.e., binding, mass transfer). Specifically, Payne et al. recently proposed reduction of the halogenated substrate via halogen-cobalt bond formation in microbial reductive dehalogenases [44]. This new mechanism suggests that a reason for the observed differences in C and Cl isotope effects between both cultures might be a distinct interaction mode between cobalamin dependent enzymes and 1,2-DCA. For reductive dehalogenases catalyzing dihaloelimination, Payne et al. proposed that formation of the Co-halogen bond occurs concomitant with leaving of the vicinal halogen atom [44]. Comparison of the isotope effects observed in this study with those obtained using enzyme extracts, corrinoids and theoretical quantum mechanical/molecular mechanical (QM/MM) modelling in future studies may help to elucidate the reaction mechanisms and the formation of VC in greater detail.

5.3.2.3. C and Cl isotope ratios of 1,2-DCA in field samples

Aside from the collaboration with Clemson University for the *Dehalococcoides*-containing microcosm, the carbon and chlorine isotopes ratios of 1,2-DCA was also compared with two industrial sites, named A and B, located in Italy and USA, respectively in collaboration with the University of Neuchâtel (Switzerland), "Sapienza" University (Italy), University of Waterloo (Canada) and the German Research Center for Environmental Health (Germany) [17].

The 1,2-DCA concentrations in groundwater samples analyzed for isotope ratios range between 2410 and 0.03 mg L^{-1} at site A and from 1810 to 0.004 mg L^{-1} at site B . At site A, samples with relatively low concentrations (< 1 mg L^{-1}) show isotopic compositions more enriched in heavy isotopes (from +28.4 to +35.6‰ for $\delta^{37}Cl$ and from +33.5 to +51.5‰ for $\delta^{13}C$) compared to those with higher concentrations (from +1.1 to +7.1‰ for $\delta^{37}Cl$ and from -21.7 to -12.2‰ for $\delta^{13}C$). Similarly, at site B, the highest isotope ratios of 1,2-DCA, up to +28.9‰ for $\delta^{37}Cl$ (only chlorine was measured in this sample) and -5.5‰ for $\delta^{13}C$, were also measured in samples with concentrations < 1 mg L^{-1} , strongly suggesting the occurrence of 1,2-DCA biodegradation at both sites.

A linear correlation between $\delta^{37}Cl$ and $\delta^{13}C$ of 1,2-DCA in groundwater samples was obtained for both sites (Figure 5.9), confirming that transformation of 1,2- DCA is an important process in the subsurface. The Λ values determined for site A (2.1 ± 0.1, R^2 = 0.997) and B (2.2 ± 2.9, R^2 = 0.84) are the same within the uncertainty. These field Λ values are strongly distinct compared to those measured in the laboratory for aerobic biodegradation pathways of 1,2-DCA (i.e. hydrolytic dehalogenation and C-H bond oxidation, Figure 5.9), which agrees with the reducing conditions observed at both sites. The field Λ values are much closer to the one determined for 1,2-DCA anaerobic dihaloelimination by the *Dehalogenimonas*-containing

culture (1.89 ± 0.02) compared to that measured for the *Dehalococcoides*-containing microcosms (6.8 ± 0.2). Taking into account the uncertainty of measurements in the field, additional data from contaminated sites under different geochemical conditions will provide the information needed to test and strengthen further the use of the dual isotope approach to evaluate the pathways involved in biodegradation of 1,2-DCA.

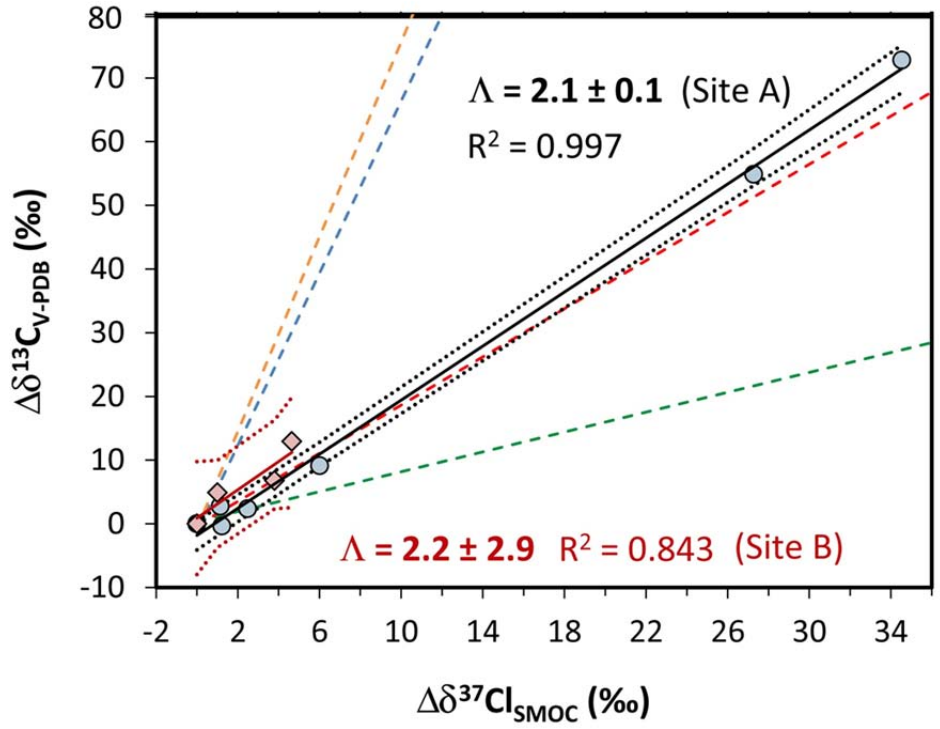


Figure 5.9: Field isotope data and dual C−Cl isotope patterns from site A (circles) and B (rhombus). Red line represents dihaloelimination by *Dehalogenimonas*, blue line represents dihaloelimination by *Dehalococcoides*, orange line represents S_N2 reaction and green line represents oxidation. Dotted lines indicate the 95% confidence intervals of the linear regression, error bars of $\Delta\delta^{13}C$ and $\Delta\delta^{37}Cl$ values are smaller than the symbols and Λ values (±95% C.I.) are given by the slope of the linear regressions [17].

In addition, the dual isotope approach may help to select the right ε_{bulk} value for calculating approximately the extent of 1,2-DCA biodegradation in the field, taking into account

that the Rayleigh equation applied to field samples tends to underestimate the actual degree of biodegradation [45]. For example, at site A and B maximal shifts in carbon isotope ratios (i.e., $\Delta\delta^{13}C_{max}$) of 73.2 ± 0.7 and 13.0 ± 0.7‰ were observed, respectively.

Table 5.4: Carbon and chlorine isotope ratios of 1,2-DCA in sites A and B. Concentrations of 1,2-DCA in each monitoring well are also indicated (LS: Long screen, n.d.: not detected) [17].

Site	Well	Sampling depth (m a.s.l.)	1,2-DCA		
			Conc. (mg/L)	$\delta^{13}C_{VPDB}$ (‰)	$\delta^{37}Cl_{SMOC}$ (‰)
A	MW-1	LS	2410	-21.3±0.5	+1.1±0.2
A	MW-2	LS	14	-21.7±0.5	+2.3±0.2
A	MW-3	LS	86	-18.6±0.5	+2.2±0.2
A	MW-4	LS	0.003	- - -	- - -
A	MW-5	LS	9.9	-12.2±0.5	+7.1±0.2
A	MW-6	LS	n.d.	- - -	- - -
A	MW-7	-9	0.03	- - -	+34.1±0.2
A	MW-7	-14	0.06	+51.5±0.5	+35.6±0.2
A	MW-7	-19	0.04	- - -	+33.1±0.2
A	MW-7	-24	0.1	+33.5±0.5	+28.4±0.2
A	MW-7	-29	1.0	-19.0±0.5	+3.6±0.2
B	MW-1	LS	5.6	-13.6±0.5	+5.6±0.2
B	MW-2	LS	1710	-11.7±0.5	+8.4±0.2
B	MW-3	LS	0.006	- - -	+5.2±0.3
B	MW-4	LS	0.1	-5.5±0.5	+9.3±0.2
B	MW-5	LS	0.004	- - -	+28.9±0.4
B	MW-6	LS	1810	-18.5±0.5	+4.6±0.2
B	MW-7	LS	0.009	- - -	- - -
B	MW-8	LS	n.d.	- - -	- - -
B	MW-9	LS	n.d.	- - -	- - -
B	MW-10	LS	n.d.	- - -	- - -
B	MW-11	LS	n.d.	- - -	- - -

The ε^{C}_{bulk} value obtained from anaerobic dihaloelimination of 1,2-DCA by *Dehalogenimonas*-containing culture was used due to the similar Λ value (Figure 5.8B), resulting in 1,2-DCA remaining fractions of 4.3 ± 1.2% (site A) and 56 ± 3% (site B) according to the Rayleigh equation (Eq. 3, the uncertainty was estimated by error propagation). It is important to note that using the ε^{C}_{bulk} value from the experiments with *Dehalococcoides*-containing culture significantly higher values would be obtained: 11.25 ± 0.4% (site A) and 67 ± 2% (site B). Estimated remaining fractions of 1,2-DCA would be even more different (< 4% in both sites) if the average ε^{C}_{bulk} value associated with aerobic oxidation of 1,2-DCA was used (-3.8 ± 0.8‰, ± 1σ, n = 6) [20], [35] leading to a critically overestimated extent of biodegradation. This example illustrates how uncertainties in the assessment of natural biodegradation at field sites can be significantly reduced if a dual element isotope approach is pursued.

Groundwater contaminant plumes are dynamic and highly heterogeneous systems subject to temporal and spatial geochemical variations that control biodegradation processes in the aquifer [46]. Gossett (2010) showed that *aerobic* VC oxidation was sustained at dissolved oxygen (DO) concentrations below 0.02 mg L^{-1} [41]. Such low DO concentrations are typically considered to indicate *anoxic* conditions in the field [41] and, therefore, this can lead to a mischaracterization of biodegradation conditions in contaminated sites. Hence, additional tools are necessary for a better characterization of chlorinated contaminants biodegradation in the environment. For 1,2-DCA, dual C-Cl isotope analysis may allow identification of either *aerobic* or *anaerobic* ongoing biodegradation in groundwater. In addition, changes in redox conditions along the plume (e.g., from reducing conditions at the source area to oxic conditions at the fringe zone) could result in two different dual C-Cl isotope correlations in sequence, e.g., anaerobic dihaloelimination and aerobic oxidation, respectively. Based on the results of this study, dual

element isotope fractionation trends of 1,2-DCA can be expected to detect such a change in transformation pathways.

5.4. Conclusions

This chapter illustrates the potential use of CSIA and 2D-CSIA to study the biodegradation pathways of chlorinated alkanes (1,2-DCP and 1,2-DCA) by *Dehalogenimonas*-containing enrichment culture. The ε^{C}_{bulk} of the *Dehalogenimonas*-containing enrichment culture was in the same order of magnitude but statistically differs from that reported for *Dehalococcoides mccartyi* strain RS and strain KC, although the *dcpA* gene encoding 1,2-DCP reductive dehalogenase was identified in both genus. Since *Dehalogenimonas* and *Dehalococcoides* presumably have similar cellular composition, the differences in the isotope fractionation can be a result of differences in reductive dehalogenase structure rather than other rate limiting effects (e.g. uptake and transport). In the light of these results, carbon stable isotope fractionation can be used to qualify 1,2-DCP dechlorination *in situ* under anoxic conditions but information on the respective responsible strain is required to quantify the extent of biodegradation. Further study involving the determination of isotope fractionation with other OHRB that can dechlorinate 1,2-DCP such as *Dehalobacter* and *Desulfitobacterium* however are necessary to validate this hypothesis.

A dual C-Cl isotope approach was used to investigate biodegradation of 1,2-DCA and the results were compared with those obtained during anaerobic degradation of 1,2-DCA by *Dehalococcoides*-containing culture, aerobic oxidation and field samples in collaboration with other universities and research centers. The different Λ values determined for *Dehalococcoides* and *Dehalogenimonas*-containing enrichment cultures in the laboratory suggest that a dual

isotope approach might even be useful to characterize degradation by different bacteria if the same apparent pathway prevails (i.e., dihaloelimination of 1,2-DCA). This could help to identify the microbial community responsible for reductive dechlorination of 1,2-DCA in the field. The present study, shows that the dual C-Cl isotope approach can be useful to distinguish among reactions with similar ε^{C}_{bulk} values (oxidation, hydrolytic dehalogenation and dihaloelimination). In addition to *Dehalococcoides* and *Dehalogenimonas*, other anaerobic dehalorespiring bacteria are able to reductively dechlorinate 1,2-DCA, including *Dehalobacter* [47] and *Desulfitobacterium* [48]. The present study is, therefore, a first step and the Λ values determined from 1,2-DCA dihaloelimination by *Dehalogenimonas*-containing enrichment cultures should be compared to data obtained with other types of microbes in future biodegradation studies. Nonetheless, the present study can already contribute to the urgent need of reducing uncertainties in the quantification of biodegradation in the field. The estimation of the extent of contaminant transformation using isotope data is one of the main applications of compound-specific isotope analysis to field studies [49]–[51]. Here, we show that identification of dual element isotope of by *Dehalogenimonas*-containing enrichment culture can facilitate the elucidation of the dihaloelimination and other degradation pathway in the field compared to CSIA of single element alone.

5.5. References

[1] C. M. Aelion and P. Höhener, "Fundamentals of environmental isotopes and their use in biodegradation," in *Environmental Isotopes in Biodegradation and Bioremediation*, C. M. Aelion, P. Höhener, D. Hunkeler, and R. Aravena, Eds. CRC Press, Boca Rato, 2009, pp. 3–22.

[2] R. U. Meckenstock, B. Morasch, C. Griebler, and H. H. Richnow, "Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated acquifers," *J. Contam. Hydrol.*, vol. 75, no. 3–4, pp. 215–255, 2004.

[3] M. Elsner, "Stable isotope fractionation to investigate natural transformation mechanisms of organic contaminants: principles, prospects and limitations.," *J. Environ. Monit.*, vol. 12, no. 11, pp. 2005–31, 2010.

[4] D. Hunkeler, "Use of compound-specific isotope analysis (CSIA) to assess the origin and fate of chlorinated hydrocarbons," in *Organohalide-Respiring Bacteria*, L.Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 587–617.

[5] J. Renpenning and I. Nijenhuis, "Evaluation of the microbial reductive dehalogenation reaction using compound-specific stable isotope analysis (CSIA)," in *Organohalide-Respiring Bacteria*, L.Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 429–453.

[6] D. Hunkeler and M. Elsner, "Principles and mechanisms of isotope fractionation," in *Environmental Isotopes in Biodegradation and Bioremediation*, C. M. Aelion, P. Höhener, D. Hunkeler, and R. Aravena, Eds. CRC Press, Boca Raton, 2009, pp. 43–77.

[7] J. Palau, S. Cretnik, O. Shouakar-Stash, M. Hoche, M. Elsner, and D. Hunkeler, "C and Cl isotope fractionation of 1,2-dichloroethane displays unique $\delta^{13}C$/ $\delta^{37}Cl$ patterns for pathway identi fi cation and reveals surprising C − Cl bond involvement in microbial oxidation," *Environ. Sci. Technol.*, vol. 48, pp. 9430–9437, 2014.

[8] M. Elsner, L. Zwank, D. Hunkeler, and R. P. Schwarzenbach, "A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants," *Environ. Sci. Technol.*, vol. 39, pp. 6896–6916, 2005.

[9] M. Schmidt, S. Lege, and I. Nijenhuis, "Comparison of 1,2-dichloroethane, dichloroethene and vinyl chloride carbon stable isotope fractionation during dechlorination by two *Dehalococcoides* strains," *Water Res.*, vol. 52, pp. 146–154, 2014.

[10] M. Elsner, M. A. Jochmann, T. B. Hofstetter, D. Hunkeler, A. Bernstein, T. C. Schmidt, and A. Schimmelmann, "Current challenges in compound-specific stable isotope analysis of environmental organic contaminants," *Anal. Bioanal. Chem.*, vol. 403, no. 9, pp. 2471–2491, 2012.

[11] T. Kuder, B. M. Van Breukelen, M. Vanderford, and P. Philp, "3D-CSIA: Carbon, chlorine, and hydrogen isotope fractionation in transformation of TCE to ethene by a *Dehalococcoides* culture," *Environ. Sci. Technol.*, vol. 47, no. 17, pp. 9668–9677, 2013.

[12] L. Zwank, M. Berg, T. C. Schmidt, and S. B. Haderlein, "Compound-Specific carbon isotope analysis of volatile organic compounds in the low-microgram per liter range," *Anal. Chem.*, vol. 75, no. 20, pp. 5575–5583, 2003.

[13] O. Shouakar-Stash, R. J. Drimmie, M. Zhang, and S. K. Frape, "Compound-specific chlorine isotope ratios of TCE, PCE and DCE isomers by direct injection using CF-IRMS," *Appl.*

Geochemistry, vol. 21, no. 5, pp. 766–781, 2006.

[14] A. Bernstein, O. Shouakar-Stash, K. Ebert, C. Laskov, D. Hunkeler, S. Jeannottat, K. Sakaguchi-Söder, J. Laaks, M. A. Jochmann, S. Cretnik, J. Jager, S. B. Haderlein, T. C. Schmidt, R. Aravena, and M. Elsner, "Compound-specific chlorine isotope analysis: a comparison of gas chromatography/isotope ratio mass spectrometry and gas chromatography/quadrupole mass spectrometry methods in an interlaboratory study," *Anal. Chem.*, vol. 83, no. 20, pp. 7624–7634, 2011.

[15] J. Renpenning, K. L. Hitzfeld, T. Gilevska, I. Nijenhuis, M. Gehre, and H.-H. Richnow, "Development and validation of an universal interface for compound-specific stable isotope analysis of chlorine (^{37}Cl/ ^{35}Cl) by GC-High-Temperature Conversion (HTC)-MS/IRMS," *Anal. Chem.*, vol. 87, no. 5, pp. 2832–2839, 2015.

[16] O. Shouakar-Stash and R. J. Drimmie, "Online methodology for determining compound-specific hydrogen stable isotope ratios of trichloroethene and 1,2- *cis* -dichloroethene by continuous-flow isotope ratio mass spectrometry," *Rapid Commun. Mass Spectrom.*, vol. 27, no. 12, pp. 1335–1344, 2013.

[17] J. Palau, R. Yu, S. Hatijah Mortan, O. Shouakar-Stash, M. Rosell, D. L. Freedman, C. Sbarbati, S. Fiorenza, R. Aravena, E. Marco-Urrea, M. Elsner, A. Soler, and D. Hunkeler, "Distinct dual C-Cl isotope fractionation patterns during anaerobic biodegradation of 1,2-dichloroethane: potential to characterize microbial degradation in the field," *Environ. Sci. Technol.*, vol. 51, pp. 2685-2694, 2017.

[18] L. Martín-González, S. H. Mortan, M. Rosell, E. Parladé, M. Martínez-Alonso, N. Gaju, G. Caminal, L. Adrian, and E. Marco-Urrea, "Stable carbon isotope fractionation during 1,2-dichloropropane-to-propene transformation by an enrichment culture containing *Dehalogenimonas* strains and a dcpA gene," *Environ. Sci. Technol.*, vol. 49, no. 14, pp. 8666–74, 2015.

[19] K. E. Fletcher, F. E. Löffler, H. H. Richnow, and I. Nijenhuis, "Stable carbon isotope fractionation of 1,2-dichloropropane during dichloroelimination by *Dehalococcoides* populations.," *Environ. Sci. Technol.*, vol. 43, no. 18, pp. 6915–6919, 2009.

[20] J. Palau, O. Shouakar-Stash, and D. Hunkeler, "Carbon and chlorine isotope analysis to identify abiotic degradation pathways of 1,1,1-trichloroethane," *Environ. Sci. Technol.*, vol. 48, no. 24, pp. 14400–14408, 2014.

[21] T. Futagami and K. Furukawa, "The genus *Desulfitobacterium*," in *Organohalide-Respiring Bacteria*, L.Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 173–207.

[22] W. M. Moe, F. A. Rainey, and J. Yan, "The genus *Dehalogenimonas*," in *Organohalide-Respiring Bacteria*, L.Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 137–151.

[23] D. Hunkeler and R. Aravena, "Evidence of substantial carbon isotope fractionation among substrate, inorganic carbon, and biomass during aerobic mineralization of 1,2-dichloroethane by *Xanthobacter autotrophicus*," *Appl. Environ. Microbiol.*, vol. 66, no. 11, pp. 4870–4876, 2000.

[24] D. Hunkeler, R. Aravena, and B. J. Butler, "Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: Microcosm and field studies," *Environ. Sci. Technol.*, vol. 33, no. 16, pp. 2733–2738, 1999.

[25] C. Aeppli, T. B. Hofstetter, H. I. F. Amaral, R. Kipfer, R. P. Schwarzenbach, and M. Berg,

"Quantifying in situ transformation rates of chlorinated ethenes by combining compound-specific stable isotope analysis, groundwater dating, and carbon isotope mass balances," *Environ. Sci. Technol.*, vol. 44, no. 10, pp. 3705–3711, 2010.

[26] D. R. Johnson, A. Nemir, G. L. Andersen, S. H. Zinder, and L. Alvarez-Cohen, "Transcriptomic microarray analysis of corrinoid responsive genes in *Dehalococcoides ethenogenes* strain 195," *FEMS Microbiol. Lett.*, vol. 294, no. 2, pp. 198–206, 2009.

[27] K. C. Harding, P. K. H. Lee, M. Bill, T. E. Buscheck, M. E. Conrad, and L. Alvarez-Cohen, "Effects of varying growth conditions on stable carbon isotope fractionation of trichloroethene (TCE) by *tceA*-containing *Dehalococcoides mccartyi* strains," *Environ. Sci. Technol.*, vol. 47, no. 21, pp. 12342–12350, 2013.

[28] M. Elsner and T. B. Hofstetter, "Current perspectives on the mechanisms of chlorohydrocarbon degradation in subsurface environments: insight from kinetics, product formation, probe molecules, and isotope fractionation," in *Aquatic Redox Chemistry,*P.G. Tratnyek, T.J. Grundl and S.B. Haderlein ACS Symposium Series vol 1071, ACS Editions, 2011, pp. 407–439.

[29] E. Marco-Urrea, I. Nijenhuis, and L. Adrian, "Transformation and carbon isotope fractionation of tetra- and trichloroethene to *trans* -dichloroethene by *Dehalococcoides* sp. strain CBDB1," *Environ. Sci. Technol.*, vol. 45, no. 4, pp. 1555–1562, 2011.

[30] W. P. Huskey, "Origins and interpretation of heavy-atom isotope effects," in *Enzyme Mechanism from Isotope Effects*, P. F. Cook, Ed., CRC Press, Boca Raton, 1991, p. 37.

[31] R. Yu, H. S. Peethambaram, R. W. Falta, M. F. Verce, J. K. Henderson, C. E. Bagwell, R. L. Brigmon, and L. Freedman, "Kinetics of 1,2-dichloroethane and 1,2-dibromoethane biodegradation in anaerobic enrichment cultures," *Appl. Environ. Microbiol.*, vol. 79, no. 4, pp. 1359–1367, 2013.

[32] D. Hunkeler, R. Aravena, and E. Cox, "Carbon isotopes as a tool to evaluate the origin and fate of vinyl chloride: laboratory experiments and modeling of isotope evolution," *Environ. Sci. Technol.*, vol. 36, no. 15, pp. 3378–3384, 2002.

