Modes of anaerobic respiration catalysed by members of the class *Dehalococcoidia*

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Summary

The bacterial class *Dehalococcoidia*, phylum *Chloroflexi,* encompasses several cultivated strains and many so far uncultivated bacteria, known from molecular detection of their 16S rRNA gene sequences in marine sediments. All cultivated representatives exclusively respire with halogenated compounds as terminal electron acceptor in a process termed organohalide respiration. However, it is not known whether organohalide respiration can explain the abundance and ubiquitous distribution of *Dehalococcoidia* in marine pristine sediments.

In the current work, electron acceptors for respiration of *Dehalococcoidia* were investigated with the model organism *Dehalococcoides mccartyi* strain CBDB1 to gain insight into which properties of halogenated compounds are required to serve as electron acceptor of *Dehalococcoidia* and might in part explain their abundance at marine sites. For this, a microtiter plate-based assay was established to measure reductive dehalogenase activity – the key enzyme in organohalide respiration. *D. mccartyi* strain CBDB1 dehalogenated chlorinated benzonitriles, chlorinated anilines and brominated phenols, benzenes, pyridines, furoic and benzoic acids and specific activities of 4.5 to 241 nkat mg^{-1} protein were determined. In cultivation experiments, growth yields of 0.1 x 10^{14} to 2.3 x 10^{14} cells mol^{-1} halogen released were obtained with strain CBDB1. Brominated electron acceptors were generally dehalogenated to a further extent than their chlorinated equivalents and showed higher specific activity rates in resting cell assays. Results of shotgun proteomics and activity assays suggest that the same reductive dehalogenases are involved in the dehalogenation of brominated and chlorinated benzenes, indicating chemical properties influence reductive dehalogenation patterns of strain CBDB1. The correlation of density functional theory calculations with microbial and biochemical experiments revealed that functional groups decreasing electron density at the halogen and not at the halogen-substituted carbon enhance reductive dehalogenation. The most positive halogen partial charge predicted the regioselective dehalogenation catalysed by strain CBDB1 for up to 96% of all evaluated molecules. These findings suggest a cobalamin–halogen interaction during reductive dehalogenation which stands in contrast to previous models of reductive dehalogenation. A halogen–cobalamin interaction could in part explain the broad electron acceptor diversity of organohalide-respiring *Dehalococcoidia* suggesting that a multitude of natural organohalogens – including brominated aromatics – could serve as natural halogenated electron acceptor for respiration and growth of *Dehalococcoidia* in marine sediments. In addition, the established prediction systems may allow for improved fate prediction of halogenated compounds from anthropogenic or natural sources in the environment. Reductive dehalogenation of brominated aromatics was also shown to occur in marine deep-sea sediment microcosms with activity assays, inhibition studies and cultivation experiments although no *Dehalococcoidia* were detected. However, the conducted experiments demonstrated that similar dehalogenation patterns observed with model organism strain CBDB1 occur in non-contaminated deep-sea sediments. To investigate the potential of *Dehalococcoidia* to respire

non-halogenated electron acceptors, the genetic information from the single amplified *Dehalococcoidia* genome SAG-C11 was used to design primers for a newly identified class of dissimilatory sulphite reduction genes (*dsr*) in *Chloroflexi*. The primers were used to study sediment samples from different locations and depths for the presence of *Chloroflexi*-related *dsr* genes. Several *dsr* genes located in a similar genetic context as observed in SAG-C11 and affiliating with *dsr* genes from SAG-C11 in phylogenetic analyses were detected in sediments from Aarhus, the Baffin Bay and in tidal flat sediments of the Wadden Sea. The obtained *dsrAB* genes shared 71–100% nucleotide sequence identities with *dsrAB* of SAG-C11 suggesting that dissimilatory sulphite reduction is a more widespread mode of respiration in the class *Dehalococcoidia* and could contribute together with organohalide respiration of brominated natural electron acceptors to the abundance and ubiquitous presence of *Dehalococcoidia* in marine sediments.

Zusammenfassung

Die vorliegende Arbeit beschreibt Formen der anaeroben Atmung der bakteriellen Klasse *Dehalococcoidia,* Abteilung *Chloroflexi*, welche zu den häufigsten und abundantesten Bakteriengruppen mariner Sedimente gehört und deren kultivierten Vertreter aus terrestrischen Habitaten ausnahmslos auf eine Atmung mit halogenierten organischen Verbindungen (Organohalid-atmung) angewiesen sind. Kultivierung und Mikrotiterplatten-basierte Aktivitätstests mit strukturell unterschiedlichen Elektronenakzeptoren zeigten, dass *Dehalococcoidia* Modellorganismus Stamm CBDB1 chlorierte Benzonitrile, chlorierte Aniline und bromierte Phenole, Benzole, Pyridine, Furon- und Benzoesäuren dehalogenierte. Wachstumsausbeuten von 0,1 x 10^{14} bis 2,3 x 10^{14} Zellen pro Mol freigesetztem Halogen Anion und spezifische Aktivitäten von 4,5 bis 241 nkat pro mg Protein wurden gemessen. Bromierte Aromaten wurden weitergehend und mit höheren spezifischen Aktivitäten dehalogeniert als ihre chlorierten Äquivalente. Aktivitätstests und Shot-Gun Proteomik deuteten darauf hin, dass die gleichen reduktiven Dehalogenasen in die Umsetzung der getesteten bromierten und chlorierten Benzole involviert sind. Bromierte aromatische Verbindungen, die in marine Habitaten vorkommen, könnten somit als natürliche Elektronenakzeptoren für Organohalid-atmende *Dehalococcoidia* in marinen Sedimenten dienen. Eine reduktive Dehalogenierung bromierter Verbindungen wurde auch in Aktivitätstests mit Zellen aus marinen Sediment-Mikrokosmen und durch Inhibitionsstudien nachgewiesen. Obwohl keine *Chloroflexi* in den marinen Sediment-Mikrokosmen detektiert werden konnten, zeigten die Experimente, dass reduktive Dehalogenierung auch in marinen nicht-kontaminierten Tiefseesedimenten vorkommen kann. Die Korrelation mikrobiologischer und biochemischer Versuche mit Dichtefunktionaltheorie-basierten Berechnungen verschiedener Partialladungs-Modelle zeigte, dass die regioselektive Dehalogenierung durch Stamm CBDB1 mit Hilfe der positivsten Halogen-Partialladung für 96% der untersuchten Moleküle vorausgesagt werden konnte. Außerdem konnte gezeigt werden, dass funktionelle Gruppen, die die Elektronendichte am Halogenatom verringern, die reduktive Dehalogenierung unterstützen. Dies weist auf eine Cobalamin–Halogen Interaktion während der reduktiven Dehalogenierung hin und steht im Gegensatz zu bisherigen Modellen der reduktiven Dehalogenierung. Eine Cobalamin–Halogen Interaktion könnte dazu beitragen, das breite Elektronenakzeptoren Spektrum Organohalid-atmender *Dehalococcoidia* zu erklären und könnte Organohalid-atmenden *Dehalococcoidia* in marinen Sedimenten erlauben, verschiedenste halogenierte Verbindungen wie z.B. bromierte komplexe Aromaten für die Atmung zu nutzen. In einem weiteren Teil dieser Dissertation wurde mit molekularbiologischen Methoden das Potential von *Dehalococcoidia* untersucht, nicht-halogenierte Elektronenakzeptoren zu nutzen. Dies könnte die Stratifikation von *Dehalococcoidia* Subgruppen entlang geochemischer Gradienten erklären. Dazu wurden mit Hilfe der Genominformation einer *Dehalococcoidia*-Einzelzelle („SAG-C11“) PCR-Primer entwickelt, die spezifisch *dsr* Gene in *Dehalococcoidia* amplifizierten. Mit diesen Primern wurden das Vorkommen und die Diversität der

dissimilatorischen Sulfitreduktase (*dsr*) in *Dehalococcoidia* in Sedimenten verschiedener Orte und Tiefen untersucht. Unterschiedliche *dsr* Gene, die sich in einem ähnlichen genetischen Kontext wie in SAG-C11 befanden und in phylogenetischen Analysen mit *dsr* Sequenzen aus SAG-C11 gruppierten, wurden in Sedimenten der Aarhusbucht, der Baffinbucht und in Wattsedimenten der Nordsee nachgewiesen. Nukleotidsequenzähnlichkeiten von 71–100% der aus marinen Sedimenten amplifizierten *dsrAB-S*equenzen zu den *dsrAB*-Sequenzen aus SAG-C11 weisen darauf hin, dass dissimilatorische Sulfitreduktion ein verbreiteter Atmungsprozess in *Dehalococcoidia* ist und zusammen mit der Organohalidatmung bromierter Verbindungen zu einer Erklärung der ubiquitären Verbreitung von *Dehalococcoidia* in marine Sedimenten in unterschiedlichen biogeochemischen Zonen beitragen kann.

List of publications and author contributions

Publication 1 (*shared first authorship):

Anke Wagner*, **Myriel Cooper***, Sara Ferdi, Jana Seifert, Lorenz Adrian: "Growth of *Dehalococcoides mccartyi* strain CBDB1 by reductive dehalogenation of brominated benzenes to benzene", Environmental Science & Technology, 2012, 46 (16), 8960–8968, DOI: 10.1021/es3003519

Contributions: Anke Wagner, Myriel Cooper and Lorenz Adrian developed the concept of the study. Cultivation studies of strain CBDB1 were done by Anke Wagner and Sara Ferdi. Cultivation for activity assays was done by Myriel Cooper, Anke Wagner and Bernd Krostitz. Myriel Cooper established and applied the activity assay and evaluated the data from activity measurements. Shotgun proteomics of bromobenzene cultures was done by Jana Seifert and data were evaluated by Myriel Cooper and Lorenz Adrian. The manuscript was drafted by Anke Wagner and Myriel Cooper and revised by Lorenz Adrian.

Publication 2:

Myriel Cooper, Anke Wagner, Dominik Wondrousch, Frank Sonntag, Andrei Sonnabend, Martin Brehm, Gerrit Schüürmann, and Lorenz Adrian: "Anaerobic microbial transformation of halogenated aromatics and fate prediction using electron density modelling", Environmental Science & Technology, 2015, 49 (10), 6018-6028, DOI: 10.1021/acs.est.5b00303.

Contributions: Myriel Cooper, Anke Wagner and Lorenz Adrian developed the concept for the study. Dominik Wondrousch calculated electron density distributions, including sigma and pi partial charges. Martin Brehm calculated the electron density distributions in solvents. The evaluation of electron density distributions for dehalogenation pathway prediction was done by Myriel Cooper and revised by Anke Wagner. The model for the newly proposed reaction mechanism was developed by Anke Wagner, Myriel Cooper, Gerrit Schüürmann and Lorenz Adrian. Cultivation of strain CBDB1 with chlorinated anilines, 2-chloro-6-fluorobenzonitrile, chlorinated pyridines, brominated furoic and benzoic acids was done by Myriel Cooper. Frank Sonntag cultivated strain CBDB1 with chlorinated benzonitriles and Andrei Sonnabend with 3-bromo-2-chloropyridine and chlorinated thiophenes. Data evaluation was done by Myriel Cooper and Anke Wagner. Activity assays and data evaluation were conducted by Myriel Cooper. The manuscript was drafted by Myriel Cooper and Anke Wagner, Gerrit Schüürmann and Lorenz Adrian revised the manuscript.

Publication 3 (in preparation, *shared first authorship):

Kenneth Wasmund*, **Myriel Cooper***, Lars Schreiber, Karen G. Lloyd, Brett Baker, Dorthe G. Petersen, Andreas Schramm, Ramunas Stepanauskas, Richard Reinhardt, Bo Barker Jørgensen, Alexander Loy, Lorenz Adrian: Single cell genome sequencing and targeted gene amplifications implicate members of the class *Dehalococcoidia* (phylum *Chloroflexi*) in sulfur cycling.

Contributions: Kenneth Wasmund and Lorenz Adrian developed the concept of the study. Lars Schreiber, Karen Lloyd, Brett Baker, Dorthe G. Petersen, Andreas Schramm and Bo Barker Jørgensen were involved in the sampling, planning and initiating single cell isolations. Single cell isolations were done by Ramunas Stepanauskas. Sequencing was done by Richard Reinhardt. Lars Schreiber and Kenneth Wasmund assembled the genome, Kenneth Wasmund analysed the assembly and annotated the genes. Pathways integration was done by Kenneth Wasmund and Lorenz Adrian. Alexander Loy contributed to annotation and analysis of *dsr*-genes. Myriel Cooper and Kenneth Wasmund developed the concept for targeted gene amplification. For this, Myriel Cooper and Lars Schreiber sampled sediments at Aarhus Bay. Experiments for targeted gene amplification and data evaluation were done by Myriel Cooper. The manuscript was written by Kenneth Wasmund, Myriel Cooper and Lorenz Adrian.

Abbreviations

16S rRNA *16S ribosomal ribonucleic acid*
APR *Adenylyl-sulphate-reductase*
Blast *Basic Local Alignment Search Tool*
BLASTn *Standard Nucleotide BLAST*
CbrA *Chlorobenzene reductive dehalogenase*
CID *Collision-induced dissociation*
cmbsf *centimetres below sea floor*
Co(I) *Cob(I)alamin*
Co(II) *Cob(II)alamin*
Co(III) *Cob(III)alamin*
CobA/HemD uroporphyrinogen III methyl transferase/synthase
CPh *Chlorophenol*
CPhate *Chlorophenolate*
DBB *Dibromobenzene*
DCB *Dichlorobenzene*
DCPh *Dichlorophenol*
DCPhate *Dichlorophenolate*
DMSO *Dimethyl sulfoxide*
dNTP *Nucleoside triphosphate*
drsAB *Dissimilatory sulphite reductase subunit A and B genes*
Dsr *Dissimilatory sulphite reductase*
emPAI *Exponentially modified protein abundance index*
FID *Flame ionization detector*
fw *Forward*
GC *Gas chromatograph*
GluRS *Porphobilinogen deaminase, glutamyl-tRNA reductase*
HCB *Hexachlorobenzene*
HPLC *High performance liquid chromatography*
LB *Lysogeny broth*
LC-ESI-MS/MS Liquid chromatography electrospray ionization mass spectrometry/ mass spectrometry
MarR *Multiple antibiotic resistance regulator*
MBB *Monobromobenzene*
MbrA *Chloroethene reductive dehalogenase*
MCB *Monochlorobenzene*
MV *Methyl viologen*
NCBI *National Center for Biotechnology Information*
nr *non-reduntant*
ODP *Ocean drilling program*
PCE *Tetrachloroethene*
PceA T*etrachlorethene reductive dehalogenase*
PeCB *Pentachlorobenzene*
PeCPh *Pentachlorophenol*
PeCPhate *Pentachlorophenolate*
rdh *Reductive dehalogenase homologous*
rdhA *Reductive dehalogenase homologous subunit A gene*
RdhA *Reductive dehalogenase subunit A*
rdhB *Reductive dehalogenase homologous subunit B gene*
rv *Reverse*
SOC *Super optimal broth*
sp. *Species*
TAE *Tris-Acetate EDTA buffer*
TAT *Twin arginine transport*
TBB *Tribromobenzene*
TCB *Trichlorobenzene*
TCE *Trichloroethene*
TceA *Trichloroethene reductive dehalogenase*
TCPh *Trichlorophenol*
TCPhate *Trichlorophenolate*
TE-buffer *Tris-EDTA buffer*
TeCB *Tetrachlorobenzene*
TeCPh *Tetrachlorophenol*
TeCPhate *Tetrachlorophenolate*
UPLC *Ultra performance liquid chromatography*
VcrA *Vinyl chloride reductive dehalogenase*
w/v *Weight per volume*
X-Gal *5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside*

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1 Introduction

1.1 The class *Dehalococcoidia*

1.1.1 Isolated and cultivated strains of the class *Dehalococcoidia*

The bacterial class *Dehalococcoidia* (previously referred to as the *Dehalococcoides*-related *Chloroflexi* (1)) belongs together with the classes *Anaerolineae, Caldilineae, Ktedonobacteria, Thermomicrobia, and Chloroflexia,* to the deeply branching phylum *Chloroflexi* (2-5). The species *Dehalococcoides mccartyi, Dehalogenimonas lykanthroporepellens*, *Dehalogenimonas alkenigignens*, *"*Dehalobium chlorocoercia*"* and many sequences of so far uncultivated microorganisms from marine and terrestrial subsurface sites constitute the *Dehalococcoidia* class (1, 6). More than seven strains of *Dehalococcoides mccartyi* have been isolated, including strain 195, CBDB1, VS, BAV1, FL2, MB and GT (7-16). For each of the species *Dehalogenimonas lykanthroporepellens* and *Dehalogenimonas alkenigignens* two isolates are cultivated (17, 18). Strain DF-1 is the only cultivated representative of "Dehalobium chlorocoercia*"* (19). Most cultivated *Dehalococcoides* and *Dehalogenimonas* strains have been isolated from terrestrial freshwater sites, among them river sediments, aquifers or digester sludge (7-13, 17, 18, 20, 21) strain MB and "Dehalobium chlorocoercia*"* DF-1 were isolated from estuarine sediments (14, 22).