[33] S. K. Hirschorn, A. Grostern, G. Lacrampe-Couloume, E. A. Edwards, L. MacKinnon, C. Repta, D. W. Major, and B. Sherwood Lollar, "Quantification of biotransformation of chlorinated hydrocarbons in a biostimulation study: Added value via stable carbon isotope analysis," *J. Contam. Hydrol.*, vol. 94, no. 3, pp. 249–260, 2007.

[34] J. Renpenning, I. Rapp, and I. Nijenhuis, "Substrate hydrophobicity and cell composition influence the extent of rate limitation and masking of isotope fractionation during microbial reductive dehalogenation of chlorinated ethenes," *Environ. Sci. Technol.*, vol. 49, no. 7, pp. 4293–4301, 2015.

[35] S. K. Hirschorn, M. J. Dinglasan, M. Elsner, S. A. Mancini, G. Lacrampe-Couloume, E. A. Edwards, and B. S. Lollar, "Pathway dependent isotopic fractionation during aerobic biodegradation of 1,2-dichloroethane," *Environ. Sci. Technol.*, vol. 38, no. 18, pp. 4775–4781, 2004.

[36] Y. Abe, J. Zopfi, and D. Hunkeler, "Effect of molecule size on carbon isotope fractionation during biodegradation of chlorinated alkanes by *Xanthobacter autotrophicus* GJ10," *Isotopes Environ. Health Stud.*, vol. 45, no. 1, pp. 18–26, 2009.

[37] S. K. Hirschorn, M. J. Dinglasan-Panlilio, E. A. Edwards, G. Lacrampe-Couloume, and B. Sherwood Lollar, “Isotope analysis as a natural reaction probe to determine mechanisms of biodegradation of 1,2-dichloroethane,” *Environ. Microbiol.*, vol. 9, no. 7, pp. 1651–1657, 2007.

[38] B. S. Lollar, S. Hirschorn, S. O. C. Mundle, A. Grostern, E. A. Edwards, and G. Lacrampe-Couloume, “Insights into enzyme kinetics of chloroethane biodegradation using compound specific stable isotopes,” *Environ. Sci. Technol.*, vol. 44, no. 19, pp. 7498–7503, 2010.

[39] M. Elsner, D. M. Cwiertny, A. L. Roberts, and B. S. Lollar, “1,1,2,2-Tetrachloroethane reactions with OH-, Cr(II), granular iron, and a copper-iron bimetal: Insights from product formation and associated carbon isotope fractionation,” *Environ. Sci. Technol.*, vol. 41, no. 11, pp. 4111–4117, 2007.

[40] T. B. Hofstetter, C. M. Reddy, L. J. Heraty, M. Berg, and N. C. Sturchio, “Carbon and chlorine isotope effects during abiotic reductive dechlorination of polychlorinated ethanes,” *Environ. Sci. Technol.*, vol. 41, pp. 4662–4668, 2007.

[41] J. M. Gossett, “Sustained aerobic oxidation of vinyl chloride at low oxygen concentrations.,” *Environ. Sci. Technol.*, vol. 44, no. 4, pp. 1405–1411, 2010.

[42] A. Badin, G. Buttet, J. Maillard, C. Holliger, and D. Hunkeler, “Multiple dual C-Cl isotope patterns associated with reductive dechlorination of tetrachloroethene,” *Environ. Sci. Technol.*, vol. 48, no. 16, pp. 9179–9186, 2014.

[43] J. Renpenning, S. Keller, S. Cretnik, O. Shouakar-Stash, M. Elsner, T. Schubert, and I. Nijenhuis, “Combined C and Cl isotope effects indicate differences between corrinoids and enzyme (*Sulfurospirillum multivorans* PceA) in reductive dehalogenation of tetrachloroethene, but not trichloroethene,” *Environ. Sci. Technol.*, vol. 48, no. 20, pp. 11837–11845, 2014.

[44] K. a P. Payne, C. P. Quezada, K. Fisher, M. S. Dunstan, F. a Collins, H. Sjuts, C. Levy, S. Hay, S. E. J. Rigby, and D. Leys, “Reductive dehalogenase structure suggests a mechanism for B12-dependent dehalogenation.,” *Nature*, vol. 517, no. 7535, pp. 513–516, 2014.

[45] Y. Abe and D. Hunkeler, “Does the Rayleigh equation apply to evaluate field isotope data in contaminant hydrogeology?,” *Environ. Sci. Technol.*, vol. 40, no. 5, pp. 1588–1596, 2006.

[46] R. U. Meckenstock, M. Elsner, C. Griebler, T. Lueders, C. Stumpp, J. Aamand, S. N. Agathos, H.-J. Albrechtsen, L. Bastiaens, P. L. Bjerg, N. Boon, W. Dejonghe, W. E. Huang, S. I. Schmidt, E. Smolders, S. R. Sørensen, D. Springael, and B. M. van Breukelen, “Biodegradation: Updating the concepts of control for microbial cleanup in contaminated aquifers,” *Environ. Sci. Technol.*, vol. 49, no. 12, pp. 7073–7081, 2015.

[47] A. Grostern and E. A. Edwards, “Characterization of a *Dehalobacter* coculture that dechlorinates 1,2-dichloroethane to ethene and identification of the putative reductive dehalogenase gene,” *Appl. Environ. Microbiol.*, vol. 75, no. 9, pp. 2684–2693, 2009.

[48] S. De Wildeman, G. Diekert, H. Van Langenhove, and W. Verstraete, “Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes,” *Appl. Environ. Microbiol.*, vol. 69, no. 9, pp. 5643–5647, 2003.

[49] D. Hunkeler, and R. Aravena, "Investigating the Origin and Fate of Organic Contaminants in Groundwater Using Stable Isotope Analysis" in *Environmental Isotopes in Biodegradation and Bioremediation*, C. M. Aelion, P. Höhener, D. Hunkeler, and R. Aravena, Eds. CRC Press, Boca

Raton, 2009, pp. 249–288.

[50] M. Thullner, F. Centler, H.-H. Richnow, and A. Fischer, "Quantification of organic pollutant degradation in contaminated aquifers using compound specific stable isotope analysis – Review of recent developments," *Org. Geochem.*, vol. 42, no. 12, pp. 1440–1460, 2012.

[51] M. Braeckevelt, A. Fischer, and M. Kästner, "Field applicability of compound-specific isotope analysis (CSIA) for characterization and quantification of in situ contaminant degradation in aquifers," *Appl. Microbiol Biot*, vol. 94, no. 6. pp. 1401–1421, 2012.

CHAPTER 6

ISOLATION ATTEMPT OF *Dehalogenimonas* sp. USING SELECTED ANTIBIOTICS AND THE DILUTION-TO-EXTINCTION APPROACH

ABSTRACT

Dehalogenimonas has been identified as the bacterium responsible for the dechlorination of halogenated alkanes in the consortia derived from Besòs River estuary sediments. To date, only three species of *Dehalogenimonas* genus have been isolated. In this chapter, the isolation of *Dehalogenimonas* was attempted using dilution to extinction method in liquid and agar medium. The DGGE analysis revealed that *Dehalogenimonas, Azonexus caeni* and *Geobacter sulfurreducens* were the predominant bacteria after applying the dilution to extinction method in liquid medium. Sequential transfers of this consortia were carried out with the antibiotic streptomycin, which specifically targeted *Azonexus caeni* and *Geobacter sulfurreducens*. DGGE analysis showed that after four sequential transfers with this antibiotic only one prominent single band was detected. Sequence analysis of this excised band revealed that it belonged to *Dehalogenimonas* sp., suggesting that it may have been isolated. A clone library of bacterial amplicons was conducted to provide additional evidence of the purity of *Dehalogenimonas* in this culture. Eighty eight clones were screened and *Dehalogenimonas sp.* constituted the predominant member, representing 65 of the 88 clones (87 %), followed by *Desulfovibrio sp.* (12 %) and unclassified *Veillonellaceae* (1.2%). Hence, further works to isolate *Dehalogenimonas sp.* with additional approach are currently attempted in our lab.

6.1. Introduction

The genus *Dehalogenimonas* belongs to the *Chloroflexi* (Phylum), *Dehalococcoidia* (Class), *Dehalococcoidales* (Order) and *Dehalococcoidaceae* (Family) [1], [2]. At present, the genus *Dehalogenimonas* contains three isolated species (*Dehalogenimoas lykanthroporepellens*, *Dehalogenimonas alkenigignens and Dehalogenimonas formicexedens*) and one species in an enriched culture (*Dehalogenimonas* sp. strain. WBC-2) [3]–[6].

Dehalogenimonas lykanthroporepellens was discovered in 2009 and it was isolated from groundwater contaminated by high concentrations of chlorinated alkanes and alkenes at a Superfund Site located near Baton Rouge, Louisiana (USA) [3], [7]. The two strains of *Dehalogenimonas lykanthroporepellens* designated BL-DC-8 and BL-DC-9 were 0.3 – 0.6 μm in diameter [3].

The second species, *Dehalogenimonas alkenigignens*, was later recovered in 2013 from a different portion of the same Superfund Site but in a contaminated groundwater plume containing much lower concentrations of chlorinated contaminants (tens rather than hundreds of mg L^{-1}) [4]. The two isolated *Dehalogenimonas alkenigignens* strains namely IP3-3T and SBP-1 had bigger diameters of 0.4 – 1.1 μm compared to *Dehalogenimonas lykanthroporepellens* species.

Most recently, a new species named D*ehalogenimonas formicexedens* was isolated from the contaminated groundwater in Louisiana (USA), the same site where *Dehalogenimonas alkenigignens* was discovered previously. The strain reductively dehalogenated polychlorinated aliphatic alkanes and can utilize either formate or hydrogen as electron donor. 16S rRNA gene sequence based phylogenetic analysis indicated that this strain was closely related to but distinct

from type strains of the species *Dehalogenimonas alkenigignens* (97.63 % similarity) and *Dehalogenimonas lykanthroporepellens* (95.05 % similarity) [6].

All three *Dehalogenimonas* species exclusively couple growth with the reductive dehalogenation of polychlorinated alkanes with chlorines on vicinal carbons including 1,2-DCA, 1,2-DCP, 1,1,2-TCA, 1,1,2,2-TeCA, and 1,2,3-TCP, when provided with hydrogen as the electron donor. These species do not utilize aliphatic alkanes that contain only a single chlorine substituent (1-chloropropane, 2-chloropropane), aliphatic alkanes with multiple chlorine substituents occurring only on one carbon atom (1,1-dichloroethane, 1,1,1-trichloroethane), chlorinated methanes [dichloromethane (methylene chloride), trichloromethane (chloroform), tetrachloromethane (carbon tetrachloride)], chlorinated benzenes (1-chlorobenzene, 1,2-dichlorobenzene) or chlorinated ethenes (tetrachlorothylene, trichloroethylene, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene (*trans*-DCE), or vinyl chloride (VC)). Their growth is not supported by acetate, butyrate, citrate, ethanol, fructose, fumarate, glucose, lactate, lactose, malate, methanol, methyl ethyl ketone, propionate, pyruvate, succinate or yeast extract in the absence of hydrogen [1], [3], [4], [7]. These species are strictly anaerobic, mesophilic, Gram-negative staining, non-spore-forming, with cells that are small, irregular cocci and resistant to ampicillin and vancomycin [1], [3], [4].

In 2012, the *Dehalogenimonas* population contained in a mixed microbial culture named WBC-2 was shown to be responsible for *trans*-DCE hydrogenolysis to VC [5], [8]. This enrichment derived from sediments contaminated with 1,1,2,2-TeCA from the West Branch Canal Creek Consortium, Maryland (USA). Their capability to transform a chloroethene is a distinctive difference and a remarkable exception among the *Dehalogenimonas* spp. isolated to date. On a 16S rRNA gene sequence basis, the *Dehalogenimonas* population from the WBC-2

culture is 96% similar to *Dehalogenimonas lykanthroporepellens*, while it is only 91% similar to *Dehalococcoides* [5], [8].

When grown in pure culture, all the isolates have been shown to carry out reductive dechlorination in the pH range of 6.0–8.0, but not at pH ≤ 5.5 or ≥8.5. The oxidation-reduction potential measured on-site at the time of groundwater collection in the samples from which *Dehalogenimonas alkenigignens* and *Dehalogenimonas lykanthroporepellens* type strains were first isolated ranged from −46 to −124 mV, indicating reducing conditions. Habitats of *Dehalogenimonas* appear to be soils and groundwater contaminated with chlorinated solvents. Their "natural" habitat devoid of anthropogenic contamination remains unknown because all electron acceptors described to date to support their growth contained halogens [1].

In this chapter, we attempted to isolate a *Dehalogenimonas* strain growing with 1,2-DCP. The enrichment and isolation procedures for organohalide-respiring bacteria involve strict anaerobic techniques because they are very sensitive to oxygen exposure. The procedures applied to the stable enrichment culture obtained in Chapter 4 included the use of different antibiotics and the dilution-to-extinction method in liquid and semisolid medium. The purity of the cultures was later assessed with 16S rRNA clone library and DGGE.

6.2. Materials and Methods

6.2.1. Chemicals

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich at the highest purity available as described in Chapter 3. The Mo Bio Ultra Clean Water DNA extraction kits were obtained from Qiagen (Carlsbad, USA). The PCR, DGGE reagents and clone library TOPO-TA cloning kit were purchased from Invitrogen (Carlsbad, USA).

6.2.2. Dilution to extinction method in semisolid and liquid medium

The isolation process was carried out in 20 mL glass vials containing 12 mL (final volume) of anoxic liquid or semisolid (soft agar) medium. Equal number of vials (dilution tubes) for semisolid and liquid were prepared. Semisolid medium contained 0.5% (weight per volume) low melting agarose in each vial before autoclaving. Once autoclaved, agar shakes were kept liquid at 35 °C until inoculated. Filter sterilized Na_2S-Cysteine, $NaHCO_3$ and vitamin solution were added through the septum to each medium with a final concentration of 20 mM, 1 M, and 0.05 mg L^{-1}, respectively. Under these conditions, sequential dilution-to-extinction series were performed up to $1:10^9$ dilution. Dilutions series were generated through serial 1 in 10 dilutions starting from either a colony-pick (agar plug) or 1 mL liquid aliquot of a previous culture (Figure 6.1). The vials were sealed with Teflon-coated butyl rubber septa and aluminum crimp caps and gassed with N_2/CO_2 (4:1, v/v, 0.2 bar overpressure) and H_2 (added to an overpressure of 0.4 bar). 1,2-DCP (50 μM) was added by syringe as electron acceptor. The vials were incubated upside down in the dark at 25 °C. The dilution-to-extinction series in semisolid and liquid medium were monitored at least biweekly for colony formation and dechlorination activity by gas chromatography, respectively. The vial with liquid medium showing dechlorinating activity at the highest dilution factor was selected as inoculum for the next serial dilution. This inoculum was also re-inoculated back to 120-mL serum bottles to monitor the microbial community transitions by DGGE. This serum bottles contained 65 mL of liquid medium described in Chapter 3 containing either 1 M bicarbonate or 10 mM HEPES buffer (CO_2 free medium), as indicated.

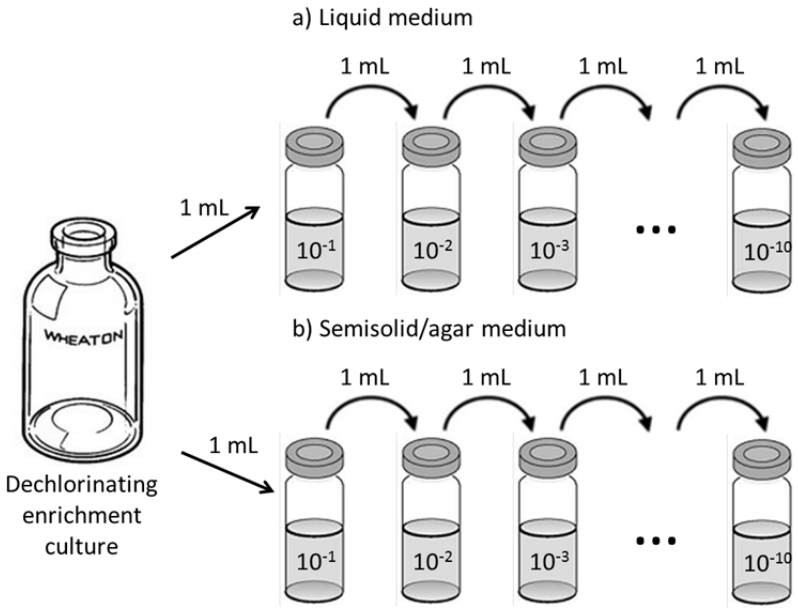


Figure 6.1: Preparation of dilution-to-extinction series in liquid and semisolid medium for obtaining isolates.

6.2.3. Resistance to antibiotics

To eliminate the undesirable bacterial populations accompanying *Dehalogenimonas* sp in the culture, the antibiotics chloroamphenicol (50 μg mL^{-1}), nalidixic acid (50 μg mL^{-1}), tetracycline (50 μg mL^{-1}), kanamycin (200 μg mL^{-1}), streptomycin (50 μg mL^{-1}), and ampicillin (400 μg mL^{-1}) were tested. All antibiotics were added from anoxic and sterile stock solutions.

6.2.4. DNA extraction

Genomic DNA was extracted from 65 mL of the cultures using an UltraClean water DNA isolation kit (MoBio, Carlsbad, USA) following the instructions of the manufacturer.

6.2.5. PCR

Each 50 μL reaction mixture contained 25 ng μL^{-1} of template DNA, 1× PCR buffer (20 mM Tris/HCl, pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer and 2.5 U of Taq DNA polymerase (Invitrogen, Carlsberg,

USA). The PCR amplification were done using a touch-down protocol: 94 °C for 5 min; 20 cycles of 94 ºC for 1 min, 65 ºC for 1 min (with a 0.5 ºC decrease for each cycle), 72 ºC for 3 min; and 15 more cycles of 94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 3 min; and a single final extension at 72 ºC for 7 min was performed to allow completion of PCR amplified fragments.

6.2.6. DGGE

DGGE was carried out using a Bio-Rad DCode Universal Mutation Detection System. 900 ng PCR products were loaded onto a 6% (w/v) polyacrylamide gel (acrylamide/bis solution 37.5:1) with 30− 70% denaturant gradient (100% denaturant contained 7 M urea and 40% v/v deionized formamide). Electrophoresis was performed at 60 °C with a constant voltage of 75 V for 16 h. The gels were stained with ethidium bromide (0.5 μg mL^{-1}), washed out with deionized water for 25 min and photographed with Universal Hood II (Bio-Rad, USA). Prominent bands were excised from the gels and amplified DNA fragments from recovered gel bands were further sequenced at external facilities (Macrogen Inc., South Korea).

6.2.7. Clone library

Fragments of 920 bp from regions V1-V4 were used to construct clone libraries amplified with the primers 27f-907r with the same reaction mixture as DGGE except 300 nM instead of 0.5 μM. Amplifications were done by 95 ºC for 2 min; 30 cycles of 94 ºC for 30 s, 52 ºC for 30 s, 72 ºC for 1.30 min (+1 s/cycle); and a single final extension at 72 ºC for 5 min. The clone library was then generated with the TOPO-TA Cloning Kit (Invitrogen) following the manufacturer´s instructions. Clones were selected using Kanamycin resistance (50 μg mL^{-1}) and picked randomly in a 96-well plate. Insert size was checked by PCR and further sequenced at Macrogen Inc., South Korea. The sequences were analyzed by performing an Operational Taxonomical Unit-based approach in MOTHUR.

6.3. Results and Discussion

6.3.1. Dilution-to-extinction approach in liquid medium

The dilution-to-extinction approach was applied to the *Dehalogenimonas*-containing cultures enriched with 1,2-DCP and maintained on hydrogen and acetate. As stated in Chapter 4, the methanogens were eliminated by BES and no further methane production was observed. The dechlorination rate was not affected by the addition of vancomycin.

With the aim of isolating *Dehalogenimonas*, three sets of dilution-to-extinction lines were performed. In the first set (Set I) and second set (Set II), the inoculum derived from the 10^{th} sequential transfer of the stable enrichment culture and was exposed to BES and vancomycin for seven transfers. Meanwhile, the inoculum for the third set (Set III) was 13^{th} sequential transfer of the enrichment culture and was exposed to BES and vancomycin for ten transfers. The graphical schemes of the isolation procedure are shown in Figure 6.2 – 6.4.

At each step/transfer of the highest dilution vials with dechlorination activity, cultures were also transferred in the serum bottles containing either bicarbonate buffer or HEPES buffer. HEPES buffer, amended in a CO_2-free medium, was used to replace bicarbonate buffer and eliminate H_2/CO_2 acetogens from our H_2-fed enrichment cultures [9].

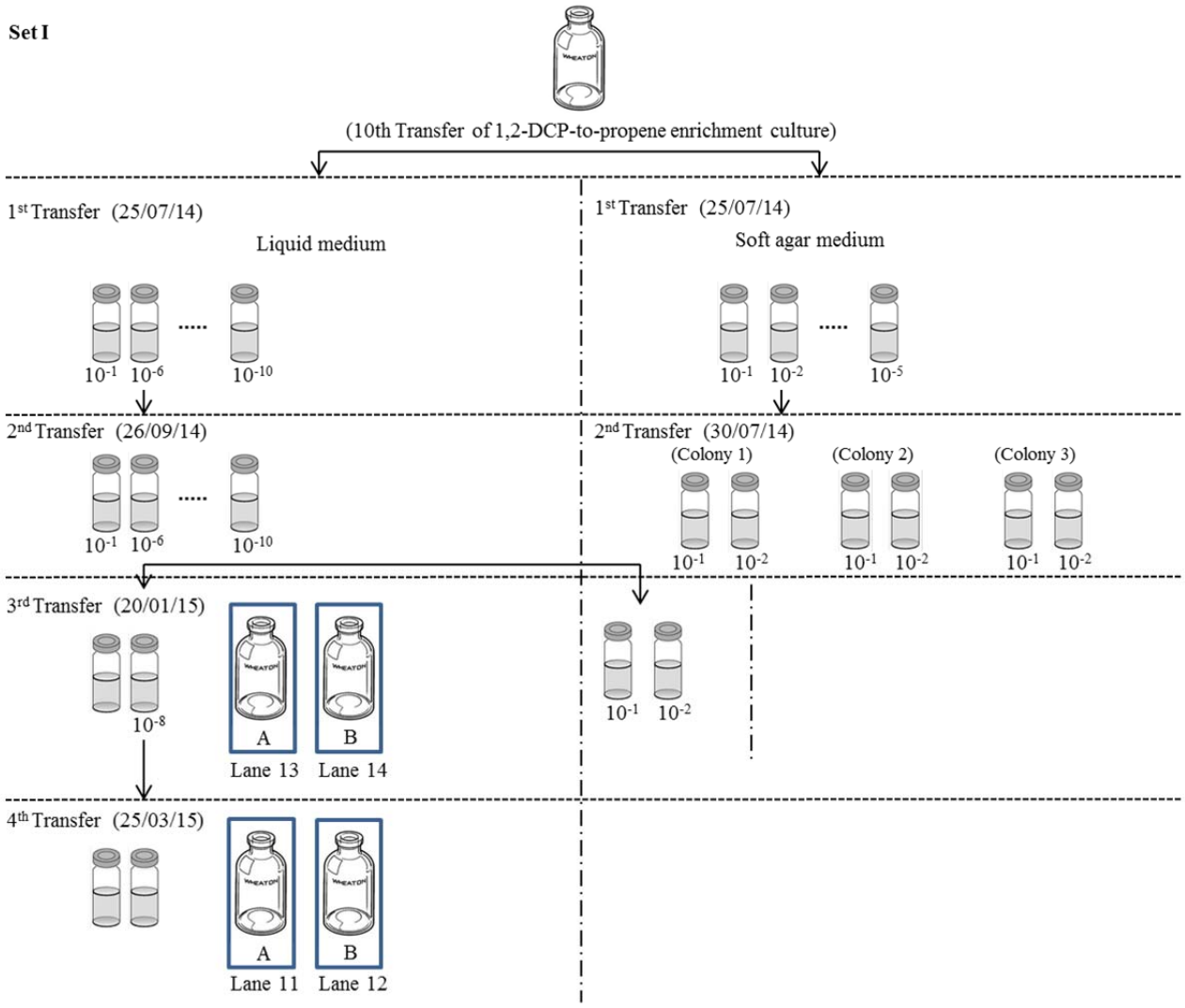


Figure 6.2: Scheme of the isolation procedure (Set I). Arrow indicates the dilution vial used as inoculum for the next serial dilution. Symbols: A: bottles with bicarbonate buffer, B: bottles with HEPES buffer. The cultures analyzed by DGGE were delimited with a square box and were marked with the lane number of the DGGE depicted in Figure 6.5.