The cultivation of these so far isolated *Dehalococcoidia* is carried out under strictly anoxic conditions in reduced synthetic mineral salts medium, amended with vitamins, except for "Dehalobium chlorocoercia*"* requiring the addition of a *Desulfovibrio* strain cell extract (19). Acetate, CO_2 or bicarbonate serve as carbon source and hydrogen or formate as the sole electron donor. Halogenated aromatic or aliphatic compounds are used as sole terminal electron acceptors allowing for energy conservation only *via* respiration with halogenated compounds. This process is termed organohalide respiration. During organohalide respiration, terminal respiratory enzymes called reductive dehalogenases transfer electrons onto the halogenated organic compound, thereby cleaving the carbon-halogen bond (for more details on the biochemistry of these enzymes see below). Many of the halogenated compounds used for organohalide respiration by *Dehalococcoidia* are highly toxic to humans and wild life and persistent in the environment. Reductive dehalogenation often renders the compound less toxic and/or more accessible for further degradation steps. Because all so far isolated and cultivated *Dehalococcoidia* are able to transform a variety of highly toxic halogenated pollutants and depend on organohalide respiration for energy conservation, cultivated strains of the class *Dehalococcoidia* are considered as key organisms for bioremediation by bioaugmentation or biostimulation of organohalogen contaminated sites (23). Additionally, for many halogenated compounds no other organisms besides members of the class *Dehalococcoidia* are known to catalyse their transformation. However, handling and cultivation of *Dehalococcoidia* strains is difficult and

time consuming. Growth of *Dehalococcoidia* strains in culture is slow with doubling times of 0.8 to 4.1 days, oxygen is not tolerated and the electron acceptors required for cultivation are often highly toxic. Despite this, strains of the class *Dehalococcoidia* seem to be robust towards starvation, as cells were observed to recover their dehalogenation activity after months of no activity at 4°C (6). Dehalogenation activity was observed at 15°C to 35° C, but highest activity was observed at about 30°C (6).

The morphology of cultivated *Dehalococcoidia* cells was revealed by transmission and scanning electron microscopy which showed disc-shaped cells less than 1 µm wide and 0.1 to 0.2 µm thick, with concave indentations at each side. Cultivated *Dehalococcoidia* cells exhibit a resistance towards antibiotics which interfere with the synthesis of a cell wall such as ampicillin and vancomycin, indicating they lack peptidoglycan. Cells were shown to be covered by a protein layer, resembling the S-layer of *Archaea* (7, 8, 10).

On the 16S rRNA gene level, *Dehalococcoides mccartyi* and *Dehalogenimonas lykanthroporepellens* strains share ~90% sequence identity and ~87.5% with "Dehalobium chlorocoercia*"* (6). *Dehalococcoides mccartyi* strains share more than 98% sequence identity among each other (6, 12). Genome sequencing and annotation revealed that *Dehalococcoides mccartyi* strains contain chromosomes of 1.34 to 1.47 Mb encoding around 1500 genes for a streamlined metabolism (24-26) while the genome of *Dehalogenimonas lykanthroporepellens* BL-DC-9 is slightly bigger with 1.69 Mb (27). Genes of *Dehalococcoides mccartyi* are highly conserved between different strains except for a high plasticity region around the origin of replication, which contains most of the reductive dehalogenase homologous genes (*rdh*) (25, 26, 28). Between 10 to 36 *rdh* genes on a single genome have been identified (24, 26, 28-30), which are located in an operon coding for *rdh* subunits. The high number of reductive dehalogenase homologues in the genome of cultivated *Dehalococcoidia* strains compared to the size of the genome, reflects the specialisation of cultivated *Dehalococcoidia* strains on reductive dehalogenation.

1.1.2 *Dehalococcoidia* in the marine subsurface

Marine sediments harbor an immensely large and diverse number of microbes. Subsurface drilling programs conducted allowed the analysis of sediment cores and thereby the detection of viable cells or membrane lipids as far as 1.9 km below the seafloor (31, 32). Recent estimations predicted between 2.9×10^{29} to 5.39×10^{29} cells in in the marine subsurface, corresponding to 0.18–3.6% of the total global biomass (33, 34). Cells were suggested to be inactive or adapted for extraordinarily low metabolic activity in deeper organic depleted sediment layers (35). However, several studies found that cells are active and able to survive on low energy fluxes (36-39). Therefore, this vast ecosystem is expected to play a crucial role in global biogeochemical element cycling, especially over geological time scales.

Insights into the bacterial composition of marine sediments were obtained by 16S rRNA gene sequence and metagenomic analyses. Sequences affiliated with the phylum *Chloroflexi* are one of the most abundant bacterial groups in marine sediments together with sequences of *Gammaproteobacteria*, *Planctomycetes* and the candidate phylum JS1, with *Chloroflexi* representing up to 15–20% of the bacterial communities (34, 40). Furthermore, *Chloroflexi* have been shown to be ubiquitously present in marine sediments from different sites and depths, ranging from shallow organic-rich to deep and organic-depleted sediment layers (41-45). Sequences affiliating with the class *Dehalococcoidia* seem to be one of the most widespread and dominant groups within the marine *Chloroflexi*, implicating a significant contribution of *Dehalococcoidia* to biogeochemical processes (46-51). Although *Chloroflexi* have been enriched from seafloor basalts, estuarine and tidal flat sediments (52-57), and two *Dehalococcoidia* isolates have been obtained from estuarine sites (14, 22), *Dehalococcoidia* from marine pristine sediments have not been cultivated in a pure culture successfully so far, narrowing the available information regarding their metabolic properties.

The closest cultivated relatives of marine subsurface *Dehalococcoidia* sequences are notably *Dehalococcoides mccartyi*, *Dehalogenimonas* spp. and *"*Dehalobium chlorocoercia*"*, with up to 89% of sequence similarity (40) (Figure 1-1). Indeed, many characteristics of the cultivated *Dehalococcoidia* spp. could be advantageous in deeper marine sediment environments. This includes their slow growth, specific metabolic pathways adapted for environments with scarce resources, a large surface to volume ratio, and an even for bacteria of extremely small cell size of 1 µm, a small streamlined genome, the absence of oxygen protection enzymes, and the usage of hydrogen as electron donor, and acetate as a carbon source. Hydrogen and acetate are both available from fermenting organisms in shallow sediments and even in deep sediments, as a result of thermal activation/maturation and aromatisation of buried organic matter (58, 59). However, little is not known to which extend reductive dehalogenation may explain the widespread distribution of *Dehalococcoidia* in pristine sediments.

Sequences of reductive dehalogenase homologous (*rdh*) genes – the key enzymes for organohalide respiration by cultivated *Dehalococcoidia* – were detected in marine sediments from the southeast Pacific off Peru, the Eastern Equatorial Pacific, the Juan de Fuca Ridge flank off Oregon, and the northwest Pacific off Japan in sediments down to 358 meters below the seafloor after whole genome amplification of DNA from sediment samples (60). In total 32 reductive dehalogenase homologues were identified, of which many affiliated with reductive dehalogenase sequences from *Dehalococcoides mccartyi* strains 195 and CBDB1 (60, 61). Moreover, experiments with enrichment cultures from tidal flat, estuarine sediments or contaminated lagoon sediments connected organohalide-respiring activity with the detection of 16S rRNA genes phylogenetically related to *Dehalococcoidia* in enrichment cultures (52-56).

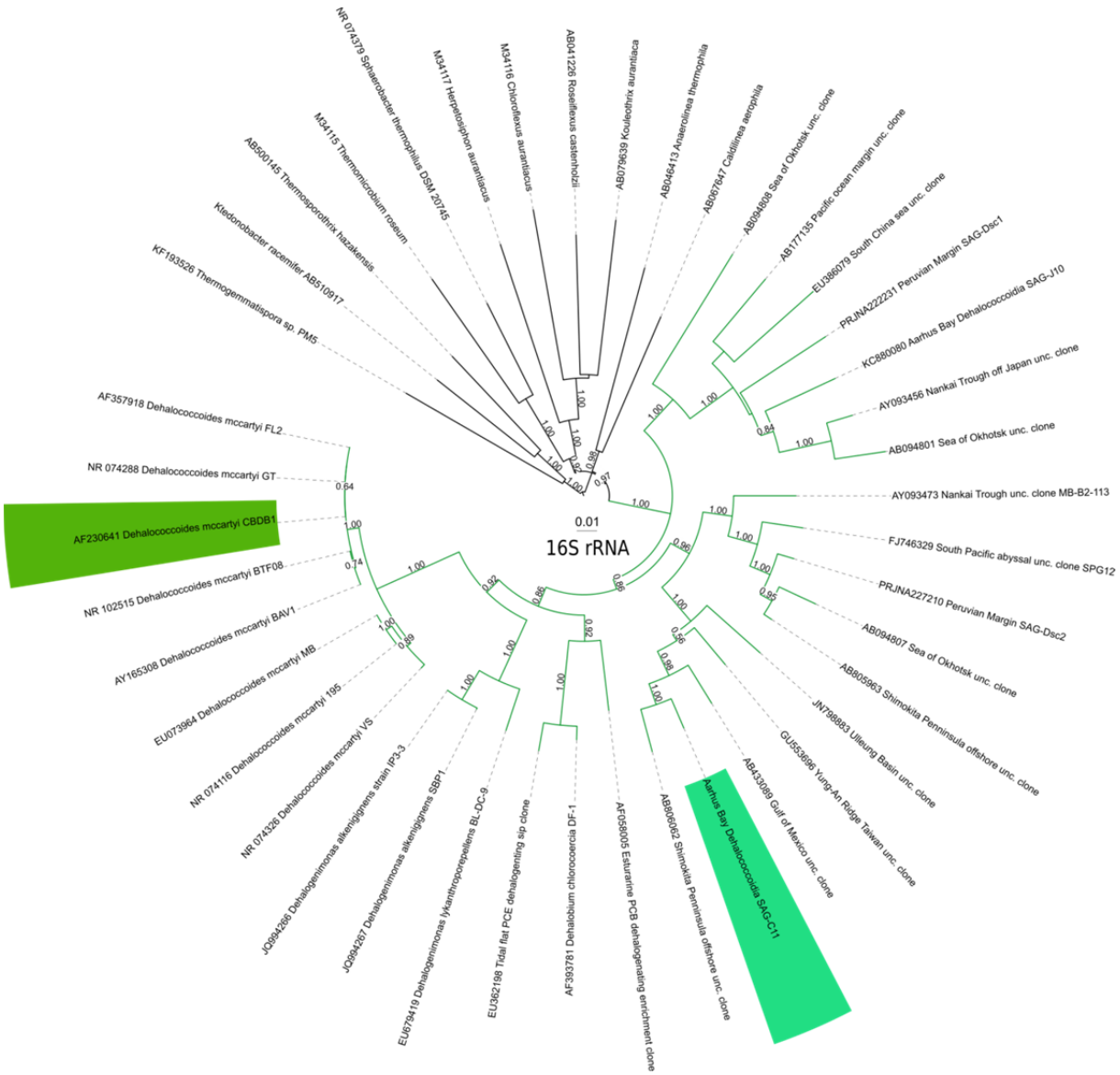

Figure 1-1: Rooted 16S rRNA gene-based phylogenetic tree of representatives of the phylum *Chloroflexi.* Green branches mark the *Dehalococcoides*-related *Chloroflexi* cluster. *Dehalococcoides mccartyi* strain CBDB1 used in the current work as model organism for organohalide-respiring *Dehalococcoidia* is highlighted in (■). Single amplified *Dehalococcoidia* genome C11 (SAG-C11), was used in the current work as model organism for *Dehalococcoidia* with an organohalide respiration-independent mode of living is highlighted in (■). The tree was constructed using the Neighbour joining algorithm, evolutionary distances were calculated using the p-distance method in Mega 6.0. Numbers show bootstrap values above 0.5 after 1000 replications.

Diverse *Dehalococcoidia* subgroups have been identified in marine sediments. All isolated organohalide-respiring strains of the class *Dehalococcoidia* affiliated with one of the subgroups termed ‘Ord-DEH’ (47). Indications of metabolic properties of *Dehalococcoidia* from other subgroups have been gained from single-cell and metagenome analyses (46, 48, 62). Annotations of *Dehalococcoidia* single-cell genome DEH-J10 (subgroup ‘GIF9-B’) (46), Dsc-1 and DscP2 (48), as well as metagenomics pan-genomes RGB-2 (subgroup GIF-9) and RGB-1351 (tentatively assigned to the GIF3 or vadinBA26 subgroup) (62) gave more detailed information about potential metabolic properties of different *Dehalococcoidia* subgroups. In the genomes of DEH-J10, DscP2, RGB-2 and RGB-1351, genes coding for enzymes of the Wood-Ljungdahl pathway were detected. For DEH-J10,

RGB-2 and RGB-1351, ATP generation during the formation of acetate from acetyl-CoA, and beta oxidation of fatty acids was furthermore predicted. In *Dehalococcoidia* single amplified genome DEH-J10, genes which confer the ability to oxidize substituted aromatics and to use dimethyl sulfoxide (DMSO) or trimethylamine N-oxide as terminal electron acceptor for respiration via a dimethyl sulfoxide reductase were found (46). No indications for organohalide-depending modes of living were detected in any of the genomes. In RBG-2 and DscP2, genes coding for an aerobic haloacid dehalogenases were detected which catalyzes dehalogenation of chlorinated and brominated substrates (63, 64). Haloacid dehalogenases however, are non-homologous to reductive dehalogenases and are not used as terminal reductases for respiration. In DscP2 a gene encoding a protein with homology over an Fe-S cluster domain of known respiratory RdhA was detected, but a TAT leader sequence and a typically adjacent *rdhB* gene coding for a membrane anchor subunit was missing (48).

1.2 Organohalide respiration

Halogenated compounds are introduced to the environment from anthropogenic sources as pesticides, flame retardants, additives to polymers and pharmaceuticals and during the production of dyes, agrochemicals and solvents (65-67). To date many halogenated compounds have been banned owing to their high toxicity and negative effects on humans and the environment. However, halogenated compounds often persist in the environment long time after their prohibition as a result of their recalcitrant properties and their potential to bioaccumulate. Accordingly, they can be widely detected not only in sediments and water, but also in tissues and milk of animals and humans (68-73). Many naturally occurring halogenated compounds are also known to exist (described below).

Transformation of halogenated compounds highly depends on the environment. In oxic environments, transformation of halogenated alkenes, short-chain alkanes and some aromatic compounds was reported involving mono- and dioxygenases and molecular oxygen in metabolic and co-metabolic processes (74-76). A preference for halogenated organic compounds with a low number of halogen substituents was observed for metabolic aerobic dehalogenation (77). Recently, the reductive dehalogenation of halogenated compounds in aerobic environments was also described (78).

In anoxic environments, several bacterial groups that are able to catalyse reductive dehalogenation reactions have been identified (7, 8, 10, 79-82). Members of the *Delta-* and *Epsilonproteobacteria* are facultative organohalide-respiring bacteria and the organohalide-respiring members of these phyla can also grow by fermentation. The phylum *Firmicutes* comprises both obligate and facultative organohalide-respiring organisms. All isolated and cultivated strains of the class *Dehalococcoidia* from the phylum *Chloroflexi* are obligate organohalide-respiring organisms. Values for changes in Gibbs free energy during reductive dehalogenation of various halogenated aromatic and aliphatic compounds suggested a similar energy yield for halogenated alkyl and aryl compounds as for the nitrate/nitrite couple when used as terminal electron acceptor for respiration in anoxic environments.

In contrary to aerobic dehalogenation, a preferential dehalogenation of higher halogenated compounds compared to less halogenated compounds as terminal electron acceptor in anoxic environments was predicted (83). In rather rare cases, halogenated compounds were reported to be transformed by other processes than reductive dehalogenation under anoxic conditions (84, 85).

Co-metabolic dehalogenation, in which other energy sources or electron acceptors are required and only low dehalogenation rates are observed, has been reported predominantly for aliphatic halogenated compounds in anoxic environments (86). The very toxic and common contaminant vinyl chloride is dehalogenated co-metabolically by *Dehalococcoides mccartyi* strain 195 but can be used for growth in a metabolic transformation by strain BAV1 (8, 10). Co-metabolic reductive dehalogenation was also observed for sulphate reducers (87), methanogenic *Archaea* (88) or acetogens (89).

For the metabolic transformation of halogenated compounds by *Dehalococcoidia* hydrogen serves as electron donor, except for 'Dehalobium chlorocoercia' using formate as electron donor (6). The electron transport between hydrogenase and reductive dehalogenase is coupled to the generation of ATP and bacterial growth. For *Dehalococcoidia* the electron shuttle between hydrogenase and reductive dehalogenase has not been identified so far. No cytochrome-containing proteins or cytochrome biosynthesis genes have been identified in *Dehalococcoides mccartyi* strains (24-26). Quinones serve as electron shuttle between hydrogenase and reductive dehalogenase in organohalide-respiring *Desulfitobacterium hafniense* (90), however, no evidence was found that quinones are involved in organohalide respiration in *Dehalococcoides mccartyi* strain CBDB1 (91).

Reductive dehalogenases are the key enzymes in organohalide respiration. A main difference between the genomes of *Dehalococcoides mccartyi* strains is the number and type of reductive dehalogenase operons. A range of 10 to 36 genes encoding *rdh* genes on a single genome have been identified (24, 26, 28-30). Generally, *rdh* genes are located in islands around the origin of replication and are located in an operon structure coding for various Rdh subunits. RdhA is a 50–65 kDa protein that functions as the catalytically active enzyme. The N-terminus of most encoded RdhA harbour twin arginine transport (TAT) signal peptide motifs for the translocation of folded proteins to or across the inner cell membrane. At the C-terminus of the RdhA two conserved motifs can be found, with four cysteine residues that can form iron sulphur clusters (92). Adjacent to the *rdhA*, the *rdhB* is localized, coding for a small hydrophobic protein of about 10 kDa with 2-3 predicted transmembrane helices. It is predicted to function as a membrane anchor protein for the RdhA subunit (29). This type of organization was not only observed in *Dehalococcoides mccartyi* but also in *Dehalogenimonas, Dehalobacter, Desulfitobacterium* and *Sulfurospirillum* (9, 11, 93-98). However, exceptions were described in which *rdhA* does not exhibit a TAT leader sequence or in which an adjacent *rdhB* gene was missing (20). Typically *rdh* genes are associated with a MarR-type or two component-type transcriptional regulator in *Dehalococcoides* species, however, there are exceptions in which the *rdh*

genes are not directly associated with a regulator as for instance in *Dehalogenimonas lykanthroporepellens* type strain BL-DC-9 (27).

The induction and substrate specificity of reductive dehalogenases towards different halogenated compounds is described for only few enzymes compared to their abundance and diversity encoded by *Dehalococcoidia* genomes. Low biomass yields and the inactivation of reductive dehalogenase enzymes by oxygen rendered purification of reductive dehalogenases a complicated step. The heterologous expression of active reductive dehalogenase was not possible until very recently (99, 100). Transcription studies were used to investigate reductive dehalogenase induction under specific cultivation conditions (97, 101, 102). In *Dehalococcoides mccartyi* strain CBDB1 for instance the transcription of cbdbA1453 and cbdbA187 was enhanced in the presence of 1,2,3-trichlorobenzene whereas the transcription of cbdbA1624 was enhanced by 1,2,4-trichlorobenzene (103). The reductive dehalogenase CbrA (cbdbA84) was isolated by native gel electrophoresis and characterized after growth of strain CBDB1 with 1,2,3-trichlorobenzene was shown to be active with 1,2,3-trichlorobenzene in methyl viologen-based activity assays (104). The tetrachloroethene reductive dehalogenase PceA and the trichloroethene reductive dehalogenase TceA were identified in *Dehalococcoides mccartyi* strain 195 which catalyse the reaction of tetrachloroethene to trichloroethene and trichloroethene to ethene, respectively (105). In strain VS the reductive dehalogenase VcrA was shown to be active with vinyl chloride (9) and the MbrA reductive dehalogenase from strain MB transformed tetrachloroethene and trichloroethene to trans-dichloroethene (101). Beyond this, proteome-based approaches were used to investigate the expression of reductive dehalogenases in presence of specific electron acceptors and to assign putative functions to the enzymes (106, 107). Similar to transcriptional studies, it is particularly difficult to elucidate the function of a single reductive dehalogenase, since multiple reductive dehalogenase are typically expressed even in the presence of a single substrate (103, 108).

The active cofactor of reductive dehalogenases was shown to be a corrinoid in purified reductive dehalogenases (93, 94, 105, 109, 110). Inhibition studies with whole cells and alkyl-iodides allowed a light reversible inhibition of dehalogenation activity, indicating the involvement of a corrinoid in the active site of the dehalogenases (105, 111). The only so far known exception is the 3-chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei,* which contains iron in the catalytic centre (112). Different types of corrinoid cofactors have been detected in organohalide-respiring organisms. For *Dehalococcoides mccartyi* strain 195, for instance, indications for a standard cobalamin cofactor were found (113), while a tetrachloroethene reductive dehalogenase isolated from *Sulfurospirillum multivorans* contained a norpseudovitamin B_{12} (114). The standard redox potential of the cob(III)alamin/cob(II)alamin couple is +204 mV and of the cob(II)alamin/cob(I)alamin -606 mV. The low redox potential of the Co(I)/Co(II) couple, together with the observation that cyanocobalamin can catalyse dechlorination reactions *in vitro* and the inhibition of reductive dehalogenation by alkyl iodides lead to the assumption that the corrinoid reacts in the Co(I) state with the organohalogen.

Corrinoids are strong nucleophils and reaction mechanisms involving nucleophilic substitutions (115) and mechanisms including radical intermediates have been proposed (116). So far considered reaction mechanisms assume an attack of the electron onto the partially positively charged carbon atom substituted by the halogen that will be removed by reductive dehalogenation. While inhibition studies with alkyl iodides suggest the formation of an organocobalt adduct, other experimental evidence suggests an electron transfer by the corrinoid onto the organohalogen without the formation of an organocobalt intermediate leading to a radical anion which is subsequently reduced by a second electron possibly from the oxidation of Co(II) to Co(III) (90). Very recently electron paramagnetic resonance spectroscopy and simulation of the electron acceptor-reductive dehalogenase interaction suggested a halogen–cobalamin interaction in the active site of a soluble oxygen tolerant reductive dehalogenase (117).

1.3 Electron acceptors for *Dehalococcoidia*

1.3.1 Electron acceptors of cultivated *Dehalococcoidia*

Whereas several strains of the class *Dehalococcoidia* reductively dehalogenate aliphatic compounds such as chlorinated ethenes (8, 10), other strains of the class *Dehalococcoidia* are specialized on reductive dehalogenation of chlorinated aromatics (7). *Dehalococcoidia mccartyi* strain CBDB1 for instance requires a conjugated pi-system with halogens as substituents that are replaced during reductive dehalogenation by hydrogen (7, 118-121). In contrast to *Dehalococcoides mccartyi* strains, *Dehalogenimonas* strains dehalogenate a variety of vicinally polychlorinated alkanes in a dihaloelimination reaction. During reductive dehalogenation via hydrogenolysis, only one halogen is removed, while during dihaloelimination two halogens are removed in one step, thereby establishing a pi-bond between the dehalogenated carbon atoms (18, 20, 27).

Reductive dehalogenation of halogenated electron acceptors follows specific pathways when catalysed by different strains of the class *Dehalococcoidia*. The reasons for the specificity of the pathways are not fully understood. To describe the preferential dehalogenation pathways, halogens have been classified as doubly flanked, singly flanked or isolated (122). *Dehalococcoides mccartyi* strain CBDB1, for instance, preferentially removes halogens that are doubly flanked in chlorinated benzenes and biphenyls (7, 118). Chlorine atoms with only one neighbouring halogen atom were dehalogenated with a lower rate or not removed at all. Isolated chlorine atoms were not dehalogenated (123, 124). For *Dehalococcoides mccartyi* strain CBDB1 a similar effect on reductive dehalogenation was described for a hydroxy substituent compared to a halogen substituent (119). 2,3,4-Trichlorophenol was dehalogenated by strain CBDB1 to 3,4- and 2,4-dichlorophenol (119). However, 3,4-dichlorophenol was dehalogenated only in the presence of a higher chlorinated phenol, while 1,2,4-trichlorobenzene was dehalogenated to 1,3- and 1,4-dichlorobenzene, showing that the effect of the hydroxy group on reductive dehalogenation was weaker than the one of a flanking

halogen (7, 119). Similarly, a positive effect of oxygen heteroatoms on reductive dehalogenation was observed for chlorinated dioxins (121).

Substituent effects might have implications for the natural electron acceptor range available for *Dehalococcoidia*. Humic acids and fulvic acids detected in sediments, natural water bodies and soils using *in-situ* X-ray spectroscopy, enclose aromatic and aliphatic organochlorines with phenolic and aliphatic groups (125). The concentration of organochlorine compounds in humified plant material was estimated to be between 0.5 and 2 mM kg^{-1}, and aromatic organochlorine compounds have been suggested to be the more abundant ones compared to aliphatic organochlorines (125). Different functional groups of natural halogenated compounds might influence whether a halogenated molecule can be used as electron acceptor by organohalide-respiring *Dehalococcoidia*. Indeed, the number of *Chloroflexi* 16S rRNA genes copies were shown previously to positively correlate with organochlorine per total organic carbon and to increase in numbers parallel to chloride release in cultures incubated with organochlorines (126).

1.3.2 Natural organohalogens as potential electron acceptors in marine sediments

Organohalogens occur naturally in the environment as a result of abiotic and enzymatic reactions (127, 128). In marine environments, brominated compounds dominate over chlorinated compounds while in terrestrial environments chlorinated compounds seem to be produced in larger quantities (129). Chlorinated compounds such as PCE and TCE are produced as secondary metabolites by marine algae. Subsurface volcanic emissions, pyrogenic activity, haloperoxidase of fungi, reaction during plant weathering and abiotic alkylation of halides during Fe(III) oxidation, produce chlorinated and brominated compounds, which can be detected in marine waters (125, 127, 130, 131). For brominated compounds, up to 2,000 natural molecules have been identified, mostly from marine origin (131). Their production has been shown for macroalgae, phytoplankton, hemichordates, sponges, and molluscs which use them most likely as chemical defence mechanisms (128, 132-136). Marine organisms seem to produce in particular a range of low molecular weight brominated aromatic and aliphatic compounds including bromophenols, brominated furanones, tribromoanisole, bromopyrroles, bromoalkanes, bromoketones and bromophycolides, and polybrominated diphenyl ether analogues such as 4,6-dibromo-2-(2,4-dibromo)phenoxyanisole (128, 132, 133, 136-138) (Figure 1-2).

The identification of the specific molecular structure of organobromines in marine sediments is difficult owing to the high diversity and low concentration of each individual compound in marine sediments. Synchrotron-based X-ray spectroscopic and spectromicroscopic techniques were used however, to investigate organobromine distribution and speciation in coastal and deep marine sediments (139). It was shown that concentrations of brominated organic compounds were high in shallow sediments but decreased with increasing sediment depth correlating with the concentration of non-halogenated organic compounds, while inorganic bromine concentrations increased with depth.

This could be due to abiotic reactions but also microbial catalysed reductive dehalogenation with a release of inorganic bromine into the pore water (140, 141).

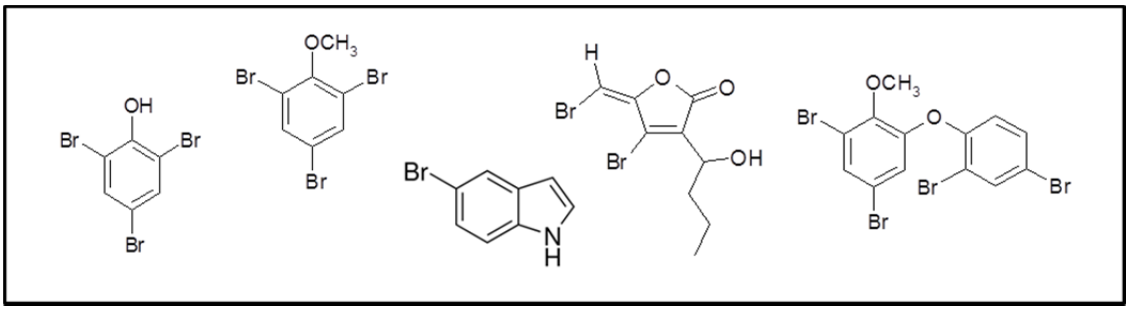

Figure 1-2: Examples of brominated compounds isolated from mussels, algae and sponges (128, 132, 133, 136-138).

Dehalogenation of brominated compounds was observed in marine and estuarine sediments or marine organobromine producing sponges (60, 140, 141), but so far debromination of aromatic compounds was not specifically connected to growth of *Dehalococcoidia* from oceanic marine sites.

Debromination of brominated aromatics by members of the class *Dehalococcoidia* class was described for a co-culture containing *Dehalococcoides* and *Desulfovibrio* species debrominating diphenyl ethers (15). However, *Desulfovibrio* species were described to dehalogenate bromo- and iodophenols (141, 142). Therefore, the participation of *Desulfovibrio* spp. in the debromination reaction could not be excluded. *D. mccartyi* strain BAV1 and 195 were shown to dehalogenate brominated biphenyl. However, debromination was incomplete and occurred only in presence of a chlorinated aliphatic compound (143). The dehalogenation of brominated aliphatic compounds by *Dehalococcoidia* strains was described for *Dehalococcoidia* strain BAV1 and vinyl bromide (10) and for alkyl bromines in activity assay-based studies (95).

1.3.3 Non-halogenated electron acceptors for *Dehalococcoidia* in marine sediments

16S rRNA gene sequences affiliating with the class *Dehalococcoidia* were detected in different sediment environments and the relative abundance of the different *Dehalococcoidia* subgroups were described to shift with geochemical gradients e.g. with the sulphate-methane transition zone (47). Moreover, reductive dehalogenase genes were absent in several *Dehalococcoidia* single-cells or metagenome-derived pan-genomes (46, 144). Therefore, other electron acceptors besides halogenated compounds may contribute to the abundance and widespread distribution of *Dehalococcoidia* in marine sediments.

In most marine sediments, with moderate to high organic inputs, oxygen is depleted rapidly by aerobic respiration within the first few millimetres or centimetres below the seafloor. With increasing sediment depth, electron acceptors for anaerobic respiration are used in a succession of decreasing energy yields. Typically, nitrate, manganese(IV), iron(III), sulphate, and CO_2 are used, while other less-studied electron acceptors such as elemental sulphur, other sulphur species (e.g. thiosulphate) and

even rare elements such as arsenate(V) or organic molecules such as dimethylsulphoxide (DMSO) or trimethylamine-N-oxide (TMAO), can be reduced in respiratory processes by prokaryotes in marine sediments.

Sulphate reduction is considered as one of the most important processes for anaerobic mineralization of organic matter in marine sediments (145). In the assimilatory sulphate reduction pathway, sulphate is reduced to sulphide, then incorporated into cysteine and subsequently processed to a range of other sulphur-containing biomolecules. Sulphate assimilation is a widespread process found in all three domains of life. In the dissimilatory pathway of sulphate reduction, sulphate is used as a terminal electron acceptor for respiration. Sulphate respiration was identified in four bacterial phyla, namely *Proteobacteria, Firmicutes, Nitrospirae, Thermodesulfobacteria*, and the two archaeal phyla *Euryarchaeota* and *Crenarchaeota* (146-150).

Because sulphate reducers are polyphyletic, 16S rRNA gene-targeting primers, which cover the 16S rRNA genes of all sulphate reducers are not available. Alternatively, functional genes of the adenylyl-sulphate-reductase adenosine-phosphosulphate reductase (APR reductase) or dissimilatory sulphite reductases (*drsAB),* both involved in sulphate reduction, have been used extensively as a functional gene marker to investigate the distribution, diversity, and evolution of sulphate reducers (151-158). APR and Dsr are both key enzymes in the anaerobic sulphate respiration and are present in all known sulphate reducing organisms. Dissimilatory sulphite reductase genes were also detected in members of the *Proteobacteria* and *Firmicutes* which are unable to respire sulphate. They have been reported to use sulphite as terminal electron acceptor (159), disproportionate inorganic sulphur compounds (160) or metabolize organosulphonates to internally produce sulphite for respiration (161). For few *dsrAB*-containing *Firmicutes* genera, the function of *dsrAB* is unknown (162).

Comparative amino acid sequence analysis of the dissimilatory sulphite reductase genes and phylogenetic congruence of the DsrAB tree with the 16S rRNA tree, revealed a mostly vertical inheritance of dissimilatory sulphite reductases (151). However, for some groups 16S rRNA and *dsrAB* phylogenies were not congruent. *Archaeoglobus,* several *Desulfotomaculum* species and the genus *Thermodesulfobacterium* likely obtained their *dsrAB* genes *via* a lateral gene transfer (157).

The usage of dissimilatory sulphite reductase genes as a functional marker has revealed high *dsr* sequence diversity in different environments. Many of the amplified sequences belong to uncultured sulphate-reducing microorganisms and have been shown to group into at least thirteen stable family-level lineages, which do not contain any cultivated representatives (150). Therefore, the potential for sulphite/sulphate reduction is expected to be more widespread in different bacterial taxa than so far recognized (150, 152, 158). For instance, sediment sites with high sulphate reduction activity but few *dsrA* gene copy numbers or 16S rRNA genes of sulphate reducing prokaryotes were described (49, 163). It was suggested that either sulphate reducing bacteria populations are small but active, or that sulphate reduction is performed by yet unknown sulphate reducing organisms with divergent genes, so far not detected by common primer and PCR methods (40, 154, 163).

1.4 Goal of this work

This work aimed at contributing to the understanding of the class *Dehalococcoidia* with respect to their widespread occurrence in marine sediments and the ability of several cultivated members of this class to transform halogenated compounds. Thereby the work was divided into two main parts which focused on one hand on the establishment of methods to investigate the potential of marine *Dehalococcoidia* to grow by organohalide respiration using *D. mccartyi* model organism CBDB1 and on the other hand on the potential of *Dehalococcoidia* to gain energy via an organohalide respiration-independent mode of living. For this purpose, electron acceptors were investigated that could support respiration of *Dehalococcoidia* in pristine marine and terrestrial sediments. This was achieved by studying reductive dehalogenation and growth of *Dehalococcoidia* model organism *D. mccartyi* strain CBDB1 with halogenated electron acceptors to elucidate fundamental chemical parameter which influence reductive dehalogenation and might therefore serve to identify potential natural electron acceptors of *Dehalococcoidia* in non-contaminated environments and to predict the fate of halogenated compounds from natural but also anthropogenic sources in anoxic environments. Additionally, a microtiter plate format activity assay was established with *D. mccartyi* strain CBDB1 to provide a fast an easy screening tool to identify new electron acceptors of organohalide-respiring *Dehalococcoidia* and marine dehalogenating bacterial strains and to determine transformation rates of electron acceptors. Furthermore, strain CBDB1 was studied for activity and enzymatic expression with brominated benzenes as model compound for commonly occurring brominated compounds in marine environments. Chemical descriptors of halogenated compounds were evaluated to gain insight into chemical parameters, governing reductive dehalogenation. The amplification of functional respiratory genes from marine sediments was used additionally to study the organohalogen-independent respiration of *Dehalococcoidia* subgroups with non-halogenated electron acceptors in marine sediments. Together the research gives insight into modes of anaerobic respiration catalysed by members of the class *Dehalococcoidia*.