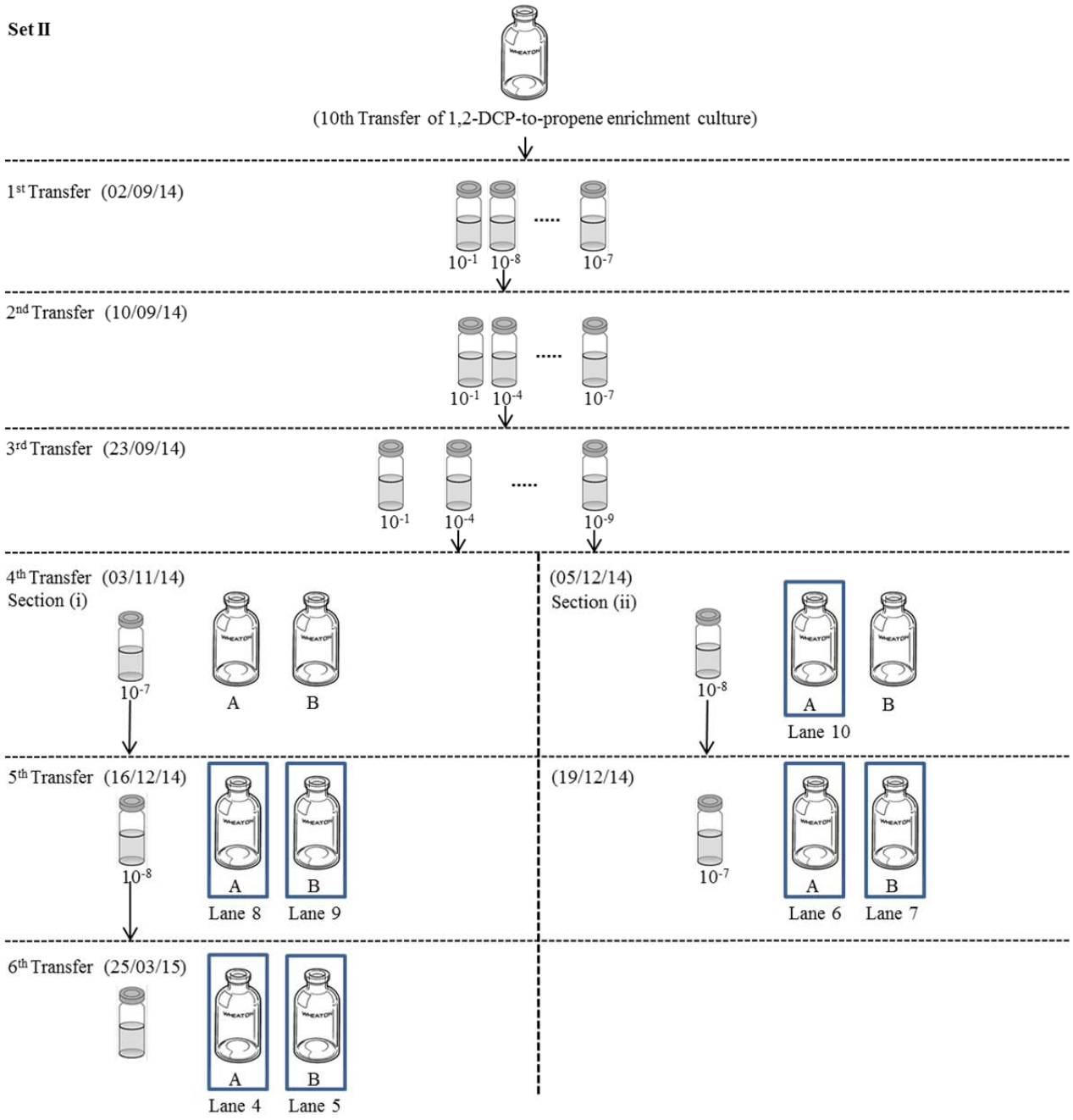


Figure 6.3: Scheme of the isolation procedure (Set II). Arrow indicates the dilution vial used as inoculum for the next serial dilution. Symbols: A: bottles with bicarbonate buffer, B: bottles with HEPES buffer. The cultures analyzed by DGGE were delimited with a square box and were marked with the lane number of the DGGE depicted in Figure 6.5.

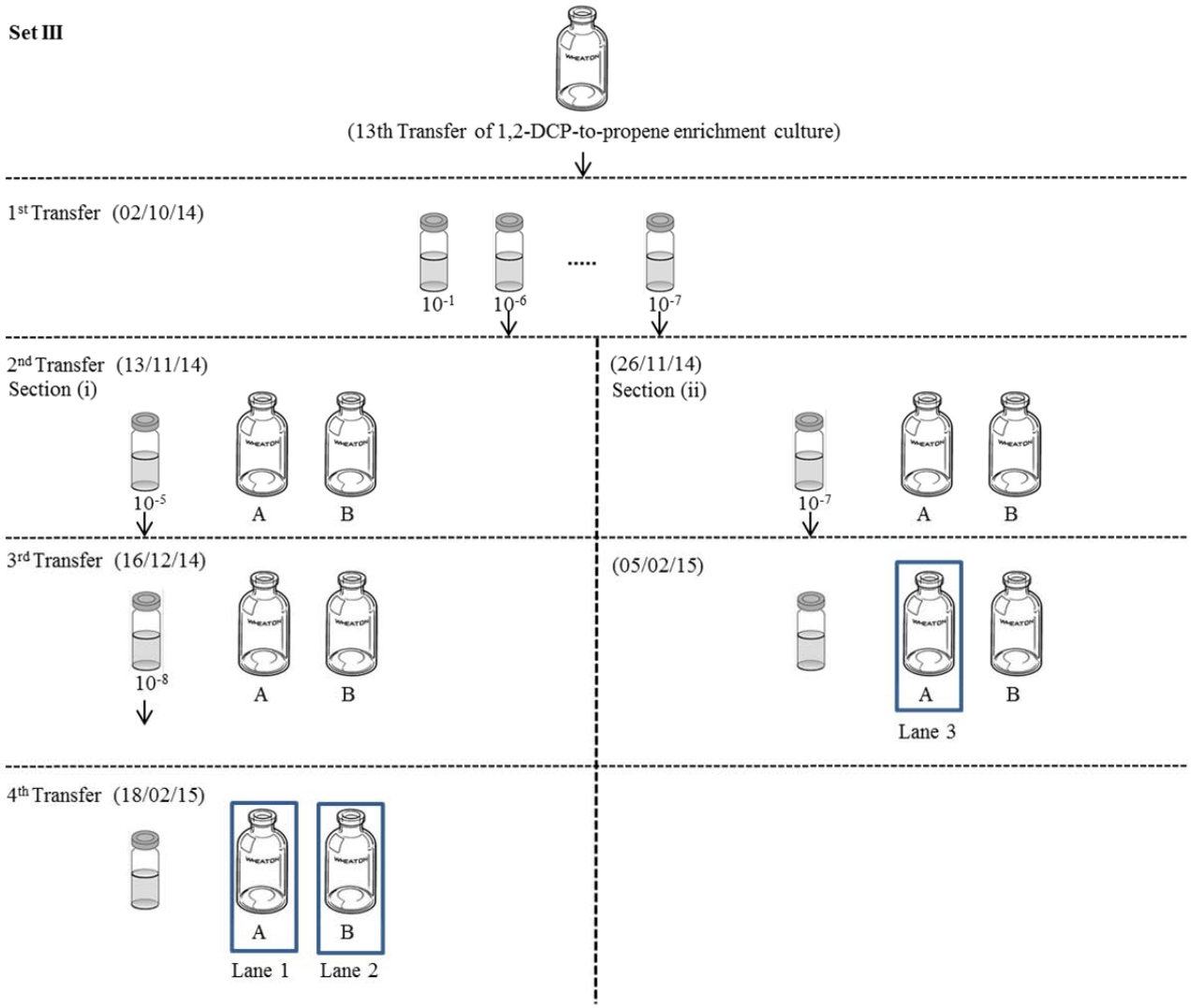


Figure 6.4: Scheme of the isolation procedure (Set III). Arrow indicates the dilution vial used as inoculum for the next serial dilution. Symbols: A: bottles with bicarbonate buffer, B: bottles with HEPES buffer. The cultures analyzed by DGGE were delimited with a square box and were marked with the lane number of the DGGE depicted in Figure 6.5.

The diversity of the bacterial community at different dilution steps was analyzed by DGGE (Figure 6.5). The DGGE with bacterial primers mainly detects the major constituents of the analyzed community overlooking the less abundant but it is a useful tool to monitor the enrichment process.

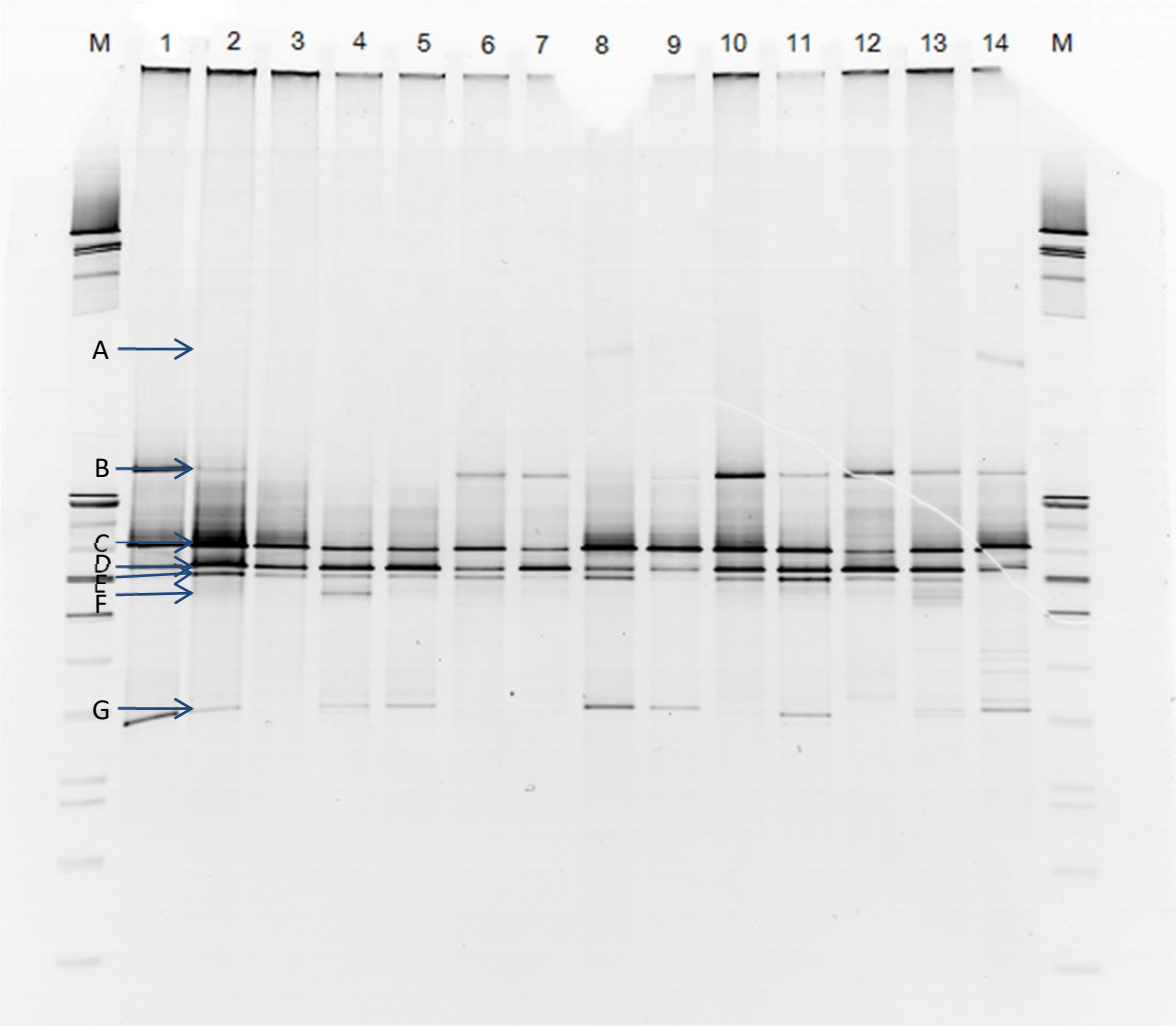


Figure 6.5: DGGE patterns of 16S rRNA fragments obtained from DNA extracted from serially diluted cultures of the enrichment. Lane M: Marker, Lane 1–14 represents the cultures that were marked in Figure 6.2 – 6.4.

A total of six genus of bacteria were detected in the DGGE bands during the dilution-to-extinction process (Table 6.1). All these bacterial genera were previously detected in the DGGE analysis during the consortium stabilization of our 1,2-DCP-to-propene *Dehalogenimonas*-containing consortia (Chapter 4). *Sphaerochaeta sp., Azonexus sp., Geobacter sp.,* and *Desulfovibrio sp.* are frequently detected in enrichment cultures containing organohalide-respiring bacteria [10]–[14], although their role in the consortium is not fully understood. For instance, the presence of the genus *Desulfovibrio* has been shown to enhance the growth and dechlorination rate of *Dehalococcoides mccartyi* strain 195, suggesting that strain 195 benefited from the organic cofactors (i.e. corrinoids) produced by *Desulfovibrio* [15], [16]. Similarly, *Geobacter sulfurreducens* has complete sets of cobamide biosynthesis genes and produce extracellular cobamide, which is an essential cofactor for organohalide-respiring bacteria such as *Dehalogenimonas* or *Dehalococcoides*, which lack the ability for *de novo* cobamide biosynthesis [17]. Also, *Sphaerochaeta* sp., commonly co-occur with organohalide-respiring bacteria and, although this association is not clear, it has been hypothesized that it may provide essential substrates (i.e. acetate and hydrogen) during fermentation of organic substrates or helps to protect them from oxygen due to their oxygen tolerance [18]. However the presence of *Acholeplasma sp.* in the cultures was surprising as this strain is normally found on animals, plants and insects though it can grew both under aerobic or anaerobic conditions [19].

Table 6.1: List of bacteria identified for the DGGE in Figure 6.5.

Label in Figure 3.5	Bacteria	% Identity	Accession Number
A	*Sphaerochaeta* sp.	91%	JN944166
B	*Acholeplasma parvum*	91%	NR_042961
C	*Dehalogenimonas alkenigignens*	100%	NR_109657
D	*Azonexus caeni*	100%	NR_041017
E	*Geobacter sulfurreducens*	99%	CP010430
F	*Desulfovibrio* sp.	99%	HE600849
G	*Sphaerochaeta globosa*	99%	CP002541

The DGGE profiles of the isolation cultures in Figure 6.5 revealed that Lane 3 had the lowest number of bands, suggesting that it was the most purified culture. This culture derived from the 13th sequential transfer of 1,2-DCP-to-propene consortia after two consecutive 10^{-7} dilutions (Figure 6.4). The three bands in Lane 3 were identified as *Dehalogenimonas alkenigignens, Azonexus caeni* and *Geobacter sulfurreducens*. Therefore, the line of this culture was chosen for the next purification steps using antibiotics.

Aside from that, we found out that using HEPES buffer instead of bicarbonate buffer did not exert any significant effect for the isolation process as the homoacetogens were absent from the culture. The absent of homoacetogens were not known until we conducted the DGGE (Table 4.4, Chapter 4) and for this reason we included this treatment. In the next steps of the isolation process, the use of HEPES buffer in the medium was ceased and only bicarbonate buffer was used.

6.3.2. Dilution to extinction in semisolid/agar medium

In the first round of dilution-to-extinction of agar shakes, three types of colonies were observed in 10^{-1} and 10^{-2} dilution (Figure 6.2, Set I, 1st transfer, agar medium) after one week of incubation. The first colony was irregular, moderate size and grey (colony type 1), the second

colony was circular, black and small (colony type 2), and the third colony was circular, white and small (colony type 3) as depicted in Figure 6.6. The two first colonies can be visually observed but become unclear in Figure 6.6. All these colonies were later transferred for a second dilution series in soft agar medium vials (see Figure 6.2).

Figure 6.6: Agar shake tubes (10^{-2} dilution) for first round of dilution-to-extinction isolation in semisolid medium. Arrows indicating/pointing to the colonies type 3 described in the paragraph above.

In the second dilution series, only colony type 3 was observed again after 2 months of incubation (Figure 6.2, Set I, 2nd transfer). However, this colony was transferred to a liquid culture medium spiked with 1,2-DCP but it did not show dechlorination activity. Further transfers to agar shakes vials also did not form any colony after several months of incubation. Therefore, we hypothesized that the colony we observed here did not belong to *Dehalogenimonas* but to other bacteria in the consortia. This is in accordance to *Dehalogenimonas lykanthroporepellens* and *Dehalogenimonas alkenigignens* isolates, which did not form any visible colonies on media solidified with agar or gellan gum even after 2 months incubation [3], [4], [7].

6.3.3. Use of selected antibiotics for further purification of the culture.

The culture that predominantly contained *Dehalogenimonas alkenigignens, Azonexus caeni* and *Geobacter sulfurreducens* achieved in section 6.3.1. was chosen for the next purification process using antibiotics specifically selected against the genus *Azonexus* and *Geobacter*.

On the one hand, the members of the *Geobacter* genus are metal-reducing microorganisms and they are obligate anaerobes. They are prominent members of the microbial community in a diversity of environments in which dissimilatory metal reduction is either naturally or artificially stimulated [10]. According to previous studies, *Geobacter sulfurreducens* can be inhibited by several antibiotics such as chloramphenicol (10 μg mL^{-1}), nalidixic acid (10 μg mL^{-1}), tetracycline (10 μg mL^{-1}), kanamycin (200 μg mL^{-1}), spectinomycin (50 μg mL^{-1}), streptomycin (400 μg mL^{-1}) and ampicillin (400 μg mL^{-1}) [10]. On the other hand, *Azonexus caeni* is a denitrifying bacterium isolated from freshwater environments [20]. *Azonexus sp.* grows under anaerobic conditions, but is slower than under aerobic conditions. All strains of the *Azonexus sp.* have shown sensitive to ampicillin (10 μg mL^{-1}), chloroamphenicol (30 μg mL^{-1}), erythromycin (15 μg mL^{-1}), gentamycin (10 μg mL^{-1}), novobiocin (30 μg mL^{-1}), penicillin G (10 μg mL^{-1}), kanamycin (30 μg mL^{-1}), nalidixic acid (30 μg mL^{-1}), streptomycin (10 μg mL^{-1}) and tetracycline (30 μg mL^{-1}) [12].

On the basis of these previous reports, chloramphenicol (50 μg mL^{-1}), nalidixic acid (50 μg mL^{-1}), tetracycline (50 μg mL^{-1}), kanamycin (200 μg mL^{-1}), streptomycin (50 μg mL^{-1}), and ampicillin (400 μg mL^{-1}) that simultaneously showed sensitivity to *Geobacter sp.* and *Azonexus sp.* were individually amended in parallel *Dehalogenimonas*-containing cultures. Dechlorination of 1,2-DCP to propene was monitored to assess if these antibiotics provoked a negative effect on

Dehalogenimonas growth. The sensitivity of *Dehalogenimonas sp.* towards the tested antibiotics except ampicillin was not known and therefore only the antibiotics allowing dechlorination activity were chosen for further transfers to isolate *Dehalogenimonas*.

The time-course dechlorination of 1,2-DCP growing with the tested antibiotics is shown in Figure 6.7. After one month of incubations, only the cultures with ampicillin, nalidixic acid and streptomycin achieved complete dechlorination of 1,2-DCP. Cultures that grew with kanamycin and tetracycline reached 30 % dechlorination but the culture loss the dechlorination capability afterwards. Meanwhile, culture with chloramphenicol did not dechlorinate within one month of incubation period.

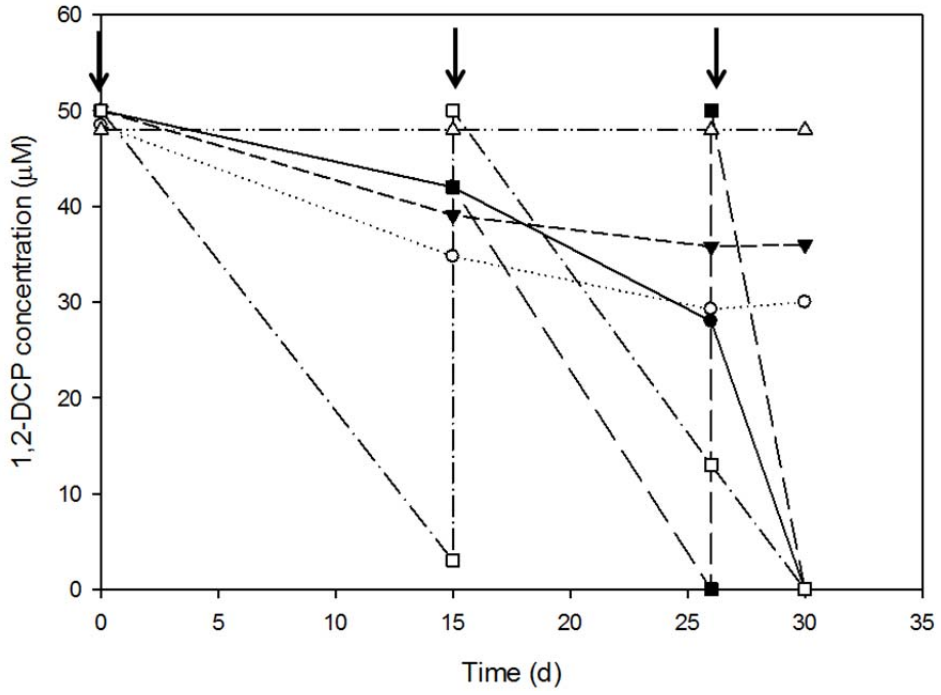


Figure 6.7: Reductive dechlorination of 1,2-DCP in the *Dehalogenimonas* enrichment culture with different antibiotics. Symbols: (●) Ampicillin; (○) Kanamycin; (▼) Tetracycline; (Δ) Chloramphenicol; (■) Nalidixic acid; and (□) Streptomycin. Each treatment was done in duplicate. Arrows indicates the addition of 1,2-DCP.