2 Material and Methods

2.1 Chemicals and gases

Chemicals were purchased from Carl ROTH (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany). Solutions and container were sterilized by autoclaving at 121°C for 20 min or by filtering through a 0.2 µm pore size Minisart filter (Sartorius, Göttingen, Germany). Container and solutions for molecular work were additionally treated by exposure to UV light for 30 min. All experiments requiring anoxic conditions were carried out within an anoxic chamber (Coy-Labs, Grass Lake, Michigan, USA) filled with nitrogen of 99.999% v/v purity and 2–4% hydrogen. TE-buffer was prepared with Millipore water and 10 mM Tris adjusted to pH 8.0 with HCl and 1 mM EDTA. For the 0.9% NaCl solution 9 g of NaCl were added to 1000 ml of Millipore water.

Table 2-1: Gases used for cultivation

Compound	***Company***
Helium 5.0 Nitrogen 4.0 Hydrogen 5.0 Biogon ® C 20 (80% N_2 20% CO_2) Aligal (20 ± 2 Vol.% CO_2 in N_2) Forming gas 80/20 (20 ± 2% H_2 in N_2)	Air Liquide Deutschland GmbH, Düsseldorf, Germany. Linde AG, Munich, Germany

2.2 Bacterial cultures and inocula

2.2.1 Cultures

Table 2-2: Different cultures and strains used in the current study

Strain	***Genotype/Phenotype***	***Reference/company***
D. mccartyi strain CBDB1	Wild type	(7)
Sediment microcosms from the coast off Chile and Peru	Uncharacterized wild type	Camelia Algora, Helmholtz Centre for Environmental Research, personal contact
NEB 5-alpha competent *E. coli* K12 strain (C2987)	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs GmbH, Frankfurt am Main, Germany

2.2.2 Sediment samples

Table 2-3: Origin of sediment samples

Sample source	*Cruise*	*Collection date*	*Site*	*Site coordinates (latitude, longitude)*	*Water depth (m)*	*Deepest sample (mbsf)*
Aarhus, Denmark	-	2011	Mimosa	56°09.60'N, 10°28.10'E	16.3	3.0
Baffin Bay, Greenland	ARK-XXV/3	2010	371	75º58.24'N, 70°34.86'W	598	4.05
Wadden Sea, Hedwigenkoog, Germany	-	2011		54°11'25.6"N 8°48'51.0"E	0–2	0.3
Black Sea	M72/1	2007	214SL	4°24.10'N, 32°51.27'E	1680	3.60
Peru	ODP Leg 201	2002	1227	8°59.5'S, 79°57.40'W	427	121.20
Chilean continental margin	SO-156/3	2001	GeoB 7155-4	34°35.00'S, 72°53.11'W	2744	6.70

Sediments of the Aarhus Bay were sampled with a gravity corer (3 m) at water depth of 16.3 m (see also Table 2-4). After retrieving, the core was cut into layers and immediately pore water was withdrawn for methane and sulphate measurements. Additionally, 10 ml of sediments were withdrawn with 10 ml plastic syringes (Braun, Melsungen, Germany) in triplicates, sealed in plastic bags and frozen at -20°C for molecular analyses in the laboratory. The rest of the sediments were stored in gas-tight sealed container or in gas-tight serum Schott bottles. Sediments for cultivation or proteomics were stored at 4 °C.

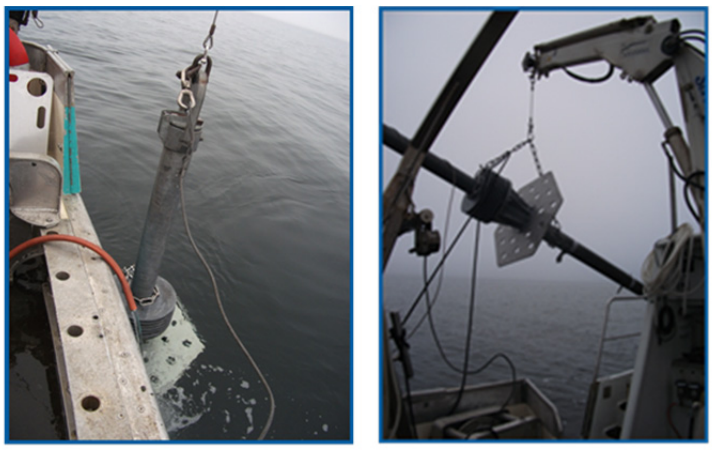

Figure 2-1: Withdrawal of a sediment core from the bay of Aarhus using a 3 m gravity corer.

Table 2-4: Sediment core samples

„Name“	*Depth [cm]*	**Purpose**	**Storage**
1	0–30	Cultivation/Proteomics	4°C and -20°C upper 10 cm
A	30–36	Molbiol[1]/Sulphate/Methane	-20°C
2	36–66	Cultivation/Proteomics	4°C
B	66–72	Molbiol/Sulphate/Methane	-20°C
3	72–102	Cultivation/Proteomics	4°C
C	102–108	Molbiol/Sulphate/Methane	-20°C
4	108–135	Cultivation/Proteomics	4°C
D	135–141	Molbiol/Sulphate/Methane	-20°C
5	141–168	Cultivation/Proteomics	4°C
E	168–174	Molbiol/Sulphate/Methane	-20°C
6	174–201	Cultivation/Proteomics	4°C
F	201–208	Molbiol/Sulphate/Methane	-20°C
7	208–235	Cultivation/Proteomics	4°C
G	235–241	Molbiol/Sulphate/Methane	-20°C
8	241–268	Cultivation/Proteomics	4°C
H	268–274	Molbiol/Sulphate/Methane	-20°C
9	274–301	Cultivation/Proteomics	4°C
I	301–308	Molbiol/Sulphate/Methane	-20°C

[1] for molecular biological applications

2.3 Cultivation

2.3.1 Medium preparation for anaerobic cultivation

2.3.1.1 Preparation of the reducing agent titanium(III) citrate

Titanium(III) citrate was prepared in nitrogen atmosphere according to Zehnder and Wuhrmann (164). For this 5.14 ml 15% titanium(III) chloride solution were added to 10 ml anoxic 1 M sodium citrate solution. Solid sodium carbonate was used to neutralize the solution to pH 7 and anoxic water was added to a final volume of 50 ml to obtain a final concentration of 0.1 M titanium(III)chloride and 0.2 M sodium chloride.

2.3.1.2 Preparation of 1 M sodium hydrogen carbonate buffer

Sodium hydrogen carbonate (7.06 g) was added to a 100 ml serum bottle and 84 ml of anoxic Millipore water was added. The bottle was sealed with a rubber septum. The mixture was flushed for several minutes with CO_2 with the help of a needle and shaken several times with the lid closed. This procedure was repeated until the carbonate was dissolved completely. The bottle was closed with an aluminium crimp cap and autoclaved. Before usage the solution was cooled to room temperature to avoid precipitation of carbonate minerals in the media.

2.3.1.3 Preparation of salt and vitamin solutions

Table 2-5: solutions for anaerobic cultivation. All solutions were prepared in Milli-Q water.

Mineral salt solution (Widdel fresh water solution)		***Trace element solution SL9 Modified from (165)***		***Vitamin 7 solution***	
Compound	***Final conc. g L^{-1}***	***Compound***	***Final conc. mg L^{-1}***	***Compound***	***Final conc. mg L^{-1}***
KH_2PO_4	10	Nitrilotriacetic acid (NTA)	12800	D(+)-Biotin	2
NH_4Cl	13.5	$FeCl_2$ x4H_2O	2000	Nicotinic acid	5
		$ZnCl_2$	70	Ca-D (+)-Pantothenic acid	5
NaCl	50	$MnCl_2$ x2H_2O	80	Pyrodoxine hydrochloride	10
$MgCl_2$ x 6H_2O	20.5	H_3BO_3	6	Thiaminechloride-dihydrochloride	5
KCl	26	$CoCl_2$ x6H_2O	190	Cyanocoalamin	50
$CaCl_2$ x 2H_2O	7.5	$CuCl_2$ x2H_2O	2	4-Amino benzoic acid	5
		$NiCl_2$ x6H_2O	24		
		Na_2MoO_4 x2H_2O	36		
		NaOH ad pH 7.0			
Sterilized by autoclaving		Sterilized by autoclaving		Filter sterilized	

2.3.1.4 Preparation of cultivation medium for *Dehalococcoides mccartyi* strain CBDB1

The medium for cultivation of strain CBDB1 was prepared by adding Widdel mineral salt solution, trace element solution SL9, sodium acetate solution, selenite-tungstate solution and resazurin to Milli-Q-water (see Table 2-6). The solution was degased for 30 min in an anoxic gas exchange chamber and purged subsequently for 30 min with nitrogen outside the anoxic glove box. In an anoxic glove box the liquid was dispensed into serum bottles or Schott bottles. Unless stated otherwise 50 ml bottles with 30 ml liquid volume were selected for cultivation to obtain a headspace to liquid ration of about 2:3. After equilibration of media for 1 h in the anoxic glove box, the bottles were sealed with Teflon-lined butyl rubber stopper, crimped with aluminium caps, and autoclaved. After the liquid was cooled to room temperature, $NaHCO_3$ buffer (1:100 (= 1% vol/vol)) and titanium(III) citrate (1:100 (= 1% vol/vol)) were added with a plastic syringe (Braun, Melsungen Germany) connected to a 0.2 µm sterile filter (Minisart®, Sartorius AG, Göttingen, Germany) under a clean bench or near a Bunsen flame. The medium was equilibrated in the flasks for at least 4 hours. Before inoculation of strain CBDB1, vitamin-7 solution was added freshly from a sterile 100x stock solution (prepared from a 1000x-concentrate in anoxic water).

Table 2-6: Medium components

Component	*Added amount or final concentration*
Milli-Q-water	800 ml
Widdel mineral salt solution (50x conc.)	20 ml
Trace element solution SL9	10 ml
Sodium acetate solution 1 M	5 ml (5 mM)
Resazurin	5–10 drops (~1 µM)
Selenit-tungstate solution	1 ml (~ 0.025 µM each)
$NaHCO_3$ 1 M	10 ml (10 mM)
15% Titanium(III) citrate	10 ml (~2 mM)
Vitamin 7 solution (100x conc.)	10 ml
Milli-Q-water	filled up to 1000 ml

2.3.1.5 Addition of halogenated compounds

The halogenated compounds were added to the medium with a 10 µl glass syringe (Hamilton, Bonaduz, Switzerland) from an acetonic stock solution or they were added as crystals. The concentration of electron acceptor in the medium was determined by gas or ion chromatography after equilibration of at least one hour.

Table 2-7: Compounds tested with strain CBDB1 in cultivation

Compound	*Solvent*	*Concentration in the stock solution [mM]*	*Final concentration [µM]*
Aromatic compounds with non-halogen substituents			
2,3-; 2,4-; 2,6-dichloroaniline	acetone	1 M	100 µM
2,3-; 2,5-; 2,6-dichlorobenzonitrile		added as crystals	
2-chloro-6-fluorobenzonitrile		added as crystals	
2,4,6-tribromophenol	acetone	1 M	10 µM
2,4-; 2,6-dibromophenol	acetone	1 M	10 µM
4-bromo-3,5-dimethoxybenzoic acid		added as crystals	
4-bromo-3,5-dihydroxybenzoic acid		added as crystals	
Heterocyclic aromatic compounds			
4,5-dibromo-2-furoic acid		added as crystals	
5-bromo-2-furoic acid		added as crystals	
2,3-; 2,6-dichloropyridine	acetone	1 M	50 µM

Cultures were initially set-up from cultures grown on 30 µM 1,2,3-TCB. A sterile 10 ml plastic syringe (Omnifix®, Braun, Melsungen, Germany) flushed previously with sterile, anoxic H_2O, was used for inoculation. An inoculum percentage of 10% was used for strain CBDB1 corresponding to starting cell densities of 5 x 10^5 to 5 x 10^6 cells ml^{-1}. The headspace was pressurized with 20% CO_2 / 80% N_2 (1.3 bar), and 0.1 bar hydrogen was added to obtain a final pressure of 1.4 bar. Cultivation was performed in the dark at 30°C without shaking. All cultivations were done at least in triplicates. Additionally, chemical controls without inoculum, negative growth controls without electron acceptor and positive controls with 30 µM 1,2,3-TCB were set up. Cultures were monitored by cell counting and gas or ion chromatography.

2.3.2 Cultivation of *E. coli*

E. coli cells used for cloning experiments were cultivated in liquid Luria-Bertani medium (1% trypton, 0.5% yeast extract, 1% NaCl, pH 7) or on Luria-Bertani agar plates (LB-medium, 1.5% agar). The medium was set up with Milli-Q-water and autoclaved. After cooling to ~50°C, ampicillin (final conc. 100 µg ml^{-1}) was added under a clean bench. For the identification of positive clones with blue-white screening X-Gal was added to the medium to a final concentration of 80 µg ml^{-1}. For agar plates, approximately 25 ml of LB-agar medium was filled into each petri dish, dried under the clean bench and stored at 4°C for further usage.

The medium was inoculated with a single colony from an ampicillin/X-Gal-containing LB-agar plate and incubated over night at 37°C and 250 rpm. *E. coli* was cultivated on LB-agar plates at 37°C for at least twelve hours. *E. coli* colonies were stored on LB-agar plates at 4°C.

2.3.3 Direct microscopic counting

Cell numbers were determined by direct epifluorescence-microscopic counting after staining with SYBR-green (Invitrogen, Carlsbad, California, USA) on agarose-coated slides. For the preparation of the slides two grams of low-melting SeaPlaque® agarose (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and 120 ml water were stirred while being heated carefully until the agarose was liquefied completely. The liquefied agarose was withdrawn with a pre-warmed 10 ml glass pipette and glass slides were covered with approximately 2 ml of agarose and dried under the clean bench (119).

The SYBR1 Green double strand DNA stain (Invitrogen, Carlsbad, California, USA) was diluted 1:100 with sterile TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.2) and stored in 0.5 ml Eppendorf tubes in 10 µl aliquots at -20°C. For counting medium was withdrawn from a culture with a 1 ml plastic syringe (BD Plastipak™, Becton Dickinson GmbH, Heidelberg, Germany) and 20 µl were mixed with 1 µl diluted SYBR green stock solution, resulting in a final concentration of 1:2000 SYBR green in the sample. The tube was incubated in the dark for 10 min. For counting, 18 µl were pipetted on a 20 mm x 20 mm cover slide. By this procedure the cells were immobilized in one defined focus level between the cover slide and an agarose-covered glass slide (119). Cell numbers were quantified by direct microscopical counting using epifluorescence of SYBR-green stained cells as described previously (119). Micrographs were taken using a Nikon fluorescence microscope at 400x magnification with a NikonDS-Ri1 digital camera. For calibration a micrometre scale was photographed with the same parameters as applied for sample micrographs. For each sample fifteen micrographs were taken from randomly chosen positions. The micrographs were taken with the NIS imaging software (Nikon, Tokyo, Japan). Cell counting and calculation of picture size were evaluated by using ImageJ (http:// http://imagej.nih.gov/ij/) mainly automated by ImageJ and Excel macros (119).

2.4 Preparation of whole cells suspensions and detachment of cells from sediments

2.4.1 Rotary evaporator

Rotary evaporation was used to concentrate cells and to remove halogenated solvents from the cell suspension. Approximately 30 ml cell culture were transferred in an anoxic glove box into a 500 ml round bottom flask and sealed with a glass stopper. The flask was removed from the glove box and connected to a rotary evaporator. The culture was concentrated to approximately half of the original volume at 50 mbar. A water bath heated to 30°C was used to accelerate evaporation. The oxygen content of the culture was monitored with the redox indicator resazurin.

2.4.2 Cell concentration via Minisart® filter

To harvest cells by filtering, culture fluid was taken from a culture within an anoxic glove box using sterile and anoxic 3–10 ml plastic syringes (Omnifix®, Braun, Melsungen, Germany) and filtered through a 0.2 µm pore size filter (Minisart®, Sartorius, Göttingen, Germany). Syringes were made anoxic by filling with sterile reduced buffer, medium or water. The filter containing the cells was then connected with the reverse side to a new syringe with the help of Luer Lock adapter. Cells were flushed into an Eppendorf reaction tube and either applied in an activity assays or used for cell counting.

2.4.3 Cell concentration via centrifugation

Centrifugation vials were filled with cell culture (max. 35 ml in 50 ml Falcon tubes or max. 11 ml in 15 ml Falcon tubes) and centrifuged for one hour at 16°C and 5,000 g. Fifty percent of the supernatant were removed in the anoxic glove box and the remaining solution was centrifuged for an additional hour at 16°C and 5,000 g. The remaining supernatant was removed and the pellet resuspended in buffer or fresh medium.

2.4.4 Density centrifugation using Nycodenz®

Density centrifugation with 80% (w/v) Nycodenz® (Nycodenz AG, Axis-Shield, Oslo, Norway) solution was done for separation of whole cells from sediment particles. This procedure was modified according to methods described previously (166-168). For the preparation of the solution Milli-Q water was filled into a beaker and stirred with a stirring bar before solid Nycodenz® was added in small amounts to facilitate the dissolving process and to avoid clumping. Finally the solution was filled up to the required volume and filter-sterilized through a 0.2 µm filter. The 80% solution was stored in a sterile Falcon tube at 4°C.