From this result, we transferred (5 % v/v) the cultures with ampicillin, nalidixic acid and streptomycin to fresh medium containing the corresponding antibiotic and the microbial composition was analyzed by DGGE at different transfer points (Figure 6.8). After six and five sequential transfers with ampicillin and nalidixic acid, respectively, these cultures still contained three predominant bands (Figure 6.8, Lane 1 and 2). However, the cultures with streptomycin contained only two bands after three transfers, and we decided to further transfer the culture with this antibiotic.

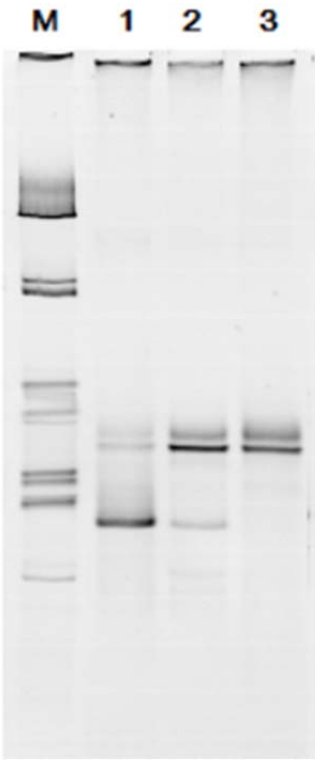


Figure 6.8: DGGE patterns of 16S rRNA fragments obtained from DNA extracted from serially diluted cultures with the antibiotics ampicillin (Lane 1, sixth transfer), nalixidic acid (Lane 2, fifth transfer) and streptomycin (Lane 3, third transfer). Lane M: marker.

After four and five sequential transfers with streptomycin, only one prominent single band was detected (Figure 6.9). The band was sequenced and identified as *Dehalogenimonas sp.* suggesting that this strain was isolated.

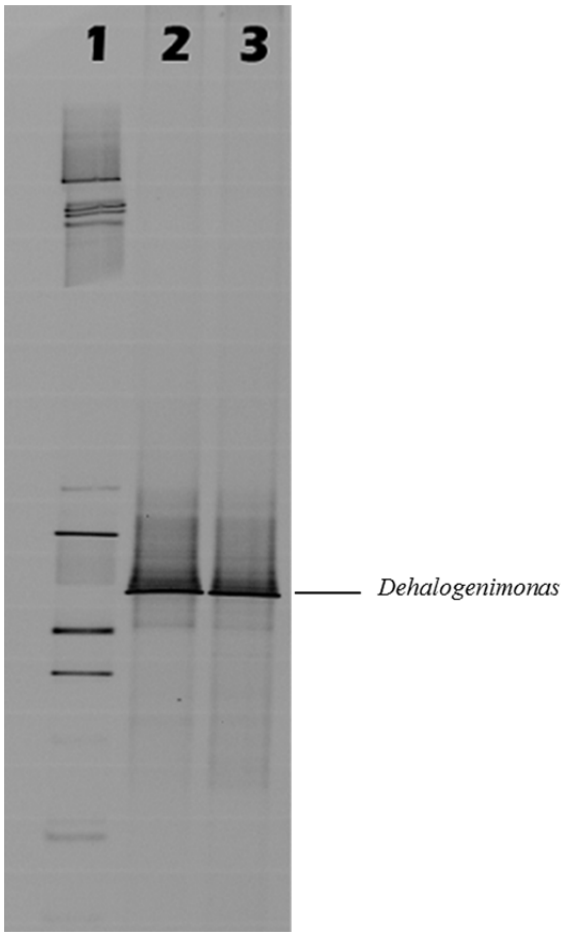


Figure 6.9: DGGE patterns of 16S rRNA fragments obtained from DNA extracted from serially diluted cultures with the antibiotic streptomycin. Lane 1: Marker; Lane 2: fourth transfer; Lane 3: fifth transfer.

To provide an additional evidence of the purity of *Dehalogenimonas* in this culture, a clone library of bacterial amplicons was conducted. Eighty-eight clones were screened and *Dehalogenimonas* sp. constituted the predominant member, representing 65 of the 88 clones (87

%), followed by *Desulfovibrio* sp. (12 %) and unclassified *Veillonellaceae* (1.2%) (Table 6.2). The traditional gel-based DGGE is estimated to detect only community members representing at least 1–2% of the microbial population in an environmental sample, and it may explain their non-detection in the previous DGGE (Figure 6.9) [21].

Table 6.2: Microbial composition of the clone library performed with the *Dehalogenimonas*-enrichment culture growing with streptomycin after five consecutive transfers.

Closest match	Abundance (%)	Clone Numbers	16S rRNA gene copies
Dehalogenimonas sp.	86.77	65	1
Desulfovibrio sp.	12.01	18	2
Unclassified *Veillonellaceae*	1.21	5	5.5
Total	100	88	

Desulfovibrio sp. is a sulfur reducing bacteria that has been commonly detected in dechlorinating communities enriched from contaminated groundwater [16], [22], [23]. Up to now, two *Desulfovibrio* spp. isolated from marine sediments have shown to use halophenols as electron acceptors [24]. *Desulfovibrio sp.* might utilized trace quantities of sulfate oxidized from sulfur-containing reductants (e.g., sodium sulfide, L-cysteine or DL-dithiothreitol) as electron acceptors for growth [25].

Though the aim of this chapter was obtaining a pure *Dehalogenimonas* strain, the presence of *Desulfovibrio* sp. may eventually bring an advantage to the cultures. As stated previously, *Desulfovibrio* may provide nutritional requirements for organohalide-respiring bacteria facilitating their co-existence [25]. *Desulfovibrio* sp. ferments a wide variety of organic compounds (e.g., hexoses, lactate, pyruvate, and butyrate) into H_2 and acetate to generate energy in the absence of exogenous terminal electron acceptors, which can be used as electron donor

and carbon source for *Dehalogenimonas*, respectively [15]. In addition, *Desulfovibrio* could use CO as carbon source, a byproduct of the metabolism of organohalide respiring bacteria that is released into the medium and can act as growth inhibitor, bringing its concentration down by an order of magnitude [26]. To illustrate the benefits of these synergistic relationships, *Dehaloccoides mccartyi* strain 195 can grow to densities 1.5 times greater under the presence of *Desulfovibrio desulfuricans* when compared to the isolate growing alone [15]. However, additional approaches are currently underway in our lab to isolate *Dehalogenimonas* sp. from this co-culture using specific antibiotics targeting *Desulfovibrio* species.

6.4. Conclusions

By using the dilution-to extinction approach in liquid medium, we obtained a culture that was predominantly constituted by three bacteria (*Azonexus*, *Geobacter* and *Dehalogenimonas* sp.) according to DGGE analysis. A screening of different antibiotics targeting *Azonexus* and *Geobacter* was performed and a co-culture mainly composed by *Dehalogenimonas* and *Desulfovibrio* was finally obtained, as evidenced by a clone library. No visible colonies of *Dehalogenimonas* were observed in dilution-to-extinction approach using soft agar medium, which is in accordance to previous described *Dehalogenimonas* isolates. Although organohalide-respiring bacteria showed better capability to grow and dechlorinate in co-culture rather than in pure isolates, obtaining a pure strain can facilitate genomic, proteomic, and biochemical analysis in the future. Hence, further work is currently underway in our lab to obtain a pure isolate of *Dehalogenimonas*.

6.5. References

[1] W. M. Moe, F. A. Rainey, and J. Yan, "The genus *Dehalogenimonas*," in *Organohalide-Respiring Bacteria*, L.Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 137–151.

[2] F. E. Löffler, J. Yan, K. M. Ritalahti, L. Adrian, E. A. Edwards, K. T. Konstantinidis, J. A. Müller, H. Fullerton, S. H. Zinder, and A. M. Spormann, "*Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and famil," *Int. J. Syst. Evol. Microbiol.*, vol. 63, pp. 625–635, 2013.

[3] W. M. Moe, J. Yan, M. F. Nobre, M. S. da Costa, and F. A. Rainey, "*Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater," *Int. J. Syst. Evol. Microbiol.*, vol. 59, no. 11, pp. 2692–2697, 2009.

[4] K. S. Bowman, M. F. Nobre, M. S. da Costa, F. A. Rainey, and W. M. Moe, "*Dehalogenimonas alkenigignens* sp. nov., a chlorinated-alkane-dehalogenating bacterium isolated from groundwater," *Int. J. Syst. Evol. Microbiol.*, vol. 63, pp. 1492–1498, 2013.

[5] M. J. Manchester, L. A. Hug, M. Zarek, A. Zila, and E. A. Edwards, "Discovery of a trans-dichloroethene-respiring *Dehalogenimonas* species in the 1,1,2,2-tetrachloroethane-dechlorinating WBC-2 consortium," *Appl. Environ. Microbiol.*, vol. 78, no. 15, pp. 5280–5287, 2012.

[6] T. A. Key, K. S. Bowman, I. Lee, J. Chun, M. da Costa, L. Albuquerque, F. A. Rainey, and W. M. Moe, "*Dehalogenimonas formicexedens* sp. nov., a chlorinated alkane respiring bacterium isolated from contaminated groundwater," *Int. J. Syst. Evol. Microbiol.*, In press, doi: 10.1099/ijsem.0.001819, 2017.

[7] J. Yan, B. A. Rash, F. A. Rainey, and W. M. Moe, "Isolation of novel bacteria within the Chloroflexi capable of reductive dechlorination of 1,2,3-trichloropropane," *Environ. Microbiol.*, vol. 11, no. 4, pp. 833–843, 2009.

[8] O. Molenda, A. T. Quaile, and E. A. Edwards, "*Dehalogenimonas* sp. strain WBC-2 genome and identification of its trans-dichloroethene reductive dehalogenase, TdrA," *Appl. Environ. Microbiol.*, vol. 82, no. 1, pp. 40–50, 2016.

[9] J. R. Graber and J. A. Breznak, "Physiology and nutrition of *Treponema primitia*, an H_2/CO_2-acetogenic spirochete from termite hindguts.," *Appl. Environ. Microbiol.*, vol. 70, no. 3, pp. 1307–14, 2004.

[10] M. V Coppi, C. Leang, S. J. Sandler, and D. R. Lovley, "Development of a genetic system for *Geobacter sulfurreducens* development," *Appl. Environ. Microbiol.*, vol. 67, no. 7, pp. 3180–3187, 2001.

[11] K. M. Ritalahti, S. D. Justicia-Leon, K. D. Cusick, N. Ramos-Hernandez, M. Rubin, J. Dornbush, and F. E. Löffler, "*Sphaerochaeta globosa* gen. nov., sp. nov. and *Sphaerochaeta pleomorpha* sp. nov., free-living, spherical spirochaetes," *Int. J. Syst. Evol. Microbiol.*, vol. 62, no. 1, pp. 210–216, 2011.

[12] J. H. Chou, S. R. Jiang, J. C. Cho, J. Song, M. C. Lin, and W. M. Chen, "*Azonexus hydrophilus* sp. nov., a nifH gene-harbouring bacterium isolated from freshwater," *Int. J. Syst. Evol. Microbiol.*, vol. 58, no. 4, pp. 946–951, 2008.

[13] J. L. Steger, C. Vincent, J. D. Ballard, and L. R. Krumholz, "*Desulfovibrio* sp. genes involved in the respiration of sulfate during metabolism of hydrogen and lactate," *Appl. Environ. Microbiol.*, vol. 68, no. 4, pp. 1932–1937, 2002.

[14] A. Grostern and E. A. Edwards, "Growth of *Dehalobacter* and *Dehalococcoides* spp. during degradation of chlorinated ethanes," *Appl. Environ. Microbiol.*, vol. 72, no. 1, pp. 428–436, 2006.

[15] J. He, V. F. Holmes, P. K. H. Lee, and L. Alvarez-cohen, "Influence of vitamin B12 and cocultures on the growth of *Dehalococcoides* isolates in defined medium," *Appl. Environ. Microb.* vol. 73, no. 9, pp. 2847–2853, 2007.

[16] Y. Men, H. Feil, N. C. Verberkmoes, M. B. Shah, D. R. Johnson, P. K. H. Lee, K. A. West, S. H. Zinder, G. L. Andersen, and L. Alvarez-Cohen, "Sustainable syntrophic growth of *Dehalococcoides ethenogenes* strain 195 with *Desulfovibrio vulgaris* Hildenborough and *Methanobacterium congolense*: global transcriptomic and proteomic analyses.," *ISME J.*, vol. 6, no. 2, pp. 410–21, 2012.

[17] J. Yan, K. M. Ritalahti, D. D. Wagner, and F. E. Löffler, "Unexpected specificity of interspecies cobamide transfer from *Geobacter* spp. to organohalide-respiring *Dehalococcoides mccartyi* strains," *Appl. Environ. Microbiol.*, vol. 78, no. 18, pp. 6630–6, 2012.

[18] A. Caro-Quintero, K. M. Ritalahti, K. D. Cusick, F. E. Löffler, and K. T. Konstantinidis, "The chimeric genome of *Sphaerochaeta*: nonspiral spirochetes that break with the prevalent dogma in spirochete biology.," *MBio*, vol. 3, no. 3, pp. e00025-12, 2012.

[19] H. Atobe, J. Watabe, and M. Ogata, "*Acholeplasma parvum*, a new species from horses," *Int. J. Syst. Bacteriol.*, vol. 33, no. 2, pp. 344–349, 1983.

[20] Z. X. Quan, W. T. Im, and S. T. Lee, "*Azonexus caeni* sp. nov., a denitrifying bacterium isolated from sludge of a wastewater treatment plant," *Int. J. Syst. Evol. Microbiol.*, vol. 56, no. 5, pp. 1043–1046, 2006.

[21] A. Nocker, M. Burr, and A. K. Camper, "Genotypic microbial community profiling: A critical technical review," *Microb. Ecol.*, vol. 54, no. 2, pp. 276–289, 2007.

[22] M. Yohda, O. Yagi, A. Takechi, M. Kitajima, H. Matsuda, N. Miyamura, T. Aizawa, M. Nakajima, M. Sunairi, A. Daiba, T. Miyajima, M. Teruya, K. Teruya, A. Shiroma, M. Shimoji, H. Tamotsu, A. Juan, K. Nakano, M. Aoyama, Y. Terabayashi, K. Satou, and T. Hirano, "Genome sequence determination and metagenomic characterization of a *Dehalococcoides* mixed culture grown on *cis*-1,2-dichloroethene," *J. Biosci. Bioeng.*, vol. 120, no. 1, pp. 69–77, 2015.

[23] M. Madigan, J. M. Martinko, K. S. Bender, D. H. Buckley and D. A. Stahl , *Brock Biology of Microorganisms, 14th edn*, Glenview, Pearson, 2012.

[24] S. Atashgahi, Y. Lu, and H. Smidt, "Overview of known organohalide-respiring bacteria—phylogenetic diversity and environmental distribution," in *Organohalide-Respiring Bacteria*, L.Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 63–105.

[25] L. K. Lee, C. Ding, K. Yang, and J. He, "Complete debromination of tetra- and penta-brominated diphenyl ethers by a coculture consisting of *Dehalococcoides* and *Desulfovibrio* species," *Environ. Sci. Technol.*, vol. 45, no. 19, pp8474-8482, 2011.

[26] S. H. Zinder, “The genus *Dehalococcoides*,” in *Organohalide-Respiring Bacteria*, L.Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 107–136.

CHAPTER 7

IDENTIFICATION OF *Dehalogenimonas* REDUCTIVE DEHALOGENASES USING SHOT-GUN AND GEL-BASED PROTEOMIC TECHNIQUES

The research work presented in this chapter was carried out during a stay at the Helmholtz-Centre for Environmental Research (UFZ) under the supervision of Dr. Lorenz Adrian.

ABSTRACT

Dehalogenimonas strains show a high substrate specialization and exclusively mediate dichloroelimination of vicinal chlorinated alkanes. A major challenge is to identify the reductive dehalogenases catalyzing these reactions. In this study, blue native polyacrylamide gel electrophoresis (BN-PAGE) combined with enzymatic assays were used to identify *Dehalogenimonas* RDase involved in 1,1,2-TCA dichloroelimination. Shotgun proteomics using LTQ-Orbitrap mass spectrometer was also used to identify the RDases expressed during growth with 1,2-dibromoethane (EDB). Despite dechlorinating activity of 1,1,2-TCA to VC was found in a gel slice using the BN-PAGE approach, reductive dehalogenases were identified by neither LC-MS/MS analysis nor SDS-PAGE. However, shotgun proteomics identified a RDase belonging to *Dehalogenimonas* identified the 1,2-DCP-to-propene RDase DcpA in this culture. Similarly, this DcpA was also identified in the *Dehalogenimonas* culture growing with EDB at low levels. This study reveals that DcpA is a candidate to catalyze 1,1,2-TCA and EDB but it needs to be biochemically confirmed in future investigations.

7.1. Introduction

Chlorinated alkanes are widespread groundwater contaminants and a viable approach for their remediation is microbial reductive dechlorination [1]. *Dehalogenimonas* strains are known for their substrate specificity towards chlorinated alkanes, with the exception of *Dehalogenimonas* strain WBC2, dechlorinating vicinally chlorinated alkanes through dihaloelimination [2]. Reductive dechlorination of these pollutants is catalyzed by reductive dehalogenases (RDase if characterized, otherwise Rdh), and their catalytic unit is encoded by the RDase subunit A gene (*rdhA*). The functional characterization of RDases is of major interest because their detection in the contaminated groundwater can be used as a biomarker to predict dechlorinating pathways, monitoring metabolically active OHRB populations, and assess the potential of the polluted site for natural monitoring attenuation [3]. However, only a few *rdhA* genes have been functionally characterized because difficulties in obtaining sufficient biomass with slow-growing OHRB and the inability to express functional Rdh heterologously [4], [5].

The preliminary characterization of the activity of Rdhs was achieved by partial purification of the *Dehalococcoides* RDase TceA, PceA and VcrA [6], [7]. Later, Adrian et al. [8] identified the first chlorobenzene Rdh, CbrA, using a combination of clear native polyacrylamide gel electrophoresis (CN-PAGE), enzymatic assays, and liquid chromatography tandem mass spectrometry (LC-MS/MS). A remarkable advantage of this approach is that enables functional attribution without requiring large amounts of biomass. This CN-PAGE strategy was substantially improved using BN-PAGE that enhanced the recovery of dechlorinating activity after electrophoresis. An alternative approach to characterize the activity and substrate range of RDases consist of inferring them from transcriptional analysis, although

an important drawback is that several RDases are usually expressed under the presence of a single chlorinated compound and biochemical confirmation is still needed.

The genus *Dehalogenimonas* comprises four cultivated species and the sequence and annotation of their genomes have revealed that they harbor multiple distinct *rdhA* genes [2]. Sequenced genomes of *Dehalogenimonas lykanthroporepellens* BL-DC-9^T, *Dehalogenimonas alkenigignens* IP3-3^T, and *Dehalogenimonas formicexedens* revealed the presence of 25, 29, and 24 *rdhA* genes, respectively [9]–[11]. Experiments employing end-point reverse transcription-PCR (RT-PCR) in conjunction with primers targeting all predicted 25 *rdhA* genes in *Dehalogenimonas lykanthroporepellens* BL-DC-9^T revealed that 19 *rdhA* genes were consistently expressed in cultures actively dechlorinating three different electron acceptors, 1,2-DCA, 1,2-DCP, and 1,2,3-TCP [2], [12]. This simultaneous transcription of many *rdhA* genes and expression of the same genes during dechlorination of several chloroalkanes hampered the elucidation of the functionality of each RDase [12]. Protein assays with BN-PAGE and LC-MS/MS confirmed that *Dehalogenimonas lykanthroporepellens* BL-DC-9^T possessed a RDase that catalyzed 1,2-DCP-to-propene dichloroelimination and was designated DcpA, encoded by the *dcpA* gene [9]. The most closely related protein sequences to the DcpA of *D. lykanthroporepellens* BL-DC-9^T (92 % amino acid identity) are two putative RDases (GenBank accession numbers JX826286 and JX826287) harbored by *Dehalococcoides mccartyi* strains KS and RC, respectively. These *Dehalococcoides* strains couple growth with 1,2-DCP to-propene dechlorination in highly enriched cultures [9], [13]. The *dcpA* gene was also detected in our consortia enriched from Besòs River Estuary sediments, as stated in Chapter 4 [14]. Recently, a novel reductive dehalogenase designated TdrA, which catalyzes the dechlorination of *trans*-DCE to VC was identified in an enrichment culture containing *Dehalogenimonas* strain WBC2.

Dehalogenimonas strain WBC2 is interestingly the first *Dehalogenimonas* species found to dechlorinate chloroethenes [15], [16]. The TdrA shares 76.4% amino acid pairwise identity with TceA (AY165309.1) from *Dehalococcoides* sp. strain FL2 [16], [17]. Interestingly, TdrA showed poor identity with other *Dehalogenimonas* RDases, sharing 31% pairwise identity with the closest *Dehalogenimonas lykanthroporepellens* dehalogenase (GenBank accession number WP013217644) [12], [16].

In this chapter, we used a combination of BN-PAGE, enzyme assays, and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with the aim of identifying the *Dehalogenimonas* RDases involved in 1,1,2-TCA dichloroelimination. In addition, shot-gun proteomics was used in an attempt to identify the RDase expressed during growth with 1,2-dibromoethane (EDB).

7.2. Materials and methods

7.2.1. Chemicals

Vinyl chloride was obtained from Linde (Munich, Germany). All the other chemicals used were reagent grade (as listed in Chapter 3) unless otherwise specified.

7.2.2. Cultures and growth conditions

The *Dehalogenimonas*-containing enrichment culture described in Chapter 4 was used for the identification of the reductive dehalogenases catalyzing EDB and 1,2-DCP (used as positive control). The culture with 1,2-DCP derived from an original culture that was maintained for over three years with this electron acceptor. The cultures growing with EDB derived from the original culture growing with 1,2-DCP and was transferred twice (10% v/v) into fresh medium with EDB

before the proteomic analysis. The cultures analyzed accounted to a final cell density of *Dehalogenimonas* about 10^6 cells mL^{-1}.

To identify the reductive dehalogenase of *Dehalogenimonas* catalyzing 1,1,2-TCA dichloroelimination, a co-culture consisted of *Dehalogenimonas* and *Dehalococcoides mccartyi* strain BTF08 was used. The construction and establishment of the co-culture are further discussed in Chapter 8. The co-culture was maintained with 1,1,2-TCA for five transfer before proteomic analysis and reached a final cell density of about 10^7 cells mL^{-1}.

7.2.3. Preparation of crude protein extract

Cells were harvested from culture volumes of 200 to 400 mL by centrifugation under anoxic conditions at 5000 × *g* and 16 °C for 1 h. Afterwards, 50% of the supernatant was removed and the cells were centrifuged again under the same conditions. The cell pellet was re-suspended in 1 × PBS buffer (pH 7.2) or 1 × BN-PAGE sample buffer (pH 7.2; Invitrogen) containing 1% (wt/vol) digitonin. Soluble and insoluble crude extract fractions were separated by centrifugation for 20 min at 14000 × *g*. Supernatants (crude protein extract), containing solubilized proteins were transferred to new 1.5 mL Eppendorf tubes.

7.2.4. BN-PAGE gel electrophoresis and staining

BN-PAGE was performed under cooled conditions inside an anaerobic chamber using NativePAGE Novex Bis-Tris gel system (Invitrogen, Carlsbad, USA). The anode and cathode buffers were prepared according to the manufacturer's instructions in the NativePAGE running buffer kit (Invitrogen, Carlsbad, USA). The buffers were degassed and prechilled to 4 °C prior to use. A precast gradient gradient Bis-Tris gel (4 to 16% Bis-Tris; 1.0 mm thick; Invitrogen) was placed in the XCell SureLock minicell, and 5 μL of NativeMark unstained protein standard (Invitrogen) was loaded into one lane to serve as the size standard. Before loading the samples

on the gel, crude protein extracts were amended with 5% (wt/vol) G-250 sample additive from the NativePAGE sample prep kit (Invitrogen) to a final concentration of 0.25% (wt/vol). Volumes of 20-25 μL of crude protein extract were loaded into each of the other lanes of the gel. The remaining crude protein extract were stored on ice to be used as positive controls in subsequent dechlorination assays. Replicate lanes were prepared for (i) staining to visualize protein bands, (ii) excision of gel slices for SDS-PAGE, (iii) excision of gel slices for activity assays. The loaded gel was run successively at 150 V for 60 min, then at 250 V for 30 min.

Once the electrophoresis was complete, the lane containing the protein ladder and one lane loaded with the crude protein extract were cut from the rest of the gel using a scalpel and silver stained by the method of Nesterenko et al. [18]. The remainder of the gel was stored in anode buffer at 4 °C during the staining procedure.

7.2.5. Dechlorination activity assays using gel slices

To determine the locations of proteins in an unstained gel lane, the corresponding lanes with silver-stained proteins were aligned, and gel slices were excised using a scalpel. Individual gel slices were cut into 0.1 to 0.5 cm square pieces and were transferred to 10 mL crimp-top glass vials. For a positive control, 10 to 25 μL of the crude protein extract from the same original sample was added to an additional glass vials. In an anoxic chamber, the 10 mL crimp top vials containing the gel slices were tested for 1,1,2-TCA dechlorination adding 2 mL assay buffer that contained 200 mM potassium-acetate (pH 5.8), 2 mM titanium citrate, 2 mM methyl viologen, and 200 μM of 1,1,2-TCA. Crimp-top vials were closed with Teflon-coated septa immediately after assay buffer addition, thoroughly mixed, and stored upside down inside the anoxic chamber for 24 to 48 h at 30 ºC prior to headspace analysis.

To determine the concentration of chlorinated substrates and their dechlorination products following incubation, 0.5 mL headspace samples were respectively taken with a 1.0 mL pressurelock precision analytical syringe (Vici, USA) from the activity assay vials. All compounds were identified using retention times of chemical standards. A gas chromatograph (GC) model 6890N (Agilent Technologies; Santa Clara, USA) equipped with a HP-5 column (30 m × 0.32 mm with 0.25 μm film thickness; Agilent Technologies) and a flame ionization detector (FID) was used to analyze all volatile organic compounds. Helium was used as the carrier gas (0.9 mL min^{-1}). The injector and detector temperatures were both set at 250 °C. After the injection of the sample (split ratio = 10:1), the initial oven temperature (40 °C), ramped at 10 °C min^{-1} to 50 °C, and then ramped at 20 °C min^{-1} to 80 °C.

7.2.6. SDS-PAGE

Parallel gel slices from the second unstained lane were excised to elute proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). BN-PAGE gel slices were cut into 1 mm square pieces and transferred to 1.5 mL Eppendorf tubes containing 250 μL of SDS elution buffer (100 mM Tris-HCl [pH 7.0] and 0.1% [wt/vol] SDS). Following 12 to 20 h of shaking at 750 rpm, the solution containing eluted proteins was concentrated to 10 to 15 μL using an Amicon ultracentrifugal filter with a 10-kDa cutoff (Millipore, Billerica, USA) [4]. The entire volume of elution buffer was loaded onto a microcon centrifugal filter unit (Millipore) with a 10 kDa molecular cutoff, and spun for 30 min at 14,000 x g. To elute the concentrated sample, the filter was turned upside-down and spun for 3 min at 1,000 × g.

Since this is the first time we performed the SDS-PAGE for this culture, another alternative protein separation process was also tried. The unstained lane was cut from a BN-PAGE gel. Protein complexes separated in the gel lane were first denatured by incubation for 15

min in 10 ml ‘pre-treatment solution’ [40 mM Tris-HCl, pH 6.8, 0.36 g mL^{-1} urea, 30% (v/v) glycerol, 10 mg mL^{-1} SDS] amended with 5 mg mL^{-1} dithiothreitol (DTT) and then carbamidomethylated by incubating for 15 min in 10 mL ‘pre-treatment solution’ containing 45 mg mL^{-1} iodoacetamide. The pre-treated gel lane was placed horizontally on top of a 10% SDS-gel to resolve individual protein complex subunits under denaturing conditions according to their molecular mass. The electrophoresis was run at 90 V for 60–90 min using the Mini-Protean Tetra Cell System (Biorad, Berkeley, USA), and the gel was silver-stained as described previously by Nesterenko et al., 1994 [18], [19].

7.2.7. LC-MS/MS analysis

Silver-stained protein bands of interest obtained from SDS-PAGE were analysed via nLC-MS/MS. Gel slices were washed with ddH_2O and de-stained in a 1:1 (v/v) mixture of 30 mM $K_3[Fe(CN)_6]$ and 100 mM $Na_2S_2O_3$. Then the gel slices were washed in 50 mM ammonium bicarbonate and reduced with 10 mM DTT (in 50 mM ammonium bicarbonate) for 30 min at room temperature in the dark. Subsequent alkylation of cysteine residues was performed by incubating gel slices in 100 mM iodoacetamide (in 50 mM ammonium bicarbonate) for 30 min at room temperature in the dark. For in-gel protein digestion, 0.1 µg of porcine trypsin (Proteomic Sequencing Grade, Promega) was added and samples were incubated at 37°C overnight. The resulting peptides were extracted from the gel slice by incubation in 50% (v/v) acetonitrile and 5% (v/v) formic acid for 10 min at least two times and extracts were combined. Extracted peptide samples were desalted using C18 Zip Tip columns (Millipore) prior to analysis via nLC-MS/MS using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nanoUPLC system (nanoAcquity, Waters) as described previously [20], [21].

Peptide identification was conducted by PROTEOME DISCOVERER (v1.4.1.14, Thermo Fisher Scientific) using the Mascot search engine. Since the genome of the *Dehalogenimonas* used in this study has not been sequenced yet, a problem arises in having the protein database to be compared with the results from LC-MS/MS. To overcome this problem, the mass spectra from these samples were searched against a constructed database consisting of all the proteins harbored by the known sequenced *Dehalogenimonas, Dehalococcoides mccartyi* and *Dehalobacter* genus (Table 7.1).

Table 7.1: List of bacteria strains included in the reference database used for reductive dehalogenase identification in Proteome Discoverer.

Organism/Name	Strain	NCBI accession number
Dehalogenimonas sp. WBC2-2	WBC2-2	CP011392.1
Dehalogenimonas lykanthroporepellens	BL-DC-9(T)	NC_014314.1
Dehalogenimonas alkenigignens	IP3-3(T)	NZ_LFDV0000000.1
Dehalococcoides mccartyi	195	NC_002936.3
Dehalococcoides mccartyi	CG5	NZ_CP006951.1
Dehalococcoides mccartyi	BAV1	NC_009455.1
Dehalococcoides mccartyi	BTF08	NC_020387.1
Dehalococcoides mccartyi	VS	NC_013552.1
Dehalococcoides mccartyi	GT	NC_013890.1
Dehalococcoides mccartyi	DCMB5	NC_020386.1
Dehalococcoides mccartyi	GY50	NC_022964.1
Dehalococcoides mccartyi	CG4	NZ_CP006950.1
Dehalococcoides mccartyi	11a5	NZ_CP0111127.1
Dehalococcoides mccartyi	KS	JX826286.1 (GenBank)
Dehalococcoides mccartyi	RC	JX826287.1 (GenBank)
Dehalobacter	DCA	NC_018866.1
Dehalobacter	CF	NC_018867.1

7.3. Results and discussion

7.3.1. Validation of the constructed database to search RDases in *Dehalogenimonas.*

The unambiguous assignment of peptides analyzed by LC-MS/MS ultimately depends on the availability of genome sequences of the bacteria concerned. As stated above, the *Dehalogenimonas* present in our culture has not been sequenced. To overcome this limitation, we constructed a reference database constituted by different OHRB, including all *Dehalogenimonas* strains sequenced to date (section 7.2.7). To validate that this approach was plausible, we analyzed by LTQ-Orbitrap (shot-gun proteomics) cultures of *Dehalogenimonas* growing with 1,2-DCP as positive control because it is known that the DcpA present in this culture catalyzes 1,2-DCP to propene [13], [14]. The most abundant RDase identified based on the number of peptide hits and coverage was DcpA, obtaining 18.39 % coverage and 7 peptide hits with the DcpA belonging to *Dehalococcoides mccartyi* strain RC (AGS15114), and 14.11 % coverage and 6 peptide hits with the one belonging to *Dehalogenimonas lykanthroporepellens* (Dehly_1524) (Table 7.2). The DcpA from *Dehalococcoides mccartyi* strain RC and *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 shared 92-95% amino acid identity.

Table 7.2: Reductive dehalogenase detected in *Dehalogenimonas* culture growing with 1,2-DCP. Coverage describes the percentage of the amino acids in a protein were identified. Unique peptides: number of peptides that are sure to come from this protein. Peptides hits: number of peptides identified to match the protein. AA: number of amino acids in the identified protein. MW (kDa): calculated mass of the proteins.

Gene Locus	NCBI Accession No.	Description	Score	Coverage (%)	Unique Peptide	Peptides hits	AAs	MW [kDa]
AGS15114	AGS15114.1	reductive dehalogenase dcpA	49.32	18.39	2	7	484	53.9
Dehly_1524	WP_013218938.1	DEHLY_RS07610 reductive dehalogenase	32.30	14.11	1	6	482	53.8
DEALK_01520	WP_058437778.1	DEALK_RS00725 reductive dehalogenase	6.38	4.41	2	2	521	57.7

Besides the detection of DcpA, another RDase belonging to *Dehalogenimonas alkenigignens* (DEALK_01520) was detected with 4.41 % coverage and 2 peptides hits. The closest homolog of this RDase is DGWBC_1268 which belongs to *Dehalogenimonas* sp. WBC-2 [10]. To date, the function of this RDase is not known.

The identification of DcpA in *Dehalogenimonas* growing with 1,2-DCP demonstrates that the constructed reference database can be used to identify putative RDase for other substrate.

7.3.2. Putative identification of a RDase catalyzing 1,1,2-TCA dihaloelimination in Dehalogenimonas using BN-PAGE, enzymatic assays and LC-MS/MS analysis.

To date, the unique reductive dehalogenase identified to dechlorinate 1,1,2-TCA was found in *Desulfitobacterium* strain PR and it was designated CtrA. The RDase CtrA in strain PR,

which also dechlorinate CF and 1,1,1-TCA, reductively dechlorinate 1,1,2-TCA to 1,2-DCA and chloroethane via hydrogenolysis. The functional RDase that catalyzes the dechlorination of 1,1,2-TCA through dihaloelimination is unknown and therefore its identification was a motivation for this study.

In an attempt to identify the RDase responsible of dichloroelimination of 1,1,2-TCA, we used a constructed co-culture consisting of our *Dehalogenimonas* and *Dehalococcoides mccartyi* strain BTF08. As stated in Chapter 8, this co-culture is able to dechlorinate 1,1,2-TCA to VC by *Dehalogenimonas* and VC to ethene by *Dehalococcoides*. The hydrogenolysis of VC is known to be catalyzed by the RDase designated VcrA, which is encoded in *Dehalococcoides mccartyi* strain BTF08 [22]. Therefore, in the optimal case, we expected to find two RDases in this co-culture, the VcrA in *Dehalococcoides* and the RDase responsible for 1,1,2-TCA dechlorination in *Dehalogenimonas*.

First of all, we aimed to obtain a list of RDase candidates expressed in the 1,1,2-TCA dechlorinating culture using the shotgun proteomic approach. Shotgun proteomics experiment revealed two RDases detectable in the 1,1,2-TCA culture (Table 7.3). The most abundant RDase matched with a RDase designated TceA (DET0079), which belongs to *Dehalococcoides mccartyi* strains. This RDase is known to reductively dechlorinate TCE, *cis*-DCE, and 1,1-dichloroethylene, but also VC and *trans*-DCE at substantially lower rates [6]. The presence of TceA instead of VcrA was unexpected in the culture since VcrA is the RDase responsible for the transformation of VC to ethene and it is encoded in the *Dehalococcoides mccartyi* BTF08 genome. However, it has been suggested that transformation of vinyl chloride by TceA occurs in cells that have uncoupled growth from dechlorination, instead of providing energy for growth when VcrA is expressed [23].

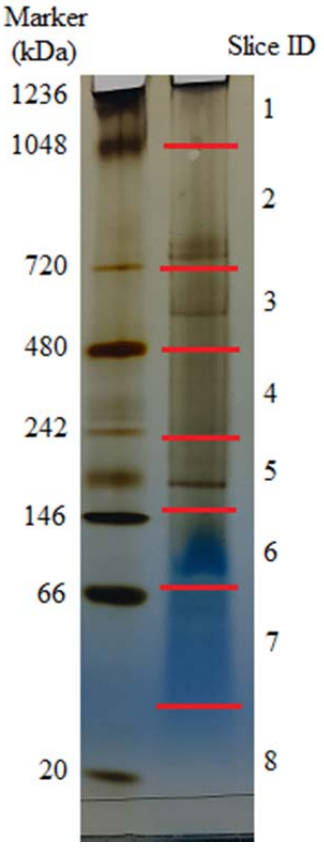
Table 7.3: Reductive dehalogenases detected in the *Dehalogenimonas* and *Dehalococcoides mccartyi* BTF08 co-culture growing with 1,1,2-TCA. Description of what each column means is described in Table 7.2.

Gene Locus	NCBI Accession No.	Description	Score	Coverage (%)	Unique Peptides	Peptides hits	AAs	MW [kDa]
DET 0079	WP_010935886.1	Trichloroethene reductive dehalogenase	39.10	13.90	5	5	554	62.1
AGS 15114	AGS15114.1	1,2-dichloropropene reductive dehalogenase	26.41	11.78	4	4	484	53.9

The second RDase detected showed 11.78% coverage and 4 peptide hits with reductive dehalogenase DcpA of *Dehalococcoides mccartyi* strain RC (GenBank JX826287) and *D. lykanthroporepellens* strain BL-DC-9 (Dehly_1524) [13]. The gene that encodes this RDase (*dcpA* gene) was previously identified in our culture (Chapter 4) and it has been shown to dichloroeliminate 1,2-DCP to propene [13]. This data suggests that this DcpA could be involved in the transformation of 1,1,2-TCA dechlorination.

To confirm the identity and catalytic function of the RDase responsible for 1,1,2-TCA dichloroelimination, the BN-PAGE approach combined with dechlorination activity assays were performed using the crude protein extracts of the co-culture. As shown in Figure 7.1, the dechlorinating activity was found for gel slice "8" in a section around 40 kDa to 20 kDa. In a previous study with *Dehalogenimonas lykanthroporepellens* BL-DC-9 using BN-PAGE gels, the 1,2-DCP dechlorinating activity and the DcpA was also found around 37 to 75 kDa [13].

(A) BN-PAGE



(B) Activity assay with 1,1,2-TCA

Gel slice	VC (µM)
1	0
2	0
3	0
4	0
5	0
6	0
7	0
8	10.9
(-) control	0
(+) control	14.8

Figure 7.1: (A) BN-PAGE of protein extracts of the co-culture growing with 1,1,2-TCA. The positions of gel slices are indicated. (B) Activity towards 1,1,2-TCA measured as micromolar of vinyl chloride produced in the different gel slices of the activity test.

The replicate silver-stained gel slice with dechlorinating activity (number 8) was then analyzed with LC-MS/MS in order to identify the 1,1,2-TCA RDase. The proteins identified are presented in Table 7.4. No RDase was detected in this gel slice. The absence of RDase can be due to different factors. First, as we are comparing the proteins with the database from other strains, there is a possibility that RDase responsible for 1,1,2-TCA dechlorination in our culture differs in some amino acid positions from the sequenced ones and therefore the peptides cannot match with the RDase included in our database. If this is true, then the DcpA identified using the shot gun proteomic approach would indicate that this RDase is also expressed in

Dehalogenimonas but it does not play a role on 1,1,2-TCA dechlorination. Second, the dechlorinating activity found in the gel slice was low, which may also indicate that the amount of protein was below the detection limit of the LC-MS/MS and it hampered its identification. Prior to this experiment, we sacrificed 200 mL of cell-cultures for BN-PAGE experiment, and we failed to see any activity in the gel pieces (data not shown). Therefore, in this experiment we sacrificed 400 mL of cell-cultures but although dechlorinating activity was found, the concentration of protein was still insufficient to detect the RDase. Due to time constraints and lack of available samples, the experiment was stopped at this point.

Aside from LC-LS/MS analysis, the replicate unstained lane from the active gel slice of the BN-PAGE was also excised to elute proteins for SDS-PAGE. However, no band was found on the gel (data not shown). As we pointed out for the LC-MS/MS results of the gel slice with dechlorinating activity, it can be due to the low amount of protein in the culture.

Table 7.4: Proteins identified in gel slice "8" exhibiting 1,1,2-TCA to VC dechlorination activity following BN-PAGE. Description of what each column means is described in Table 7.2.

Gene Locus	NCBI Accession No.	Strain name	Description	Score	Coverage (%)	Unique Peptides	Peptides hits	AAs	MW [kDa]
DHBDCA_p170	WP_015042245.1	*Dehalobacter* sp. DCA	DHBDCA_RS00875 hypothetical protein	2.20	12.33	1	1	73	8.1
DEALK_16170	WP_058439706.1	*Dehalogenimonas alkenigignens*	DEALK_RS07995 hypothetical protein	2.15	12.86	1	1	70	7.7
DEALK_03090	WP_058438057.1	*Dehalogenimonas alkenigignens*	adenylate kinase	2.10	5.19	1	1	212	22.6

7.3.3. Identification of the reductive dehalogenase expressed during EDB dihaloelimination in Dehalogenimonas using shotgun proteomics

Reductive dehalogenase responsible to catalyze the debromination of EDB to ethene has not yet being described yet in any of the known OHRB to date. Very few RDases that functionally catalyzed the reductive dehalogenation of brominated compounds have been described. Two RdhA proteins (CbdbA1092 and CbdbA1503) were expressed during cultivation with tetrabromobisphenol A (TBBPA) to bisphenol A and bromophenol blue to phenol, respectively by *Dehalococcoides mccartyi* CBDB1 [24]. PCE dehalogenase (PceA) of *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* PCE-S mediated the debromination of tribromoethene and both isomers of EDB to end product vinyl bromide, indicating that this enzyme was responsible for the reductive dehalogenation of brominated ethenes [25]. Though reductive dehalogenation is originally thought to rarely occur in aerobes, a novel RDase namely BhbA was expressed in aerobic strain *Comamonas* sp. 7D-2. The enzymes involved in the debromination of bromoxynil to 4-carboxy-2-hydroxymuconate-6-semialdehyde have key features of anaerobic respiratory RdhAs, including two predicted binding motifs for [4Fe-4S] clusters and a close association with a hydrophobic membrane protein (BhbB) [26]. Whereas several studies on the RDase of chlorinated compounds exist, limited information is known so far on the RDase with assigned functions for brominated compounds.

In our *Dehalogenimonas* culture growing with EDB, the unique RDase detected was the DcpA belonging to *Dehalogenimonas lykanthroporepellens* (Dehly_1524) with only 1 peptide hit and 2.28% coverage (Table 7.5). As stated in the previous section, this RDase is known to catalyze the dechlorination of 1,2-DCP to propene but it is not discarded that can transform brominated alkanes. This does not exclude the possibility that the RDase responsible for EDB

transformation was not encoded in the bacteria listed in the reference database. However, it is still interesting to obtain a list of RdhA candidates expressed in the debromination of EDB to ethene through this shotgun proteomic approach and provide additional information for future works.

Table 7.5: Reductive dehalogenase detected in *Dehalogenimonas* culture growing with EDB. Description of what each column means are described in previous Table 7.2.

Gene Locus	NCBI Accession No.	Description	Score	Coverage (%)	Unique Peptides	Peptides hits	AAs	MW [kDa]
Dehly_1524	WP_013218938.1	DEHLY_RS07610 reductive dehalogenase	3.64	2.28	1	1	482	53.8

7.4. Conclusions

In this study, we apply proteomic techniques to get insights into the RdhA expressed during growth of *Dehalogenimonas* with EDB and 1,1,2-TCA. The assignment of peptides analyzed by LC-MS/MS was done by constructing a database containing several sequenced OHRB, and this approach was validated identifying the DcpA in a *Dehalogenimonas* culture growing with 1,2-DCP using shot gun proteomics. In the case of 1,1,2-TCA, we aimed to identify the RDase involved in the transformation of 1,1,2-TCA using a combination of BN-PAGE, dechlorination activity assays of gel slices and LC-MS/MS for protein identification. Dechlorination activity was detected in a gel slice of the BN-PAGE of the co-culture growing with 1,1,2-TCA, but RDase were identified by neither LC-MS/MS analysis nor SDS-PAGE. This was due probably to low abundance of protein in the culture. The analysis of peptides derived from a *Dehalogenimonas* culture growing with EDB using shot gun proteomics only identified a RDase (DcpA) at low levels. Further experiments with *Dehalogenimonas* cultures containing higher cell densities need to be tested. In addition, the availability of the genome sequence of this *Dehalogenimonas* would allow to unambiguously identify RDase that are expressed in the culture and that might be hidden using a database made from other OHRB.

7.5. References

[1] J. A. Field and R. Sierra-Alvarez, "Biodegradability of chlorinated solvents and related chlorinated aliphatic compounds," *Rev. Environ. Sci. Bio/Technology*, vol. 3, pp. 185–254, 2004.

[2] W. M. Moe, F. A. Rainey, and J. Yan, "The genus *Dehalogenimonas*," in *Organohalide-Respiring Bacteria*, L.Adrian and F.E. Löffler Eds., Springer Berlin, Heidelberg, 2016, pp. 137–151.

[3] F. Maphosa, W. M. de Vos, and H. Smidt, "Exploiting the ecogenomics toolbox for environmental diagnostics of organohalide-respiring bacteria," *Trends Biotechnol.*, vol. 28, no. 6, pp. 308–316, 2010.

[4] S. Tang, W. W. M. Chan, K. E. Fletcher, J. Seifert, X. Liang, F. E. Löffler, E. A. Edwards, and L. Adrian, "Functional characterization of reductive dehalogenases by using blue native polyacrylamide gel electrophoresis," *Appl. Environ. Microbiol.*, vol. 79, no. 3, pp. 974–981, 2013.

[5] L. A. Hug, F. Maphosa, D. Leys, F. E. Löffler, H. Smidt, E. A. Edwards, and L. Adrian, "Overview of organohalide-respiring bacteria and a proposal for a classification system for reductive dehalogenases," *Philos. Trans. R. Soc. B,* vol. 368, no. 1616, pp. 1–10, 2013.

[6] J. K. Magnuson, M. F. Romine, D. R. Burris, and M. T. Kingsley, "Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: Sequence of tceA and substrate range characterization," *Appl. Environ. Microbiol.*, vol. 66, no. 12, pp. 5141–7, 2000.

[7] J. A. Müller, B. M. Rosner, G. Von Abendroth, G. Meshulam-Simon, P. L. McCarty, and A. M. Spormann, "Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution," *Appl. Environ. Microbiol.*, vol. 70, no. 8, pp. 4880–4888, 2004.