For the separation of cells from sediments, 10 g of sediment was filled into a 50 ml sterile centrifugation tube. Thirty ml of a sterile 0.9% (w/v) NaCl solution was added and incubated for at

least three hours, shaking horizontally at 450 rpm and room temperature. To further detach cells from sediment particles, the tube was sonified for 1 minute in a sonification bath. Subsequently, the tube was centrifuged for 6 min at 800 g, 4°C. The supernatant was transferred into sterile 15 ml centrifugation tubes. The remaining sediments were stored at 4°C for a second extraction. A layer of 2 ml 80% (w/v) Nycodenz® was carefully injected to the bottom of the centrifugation tube below the 10 ml aqueous phase of the supernatant with a long needle. The tube was transferred to a centrifuge without disturbing the Nycodenz®-liquid interface and centrifuged at 14,000 g, at 4°C, for 45 min. Supernatant and Nycodenz® layer were transferred into a new 15 ml centrifugation tube without disturbing the sediment pellet. The water-Nycodenz-mixture was thoroughly mixed by using a vortex mixer until no phase separation was visible to lower the Nycodenz® concentration and to allow for sedimentation of cells at the bottom of the centrifugation tube. Cells were pelleted by a third centrifugation step at 14,000 g at 4°C for 15 min and the supernatant was discarded. To remove remaining traces of Nycodenz®, the pellet was washed with 1 ml of a 0.9% NaCl solution and centrifuged at 14,000 g at 4°C for 15 min. The dried pellet was resuspended in 100 µl 0.9% NaCl solution. For parallel or subsequent extractions, the resuspended pellets were combined into one centrifugation tube. Twenty µl cell suspension were withdrawn for cell counting (see section 2.3.3). The cell pellet was obtained by centrifugation at 14,000 g at 4°C for 15 min and was used immediately for activity assays, DNA extraction or was stored dry at -20°C for further usage.

2.5 Resting-cell activity assay

Methyl viologen and other viologens can be used in their reduced state as artificial electron donors for reductive dehalogenase in an *in vitro* activity assay (105, 169). Titanium(III) citrate as described above (section 2.3.1.1) was used to reduce viologens. In their reduced state viologens are blue while in their oxidized state viologens are colourless. The catalytic activity of strain CBDB1 or marine microcosms towards halogenated compounds was analysed by using whole non-disrupted cells and methyl viologen or in specific cases benzyl and ethyl viologen as an artificial electron donor.

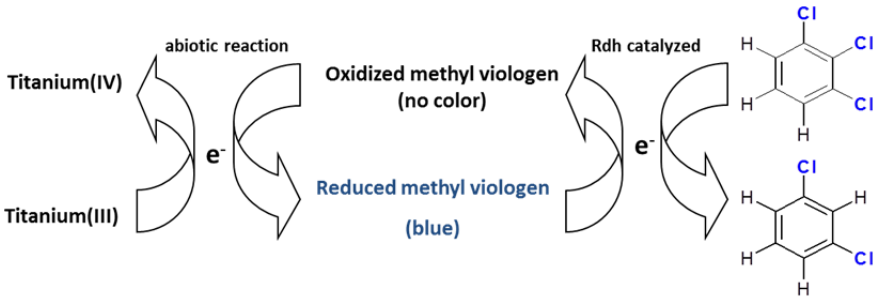

Figure 2-2: Principle of the viologen-based activity assay

2.5.1 Preparation of solutions and cell suspensions for the activity assay

For the preparation of potassium acetate buffer 46 ml of a 2 M potassium acetate stock solution and 4 ml of a 2 M acetic acid stock solution were filled into a 100 ml Schott bottle and adjusted to pH 5.8 with a potassium acetate solution or acetic acid (170). The solution was then filled to a volume of 100 ml with Milli-Q water and filtered through a 0.2 µm sterile filter. Methyl viologen was prepared as a 10 mM solution (111). Methyl viologen solution and potassium acetate buffer were degased by applying vacuum in the air lock of an anoxic chamber for 30 min and subsequently equilibrated in the anoxic glove box for at least 24 hours prior to usage.

Halogenated compounds were dissolved in acetone to obtain a final concentration of 50 mM. Titanium(III) citrate was used from a stock solution (see section 2.3.1.1). Cells were concentrated to at least 1 x 10^7 cells ml^{-1} using either a rotary evaporator or a 0.2 µm filter (see section 2.4.1 and 2.4.2). Twenty µl were removed from the whole cell suspension for cell counting (see section 2.3.3). The protein concentration was calculated by using the cell numbers and protein amount determination by Anja Kublik (personal communication) with 30 fg per cell. All solutions and cell suspensions were prepared in an anoxic glove box.

The activity assay solution contained potassium acetate buffer (pH 5.8), titanium(III) citrate, methyl viologen and halogenated electron acceptors (see Table 2-8). Halogenated electron acceptors were added from a 50 mM stock solution in acetone. For every tested compound a separate reaction mix was prepared in a glass reaction vial, sealed and equilibrated for at least 10 min before a catalyst was added. The negative control contained acetone without electron acceptor and the assay solution. The positive control contained 1,2,3-trichloro- or 1,2,3,4-tetrachlorobenzene.

Table 2-8: Activity assay solution for 10 ml

Compound	***added volume (ml)***	***Final conc. (mM)***
Anoxic H_2O	7	
Methyl- ethyl- or benzyl viologen (10 mM in anoxic H_2O)	1.25	1.25
Potassium acetate buffer pH 5.8 (1 M in anoxic H_2O)	1.25	125
Titanium(III) citrate (~ 100 mM)	~ 0.01	~2
Electron acceptor (50 mM in acetone)	0.125	0.625
Anoxic H_2O	Ad 10	

2.5.2 Gas chromatography-based activity assay

For the GC-FID-based activity assay 80 µl cell suspension and 800 µl assay solution were filled into 2 ml HPLC vials in an anoxic chamber, sealed with Teflon-lined rubber septa and incubated at 30°C. At time zero and after 1 to 15 hours of incubation the reaction was stopped by hexane extraction. Extracts were analysed for dehalogenation products using a GC-FID (see section 2.6.1). The activity was calculated from the amount of electron acceptor which reacted after one to fifteen

hours compared to the amount of electron acceptor measured at time zero or from the amount of products formed.

2.5.3 Photometer-based microtiter plate activity assay

The microtiter plate activity assay was set up in an anoxic chamber using a 96-round bottom well glass microtiter plate (Zinsser Analytic, Frankfurt, Germany). Each plate contained negative controls with cells and acetone but without an halogenated electron acceptor and positive controls with cells and 1,2,3-trichloro- or 1,2,3,4-tetrachlorobenzene. A chemical control was set up to test for abiotic reactions of the electron acceptors with compounds from the reaction mix. The chemical control contained an electron acceptor, the reaction mix and cell-free media. It was measured either in a separate plate or was included with three parallels on the same plate in which resting cell activity was measured. Every electron acceptor was tested for activity with resting cells in at least three parallel wells (see **Figure 2-3**). Twenty or forty µl of whole cell suspension with approximately 1×10^7 cells ml^{-1} and 200 µl assay solution were filled into the designated wells. The plate was immediately sealed with a transparent PCR-foil seal (VWR, Dresden, Germany).

	1	2	3	4	5	6	7	8	9	10	11	12
A	R	R	R	R	R	R	R	R	R	R	R	R
B	R	PC	E	E	E	E	E	E	E	E	E	R
C	R	PC	E	E	E	E	E	E	E	E	E	R
D	R	PC	E	E	E	E	E	E	E	E	E	R
E	R	NC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	R
F	R	NC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	R
G	R	NC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	AEC	R
H	R	R	R	R	R	R	R	R	R	R	R	R

Figure 2-3: Example for a microtiter well plate set-up with negative controls (NC), positive controls (PC), electron acceptor, reaction mix and resting cells (E), abiotic electron acceptor controls (AEC) and reaction mix without cells and electron acceptor (R). The latter were included to avoid any influence of oxygen traces diffusing from the atmosphere of the tent below the transparent film into the plate wells, so that only the inner wells of the plate were used for measurements.

The activity was measured photometrically by following the oxidation of methyl viologen at its absorbance maximum of 578 nm (extinction coefficient: 9.78 mM^{-1} cm^{-1}) with a microtiter plate reader from Synergy HT, BioTek (Bad Friedrichshall, Germany). To monitor background absorbance originating from a condensate formed on the plastic film, the absorbance at 450 nm was measured additionally to off-set the absorbance at 450 nm with the absorbance measured at 578 nm. The initial absorbance of the assay solution at 578 nm was adjusted to 1.5 to 3.5 with titanium(III) citrate. The absorbance was monitored for 15 h at 30°C. Data were recorded using the Gen5™ program 1.06 (BioTek, Winooski, VT, USA) with the following program settings:

Table 2-9: Settings for the photometric activity assay with the Gen5™ program

Parameter	***Setting***
Temperature set point	30°C,
Start kinetic	Run 15 h, Interval 7.5 min
Shake	Medium for 3 s
Read	(A)450, 578, 650

The specific activity was calculated from the slope of the linear part of measured curves (Figure 2-4). To obtain activity rates in nkat mg^{-1} of protein of strain CBDB1 cells a conversion factor according to Tina Hölscher was used (170) and then recalculated according to new protein determination by Anja Kublick, UFZ Leipzig of 30 fg protein per cell (personal communication).

$$\text{Spec. Act. } \frac{nkat}{mg} = \frac{\left(\Delta E_{e-acceptor} - \Delta E_{blank}\right) * V_{total}\ (\mu l) * 16.67\ (nkat * U^{-1})}{t\ (\text{min}) * \varepsilon_{mv}\ (mM^{-1} * cm^{-1}) * d\ (cm) * V_{sample}\ (\mu l) * N_{mv} * P\ (mg\ ml^{-1})}$$

Figure 2-4: Formula for the calculation of the specific activity of resting cells based on the decrease of methyl viologen absorption. $\Delta E_{e\text{-}acceptor}$: extinction measured in a sample with electron acceptor and cells; ΔE_{blank}: extinction measured in negative controls without electron acceptor but with cells, t: extinction measuring time in minutes, always a time in the linear range of the curve was chosen; V total: combined volume of reaction mix and resting cell sample (µl); εMV: extinction co-efficient of methyl viologen (9.78 cm^{-1} mM^{-1}); d: pathway of light through the sample (cm); V sample: volume of the sample (µl), N_{mv}: number of methyl viologen molecules consumed per halogen released, P: protein concentration (cell density (cells ml^{-1}) x cellular protein content (mg protein $cell^{-1}$)). A value of 30 fg $cell^{-1}$ was used as a cellular protein content. The factor 16.67 compensates for the calculation from µkat to nkat and from minutes to seconds.

2.5.4 Heat inactivation of cells for activity assay

To investigate background activity detected in activity assays, cells were harvested in an anoxic glove box and transferred into gas-tight crimp vials. The vials were incubated for thirty minutes in a water bath, either at room temperature, at 60°C, or at 80°C. Cells were transferred to the anoxic glove box and used for comparative activity assays.

2.5.5 Reversible inhibition of reductive dehalogenases

The reversible inhibition of dehalogenase activity with alkyl iodides was used to investigate i) the background activity in the microtiter plate activity assay with strain CBDB1 and ii) the involvement of a cobalamin-dependent reductive dehalogenase in marine sediment microcosms (169). Resting cell suspensions were reduced with titanium(III) citrate (2 mM final conc.) in an anoxic glove box. Propyl iodide was added from an acetonic stock solution to obtain a final concentration of 1 mM for cell suspensions of strain CBDB1 or 10 µM for cell suspensions of marine sediment microcosms. The same amount of acetone without propyl iodide was added to a negative control. The cell suspensions were incubated for one hour in 2 ml HPLC vials, covered with aluminium. All vials were sealed with Teflon-lined rubber stopper. After one hour of incubation at room temperature the aluminium foil was removed from selected vials with propyl iodide and vials without propyl iodide and exposed to a 250

watt light source for 30 min. Three different experimental set-ups, each in triplicates were compared: a) Propyl iodide treated cell suspensions incubated in the dark without exposure to a light source, b) propyl iodide treated cell suspensions exposed to light and c) controls incubated without alkyl iodide exposed to light. All were tested for dehalogenase activity by the microtiter plate activity assay and/or a GC-FID-based activity assay (see section 2.5).

2.5.6 Oxygen sensitivity tests with marine cultures and activity assay

Culture liquid was retrieved from cultures in an anoxic glove box using a 5 ml syringe (Omnifix®-F, Braun AG, Melsungen, Germany) previously flushed with anoxic water. The cell suspension was transferred into 2 ml HPLC vials, each time point in triplicates. Cells were stirred with stirring bars to oxygenize the cell suspension and incubated at room temperature for 0.5, 1, 2 and 4 hours in the anoxic glove box and in a parallel set-up outside the anoxic glove box to expose the samples to oxygen. After the incubation time vials incubated outside the anoxic glove box were transferred into the anoxic chamber. Cells incubated the same time in the anoxic chamber and cells exposed to oxygen were both used for setting up GC-FID-based activity assays. The samples were incubated at 30°C for 12 hours in the activity assay solution and subsequently analysed for reaction products.

2.5.7 Activity assays with cells from sediments

Sediments stored at 4°C from the Baffin Bay, from tidal flat sediments and from the Aarhus bay were used for activity assays in HPLC vials. Sediments stored in gas-tight Schott bottles at 4°C were introduced into the anoxic glove box and 0.5–3 g were transferred into 2 or 5 ml HPLC vials. The reaction mix for activity assays was prepared with 1,2,3,4-tetrachlorobenzene, 2,4,6-tribromophenol, 1,2,3-trichlorobenzene or 1,2,4-dibromobenzene. The assay solution (see section 2.5.1) was added to the sediments and the vials were incubated at room temperature or at 30°C for 12 to 60 hours. After incubation the assay solution was transferred to a new glass reaction vial and the reaction was stopped by extraction with hexane in a ratio of 1:2 assay solution to hexane. Alternatively, cells were extracted from sediments using Nycodenz® and were set up with the activity assay as described above.

2.6 Analytical methods

2.6.1 Gas chromatography

Brominated and chlorinated benzenes, chlorinated and fluorinated benzonitriles, chlorinated thiophenes, anilines and pyridines from cultivation and resting cell activity assays were extracted from 0.5 ml samples with 1 ml of hexane. All compounds and reaction products except for benzene and thiophene were analysed with a Shimadzu GC 14A equipped with a flame ionization detector and a Permabond-FFAP capillary column (25 m, 0.25 mm i.d., 0.25 µm film thickness, Macherey & Nagel, Düren, Germany). Generally, 1.5 µl samples were injected in splitless mode. Nitrogen (99.999% purity) was used as carrier and make-up gas. The gas pressure settings were the following: Air 0.20 kg cm_2^{-1}, hydrogen 0.6 kg cm_2^{-1}, nitrogen 0.65 kg cm_2^{-1} as carrier gas and 1.00 kg cm_2^{-1} as make up gas. The temperature program started with an initial hold at 55°C for 1 min, an increase of temperature at a rate of 6°C min^{-1} to 225°C with a final hold for 2 min. The injector temperature was 250°C and the detector temperature 300°C.

Benzenes and thiophenes could not be detected with the same program as both reaction products were covered by the hexane solvent peak. Therefore, these compounds were analysed from the headspace of the reaction vial. Two hundred µl of the headspace were removed from the sealed reaction vial using a gas-tight 200 µl glass syringe (Hamilton, Bonaduz, Switzerland) and transferred directly into the injector of a HP5890II gas chromatograph equipped with a flame ionization detector and a DB-5 column (30 m, 0.32 mm i.d., 0.25 µm film thickness, J&W Scientific, Köln, Germany). Nitrogen (99.999% purity) served as carrier and make-up gas. The column temperature was 35°C when the sample was injected. After 5 min the column temperature was increased at a rate of 5°C $minute^{-1}$ to 60°C and then at a rate of 30°C $minute^{-1}$ to 300°C, with a final hold of 10 min at 300°C. The injector temperature was 240°C and the detector temperature was 320°C.

To allow gas chromatographic analysis, bromophenols were acetylated with 50 mM acetic anhydride in 120 mM sodium bicarbonate. Therefore, sodium bicarbonate and acetic anhydride were added to 1 ml culture volume in a glass reaction vial, sealed and placed on a rotary shaker set to 350 rpm. The sample was mixed for 10 min at room temperature before extracting reaction products with 1 ml hexane. Derivatized bromophenols were analysed using a Hewlett Packard gas chromatograph 6890 equipped with a flame ionization detector and a HP-5 capillary column (30 m, 0.25 mm inner diameter, 0.25 µm film thickness, Agilent Technologies, Böblingen, Germany). The temperature program was as follows: initial hold at 55°C for one minute, the temperature then increased by 10°C $minute^{-1}$ until it reached 150°C and continued by an increase of 10° $minute^{-1}$ to 230°C and then 30°C $minute^{-1}$ to 260°C with a final hold of 2 min. Injector and detector temperatures were both 250°C. Injection was done splitless.

All compounds and most reaction products were identified and quantified by injecting single reference compounds as standards in concentrations from 5 to 100 µM. For the benzene standard curve

cultivation bottles with 60 ml anoxic water sealed with Teflon lined rubber septa were spiked with benzene in concentrations between 5 and 100 µM. The detection limit for all compounds was 1–5 µM.

2.6.2 Ion chromatography

Transformation of brominated furoic acids and benzoic acids was analysed by taking 1 ml samples of the cultures for measuring the bromide concentration using a DIONEX-ISC2000 Ion Chromatography System equipped with an IonPacAS18/AG18 column (4 mm inner diameter, Thermo Fisher Scientific, Bremen, Germany). Samples were measured according to the ISO 10304-2: 1995 protocol part 2 for the determination of bromide, chloride, nitrate, nitrite, ortho-phosphate and sulphate in waste water.

2.6.3 NanoLC-ESI-MS/MS (LTQ-Orbitrap) analysis

For the analysis of expressed proteins by nano LC-ESI-MS/MS (LTQ-Orbitrap) of protein digests strain CBDB1 was cultivated in 50 ml serum bottle flasks with mono- 1,2-, 1,3-, or 1,4-di- or 1,2,4-tribromobenzene. When cultures were harvested for protein analysis they contained cell numbers from 2 to 4 $x10^7$ cells ml^{-1}. Cells were obtained by filtering 30 ml of culture volume through a 0.2 µm filter (see also 2.4.2). Subsequently cells were lysed, treated with DTT and iodoacetamide and digested with trypsin as described previously (171). The obtained peptides were purified, desalted and concentrated by ZipTip® pipette tips (Millipore, Eschborn, Germany). The peptides were reconstituted in 0.1% formic acid for nanoLC-ESI-MS/MS measurements. The samples were trapped with 0.1% formic acid in water and at flow rates of 15 µl min^{-1} (nanoAcquity UPLC column, C18, 180 µm x 2 cm, 5 µm, Waters, Eschborn, Germany). The peptides were eluted after eight minutes onto the separation column (nanoAcquity UPLC column, C18, 75 µm x 100 mm, 1.7 µm, Waters, Eschborn, Germany). Chromatography was done with a nano-UPLC system (nanoAquity) and the solvents used for chromatography (solvent A 100% water, solvent B 100% acetonitrile) contained 0.1% formic acid. For the elution of the peptides a 6–20% solvent B gradient was applied over 90 min and continued with a 20–85% solvent B gradient over 60 min. A TriVersa NanoMate LC-coupler (Advion) was used for coupling of the nano-UPLC system to the LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The eluted peptides were scanned continuously at 300–1600 m/z, automatically switching to MS/MS CID mode with normalized collision energy of 35% and an activation time of 30 ms on top six peptide ions exceeding an intensity of 3,000. An exclusion mass width of ±4 ppm over 3 min enabled dynamic exclusion. Obtained data were evaluated using the tandem mass spectrometry ion search algorithms from the Mascot house server (v2.2.1). For the search, a taxonomy ID 255470 (*Dehalococcoides mccartyi* strain CBDB1) of NCBI nr (National Centre for Biotechnology Information, Rockville, MD), tryptic cleavage and a maximum of two missed cleavage sites was selected. Additionally carbamidomethylation at cysteines was given as a fixed and oxidation of methionines as a variable modification. The peptide tolerance threshold was set at ±10 ppm and the

MS/MS tolerance at ±0.8 Da and peptides were considered to be identified by Mascot when a false positive probability of 0.05 (probability-based ion score threshold of 40) was obtained. For identified peptides and their respective proteins the protein score and the emPAI (exponentially modified protein abundance index (172)) were calculated by Mascot. A higher protein score corresponds to a more confident match between the ion score and the amino acid sequence of the protein while the emPAI allows an approximate quantitation of the protein by dividing the number of detected peptides by the number of expected peptides and subsequent exponential modification of the calculated value.

2.7 Molecular methods

2.7.1 Chloroform-based DNA extraction

DNA was extracted according to a modified protocol described by Miller and colleagues (173) from crude cell pellets obtained via Nycodenz® density centrifugation from 40 g of sediments (see section 2.4.4). Cells were resuspended in 1.35 ml extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 100 mM sodium hydrogen phosphate, 1.5 M NaCl, pH 8.0) and transferred to a fresh 2 ml centrifugation vial. Five µl of fresh Proteinase K (20 mg ml^{-1}) was added and carefully mixed by gently flicking the tube. The tube was incubated at 37°C for 30 min and carefully mixed every ten minutes. 150 µl of 20% (w/v) SDS were added, incubated at 65°C for 2 hours and mixed by inversion of the tube every fifteen minutes. After incubation the solution was centrifuged for 10 min at 6,000 g, room temperature and the supernatant was transferred to a fresh sterile 2 ml centrifugation vial. This step was repeated until the supernatant was clear. To the crude extract an equal volume of chloroform-containing 4% (v/v) isoamyl alcohol was added and gently mixed by inversion. The aqueous phase was separated from the chloroform phase by centrifugation at 16,000 g 10 min at room temperature. The upper aqueous phase was transferred with a cut wide bored 1000 µl pipette tip (~3 mm opening) while leaving the interface undisturbed. The DNA was precipitated by adding 0.6 ml of isopropanol to one ml of aqueous phase (ratio of 0.6:1). Isopropanol was mixed thoroughly with the aqueous phase and incubated for one hour at room temperature to precipitate DNA. The tubes were centrifuged for 20 min at 16,000 g for 20 min at room temperature. After removing the supernatant the pellet was washed with 500 µl cold 80% (v/v) ethanol and centrifuged for 10 min at 16,000 g and room temperature. The ethanol was removed while leaving the pellet undisturbed. The pellet was air-dried for one hour in the dark and resuspended in 30 µl PCR grade H_2O or in 0.1 x concentrated TE-buffer. The DNA concentration and quality was measured. In case of remaining protein contaminations the DNA was additionally purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and eluted with 30 µl PCR grade H_2O. The DNA was stored at -80° for further usage.

2.7.2 DNA extraction with the FastDNA™ Spin Kit for Soil

DNA was extracted from 0.5 g of sediments with the FastDNA™ Spin Kit for Soil (Biomedicals, Eschwege, Germany) according to the manufactures instructions in combination with the FastPrep FP120 cell disrupter (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.7.3 Whole genome amplification

Whole genome amplification of SAG-C11 was done using the Phi29 whole genome amplification kit (GE Healthcare, Chalfont St Giles, Buckinghamshire, Great Britain) according to the instruction of the manufacturer.

2.7.4 Determination of DNA concentration and purity

The concentration and purity of nucleic acids were determined by measuring the absorbance at 260 nm and 280 nm with a NanoDrop ND-1000 spectrophotometer. Additionally the concentration of nucleic acids was estimated by comparing the amplified fragments with defined fragments of commercially available DNA ladder.

2.7.5 Primer combinations and expected products

Dissimilatory sulphite reductase (*dsr*) related genes were identified in the *Chloroflexi* single amplified genome C11 (SAG-C11). SAG-C11 was annotated in a parallel project by Dr. Kenneth Wasmund. To investigate the distribution of *dsr* in marine sediments, primers were obtained from Eurofins MWG Operon (Ebersberg, Germany) and diluted with PCR grade water to obtain a stock solution with a concentration of 100 pmol μl^{-1}. For PCR reactions, primers were diluted to concentrations of 5 or 10 pmol μl^{-1}. Primers for long range PCR of 3.9 to 10.1 kb were purchased with a phosphorothioate 3 prime end modification to protect primers from exonuclease activity of the phusion DNA polymerase during long amplification cycles (174). Primers dsr1F-DHC-2-pt and dsr4r-C11 (Table 2-10) were based on the primers DSR4R and DSR1F used by Wagner and colleagues (151). They were modified according to *Chloroflexi* SAG-C11. All other primers were based on the sequence of SAG-C11 and degenerated positions were introduced after comparison of SAG-C11 sequences with BlastN hits from the NCBI nr database by aligning with these sequences using MUSCLE implemented in Mega 6.0 (175).

Table 2-10: Primer combinations used for the amplification of *dsr*-related genes from sediments (the phosphorothioate modification is indicated in small letters). For several fragments different primer combinations were used yielding fragments with a similar size.

type	*Name*	*Sequence 5'→ 3'*	*Targeted gene/s*	*Size (kb)*
fw	Dsr1F-DHC-2-pt	GATTACSCACTGGAAACAtg		
rv	SAG-D_Glut	TTGGCATTAATCATAAGAC	*dsrABDNCMK,*	10.185
rv	SAG-D_Glut_plus	TTGGCATWAAYCATMAKAC	*cobA/hemD,*	10.185
rv	SAG-D_Glut-pt	TTGGCATTAATCATAAGac	*hemC, hemA*	10.185
rv	SAG-D_Glut_v2	TACCTGTAACCGAACCga		10.064
rv	SAG-D_Glut_v3	CTTTTTCGAGTGGCATCcg		9.913
rv	SAG-D_Glut_v4	ATTGCTGTTCCTCCAGat		9.376
fw	Dsr1F-DHC-2-pt	GATTACSCACTGGAAACAtg		
rv	SAG-D_UropF1	AGGGAAAGGTCTATCTGgt	*dsrABDNCM,*	8.108
rv	SAG-D_UropF2	TGATAACAGTAAAGGGCct	*cobA/hemD*	8.063
rv	SAG-D_UropF3	AAAGGTGGTGACCCTTTTgt		7.854
fw	C11_luxr1_out1	CTCTAGAACTTTTCATCCG		
rv	SAG_D_gammaF_C	AAGCCATCCTCATCAASyt	*luxR, dsrABDN*	4.687
fw	Dsr1F-DHC-2-pt1	GATTACSCACTGGAARCAyg		
rv	SAG_D_gammaF_A	AAGCCATCCTCRTCMASyt	*dsrABDNC*	3.896
fw	Dsr1F-DHC-2-pt2	GATTACACACTGGAARCAyg		
rv	SAG_D_gammaF_B	AAGCCATCCTCATCMASyt	*dsrABDNC*	3.896
fw	Dsr1F-DHC-2-pt3	GATTACACACTGGAATCAyg		
rv	SAG_D_gammaF_C	AAGCCATCCTCATCAASyt	*dsrABDNC*	3.896
fw	C11_luxr1_out1	CTCTAGAACTTTTCATCCG		
rv	Dsr4R-C11	GTAAAGCAATTGGCACA	*luxR, dsrAB*	2.589
rv	Dsr4R-C11plus	GTRWAGCARTTRSCRCA		
rv	Dsr4R-C11plus2	GTAAAGCAATTRSCRCA		
rv	Dsr4R-C11plus3	GTAAAGCAATTGGCRCA		
rv	Dsr4R-C11plus4	GTAAAGCAATTRGCRCA		
rv	Dsr4R-C11plus5	GTAAAGCAATTGSCRCA		
fw	C11_luxr1_out2	GCCCACCATGTGTATTCC		
rv	Dsr4R-C11	GTAAAGCAATTGGCACA	*luxR, dsrAB*	2.667
rv	Dsr4R-C11plus	GTRWAGCARTTRSCRCA		
rv	Dsr4R-C11plus2	GTAAAGCAATTRSCRCA		
rv	Dsr4R-C11plus3	GTAAAGCAATTGGCRCA		
rv	Dsr4R-C11plus4	GTAAAGCAATTRGCRCA		
rv	Dsr4R-C11plus5	GTAAAGCAATTGSCRCA		
fw	C11_luxr1	GAACTTATCTTGGCTCTG		
fw	C11_luxr1_deg	GAACTTATCTTGRCYCWG		
rv	Dsr4R-C11	GTAAAGCAATTGGCACA	*luxR, dsrAB*	2.502
rv	Dsr4R-C11plus	GTRWAGCARTTRSCRCA		
rv	Dsr4R-C11plus2	GTAAAGCAATTRSCRCA		
rv	Dsr4R-C11plus3	GTAAAGCAATTGGCRCA		
rv	Dsr4R-C11plus4	GTAAAGCAATTRGCRCA		
rv	Dsr4R-C11plus5	GTAAAGCAATTGSCRCA		
fw	Dsr1F-DHC-2-pt	GATTACSCACTGGAAACAtg		
rv	Dsr4R-C11	GTAAAGCAATTGGCACA	*dsrAB*	1.978

For the amplification of fragments cloned into the commercially available vectors pJET 1.2 and pGEM T-easy specific primers binding within the sequence of the vector have been used (see Table 2-11). For testing marine dehalogenating microcosms for cross-contamination with strain CBDB1 primers targeting the reductive dehalogenase homologous gene cbdbA1453 were used (Table 2-12).

Table 2-11: Primers for the amplification of inserts from vectors using the flanking regions of the vector as primer targets

combination	***Name***	***Sequence 5'→ 3'***
1	pJET 1.2 fw	CGACTCACTATAGGGAGAGCGGC
	pJET 1.2 rv	AAGAACATCGATTTTCCATGGCAG
2	pGEM T-easy M13 fw	GTAAAACGACGGCCAGT
	pGEM T-easy M13 rv	GCGGATAACAATTTCACACAGG

Table 2-12: Primers used for testing for contamination by strain CBDB1 by amplification of cbdbA1453

type	***Name***	***Sequence 5'→ 3'***
fw	cbdbA1453_f	AATCTCTCGAGGGCACTC
rv	cbdbA1453_r	CCAGAGGGCTGGTAAGG

2.7.6 Polymerase chain reaction

Polymerase chain reactions were carried out in an Eppendorf Mastercycler® Personal (Eppendorf, Hamburg, Germany). For long range PCRs the Phusion®High-Fidelity DNA polymerase master mix with HF buffer (New England Biolabs, Ipswich, Massachusetts, USA) was used. The final primer concentration in the PCR reaction mix was 400 nM. The concentration of the template was typically ~10 ng μl^{-1}. One µl template was added to a PCR reaction with a total volume of 25 µl. SAG-C11 DNA previously amplified using a Phi29 whole genome amplification kit (see section 2.7.3) was used as a positive control for the amplification of *dsr*-related genes from sediments. For shorter fragments up to 1.8 kb the Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA) was used. The elongation time depended on the used polymerase and the size of the amplified fragment. The binding temperature of the applied primers was estimated according to the GC content and optimized by a temperature gradient PCR.

Table 2-13: PCR reaction components and concentrations

Component	***Taq DNA polymerase (NEB)***	***Phusion™ High Fidelity DNA polymerase (NEB)***
PCR-buffer 1x	2.5 µl	12.5 µl
$MgCl_2$	1.5 mM	1.5 mM
Forward primer 10 µM	0.4 µM	0.4 µM
Reverse primer 10 µM	0.4 µM	0.4 µM
dNTPs (2.5 mM each)	250 µM	200 µM
DNA-polymerase	0.025 U μl^{-1}	0.02 U μl^{-1}
DNA template	1 µl	1 µl
H_2O add to a total volume of	25 µl	25 µl

2.7.6.1 PCR temperature programs

The following programs were used for the amplification of DNA fragments from DNA isolated from marine sediments (Table 2-14 to Table 2-16).

Table 2-14: PCR temperature programs for 1.8 and 2.5 kb fragments.

Fragment	***1.8 kb***		***2.5 kb***	
Polymerase	***Taq***		***Phusion***	
	Temperature	Time	Temperature	Time
Initial denaturation	98°C	15 sec	98°C	15 sec
Denaturation	98°C	10 sec	98°C	15 sec
Annealing	54°C	20 sec	54°C	20 sec
Elongation	72°C	45 sec	72°C	45 sec
Cycles		35		35
Final elongation	72°C	5 min	72°C	5 min
Hold	4°C	∞	4°C	∞

Table 2-15: PCR temperature program for a touch-down PCR for a 2.5 kb fragment and degenerated primers C11_luxr1_deg and Dsr4R-C11 and the Phusion Polymerase

Step	***Temperature***	***Time***
Initial denaturation	98°C	15 sec
Denaturation	98°C	10 sec
Annealing	60°C	20 sec
	-0.5°C	
Elongation	72°C	45 sec
Cycles		12
Denaturation	98°C	10 sec
Annealing	54°C	20 sec
Elongation	72°C	45 sec
Cycles		28
Final elongation	72°C	5 min
Hold	4°C	∞

Table 2-16: PCR temperature program for a 3.9 kb DNA fragment amplified with the Phusion DNA polymerase

Step	***Temperature***	***Time***
Initial denaturation	98°C	30 sec
Denaturation	98°C	10 sec
Annealing	58°C	20 sec
Elongation	72°C	4 min
Cycles		30
Denaturation	98°C	10 sec
Annealing	58°C	20 sec
Elongation	72°C	5 min
Cycles		10
Final elongation	72°C	10 min
Hold	4°C	∞

The following PCR program was used to test marine microcosms for the presence of contaminations by strain CBDB1 (Table 2-17).

Table 2-17: PCR temperature program for the amplification of the cbcb1453 of strain CBDB1 using the Hot start Taq polymerase (NEB).

Step	***Temperature***	***Time***
Initial denaturation	95°C	15 min
Denaturation	95°C	30 sec
Annealing	59°C	30 sec
Elongation	72°C	1 min
Cycles		35
Final elongation	72°C	10 min
Hold	4°C	∞

2.7.7 Agarose gel electrophoresis

For agarose gel electrophoresis 1% (w/v) agarose was dissolved in TAE buffer with a 0.5 x final concentration (20 mM Tris-Acetat, 0.5 mM EDTA, pH 8.3). As running buffer TAE buffer with a 0.5 x final concentration was used as a running buffer. Separation of DNA fragments was done at 60–90 V. Agarose gels were stained with ethidium bromide and DNA fragments were visualized using an UV-light detector (BioRad Gel Doc™ XR+Imager, Munich, Germany). The following DNA ladder was used to estimate size and concentration of the amplified PCR products: MassRuler High Range DNA Ladder (Thermo Scientific, Waltham, Massachusetts, USA).

2.7.8 Elution of DNA from agarose gels

PCR amplified fragments were separated on a 1% agarose gel and visualized by ethidium bromide staining and UV-light. Gel pieces which contained DNA fragments with the required size were excised with a scalpel. Gel pieces were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA). When PCR product amounts were very low, up to10 gel pieces with DNA products of the same size were combined before gel purification. The purification of PCR products via the PCR product wizard was done according to the manufacturer's recommendations. The PCR products were eluted in 30 to 50 µl of PCR grade water.

2.7.9 DNA and plasmid preparation and purification

Plasmids were purified with the NucleoSpin® Plasmid Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

2.7.10 Addition of an poly adenosine tail

Prior to cloning, DNA fragments obtained by the Phusion DNA polymerase were incubated with deoxyadenosine triphosphate and Taq polymerase to add an additional adenosine overhang to the three prime for TOPO TA cloning.