[8] L. Adrian, J. Rahnenführer, J. Gobom, and T. Hölscher, "Identification of a chlorobenzene reductive dehalogenase in *Dehalococcoides* sp. strain CBDB1," *Appl. Environ. Microbiol.*, vol. 73, no. 23, pp. 7717–7724, 2007.

[9] S. Siddaramappa, J. F. Challacombe, S. F. Delano, L. D. Green, H. Daligault, D. Bruce, C. Detter, R. Tapia, S. Han, L. Goodwin, J. Han, T. Woyke, S. Pitluck, L. Pennacchio, M. Nolan, M. Land, Y.-J. Chang, N. C. Kyrpides, G. Ovchinnikova, L. Hauser, A. Lapidus, J. Yan, K. S. Bowman, M. S. da Costa, F. a. Rainey, and W. M. Moe, "Complete genome sequence of *Dehalogenimonas lykanthroporepellens* type strain (BL-DC-9T) and comparison to '*Dehalococcoides*' strains," *Stand. Genomic Sci.*, vol. 6, no. 2, pp. 251–264, 2012.

[10] T. A. Key, D. P. Richmond, K. S. Bowman, Y.-J. Cho, J. Chun, M. S. da Costa, F. A. Rainey, and W. M. Moe, "Genome sequence of the organohalide-respiring *Dehalogenimonas alkenigignens* type strain (IP3-3T)," *Stand. Genomic Sci.*, vol. 11, no. 1, p. 44, 2016.

[11] T. A. Key, K. S. Bowman, I. Lee, J. Chun, M. da Costa, L. Albuquerque, F. A. Rainey, and W. M. Moe, "*Dehalogenimonas formicexedens* sp. nov., a chlorinated alkane respiring bacterium isolated from contaminated groundwater," *Int. J. Syst. Evol. Microbiol.*, In press, doi: 10.1099/ijsem.0.001819 2017.

[12] K. Mukherjee, K. S. Bowman, F. A. Rainey, S. Siddaramappa, J. F. Challacombe, and W. M. Moe, "*Dehalogenimonas lykanthroporepellens* BL-DC-9T simultaneously transcribes many rdhA genes during organohalide respiration with 1,2-DCA, 1,2-DCP, and 1,2,3-TCP as electron

acceptors," *FEMS Microbiol. Lett.*, vol. 354, no. 2, pp. 111–118, 2014.

[13] E. Padilla-Crespo, J. Yan, C. Swift, D. D. Wagner, K. Chourey, R. L. Hettich, K. M. Ritalahti, and F. E. Löffler, "Identification and environmental distribution of dcpA, which encodes the reductive dehalogenase catalyzing the dichloroelimination of 1,2-dichloropropane to propene in organohalide-respiring Chloroflexi," *Appl. Environ. Microbiol.*, vol. 80, no. 3, pp. 808–818, 2014.

[14] L. Martín-González, S. H. Mortan, M. Rosell, E. Parladé, M. Martínez-Alonso, N. Gaju, G. Caminal, L. Adrian, and E. Marco-Urrea, "Stable carbon isotope fractionation during 1,2-dichloropropane-to-propene transformation by an enrichment culture containing *Dehalogenimonas* strains and a *dcpA* gene.," *Environ. Sci. Technol.*, vol. 49, no. 14, pp. 8666–74, 2015.

[15] M. J. Manchester, L. A. Hug, M. Zarek, A. Zila, and E. A. Edwards, "Discovery of a trans-dichloroethene-respiring *Dehalogenimonas* species in the 1,1,2,2-tetrachloroethane-dechlorinating WBC-2 consortium," *Appl. Environ. Microbiol.*, vol. 78, no. 15, pp. 5280–5287, 2012.

[16] O. Molenda, A. T. Quaile, and E. A. Edwards, "*Dehalogenimonas* sp. strain WBC-2 genome and identification of its trans-dichloroethene reductive dehalogenase, TdrA," *Appl. Environ. Microbiol.*, vol. 82, no. 1, pp. 40–50, 2016.

[17] J. He, Y. Sung, R. Krajmalnik-Brown, K. M. Ritalahti, and F. E. Loffler, "Isolation and characterization of *Dehalococcoides* sp. strain FL2, a trichloroethene (TCE)- and 1,2-dichloroethene-respiring anaerobe," *Environ. Microbiol.*, vol. 7, no. 9, pp. 1442–1450, 2005.

[18] M. V Nesterenko, M. Tilley, and S. J. Upton, "A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels.," *J. Biochem. Biophys. Methods*, vol. 28, no. 3, pp. 239–42, 1994.

[19] A. Kublik, D. Deobald, S. Hartwig, C. L. Schiffmann, A. Andrades, M. von Bergen, R. G. Sawers, and L. Adrian, "Identification of a multi-protein reductive dehalogenase complex in *Dehalococcoides mccartyi* strain CBDB1 suggests a protein-dependent respiratory electron transport chain obviating quinone involvement," *Environ. Microbiol.*, vol. 18, pp. 3044–3056, 2016.

[20] E. Marco-Urrea, S. Paul, V. Khodaverdi, J. Seifert, M. von Bergen, U. Kretzschmar, and L. Adrian, "Identification and characterization of a re-citrate synthase in *Dehalococcoides* strain CBDB1.," *J. Bacteriol.*, vol. 193, no. 19, pp. 5171–8, 2011.

[21] A. Wagner, M. Cooper, S. Ferdi, J. Seifert, and L. Adrian, "Growth of *Dehalococcoides mccartyi* strain CBDB1 by reductive dehalogenation of brominated benzenes to benzene," *Environ. Sci. Technol.* vol 46, no 16, pp 8960-8968, 2012.2012.

[22] M. Pöritz, T. Goris, T. Wubet, M. T. Tarkka, F. Buscot, I. Nijenhuis, U. Lechner, and L. Adrian, "Genome sequences of two dehalogenation specialists - *Dehalococcoides mccartyi* strains BTF08 and DCMB5 enriched from the highly polluted Bitterfeld region," *FEMS Microbiol. Lett.*, vol. 343, no. 2, pp. 101–104, 2013.

[23] D. R. Johnson, P. K. H. Lee, V. F. Holmes, A. C. Fortin, and L. Alvarez-Cohen, "Transcriptional expression of the *tceA* gene in a *Dehalococcoides*-containing microbial enrichment.," *Appl. Environ. Microbiol.*, vol. 71, no. 11, pp. 7145–51, 2005.

[24] C. Yang, A. Kublik, C. Weidauer, B. Seiwert, and L. Adrian, "Reductive dehalogenation of oligocyclic phenolic bromoaromatics by *Dehalococcoides mccartyi* strain CBDB1," *Environ. Sci.*

Technol., vol. 49, no. 14, pp. 8497–8505, 2015.

[25] L. Ye, A. Schilhabel, S. Bartram, W. Boland, and G. Diekert, "Reductive dehalogenation of brominated ethenes by *Sulfurospirillum multivorans* and *Desulfitobacterium hafniense* PCE-S," *Environ. Microbiol.*, vol. 12, no. 2, pp. 501–509, 2010.

[26] K. Chen, L. Huang, C. Xu, X. Liu, J. He, S. H. Zinder, S. Li, and J. Jiang, "Molecular characterization of the enzymes involved in the degradation of a brominated aromatic herbicide," *Mol. Microbiol.*, vol. 89, no. 6, pp. 1121–1139, 2013.

CHAPTER 8

FED BATCH GROWTH OF A *Dehalogenimonas* AND *Dehalococcoides mccartyi* CO-CULTURE IN A BIOREACTOR TO ACHIEVE 1,1,2-TRICHLOROETHANE DETOXIFICATION

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ABSTRACT

1,1,2-Trichloroethane (1,1,2-TCA) is a non-flammable organic solvent and common environmental contaminant in groundwater. Organohalide-respiring bacteria are key microorganisms to remediate 1,1,2-TCA because they can gain metabolic energy during its dechlorination under anaerobic conditions. However, all current isolates produce hazardous end products such as vinyl chloride, monochloroethane or 1,2-dichloroethane that accumulate in the medium. Here, we constructed a syntrophic co-culture of *Dehalogenimonas* and *Dehalococcoides mccartyi* strains to achieve complete detoxification of 1,1,2-TCA to ethene. In this co-culture, *Dehalogenimonas* transformed 1,1,2-TCA via dihaloelimination to vinyl chloride, whereas *Dehalococcoides* reduced vinyl chloride via hydrogenolysis to ethene. Molasses, pyruvate, and lactate supported full dechlorination of 1,1,2-TCA in serum bottle co-cultures. Scale up of the cultivation to a 5-L bioreactor operating for 76 d in fed-batch mode was successful with pyruvate as substrate. This synthetic combination of bacteria with known complementary metabolic capabilities demonstrates the potential environmental relevance of microbial cooperation to detoxify 1,1,2-TCA.

8.1. Introduction

Polychlorinated ethanes such as,1,2-TCA and 1,1,2,2-TeCA have been used for decades as chemical intermediates, solvents, degreasing agents, and paint removers [1], [2]. Both compounds have adverse health effects on the liver, the kidneys, and the nervous and immune systems [3]. Due to improper disposal practices and accidental releases, these contaminants are widely distributed in groundwater and soils [4]. For instance, 1,1,2-TCA and 1,1,2,2-TeCA have been found in at least 157 and 112 out of the 1,774 National Priorities List sites identified by the Environmental Protection Agency (EPA), respectively [5].

Highly chlorinated ethanes are considered poorly biodegradable under aerobic conditions, and little evidence exists of single bacterial strains or mixed microbial populations able to transform either 1,1,2-TCA or 1,1,1,2-TeCA during aerobic cometabolism [6]. Several major drawbacks such as the obligate presence of a growth substrate, competition between the chlorinated compounds and primary substrates for binding to the active site of the responsible enzyme, the toxic effect of some transformation products, and frequent anoxic conditions in contaminated aquifers, limit *in situ* application of aerobic cometabolic bacteria as bioremediation agents. Conversely, organohalide-respiring bacteria can use chlorinated ethanes as terminal electron acceptors deriving energy for growth during dechlorination. Dichloroelimination of 1,1,2-TCA to VC has been the main dechlorination pathway observed in uncharacterized anaerobic mixed cultures and organohalide-respiring isolates (i.e. *Dehalobacter* sp., *Dehalogenimonas alkenigignens* strain IP-3*, Dehalogenimonas lykanthroporepellens* strain BL-DC-9, and *Desulfitobacterium dichloroeliminans* strain DCA1) [7]–[10]. 1,1,2,2-TeCA was dechlorinated by *Dehalogenimonas* isolates to *cis*-DCE and *trans*-DCE [11]–[13], whereas *Dehalobacter* spp were shown to grow during transformation of 1,1,2,2-TeCA to *trans*-DCE in

an anaerobic enrichment culture [14]. Dechlorination of 1,1,2-TCA to predominantly 1,2- DCA and monochloroethane via hydrogenolysis is an alternative pathway observed in *Desulfomonile tiedjei* strain DCB-1 and *Desulfitobacterium* sp. strain PR [10], [15], and the responsible 1,1,2-TCA reductive dehalogenase gene was recently reported in the latter strain [10]. As a result, accumulation of VC, DCA, *trans*- and *cis*-DCE was often observed at Superfund sites contaminated with 1,1,2-TCA or 1,1,2,2-TeCA [5].

No single bacterial strains are known to fully dechlorinate 1,1,2-TCA or 1,1,2,2-TeCA, and detoxification therefore appears to always require the syntrophic action of different organohalide-respiring bacterial species. For instance, a mixed microbial culture named WBC-2, derived from sediments contaminated with 1,1,2,2-TeCA, completely dechlorinated 1,1,2,2TeCA to ethene involving three microorganisms catalyzing consecutive steps: 1,1,2,2-TeCA to trans-DCE catalyzed by *Dehalobacter spp*, *trans*-DCE to VC catalyzed by *Dehalogenimonas* and *Dehalococcoides spp*, and VC to ethene catalyzed by *Dehalococcoides spp* [14]. Similarly, full dechlorination of 1,1,2-TCA in an uncharacterized anaerobic enrichment culture implicated *Dehalobacter* in the dechlorination of 1,1,2-TCA to VC, and *Dehalococcoides mccartyi* in the dechlorination of VC to ethene [7]. Recently, a co-culture was constructed with *Desulfitobacterium sp.* strain PR and *Dehalococcoides mccartyi* strain 11a to fully dechlorinate 1,1,2-TCA to ethene. In this case, however, trichloroethene (TCE) was required to suppress monochloroethane production from strain PR, and DCA was dechlorinated to ethene by strain 11a [10].

To date, most of the research reported on degradation of these chloroethanes has been conducted in serum bottles. However, the implementation of effective engineering bioremediation approaches to clean up contaminated groundwater frequently involves the

introduction of microbial consortia into the aquifer (bioaugmentation) and it is desirable to develop suitable lab-scale bioreactor processes as a first step for large-scale production of high cell density cultures. The production of organohalide-respiring bacteria in a bioreactor presents challenges due to the strict anaerobicity required, the low growth rate of the strains, and the need of maintenance of other consortium members that provide cofactors and/or electron donors/carbon source to the dechlorinating bacteria [16].

In this study, we aimed to construct a co-culture consisting of a *Dehalogenimonas* and *Dehalococcoides mccartyi* to provide experimental evidence that cooperation of both strains lead to full dechlorination of 1,1,2-TCA in a lab-scale bioreactor. The *Dehalogenimonas sp.* was enriched from sediments collected in the Besòs River estuary (Spain) and only transforms chloroalkanes containing chlorine substituents located on adjacent carbon atoms via dichloroelimination (vicinal reduction) [17]. Thus, 1,1,2-TCA is transformed to VC, which accumulates in the medium. Conversely, *Dehalococcoides mccartyi* strain BTF08, isolated from a contaminated aquifer in Bitterfeld (Germany), dechlorinates VC but not 1,1,2-TCA [18], [19]. Taken together, the combined catalytic activity of both strains can potentially detoxify 1,1,2-TCA to ethene . This approach, based on the combination of two bacteria with known complementary dechlorination activities, differs from previous studies characterizing anaerobic mixed cultures that assigned a role to different bacteria based exclusively on differences in their dechlorination-dependent growth on each dechlorination step [7], [14]. Here, we selected an appropriate medium to grow both organohalide-respiring bacteria and tested different primary growth substrates to be fermented by other members of the consortium to provide hydrogen (electron donor) and acetate (carbon source) to *Dehalococcoides* and *Dehalogenimonas* strains.

Finally, we investigate the potential to scale up the production of this co-culture to an anaerobic 5-L bioreactor operating in fed batch mode.

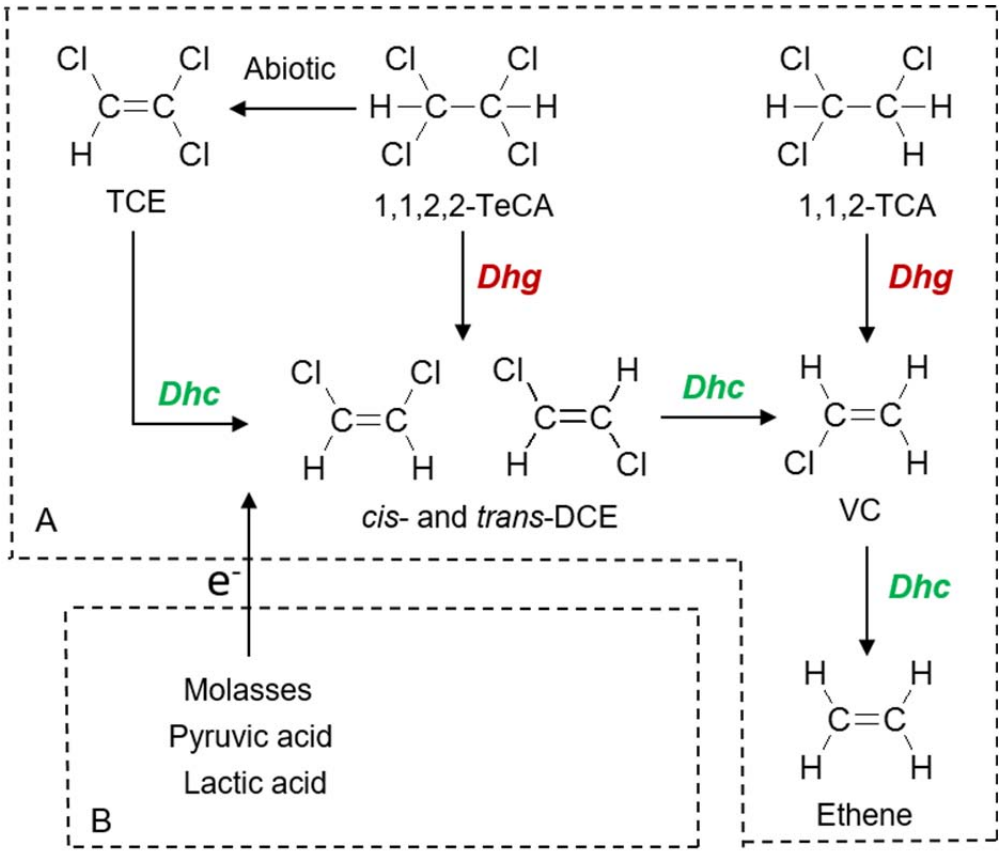

Figure 8.1: Organohalide respiration of 1,1,2,2-TeCA and 1,1,2-TCA to ethene by a co-culture of *Dehalogenimonas* (Dhg) and *Dehalococcoides* (Dhc) (A) and supply of electrons from fermentation of reduced organic compounds (B) [20].

8.2. Materials and methods

8.2.1. Chemicals

All the chemicals used were reagent grade and described in Chapter 3 unless otherwise specified.The characteristics of the sugar beet molasses used in this study were pH: 6.79±0.06;

moisture (%): 16.9±0.8; organic matter (%, dry basis): 87.3±2.1; carbohydrate content (%, dry basis): 78.4±2.8; and fat content (%, dry basis): not detectable [21].

8.2.2. Establishment of the co-culture in serum bottles

Dehalococcoides mccartyi strain BTF08 was kindly provided by Ivonne Nijenhuis from the Helmholtz-Centre for Environmental Research (UFZ), Leipzig (Germany). *Dehalogenimonas*-containing mixed culture and *Dehalococcoides mccartyi* strain BTF08 were co-cultivated in 120 mL serum bottles containing 65 mL of anaerobic defined medium as described in Chapter 3. Inoculation was done with a volume of 5-10% (v/v) of pre-grown cultures with cell numbers of around 5×10^7 cells mL^{-1}. To test organic fermentable substrates as electron donor and carbon source in the co-culture, pyruvate, lactate or sugar beet molasses were added to the original medium instead of acetate from filter-sterilized 100× aqueous stock solutions to achieve final concentrations of 4.5 mM, 7.5 mM, and 200 mg L^{-1}, respectively. 1,1,2-TCA was added with a syringe from a 3.2 mM stock solution in acetone to a concentration of 20 μM. Microcosms were prepared in triplicate and incubated statically at 25 °C in the dark.

8.2.3. Bioreactor experiment

The bioreactor experiment was carried out in a 5 L jacketed glass reactor connected to a thermostatic bath through which the temperature was maintained at 30 °C (see photograph of the reactor setup in Figure 8.2). Stirring at 100 rpm was regularly provided for 15 min once every hour. The basal medium described above with pyruvate (5 mM) instead of acetate was added to the reactor and steam sterilized at 121 ºC for 30 min. After sterilization, anoxic conditions were achieved by flushing nitrogen through a gas distributor, which purged the gas in the form of bubbles into reactor, until the dissolved oxygen levels in the liquid medium were below the limit of detection of the oxygen electrode. Then, $Na_2S \times 9\ H_2O$ and L-cysteine (0.2 mM each) and

$NaHCO_3$ (10 mM) were added aseptically through a feeding port using a sterile anoxic syringe and conditions were equilibrated overnight. This feeding port was equipped with a removable Teflon-coated butyl rubber septum that was changed periodically. The reactor was gassed with N_2/CO_2 (70%/30% v/v, 0.4 bar overpressure) and vitamins [17] were added aseptically. The reactor was then inoculated (5% v/v) from a co-culture grown in serum bottles that had consumed a total of about ~90 µM 1,1,2-TCA during an incubation time of 11 days. The final volume of the liquid medium in the reactor was 5 L. The reactor was maintained in fed-batch mode adding 1,1,2-TCA (30 µM nominal concentration at each addition) when exhausted.

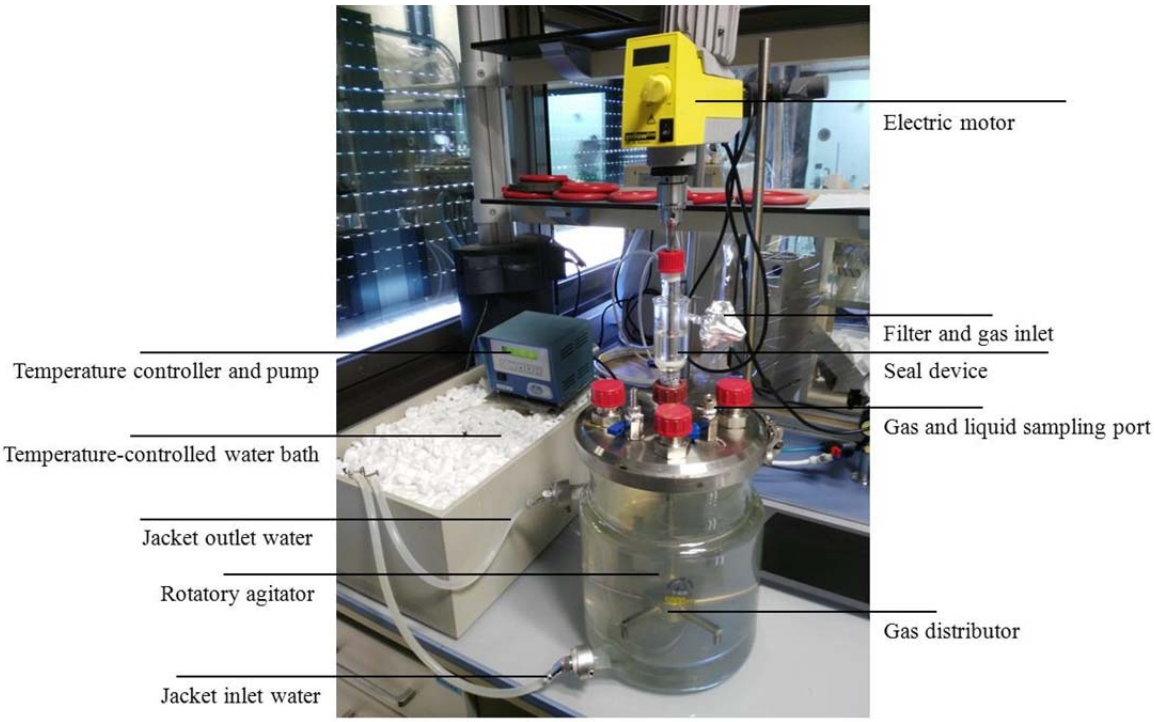

Figure 8.2: Photograph and description of the bioreactor employed in this study.

8.2.4. Sampling and analytical methods

In the bioreactor, the sampling was done through ports located in the stainless steel cap. The concentrations of 1,1,2-TCA and VC were determined by transferring 1 mL of liquid medium to a 2 mL vial and sealed immediately with a Teflon-coated stopper. The vials were heated to 85 ºC for 30 min to volatize all target compounds, and subsequently 1 mL headspace samples were taken for analysis by GC. The concentrations of gases (ethene and hydrogen) and organic acids in the bioreactor were determined similarly to the serum bottles analyzing 0.5 mL of the headspace and 1 mL liquid sample, respectively.

8.2.5. Molecular analyses

For DNA extraction, 950 µL of sample were collected at the beginning of the experiment (t=0) and at different time points, as indicated. The DNA was extracted with the NucleoSpin Tissue DNA extraction kit following the instructions of the manufacturer. The gene copies of *Dehalogenimonas* and *Dehalococcoides spp.* 16S rRNA genes in the extracted DNA were analyzed by quantitative PCR. Reactions were set up in 10 µl of Kapa Sybr Fast qPCR master mix (ABI), PCR-grade water, and primers. The primers used were BL-DC-142f (5GTGGGGGATAACACTTCGAAAGAAGTGC-3') and BL-DC-1243r (5'CCGGTGGCAACCCATTGTACCGC-3') for *Dehalogenimonas sp.* [22] and 5′ AGGAAGCAAGCGTTATCC-3′) and 731r (5′-GACAACCTAGAAAACCGC-3′) for *Dehalococcoides sp.* [23]. Amplifications were done in triplicate samples using a StepOne qPCR instrument (StepOnePlus, Applied Biosystems) under the following conditions: initial denaturation at 95 °C for 2 min, annealing at 56 °C for 20 s, elongation at 72 °C for 20 s and denaturation at 95 °C for 3 s. A total of 40 cycles were done and quantification was done via

SYBR-green fluorescence detection. All assays were followed by a melting curve between 60 and 95 °C in 0.3 °C steps, checking for amplicon specificity.

Standard curves were done by 10-fold serial dilutions of cloned 16S rRNA genes of *Dehalococcoides* strain BTF08 [19] and the *Dehalogenimonas* strain in our mixed culture using the NEB PCR cloning kit (NEB). Amplification efficiencies were calculated from the slope of each calibration curve according to the formula 10(-1/slope). Amplification efficiencies ranged between 85 and 115%. All analyses were done at least in triplicate and measurements were repeated at least once.

8.3. Results and discussion

8.3.1. Establishment of the co-culture in serum bottles for complete 1,1,2-TCA dechlorination

Dehalococcoides mccartyi strain BTF08 and an enrichment culture containing a *Dehalogenimonas* sp. were combined and amended with different fermentable substrates that provide the required electrons stem to dechlorinate 1,1,2-TCA to ethene, as depicted in Figure 8.3. A stable co-culture was established after three subsequent transfers (5% v/v) with the respective organic fermentable substrates (lactate, pyruvate, and molasses) and consuming each at least three amendments of 1,1,2-TCA at 20 μM.

The time-course of 1,1,2-TCA dechlorination to ethene for the fourth transfer is shown in Figure 8.3. All microcosms reached complete removal of 1,1,2-TCA within 21 days. In microcosms with pyruvate or sugar beet molasses, VC reached its highest concentration on day 14 and was completely dechlorinated to ethene after 28 days (Figure 8.3A and 8.3E). The same dechlorination pattern was observed in microcosms amended with lactate, but the highest

accumulation of VC was observed after 21 days (Figure 8.3C). The sum of moles of 1,1,2-TCA, VC and ethene during dechlorination was within 10 % of the initial moles of 1,1,2-TCA added at the beginning of the experiment, indicating quantitative conversion.

We also tested the ability of our co-culture for dechlorination of 1,1,2,2-TeCA to ethene (data not shown). In this case, we observed three stepwise reactions: dichloroelimination of 1,1,2,2-TeCA to *trans*-DCE and *cis*-DCE, hydrogenolysis of *cis*-DCE to VC and subsequent dechlorination to ethene. Transformation of 1,1,2,2-TeCA was assigned to *Dehalogenimonas* and dechlorination of *cis*-DCE and VC was assigned to strain BTF08 because their dechlorination only occurred in parallel cultures when these bacteria were present. In the abiotic controls, 1,1,2,2-TeCA (20 μM) was partially degraded abiotically by dehydrochlorination to TCE, accounting for up to 40% of the initial 1,1,2,2-TeCA added after one month. This abiotic reaction pathway was consistent with previous studies [12], [24]. In all microcosms containing *Dehalogenimonas,* dechlorination of 1,1,2,2-TeCA proceeded faster than in abiotic controls, indicating that this reaction was biotically catalyzed. However, due to substantial interference from the abiotic reaction of 1,1,2,2-TeCA, we decided to focus on 1,1,2-TCA for further experiments.

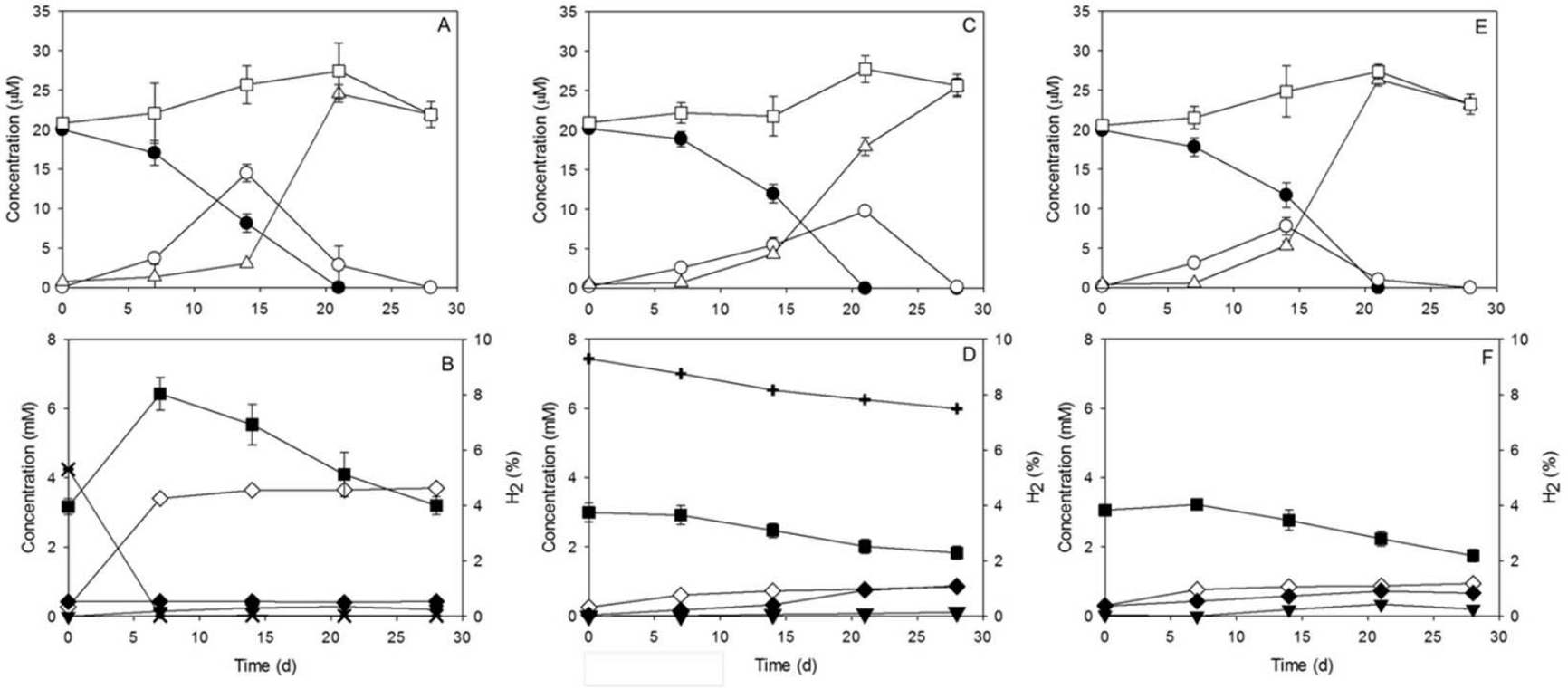

Figure 8.3: Dechlorination of 1,1,2-TCA to ethene by *Dehalogenimonas* and *Dehalococcoides* coculture (upper panels) and fermentation of the different organic substrates amended (lower panels) in serum bottles. Batch cultures established with pyruvate (A,B), lactate (C,D), and molasses (E,F). Symbols: sum of 1,1,2-TCA, VC and ethene (□), 1,1,2-TCA (●), VC (○), ethene (Δ), pyruvate (×), lactate (+), hydrogen (■), acetate (◊), formate (▼), and propionate (♦). Error bars represent standard deviation of triplicate bottles.

One of the most remarkable advantages of our co-culture in comparison to the previously reported co-culture containing *Desulfitobacterium* strain PR and *Dehalococcoides mccartyi* strain 11a [10] is that accumulation of toxic chlorinated compounds does not occur from 1,1,2-TCA degradation. Strain PR sequentially dechlorinated 1,1,2-TCA predominantly to 1,2-DCA and monochloroethane via hydrogenolysis, while strain 11a converted 1,2-DCA to ethene in the co-culture. To avoid monochloroethane accumulation, TCE had to be present in the medium to inhibit dechlorination of 1,2-DCA to monochloroethane. Only then both TCE and 1,2-DCA were fully dechlorinated to ethene by strain 11a. Therefore, the application of this co-culture would only detoxify 1,1,2-TCA when TCE is present as co-contaminant in groundwater.

The dechlorination pathway catalyzed by our consortium is similar to that catalyzed by an enrichment culture containing *Dehalobacter* and *Dehalococcoides* [7]. Since chloroorganics commonly occur as complex mixtures in groundwater, here it is interesting to compare the array of contaminants that can be transformed by each co-culture besides 1,1,2-TCA. Interestingly, *Dehalogenimonas* and *Dehalobacter* strains reported in these co-cultures have complementary dechlorination activities towards some chloroalkanes such as 1,2-DCA, 1,1,2-TCA, and 1,1,2,2TeCA [7], [14]; however, *Dehalogenimonas* can extend the number of chloroalkanes dichloroeliminated that are not transformed by *Dehalobacter spp.* (e.g. 1,2,3-trichloropropane and 1,2-dichloropropane) [17].

In regard to the fermentation of the organic substrates to generate hydrogen and acetate, the most commonly used compounds to support organohalide-respiring bacteria include alcohols, low-molecular-weight fatty acids, and vegetable oils. Sugar beet molasses were included in this study to explore the potential of reusing this by-product of the sugar manufacturing process for bioremediation purposes. As it is a relatively inexpensive and readily available raw material,

molasses can be a cheap alternative for a larger scale operation[24]. Lactate and pyruvate were also tested because their good water solubility would facilitate a better distribution and mass transfer of these substrates in the reactor. Especially lactate additions would prevent extensive fermentative growth which can lead to plugging of reactor structures. As shown in Figure 8.3B, pyruvate was completely fermented within 7 days to predominantly acetate and minor amounts of formate and propionate. Fermentation of lactate proceeded slowly and only 30% of the initial concentration was fermented after 28 days (Figure 8.3D), producing mostly acetate and propionate and minor amounts of formate. Molar balance closure revealed that consumed pyruvate and lactate were fully converted to the acetate, propionate and formate, although the presence of other nondetected organic acids at low concentrations cannot be ruled out. In the case of molasses, the concentration of acetate produced was five times less than that produced with pyruvate (Figure 8.3F). As expected from these results, the production of hydrogen was higher in the microcosms amended with pyruvate, accounting to ~8% (v/v) of the gas phase and then progressively decreased during the incubation time due to the consumption by hydrogenotrophic microorganisms (Figure 8.3B).

In our medium, lacking non-halogenated acceptors such as oxygen, nitrate or sulfate, acetate and H_2 can still be used by other organisms than organohalide-respiring bacteria. Methanogens and homoacetogens are considered to be major competitors of organohalide respiring bacteria for H_2. However, methanogenic activity in the *Dehalogenimonas*-containing culture was completely suppressed after several transfers with the specific inhibitor bromoethanesulfonate as described in Chapter 4. H_2 consumption by autotrophic homoacetogens was discarded because they were not identified in the DGGEs performed during the enrichment and isolation process in Chapter 4 and 6.

Lactate fermentation proceeded slower than pyruvate fermentation and, hence, the hydrogen concentration stayed at a lower level. This might indicate an advantage of lactate in comparison to pyruvate because organohalide-respiring bacteria are more competitive than carbon dioxide-reducing homoacetogens and methanogens for the obligate electron donor (H_2) at low hydrogen concentrations [25]. However, the dechlorination rate of 1,1,2-TCA in microcosms with lactate decreased dramatically in the next transfers and therefore disfavored the use of lactate in the following experiments. In the case of sugar beet molasses, the co-culture showed robust dechlorination rates during several transfers but we observed a gradual increase in the turbidity and viscosity of the medium. This was probably due to the fact that this complex substrate heavily stimulates the growth of non-organohalide-respiring bacteria, leading to increased biomass that would potentially provoke operational problems in the bioreactor (e.g. biomass conglomerates growing on devices). Because these obstructions were not observed with pyruvate, pyruvate was selected as organic substrate for further studies in the bioreactor.

8.3.2. Scale-up of the co-culture in a fed-batch bioreactor

To scale up the batch experiments to a batch reactor volume we performed an air tightness and leakage test of the bioreactor to ensure that 1,1,2-TCA was not removed by abiotic means (volatilization, sorption, liquid leaks, etc). As shown in Figure 8.4, the concentration of 1,1,2-TCA remained constant under operating conditions (intermittent mixing, 30 ºC) in a non-inoculated reactor for 11 d, indicating that the reactor was suitable for this application.

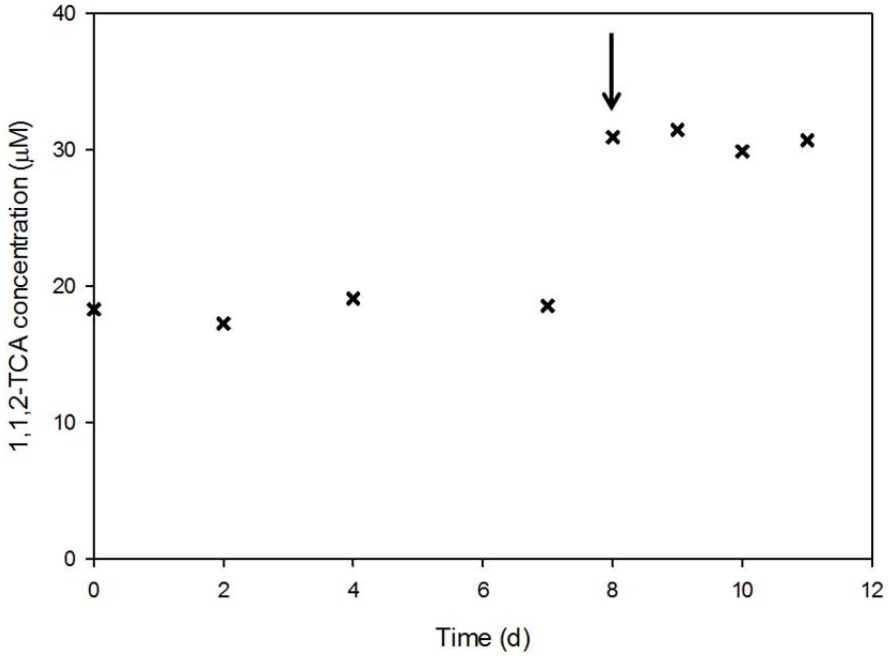

Figure 8.4: Concentration of 1,1,2-TCA in the bioreactor at operating conditions through the time without bacterial inoculum. Arrow indicate the addition of 1,1,2-TCA

Once the co-culture was inoculated into the reactor, the first addition of 1,1,2-TCA (30 µM) took 7 days to be dechlorinated to VC (Figure 8.5A). The repeated addition of 1,1,2-TCA led to faster dechlorination rates, consuming each of these feedings within 2-3 days. Pyruvate fermented in four days producing acetate as major product, formate, and hydrogen (Figure 8.5B). Unlike the hydrogen concentration in the headspace, the acetate concentration did not show a noticeable decrease during the incubation time, which is consistent with the low acetate consumption rate described for organohalide-respiring bacteria [26].

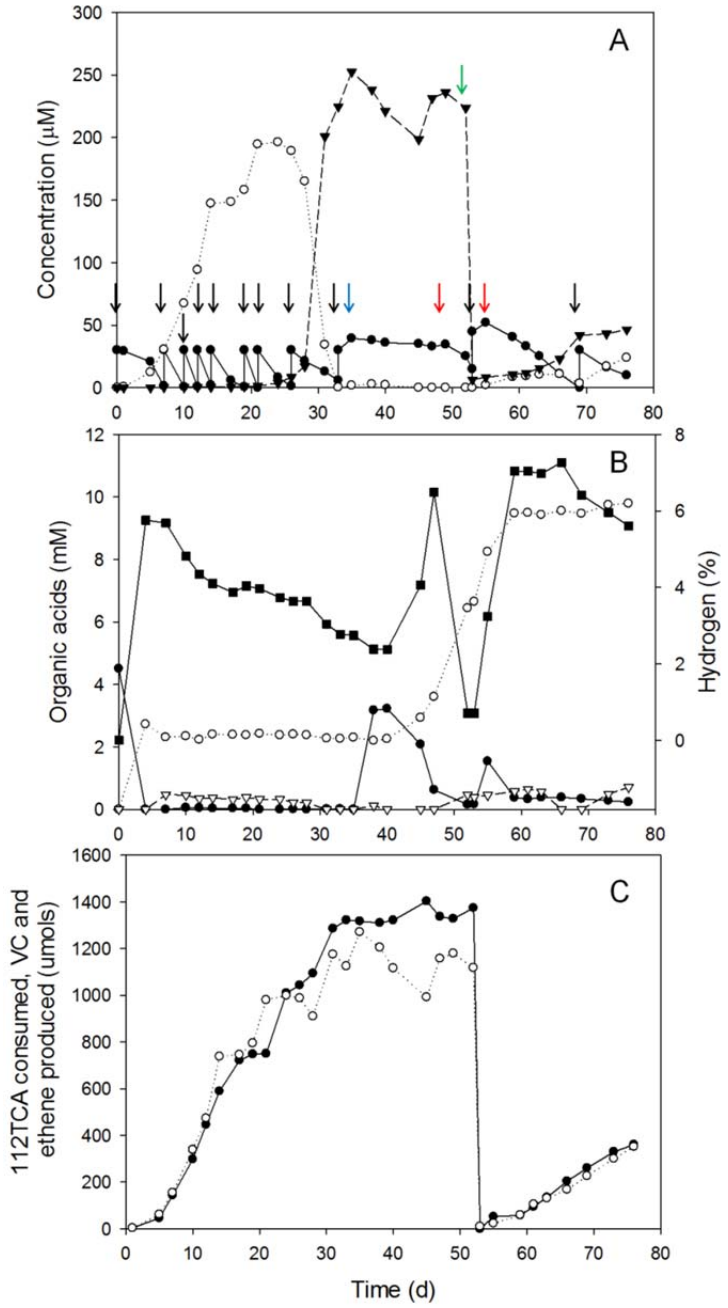

Figure 8.5: Transformation of 1,1,2-TCA to ethene by a co-culture containing Dehalogenimonas and Dehalococcoides in a 5-L anaerobic reactor. (A) Dechlorination of 1,1,2-TCA (●) to vinyl chloride (○) and ethene (▼). (B) Fermentation of pyruvate (●) into acetate (○), formate (Δ) and hydrogen (■). (C) Micromoles of 1,1,2-TCA dechlorinated (●) and vinyl chloride plus ethene produced (○). Arrows indicate addition of 1,1,2-TCA (black), vitamins solution (blue), pyruvate (red), N2/CO2 flushing (green).

A total of 210 μM 1,1,2-TCA was dechlorinated in 24 days, with concomitant accumulation of VC during this period before dechlorination to ethene started at day 26. This substantial delay of VC dechlorination was not observed in microcosms and it was therefore unexpected in the reactor, but the concentration of VC significantly dropped afterwards and it was completely dechlorinated to ethene within 5 days (Figure 8.5A). On day 35, the dechlorination of 1,1,2-TCA stopped. Firstly, we hypothesize that this could be due to a lack of nutrients. As organic cofactors such as vitamin B12 are essential constituents in the medium to support metabolic dechlorination [27], vitamins were re-spiked on day 33, however, no effect on dechlorination was found within an observation period of two weeks (Figure 8.5A). Acetate and hydrogen produced from pyruvate fermentation were still present at suitable concentrations to support organohalide respiration (Figure 8.5B), but we added pyruvate again at 5 mM to test if fermentative bacteria play a role on stimulating the activity of *Dehalogenimonas* and *Dehalococcoides*. After pyruvate addition, hydrogen and acetate concentration increased but 1,1,2-TCA dechlorination was still stalled (Figure 8.5A and 8.5B). Then, we investigated if the presence of ethene might inhibit 1,1,2-TCA dechlorination. This hypothesis was based on the observation that ethene production was accompanied with a slight decrease on 1,1,2-TCA dechlorination rate in the bioreactor, but when ethene reached its maximum concentration, 1,1,2-TCA dechlorination stopped (day 35) (Figure 8.5A). To clarify this point, we set up parallel *Dehalogenimonas* cultures in 120-mL serum bottles containing 1,1,2-TCA plus ethene and controls solely containing 1,1,2-TCA in triplicate. Our results indicate that ethene was not exerting an inhibitory effect on 1,1,2-TCA dechlorination (Figure 8.6). In parallel, the reactor was purged with nitrogen for 15 min to remove volatile compounds in the bioreactor that could inhibit the dechlorination of 1,1,2-TCA to VC. After adding pyruvate again, dechlorination of

1,1,2-TCA was recovered after a relatively short time, consuming the first feeding of 52 μM of 1,1,2-TCA within 14 d and consuming the next even faster (Figure 8.5A). One hypothesis is that purging with nitrogen could benefit *Dehalogenimonas* in acidic medium by removing the dissolved carbon dioxide and increasing the pH, but the values measured at each time point of analysis using pH indicator showed that it was maintained within the dechlorination range described for *Dehalogenimonas* (pH 6-8) [11]. Although the reason for this 1,1,2-TCA dechlorination inhibition is not known, accumulation of non-identified volatile components may explain this phenomenon. A feasible candidate is carbon monoxide, an obligate by-product accumulating in the headspace during a peculiar process in *Dehalococcoides mccartyi* in which the C-2 of acetyl-CoA is transferred to tetrahydrofolate for methionine biosynthesis [28]. It has been shown that carbon monoxide accumulation caused the cessation of dechlorination activity in *Dehalococcoides mccartyi* strain 195 after several dose amendments at concentrations as low as ~0.1 % (v/v) [28]. However, carbon monoxide concentration was not measured in the headspace of the bioreactor and this issue needs to be studied in more detail in future experiments.

The mass balance of the total amount of 1,1,2-TCA dechlorinated and total amount of VC and ethene produced was closed throughout the cultivation process (Figure 8.5C).