2.7.11 TA cloning

DNA fragments containing an additional adenosine overhang were ligated in the pGEM®-T-easy vector for 12 hours at 4°C using the pGEM®-T-easy Vector System (Promega, Madison, WI, USA) according to the manufactures instructions.

Table 2-18: Blunt end PCR products were incubated for 20 minutes at 72°C with following components

Component	***Volume in µl***
Buffer (10x)	0.5
Taq Polymerase	0.5
dATPs (10 mM)	1
Template	3

Table 2-19: Reaction mix for ligation

Component	***Volume in µl***
Buffer	2.5
Vector	0.5
Ligase	0.5
Template	1.5

2.7.12 Blunt end cloning

For blunt end cloning of fragments obtained with the Phusion DNA polymerase were ligated into the pJET 1.2 blunt end cloning system (ThermoScientific, Waltham, Massachusetts, USA) according to the manufactures instructions. Vector and insert were ligated for 30 min at 37°C.

2.7.13 Transformation

Clone libraries were constructed by transformation of ligation products into *E. coli* K12 cells (NEB C2987, New England Biolabs, Ipswich, Massachusetts, USA). Cells were thawed on ice for ten minutes. Five µl plasmid DNA was added to 50 µl competent cells and gently stirred with the pipette tip. The cells were incubated for 30 min on ice. Subsequently, the cells were exposed for 30 s to 42°C and then incubated 5 min on ice. After this 950 µl of SOC outgrowth medium (New England Biolabs, Ipswich, Massachusetts, USA) were added and the cells were incubated at 37°C for one hour at 250 rpm. The mix was plated on selective LB-agar plates (80 µg ml^{-1} X-Gal, 100 µg ml^{-1} ampicillin).

2.7.14 Colony PCR

After ligation of specific fragment into the pGEM®-T-easy vector or the pJET 1.2 vector transformation of *E.coli* cells and identification of positive clones with the blue white screening, the

plasmid insert was re-amplified with the vector-specific primers M13 from pGEMT easy and pJET (see Table 2-11) with an annealing temperature of 55°C. White colonies were resuspended in 100 µl sterile water, incubated for 2 h at 37°, mixed on a Vortex mixer and centrifuged for 1 min at 6,000 rcf. The supernatant was applied into the reaction mix and the solution was stored at -20°C.

2.7.15 Screening and sequencing of inserts

White colonies were picked, suspended in PCR grade water and screened for the correct insert size by PCR and agarose gel electrophoresis using M13 forward and reverse vector primer or pJET 1.2 fw and rv primer (Table 2-11). For sequencing PCR fragments obtained via colony PCR were separated on an agarose gel. The obtained fragment was purified using the PCR purification wizard (Promega, Fitchburg, Wisconsin, USA). For sequencing at the GATC European Custom Sequencing Centre (Köln, Germany), 5 µl of a 20–80 ng μl^{-1} PCR amplified DNA fragment were mixed with 5 µl of vector primer (5 pmol μl^{-1}) (see Table 2-11 page 30). For sequencing, plasmids were purified with the NucleoSpin® Plasmid Kit (Macherey-Nagel, Düren, Germany). For sequencing, plasmids were used at 80–100 ng μl^{-1}. The full-length sequence of several selected clones was sequenced by using vector primer and internal primers (see Table 2-20). For internal primers a sequence with a length of 19 to 21 bases and a GC content of 40–60% was selected in a position of approximately 150 bases before the 3´ end of the sequenced fragment. For obtaining full-length sequences of long-range PCR fragments, primers were selected based on the sequences amplified from sediments.

Table 2-20: The following primers were used for internal sequencing of inserts

Primer name	***Sequence 5'–3'***	***Target gene***
PW_dsrB_75	ACCTCAGATAGCCATCACAG	*dsrB*
PW2_dsrB_75	GTTGATACAGTGCATACATCT	*dsrB*
PW_dsrB_11	TCCTGAGGCATCTGTTGCCG	*dsrB*
PW2_dsrB_11	ATCGTAAGGTGGCTCCATCT	*dsrB*
PW_dsrB_20	CCGTATCGAATCGACACTTA	*dsrB*
PW2_dsrB_20	TTCTCATCCCAGTTCAGGGC	*dsrB*
PW3_dsrA_11 u 20	AGTTCCTTAGCTGCCGGTT	*dsrA*
PW_dsrA_290	TGCCCCAATGACTGTGTTGC	*dsrA*
PW2_dsrA_290	CAGTTGAGGAGAGCGCTTTG	*dsrA*
PW3_dsrB_290	TCACCGACCAGCATATTGAT	*dsrB*
PW4_cobyrinic_290	ATCGACAATATTTGCCATC	*dsrN*
PW_dsrA_252	CGATGCCCTGACTGATGAGG	*dsrA*
PW2_dsrA_252	CACCATATACTGAGTTGAAAG	*dsrA*
PW3_dsrB_252	AAGGCGATGGCATCTCCATC	*dsrB*
PW4_cobyrinic_252	GCTATGGTAACAGGTTATCA	*dsrN*
PW_dsrA_265	AGGATGCCCAAGGCACTTAG	*dsrA*
PW2_dsrA_265	GGCTATCTGAGGTTTACCAGC	*dsrA*
PW3_dsrD_265	GCTGGTTGATAAGGCAATAG	*dsrD*
PWb_dsrA_285	TATCCAGAATGAAGTTATCA	*dsrA*
PW2_dsrA_285	TTAAGCGTCGATTCTGTACGG	*dsrA*
PW3_dsrB_285	GACTTCGACGCAGTTCAGG	*dsrB*
PW5_cobyrinic_285	GCATATAGCGCAGGCTATT	*dsrN*
PW_dsrA_271	CTCAACCTGAAGCCAGAGGA	*dsrA*

Primer name	*Sequence 5'–3'*	*Target gene*
PW2_dsrA_271	GTTGAGTATTGATACAGTGCG	*dsrA*
PW3_dsrB_271	CAACTACACAGTTCAAGTGG	*dsrB*
PWb_dsrA_215b	GTTCTACTCTACCAAAGCTCT	*dsrA*
PW2_dsrA_215	TATAGAACCATGCTTCACC	*dsrA*
PW3_dsrB_215	CGTGACAACCTTCCGGAGT	*dsrB*
PW4_cobyrinic_215	GTAATGCTTGACCACGTCT	*dsrN*

2.8 Bioinformatic tools

Sequences obtained from the GATC sequencing service were evaluated for their quality using Chromas Lite 2.1.1 (Technelysium Pty Ltd, South Brisbane, Australia). Functional annotation of genes was done by Blast (Basic Local Alignment Search Tool (176)) and the NCBI non-redundant database (nr) supplemented with the single-cell genome sequence of *Dehalococcoidia* C11. Sequences were aligned using the muscle program (177). Sequences obtained with internal primers from long range PCR fragments were assembled with the Sequencher program (Gene Codes Corporation, Ann Arbor, USA). For these assembled sequences initial annotations were done using RAST 2.0 (178) and Artemis (179).

For the construction of phylogenetic trees, DNA or amino acid sequences of functional genes were aligned by using Muscle implemented in the Mega 6.0 program and nucleotide or amino acid sequences retrieved from the NCBI non-redundant database. The obtained alignment was used to construct phylogenetic trees using the Maximum Likelihood (180), Neighbor Joining (181) and Minimum evolution (182) algorithm implemented in Mega 6.0 (175) and compared for achieved topology. Bootstrap analysis was done with 1000 repetitions to test the stability of the tree topology. Pairwise deletion was selected for gaps and missing data treatment and the p-distance method was used for evolutionary distance calculation (183). The visualization of topologies were optimized with the online tool "iTOL" (184, 185).

2.9 Electron density distributions for halogenated aromatics

To calculate the chemical parameters geometry optimization of halogenated aromatics were carried out. Density Functional Theory level B3LYP/6-31G(d,p) was used to obtain a minimum energy structure for each molecule and correct ground-state geometries were confirmed by frequency analysis. The Gaussian 09 revision C.0149 (186) was used to carry out Mulliken (187), Hirshfeld (188) (keyword IOP(6/79=1)) and NBO (189) population analysis. The analyses were done together with D. Wondrousch, Department Ecological Chemistry, UFZ Leipzig.

3 Results

3.1 Respiration and growth of *Dehalococcoides mccartyi* strain CBDB1 with halogenated aromatic and heteroaromatic electron acceptors

Dehalococcoidia are ubiquitously distributed in marine sediments and reductive dehalogenase homologues genes have been detected in non-contaminated marine sediments (60, 61). Because so far all attempts to cultivate *Dehalococcoidia* from marine sediments remained unsuccessful, *D. mccartyi* strain CBDB1 was used as a model organism for *Dehalococcoidia* to investigate potential electron acceptors for respiration beyond known halogenated contaminants from anthropogenic sources. For this strain CBDB1 was cultivated with halogenated electron acceptors, containing different halogen and non-halogen substituents or heteroatoms: The compounds were selected based on structural considerations to elucidate chemical parameters influencing reductive dehalogenation. The information might help to clarify which functional groups of natural haloorganic molecules and haloorganics from anthropogenic sources allow for reductive dehalogenation and growth of organohalide-respiring members of the class *Dehalococcoidia* and may thereby help future attempts for cultivation of *Dehalococcoidia* from marine sediments. In addition, the selected molecules contain structures present in currently used halogenated xenobiotics and may therefore also contribute to a better understanding of the role of *Dehalococcoidia* in fate prediction of halogenated xenobiotics in anaerobic environments.

3.1.1 Chlorinated anilines as electron acceptor

To investigate the influence of amino substituents onto reductive dehalogenation, and growth with halogenated anilines, *D. mccartyi* strain CBDB1 was cultivated in triplicates with 2,3-, 2,4- and 2,6-dichloroanilines. For this, culture medium was inoculated with a 10% inoculum of strain CBDB1 and dichloroanilines were added to an initial concentration of approximately 100 µM. After 70 days of incubation at 30°C, 100 µM 2,3-dichloraniline were transformed. Additional 200 µM 2,3-dichloroaniline were transformed after 104 days of cultivation. (Figure 3-1, left). In chemical controls with 2,3-dichloroaniline but no inoculum, no transformation of 2,3-dichloroaniline was observed. In a second transfer with 2,3-dichloraniline and an inoculum of 10%, approximately 87 µM of 2,3-dichloroaniline were transformed within 14 days in six parallel cultures. Additional 168 µM 2,3-dichloroaniline were transformed within 43 days. In cultures grown with 100 µM 2,4- or 2,6-dichloroaniline no transformation was detected (Figure 3-1; left). In chemical controls with 2,4- or 2,6-dichloroaniline, no transformation was detected.

Triplicate cultures with 2,3-dichloroaniline present as electron acceptor were inoculated with starting concentration of 1.5 x 10^6 to 3.2 x 10^6 cells ml^{-1}. Cell numbers increased constantly (5.2 x 10^6 cells ml^{-1} after 35 days and 1.10 x 10^7 cells ml^{-1} after 56 days) and reached final cell numbers of 1.5 to

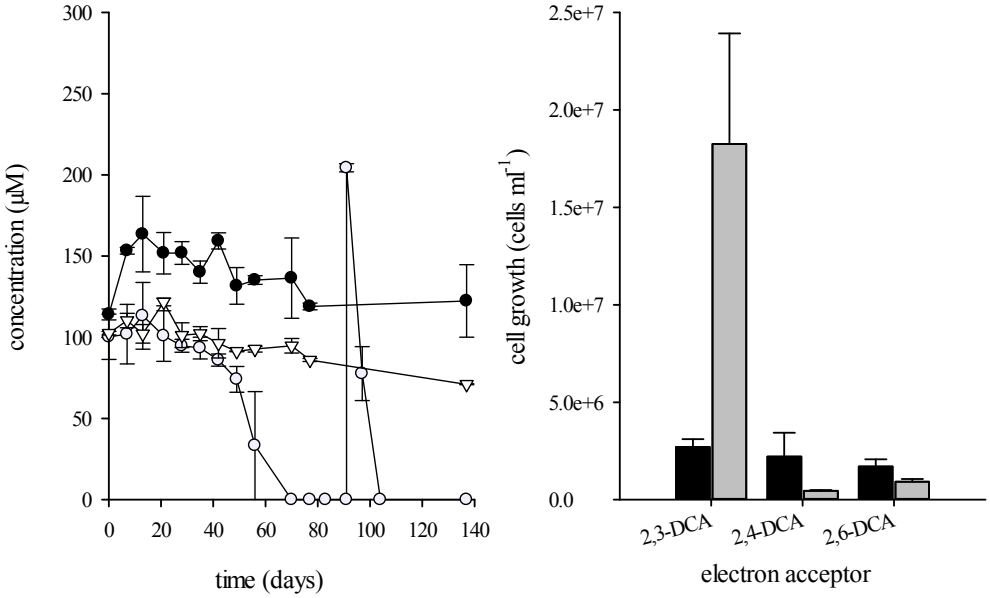

Figure 3-1: Transformation of chlorinated anilines and concomitant growth of *D. mccartyi* strain CBDB1. Left: Transformation of dichloroanilines in cultures of strain CBDB1 (○ – 2,3-dichloroaniline, ● – 2,4-dichloroaniline; ▽ – 2,6-dichloroaniline). Approximately 200 µM 2,3-dichloroaniline was added after 95 days to the cultures set up with 2,3-dichloroaniline. Right: Cell numbers of *D. mccartyi* strain CBDB1 in cultures with one of the tested chlorinated anilines immediately after inoculation (black bars), and cell numbers after 140 days of incubation (grey bars). Shown are mean values of triplicate cultures ± SD.

2.8 x 10^7 cells ml^{-1} after 140 days of cultivation (Figure 3-1, right). This corresponds to a cell growth of 0.6 x 10^{14} cells mol^{-1} of halogen released. Cultures with 2,4-dichloroaniline as electron acceptor were inoculated with 1.8 to 2.4 x 10^6 cells ml^{-1}. After a cultivation period of 140 days, cell numbers had decreased to 4.3 to 5.0 x 10^5 cells ml^{-1} (Figure 3-1, right). Similarly, in cultures with 2,6-dichloroaniline as electron acceptor, cell numbers had decreased from 1.2 to 2.1 x 106 cells ml-1 initial cell number to 0.7 to 1.1 x 10^6 cells ml^{-1} after 140 days of incubation (Figure 3-1, right). In the second transfer with six parallel cultures of strain CBDB1 and 2,3-dichloroaniline as electron acceptor with initial cell numbers of 4–10 x 10^5 cells ml^{-1}, growth was detected in all cultures. Cell numbers reached 2–4 x 10^7 cells ml^{-1} after 83 days of incubation, corresponding to a growth yield of 0.9 x 10^{14} cells mol^{-1} halogen released.

3.1.2 2,3-Dichloronitrobenzene as electron acceptor

To investigate the influence of nitro substituents onto reductive dehalogenation, and growth with halogenated nitrobenzenes, 2,3-dichloronitrobenzene was tested as an electron acceptor for *D. mccartyi* strain CBDB1. However, 2,3-dichloronitrobenzene reacted abiotically with the reducing agent during the equilibration time of 12 hours to 2,3-dichloroaniline. One culture transformed 70 µM 2,3-dichloroaniline after 28 days and additional doses of 110 µM 1,2-dichloronitrobenzene (which reacted to 2,3-dichloroaniline) after 49 days (see 3.1.1). Initial *D. mccartyi* strain CBDB1 cell numbers of 2 x 10^6 cells ml^{-1} increased 10-fold to 1.7 x 10^7 cells ml^{-1} after 56 days of incubation. A second

culture transformed 70 µM 2,3-dichloroanilines after 77 days of incubation and cell numbers increased from initial cell numbers of 2.9 x 10^6 to 9.8 x 10^6 cells ml^{-1}.

3.1.3 Dehalogenation of and growth with 2-chloro-6-fluorobenzonitrile

To investigate growth with halogenated benzonitriles and the influence of the cyano group onto reductive dechlorination and defluorination, strain CBDB1 was cultivated in triplicates with 2-chloro-6-fluorobenzonitrile. 2-Chloro-6-fluorobenzonitrile was added in crystals to the medium. Bottles were incubated overnight to equilibrate before inoculation was done with strain CBDB1 previously grown with 1,2,3-trichlorobenzene as electron acceptor. Initial 2-chloro-6-fluorobenzonitrile concentrations were approximately 260 µM. After 70 days 2-chloro-6-fluorobenzonitrile was completely transformed to 2-fluorobenzonitrile (Figure 3-2). The product was identified by parallel cultivation of 2,6-dichlorobenzonitrile and comparison of measured retention times of reaction products. Defluorination to benzonitrile was not observed. In chemical controls with 2-chloro-6-fluorobenzonitrile no transformation products were detected. A second transfer of three cultures was inoculated with a volume of 10% of strain CBDB1 culture pregrown on 2-chloro-6-fluorobenzonitrile and contained an initial concentration of 467 ± 128 µM of 2-chloro-6-fluorobenzonitrile. Only one of three cultures containing 532 µM 2-chloro-6-fluorobenzonitriles showed transformation of 155 µM 2-chloro-6-fluorobenzonitrile to 2-fluorobenzonitrile within 111 days of cultivation.