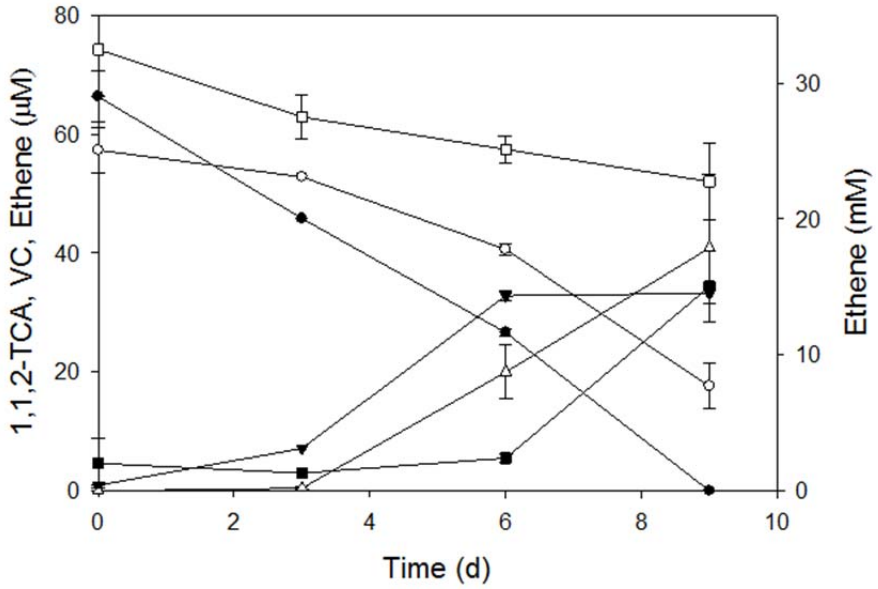

Figure 8.6: Effect of ethene on dechlorination of 1,1,2-TCA by *Dehalogenimonas* in serum bottles. Black symbols refer to cultures without added ethene, white symbols refer to parallel cultures containing ethene at an initial concentration of ~30 mM. Symbols: 1,1,2TCA (circle), VC (triangle), ethene (square). Y-axis refer to ethene concentration in the treatments that contained initially ethene.

The numbers of 16S rRNA gene copies of both *Dehalogenimonas* and *Dehalococcoides* were both monitored during the complete dechlorination of 1,1,2-TCA to ethene in the bioreactor for the first 38 days (Figure 8.7). During the first week of incubation, the slow growth of *Dehalogenimonas* was consistent with the consumption of a single amendment of 1,1,2-TCA (30 µM). As would be expected from growth via 1,1,2-TCA respiration, the consumption of five consecutive feeding doses of 1,1,2-TCA during the following two weeks was accompanied by a marked increase in *Dehalogenimonas* cell numbers. At this point, dechlorination of 1,1,2-TCA proceeded much more slowly and the relationship between *Dehalogenimonas* growth and 1,1,2-TCA consumption was more difficult to establish until the end of the monitoring.

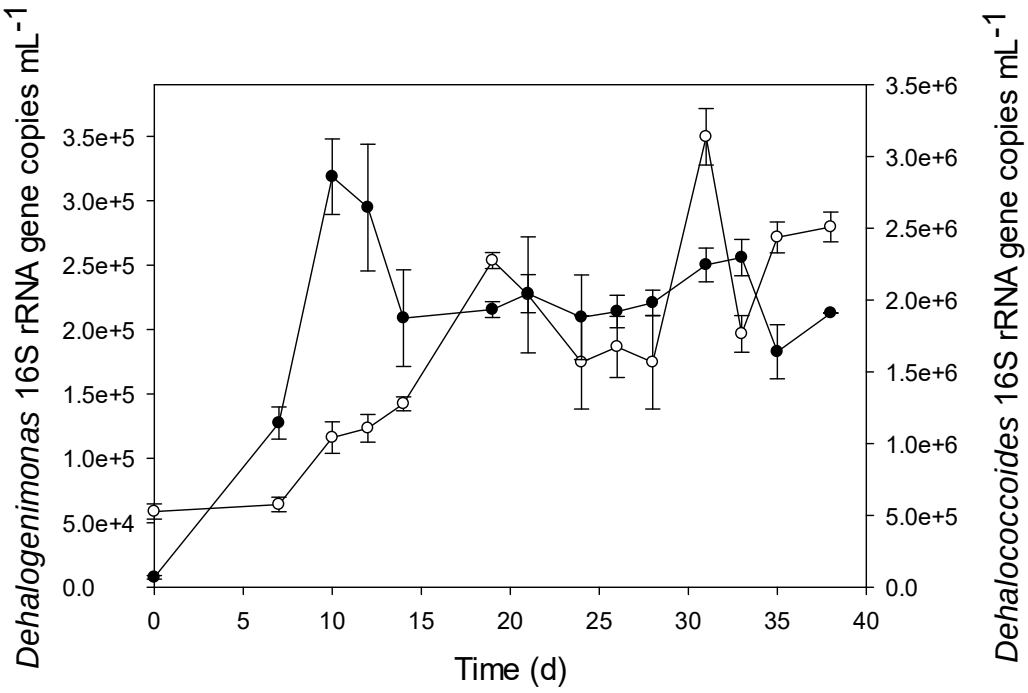

Figure 8.7: *Dehalogenimonas* (○) and *Dehalococcoides* (●) 16S rRNA gene copies per mL of culture during 1,1,2-TCA dechlorination in the bioreactor experiment depicted in Figure 8.5.

In the case of *Dehalococcoides,* cell growth was not correlated with the dechlorination of VC to ethene. During the first ten days of cultivation, *Dehalococcoides* grew unexpectedly without consumption of VC despite previous investigations neither observe dechlorination of 1,1,2-TCA nor growth with non-halogenated compounds in strain BTF08 [19]. The lack of growth of *Dehalococcoides* observed in the subsequent days was consistent with the accumulation of VC until day 26, but at this point conversion of VC to ethene started but the number of cells remained constant until the end of the monitoring period. Uncoupling of VC dechlorination from growth was unexpected because the VC reductive dehalogenase gene *vcrA* was encoded in the genome of strain BTF08 [29]; however, this is not the first description of growth-uncoupled dechlorination of VC in *Dehalococcoides mccartyi* strains [30]. Recently, the exposure of 1,2-DCA on to *Dehalococcoides* populations growing with TCE has been shown to

provoke a decrease of the *Dehalococcoides-vcrA*-containing populations in a mixed culture, reducing the maximum rate of VC transformation to ethene by an order of magnitude [31]. Therefore, there are possibilities that the chlorinated alkane 1,1,2-TCA also inhibited the expression of VcrA in the co-culture as no VC dechlorination was observed in the first 30 d during the repeated addition of 1,1,2-TCA (Figure 8.5A). This hypothesis is consistent with the absence of the VC reductive dehalogenase (VcrA) in this co-culture when analyzed by shotgun proteomics in Chapter 7 (Table 7.3). Interestingly, the only RDase identified for strain BTF08 was TceA, which can transform VC to ethene at lower rates without the growth of *Dehalococcoides* [31]. Induction of reductive dehalogenases with dechlorination capability towards compounds that did not support respiratory growth have been described for several *Dehalococcoides mccartyi* strains. For instance, expression of the *tceA* gene in the *Dehalococcoides*-containing enrichment ANAS increased after exposure to *trans*-DCE, but the dechlorination of this compound was unable to support growth of the *tceA*-containing bacteria [32]. In all, further studies are needed to elucidate the apparent nonmetabolic transformation of VC by strain BTF08 in the presence of 1,1,2-TCA.

8.4. Conclusions

The characterization of mixed enrichment cultures and growth monitoring of different organohalide-respiring bacteria during the dechlorination of halogenated compounds provide valuable insight into the microorganisms responsible for these transformations. An alternative approach to support these observations is the synthetically combination of co-cultures that allow to unequivocally demonstrate functional interdependences and broadening of substrate spectra. This study shows the potential of the constructed co-culture composed by *Dehalococcoides* and *Dehalogenimonas* to completely dechlorinate 1,1,2-TCA to ethene which cannot be catalyzed by a single anaerobic bacterium to date. Indeed, our syntrophic consortium overcomes the accumulation of hazardous intermediates as observed in other organohalide-respiring co-cultures during 1,1,2-TCA dechlorination. Our results show that under the tested conditions, dechlorination of VC was uncoupled from *Dehalococcoides mccartyi* strain BTF08 growth and alternative electron acceptors need to be tested in the future to produce high cell density cultures of this strain in bioreactors.

8.5. References

[1] Agency for Toxic Substances and Disease Registry, "Toxicological profile on 1,1,2-trichloroethane," 1999. [Online]. Available: http://www.atsdr.cdc.gov/toxfaqs/tfacts148.pdf. [Accessed: 30-Jul-2016].

[2] Agency for Toxic Substances and Disease Registry, "Toxicological profile for 1,1,2,2-tetrachloroethane," 2008. [Online]. Available: https://www.atsdr.cdc.gov/toxprofiles/tp93.pdf. [Accessed: 22-Nov-2016].

[3] Agency for Toxic Substances and Disease Registry, "2015 Priority List of Hazardous Substances | ATSDR," 2015. [Online]. Available: https://www.atsdr.cdc.gov/spl/. [Accessed: 21-Nov-2016].

[4] P. Bhatt, M. S. Kumar, S. Mudliar, and T. Chakrabarti, "Biodegradation of chlorinated compounds-A review," *Crit. Rev. Environ. Sci. Technol.*, vol. 37, pp. 165–198, 2007.

[5] United States Environmental Protection Agency, "Superfund site information," 2016. [Online]. Available: https://cumulis.epa.gov/supercpad/cursites/. [Accessed: 22-Aug-2016].

[6] D. Frascari, M. Cappelletti, S. Fedi, D. Zannoni, M. Nocentini, and D. Pinelli, "1,1,2,2-tetrachloroethane aerobic cometabolic biodegradation in slurry and soil-free bioreactors: A kinetic study," *Biochem. Eng. J.*, vol. 52, no. 1, pp. 55–64, 2010.

[7] A. Grostern and E. A. Edwards, "Growth of *Dehalobacter* and *Dehalococcoides* spp. during degradation of chlorinated ethanes," *Appl. Environ. Microbiol.*, vol. 72, no. 1, pp. 428–436, 2006.

[8] A. D. Maness, K. S. Bowman, J. Yan, F. A. Rainey, and W. M. Moe, "*Dehalogenimonas* spp. can reductively dehalogenate high concentrations of 1,2-dichloroethane, 1,2-dichloropropane, and 1,1,2-trichloroethane," *AMB Express*, vol. 2, no. 1, p. 54, 2012.

[9] J. L. Dillehay, K. S. Bowman, J. Yan, F. A. Rainey, and W. M. Moe, "Substrate interactions in dehalogenation of 1,2-dichloroethane, 1,2-dichloropropane, and 1,1,2-trichloroethane mixtures by *Dehalogenimonas* spp.," *Biodegradation*, vol. 25, no. 2, pp. 301–312, 2014.

[10] S. Zhao, C. Ding, and J. He, "Detoxification of 1,1,2-trichloroethane to ethene by *Desulfitobacterium* and identification of its functional reductase gene," *PLoS One*, vol. 10, no. 4, pp. 1–13, 2015.

[11] K. S. Bowman, M. F. Nobre, M. S. da Costa, F. A. Rainey, and W. M. Moe, "*Dehalogenimonas alkenigignens* sp. nov., a chlorinated-alkane-dehalogenating bacterium isolated from groundwater," *Int. J. Syst. Evol. Microbiol.*, vol. 63, pp. 1492–1498, 2013.

[12] J. Yan, B. A. Rash, F. A. Rainey, and W. M. Moe, "Isolation of novel bacteria within the Chloroflexi capable of reductive dechlorination of 1,2,3-trichloropropane," *Environ. Microbiol.*, vol. 11, no. 4, pp. 833–843, 2009.

[13] W. M. Moe, J. Yan, M. F. Nobre, M. S. da Costa, and F. A. Rainey, "*Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater," *Int. J. Syst. Evol. Microbiol.*, vol. 59, no. 11, pp. 2692–2697, 2009.

[14] M. J. Manchester, L. A. Hug, M. Zarek, A. Zila, and E. A. Edwards, "Discovery of a trans-

dichloroethene-respiring *Dehalogenimonas* species in the 1,1,2,2-tetrachloroethane-dechlorinating WBC-2 consortium," *Appl. Environ. Microbiol.*, vol. 78, no. 15, pp. 5280–5287, 2012.

[15] B. Z. Fathepure and J. M. Tiedje, "Reductive dechlorination of tetrachloroethylene by a chlorobenzoate-enriched biofilm reactor.," *Environ. Sci. Technol.*, vol. 28, no. 4, pp. 746–52, 1994.

[16] R. Steffan, C. Schaefer, and D. Lippincott, "Bioaugmentation for groundwater remediation: an overview," in *Bioaumentation for Groundwater Remediation*, H. F. Stroo, A. Leeson, and C. H. Ward, Eds. Springer Science Business Media, New York:, 2010, pp. 1–138.

[17] L. Martín-González, S. H. Mortan, M. Rosell, E. Parladé, M. Martínez-Alonso, N. Gaju, G. Caminal, L. Adrian, and E. Marco-Urrea, "Stable carbon isotope fractionation during 1,2-dichloropropane-to-propene transformation by an enrichment culture containing *Dehalogenimonas* strains and a dcpA gene," *Environ. Sci. Technol.*, vol. 49, no. 14, pp. 8666–74, 2015.

[18] D. Cichocka, M. Nikolausz, P. J. Haest, and I. Nijenhuis, "Tetrachloroethene conversion to ethene by a *Dehalococcoides*-containing enrichment culture from Bitterfeld," *FEMS Microbiol. Ecol.*, vol. 72, no. 2, pp. 297–310, 2010.

[19] T. Kaufhold, M. Schmidt, D. Cichocka, M. Nikolausz, and I. Nijenhuis, "Dehalogenation of diverse halogenated substrates by a highly enriched *Dehalococcoides*-containing culture derived from the contaminated mega-site in Bitterfeld," *FEMS Microbiol. Ecol.*, vol. 83, no. 1, pp. 176–188, 2013.

[20] S. H. Mortan, L. Martín-González, T. Vicent, G. Caminal, I. Nijenhuis, L. Adrian, and E. Marco-Urrea, "Detoxification of 1,1,2-trichloroethane to ethene in a bioreactor co-culture of *Dehalogenimonas* and *Dehalococcoides mccartyi* strains," *J. Hazard. Mater.*, vol. 331, pp. 218–225, 2017.

[21] P. Jiménez-Peñalver, T. Gea, A. Sánchez, and X. Font, "Production of sophorolipids from winterization oil cake by solid-state fermentation: Optimization, monitoring and effect of mixing," *Biochem. Eng. J.*, vol. 115, pp. 93–100, 2016.

[22] J. Yan, B. A. Rash, F. A. Rainey, and W. M. Moe, "Detection and quantification of *Dehalogenimonas* and ' *Dehalococcoides*' populations via PCR-based protocols targeting 16S rRNA genes," *Appl. Environ. Microbiol.*, vol. 75, no. 23, pp. 7560–7564, 2009.

[23] E.-M. Ewald, A. Wagner, I. Nijenhuis, H.-H. Richnow, and L. Ute, "Microbial dehalogenation of trichlorinated dibenzo-p-dioxins by a *Dehalococcoides*-containing mixed culture is coupled to carbon isotope fractionation," *Environ. Sci. Technol*., vol. 41 pp. 7744–7751, 2007.

[24] F. Aulenta, M. Potalivo, M. Majone, M. P. Papini, and V. Tandoi, "Anaerobic bioremediation of groundwater containing a mixture of 1,1,2,2-tetrachloroethane and chloroethenes," *Biodegradation*, vol. 17, no. 3, pp. 193–206, 2006.

[25] F. E. Löffler, J. M. Tiedje, and R. A. Sanford, "Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology," *Appl. Environ. Microbiol.*, vol. 65, no. 9, pp. 4049–4056, 1999.

[26] E. Marco-Urrea, J. Seifert, M. von Bergen, and L. Adrian, "Stable isotope peptide mass spectrometry to decipher amino acid metabolism in *Dehalococcoides* strain CBDB1," *J. Bacteriol.*, vol. 194, no. 16, pp. 4169–4177, 2012.

[27] C. J. Schipp, E. Marco-Urrea, A. Kublik, J. Seifert, and L. Adrian, "Organic cofactors in the metabolism of *Dehalococcoides mccartyi* strains.," *Philos. Trans. R. Soc. B,* vol. 368, no. 1616, p. 20120321, 2013.

[28] W.-Q. Zhuang, S. Yi, M. Bill, V. L. Brisson, X. Feng, Y. Men, M. E. Conrad, Y. J. Tang, and L. Alvarez-Cohen, "Incomplete Wood-Ljungdahl pathway facilitates one-carbon metabolism in organohalide-respiring *Dehalococcoides mccartyi*," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 17, pp. 6419–24, 2014.

[29] M. Pöritz, T. Goris, T. Wubet, M. T. Tarkka, F. Buscot, I. Nijenhuis, U. Lechner, and L. Adrian, "Genome sequences of two dehalogenation specialists - *Dehalococcoides mccartyi* strains BTF08 and DCMB5 enriched from the highly polluted Bitterfeld region," *FEMS Microbiol. Lett.*, vol. 343, no. 2, pp. 101–104, 2013.

[30] X. Maymó-Gatell, T. Anguish, and S. H. Zinder, "Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by '*Dehalococcoides ethenogenes*' 195," *Appl. Environ. Microbiol.*, vol. 65, no. 7, pp. 3108–3113, 1999.

[31] K. Mayer-Blackwell, M. Fincker, O. Molenda, B. Callahan, H. Sewell, S. Holmes, E. A. Edwards, and A. M. Spormann, "1,2-dichloroethane exposure alters the population structure, metabolism, and kinetics of a trichloroethene-dechlorinating *Dehalococcoides mccartyi* consortium," *Environ. Sci. Technol.*, vol. 50, no. 22, pp. 12187–12196, 2016.

[32] D. R. Johnson, P. K. H. Lee, V. F. Holmes, A. C. Fortin, and L. Alvarez-Cohen, "Transcriptional expression of the tceA gene in a *Dehalococcoides*-containing microbial enrichment.," *Appl. Environ. Microbiol.*, vol. 71, no. 11, pp. 7145–51, 2005.

CHAPTER 9

GENERAL CONCLUSIONS AND FUTURE WORK

9.1. Conclusions

The principal motivation for this study was the cultivation and characterization of organohalide respiring bacteria capable of transforming chlorinated alkanes. During this thesis, a stable *Dehalogenimonas*-containing enrichment culture derived from Besòs River estuary sediments was established. Compound-specific isotope analysis, molecular techniques (PCR, qPCR, and DGGE), and proteomic analysis were performed to get insight into the identity, physiology, biochemistry, and degradation pathways of the *Dehalogenimonas* strain contained in this consortium. The main findings of this study are described as follows:

1) The *Dehalogenimonas* contained in this culture exclusively dehalogenates vicinally chlorinated and brominated alkanes via dihaloelimination which is in agreement with other previously described *Dehalogenimonas* strains with the exception of strain WBC-2. In the enrichment stage of the consortia, the presence of methanogens was ceased with BES addition and the culture also was resistant to vancomycin. The stable culture was predominantly constituted by bacteria belonging to seven different genera, with *Dehalogenimonas* and *Azonexus* being the most abundant bacteria. The *dcpA* gene encoding 1,2-DCP reductive dehalogenase was also identified in the consortia.
2) As compound-specific stable isotope analysis has evolved as one of the tools to assess the organohalide transformation, the carbon stable isotope fractionation (ε^{C}_{bulk}) of 1,2-DCP and dual isotope enrichment factor (Λ) of carbon and chlorine for the 1,2-DCA by *Dehalogenimonas*-containing enrichment culture were determined. The ε^{C}_{bulk} obtained during 1,2-DCP dichloroelimination was in the same order of magnitude but statistically differs from that reported for *Dehalococcoides mccartyi* strain RS and

strain KC, although the *dcpA* gene encoding 1,2-DCP reductive dehalogenase was also identified in both genus. The Λ values for 1,2-DCA dichloroelimination in *Dehalogenimonas* permits to distinguish among different degradation pathways (oxidation, hydrolytic dehalogenation and dihaloelimination). Interestingly, the value of Λ obtained for *Dehalogenimonas* is similar to those obtained in two groundwaters contaminated with 1,1,2-TCA in Italy and U.S.A.

3) The isolation the *Dehalogenimonas* was attempted using dilution to extinction method in liquid and agar medium. According to previous studies, *Dehalogenimonas* apparently do not form colonies in semisolid medium. After applying the dilution-to-extinction approach in liquid medium and using selected antibiotics, a clone library revealed that our consortia was composed by *Dehalogenimonas sp.* (87 %), *Desulfovibrio sp.* (12 %), and unclassified *Veillonellaceae* (1.2 %).
4) Proteomic techniques were applied to get insights into the RdhA expressed during growth of *Dehalogenimonas* with EDB and 1,1,2-TCA. Shotgun proteomics (LTQ-Orbitrap) identified the RDase DcpA in both halogenated compounds, indicating that it is a feasible candidate although biochemical confirmation is required. Application of BN-PAGE was not successful in identifying the RDase catalyzing 1,1,2-TCA probably due to the low abundance of protein in the culture.
5) A co-culture composed of *Dehalococcoides mccartyi* strain BTF08 and *Dehalogenimonas* was constructed with the aim to detoxify 1,1,2-TCA. Preliminary studies were performed in serum bottles and then scale-up to 5-L bioreactor. Though the complete dechlorination of 1,1,2-TCA to ethene was successfully achieved, the

dechlorination of VC was found out to be uncoupled from *Dehalococcoides mccartyi* strain BTF08 growth.

9.2. Future work

1) The isolation of *Dehalogenimonas* are underway in our lab using selected antibiotics targeting *Desulfovibrio* species. The isolation of *Dehalogenimonas* facilitates a better understanding of their physiological, biochemical and molecular characteristics.
2) The genome of *Dehalogenimonas* present in our culture is currently sequenced. The annotation of the genome and the comparison with previous sequenced genomes of *Dehalogenimonas* can provide important conclusions about the function and regulation of reductive dehalogenases and other key enzymes.
3) The annotated genome of this *Dehalogenimonas* can guarantee a reliable identification of proteins without the need of the constructed database used in this study. In addition, Blue-Native PAGE and gel activity tests should be conducted again with cultures of *Dehalogenimonas* at higher cell densities to identify functional RDases.
4) Compound specific isotope analysis during the reductive dehalogenation of other halogenated compounds by *Dehalogenimona*s should also be conducted as nowadays the information on isotopic fractionation by *Dehalogenimonas* is very limited (this research was the first isotopic study done on *Dehalogenimonas* species). In this sense, we are currently determining the dual isotope fractionation of carbon and chlorine during 1,1,2-TCA dichloroelimination by *Dehalogenimonas* and comparing this value with groundwaters impacted with 1,1,2-TCA in the region of Barcelona.

5) Eventhough the work described in part of the thesis focused on the isolation of the *Dehalogenimonas* present in the consortia, in practice these organisms exist in mixed cultures and it is desirable to supply them as mixed cultures for future bioaugmentation endeavors because they are more robust. In order to scale up the production of *Dehalogenimonas* in bioreactors, more studies are needed to select an electron acceptor that provide a higher growth rate and a better knowledge on the growth conditions (i.e. optimal temperature, nutrients, hydrogen levels, etc).
6) As chlorinated alkanes are often occur in mixtures in groundwater pollutants, the study on dechlorination of several chlorinated alkanes in mixtures such 1,2-DCP, 1,2-TCP, 1,2-DCA and 1,1,2-TCA by *Dehalogenimonas* can also being carried out in the future. This may provide insights into the chlorinated alkane transformation processes by *Dehalogenimonas* sp. when multiple electron acceptors instead of sole electron acceptor are present in environments.
7) Recently, our research group has been involved in a project dealing with *in situ* biodegradation of groundwaters contaminated with chlorinated compounds. In the case that these sites do not have the right bacteria to detoxify the environment, it could be an opportunity to apply this *Dehalogenimonas* consortia to degrade specific chlorinated alkanes. Further research into the cost of growing large volumes of *Dehalogenimonas* for bioaugmentation would be invaluable for such applied projects.