In the first transfer three serum bottles containing culture medium and 2-chloro-6-fluoro-benzonitrile were inoculated with starting concentration of 6.8–7.5 x 10^6 cells ml^{-1}. Cell numbers increased constantly over time and reached final cell numbers of 4.7 to 6.9 x 10^7 cells ml^{-1} after 62

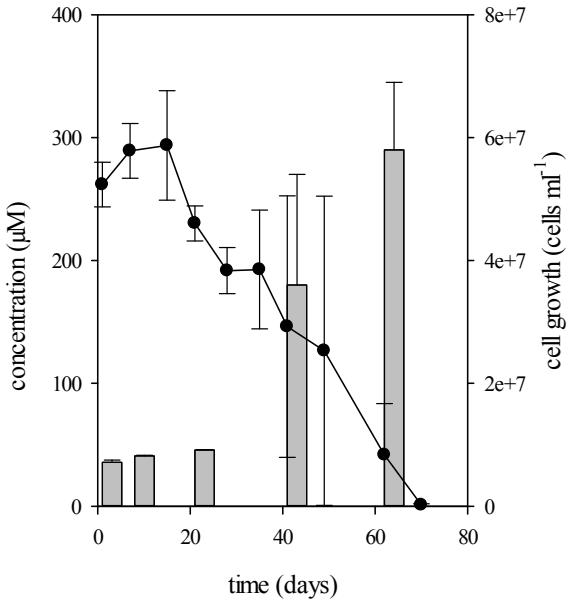

Figure 3-2: Transformation of 2-chloro-6-fluorobenzonitrile (●) and concomitant growth of *D. mccartyi* strain CBDB1 (grey bars). Shown are means of triplicate cultures from the first transfer ± SD.

days of cultivation (Figure 3-2), corresponding to a cell growth of 2.3 x 10^{14} cells mol^{-1} of halogen released. In the second transfer with three parallel cultures with initial cell numbers of 2.1 x 10^6 to 2.9 x 10^6 cells ml^{-1}, growth was detected only in the culture, which transformed 2-chloro-6-fluoro-benzonitrile. For this culture, cell numbers had increased from 2.4 x 10^6 cells ml^{-1} to 3.3 x 10^7 after 111 days of incubation, corresponding to a growth yield of 0.9 x 10^{14} cells mol^{-1} halogen released.

3.1.4 Chlorinated pyridines as electron acceptor

Strain CBDB1 was cultivated with chlorinated pyridines to investigate the influence of nitrogen heteroatoms onto reductive dehalogenation, and growth with halogenated pyridines. For this purpose, cultures were set-up in triplicates containing a 10% inoculum of strain CBDB1 and either 2,3- or 2,6-dichloropyridine as electron acceptor. The concentration of 2,3-dichloropyridine decreased from 48 ± 5 µM to 33 ± 0.2 µM after 97 days of incubation, however, no transformation products were detected (Figure 3-3, left). For cultures with 2,6-dichloropyridine, the concentration of 2,6-dichloropyridine decreased slightly to 17 ± 1.3 µM after 97 days of incubation but no transformation products were observed (Figure 3-3, left). In a second set-up with the same culture and inocula volumes, 2,3-dichloropyridine and 2,6-dichloropyridine were added with initial concentrations of 35 ± 4 µM and 40 ± 3 µM, respectively. The concentration of the two tested dichloropyridines did not decrease over 60 days and no transformation products were detected.

Initial cell numbers of cultures incubated with approximately 48 µM 2,3-dichloropyridines were 1.5 to 2 x 10^6 cells ml^{-1}. After 97 days of cultivation 0.9 to 2.3 x 10^6 cells ml^{-1} were detected (Figure

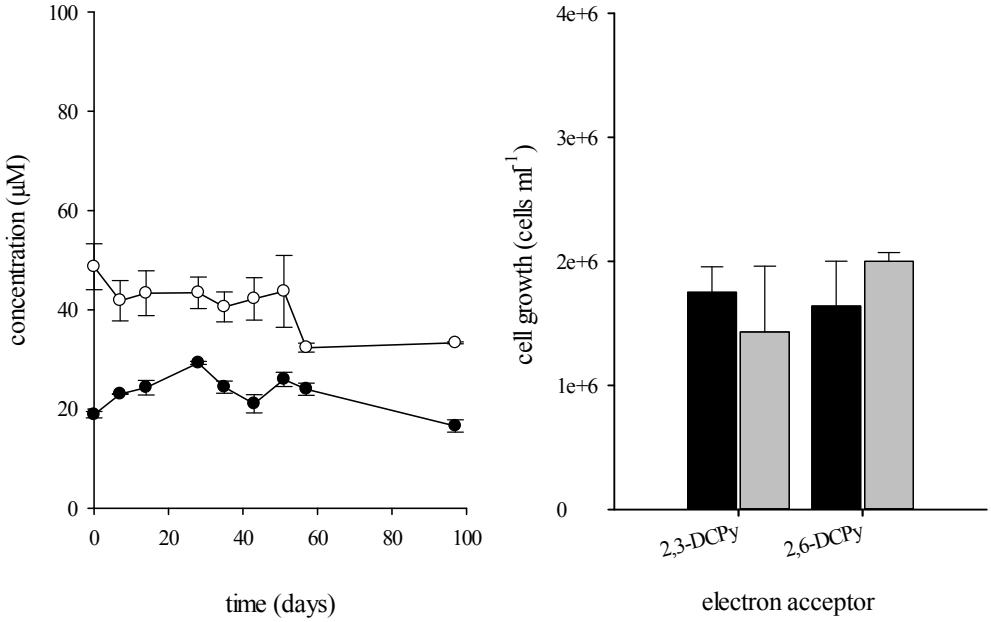

Figure 3-3: Transformation of chlorinated pyridines and cell numbers of *D. mccartyi* strain CBDB1. Left: Concentration of 2,3-dichloropyridine (○) and 2,6-dichloropyridine (●) during cultivation of strain CBDB1. Right: Cell numbers of strain CBDB1 in cultures with one of the chlorinated pyridines after inoculation (black bars) and after 97 days of incubation (grey bars). Shown are means of triplicate cultures ± SD.

3-3, right). Cultures with 2,6-dichloropyridine had initial cell numbers of 1.1 to 2.2 x 10^6 cells ml^{-1}. After 97 days of cultivation 1.9 to 2.1 x 10^6 cells were observed (Figure 3-3, right). In the second set-up cultures with 2,3-dichloropyridine had initial cell numbers of 1 to 1.1 x 10^6 cells ml^{-1} and cultures with 2,6-dichloropyridine had cell numbers of 0.9 to 1.2 cells ml^{-1}. After 60 days of incubation cell numbers ranged from 0.8 x 10^6 to 1 x 10^6 cells ml^{-1} for cultures with 2,3-dichloropyridines or 2,6-dichloropyridines, respectively.

3.1.5 Brominated phenols as electron acceptor

Strain CBDB1 was cultivated with brominated phenols to investigate growth and the influence of hydroxy substituents onto reductive debromination. For this, cultures with *D. mccartyi* strain CBDB1 and 2,4-dibromophenol, 2,6-dibromophenol or 2,4,6-tribomophenol were set up. First attempts to cultivate strain CBDB1 with initial bromophenol concentrations of 50 µM were not successful. Dehalogenation was slow and cell growth was low. Therefore, lower initial bromophenol concentrations were tested. Successful cultivation of strain CBDB1 was achieved by applying initial bromophenol concentrations of 10 µM and addition of further 20 µM of either 2,4-, 2,6- dibromo-, or 2,4,6-tribromophenol in repeated steps with continuing growth of strain CBDB1. *D. mccartyi* strain CBDB1 was cultivated over 89 days with 2,4-dibromophenol, 2,6-dibromophenol and 2,4,6-tribromophenol in separate cultures. First transformation products were detected after 7 days in cultures with 2,4- and 2,6-dibromophenol and after 7 to 14 days in cultures with 2,4,6-tribromophenol. Additional 20 µM of the respective bromophenols were added after 20 days, 40 days and 69 days to cultures with strain CBDB1. In cultures with strain CBDB1 2,4-dibromophenol was transformed to 4-bromophenol and 2,6-dibromophenol to 2-bromophenol. Both monobromophenol congeners were transformed subsequently to phenol (Figure 3-4, left). The first transformation product detected for 2,4,6-tribromophenol was 2,4-dibromophenol, which was transformed successively to 4-bromophenol and phenol (Figure 3-4, left). Chemical controls contained 10 µM 2,4,6-tribromophenol, 2,4-dibromophenol or 2,6-dibromophenol. Additional 20 µM of the respective bromophenol were added after 20 days and after 40 days of incubation. No reaction products were detected in chemical controls with 2,4- or 2,6-dibromophenol. 2,4,6-Tribromophenol reacted in chemical controls abiotically to 21.3 µM of 2,4-dibromophenol after 89 days of incubation. This corresponds to approximately 50% of 2,4,6-tribromophenol transformed in cultures incubated with strain CBDB1. No further abiotic transformation of 2,4-dibromophenol or monobromophenol was observed in the chemical control.

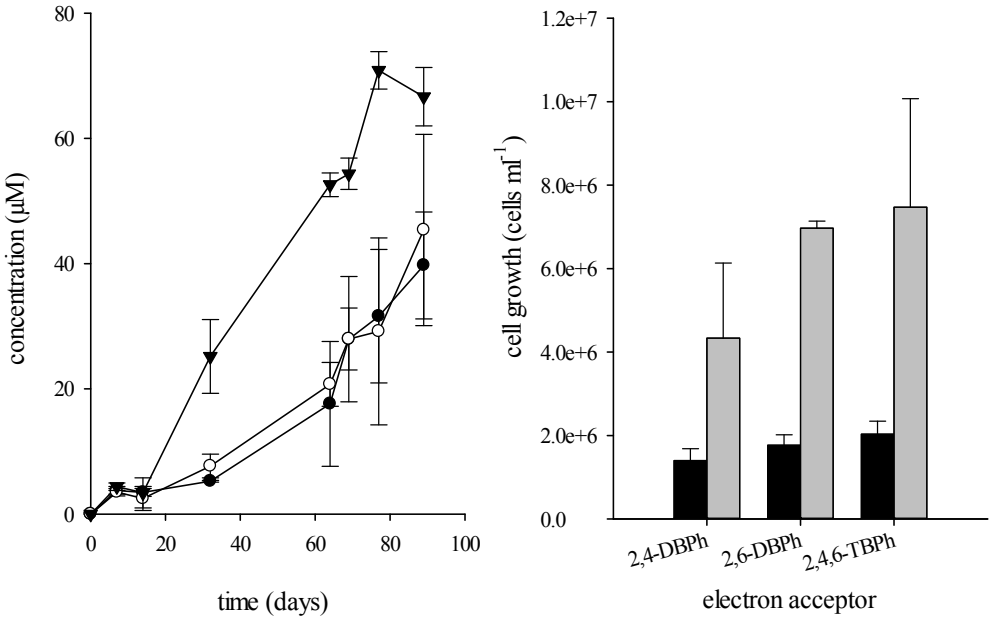

Figure 3-4: Transformation of brominated phenols and cell numbers of strain CBDB1. Left: Phenol production in cultures of strain CBDB1 amended with 2,4-dibromophenol (○), 2,6-dibromophenol (▼) or 2,4,6-tribromophenol (●). Right: Cell numbers of strain CBDB1 in cultures with one of the three tested bromophenols after inoculation (black bars) and after 89 days of incubation (grey bars). Shown are means of triplicate cultures ± SD.

Cultures with 2,4-dibromophenol had initial cell numbers of 1 to 1.6 x 10^6 and final cell numbers of 2.2 to 6.6 x 10^6 cells ml^{-1} (Figure 3-4, right). Growth yields of 0.3 x 10^{14} cells mol^{-1} halogen released were calculated. Cultures with 2,6-dibromophenol had initial cell numbers of 1.5 to 2.1 x 10^6 cells ml^{-1} and final cell numbers of 6.8 to 7.2 x 10^6 cells ml^{-1} after 89 days of incubation (Figure 3-4, right), corresponding to a growth yield of 0.37 x 10^{14} cells mol^{-1} of bromide released. Cultures with 2,4,6-tribromophenol had initial cell number of 1.6 to 2.3 x 10^6 cells ml^{-1}. After 89 days of cultivation 0.5 to 1.1 x 10^7 cells ml^{-1} were obtained (Figure 3-4, right). After subtraction of the abiotic background reaction of 2,4,6-tribromophenol measured in the chemical control, growth yields of 0.4 x 10^{14} cells mol^{-1} halogen released were calculated.

3.1.6 Brominated furoic acids as electron acceptor

Strain CBDB1 was cultivated with brominated furoic acids to investigate growth with brominated furoic acids and the influence of the oxygen heteroatom onto reductive debromination. The transformation of 4,5-dibromo-2-furoic acid and 5-bromo-2-furoic acid was measured by the release of bromide. First attempts to measure the release of bromide with an ion selective electrode were hampered by the high chloride concentration in the medium. Therefore, bromide release was measured by ion chromatography. Ion chromatography did not allow for the detection of the dehalogenated reaction products. However, 5-bromo-2-furoic acid contains only one halogen substituent, therefore dehalogenation will yield a non-halogenated reaction product. 4,5-Dibromo-2-furoic acid was not stable under the chosen cultivation conditions and bromide was released also in the abiotic chemical

controls. Therefore the release of bromide in the abiotic control was subtracted from the release of bromide in cultures with strain CBDB1. In the first cultivation set-up with 4,5-dibromo-2-furoic acid, the abiotic chemical control contained 164 µM of bromide after 24 hours of incubation. In cultures with strain CBDB1 set up in triplicates, 100 µM to 172 µM of bromide were measured after 24 hours of incubation. After 31 days of incubation 276 µM bromide were detected in the abiotic chemical control. In cultures with strain CBDB1 503 ± 90 µM bromide were detected. A bromide release of 250 ± 60 µM by microbial reductive dehalogenation was calculated after subtraction of the abiotic bromide release in the chemical control and subtraction of the initial bromide concentration measured after 24 hours of incubation in cultures with strain CBDB1. In a second transfer, bromide concentrations after addition of 4,5-dibromo-2-furoic acid and 24 h equilibration were 80 ± 17 µM. After 34 days of incubation 140 ± 10 µM were measured in cultures with strain CBDB1, however in the chemical control 130 µM of bromide were released abiotically. After 127 days, only two of three parallel cultures showed a high bromide release of 740 and 1240 µM compared to the chemical control, in which 590 µM of bromide were detected, corresponding to a release of 402 ± 252 µM bromide by reductive dehalogenation (Figure 3-5, left).

In the first cultivation set-up with 5-bromo-2-furoic acid, bromide concentrations after 24 hours of incubation were 85 to 116 µM in triplicate cultures. The abiotic chemical control contained 131 µM bromide after 24 hours of incubation. After 31 days of incubation, 110 to 178 µM bromide were detected in cultures with strain CBDB1. In abiotic chemical controls no abiotic reaction of 5-bromo-2-

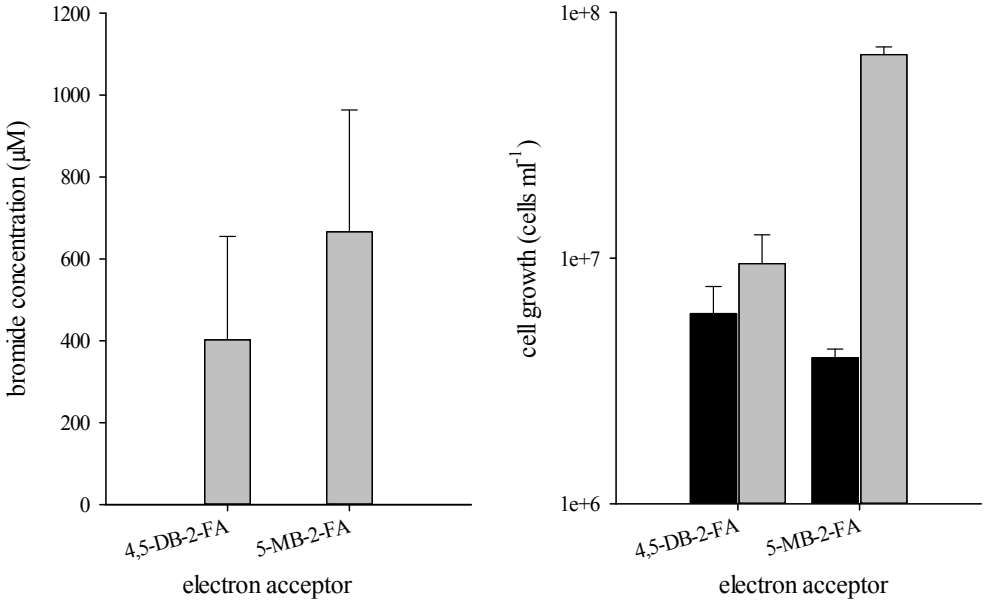

Figure 3-5: Transformation of brominated furoic acids and concomitant growth of strain CBDB1. Left: Bromide release in cultures of strain CBDB1 amended with 4,5-dibromo-2-furoic acid or 5-bromo-2-furoic acid. The initial bromide concentration measured in cultures with strain CBDB1 and the bromide release detected in the abiotic chemical controls after 127 or 63 for 4,5-dibromo- or 5-bromo-2-furoic acid respectively, were subtracted from the final bromide concentration measured in cultures with strain CBDB1. Right: Cell numbers of strain CBDB1 in cultures with one of the brominated furoic acids after inoculation (black bars) and after 127 days (4,5-dibromo-2-furoic acid) or 63 days (5-bromo-2-furoic acid) of incubation (grey bars). Data for 4,5-dibromo-2-furoic acid show two parallel cultures ± SD and for 5-bromo-2-furoic acid three parallel cultures ± SD.

furoic acid was observed and the bromide concentration remained at 131 µM. In a second transfer with 5-bromo-2-furoic acid, initial concentrations of 156 ± 19 µM of bromide were measured in cultures and 165 µM bromide in the abiotic chemical control. After 63 days of incubation, 823 ± 300 µM bromide were detected in cultures with strain CBDB1 and 108 µM bromide in the abiotic chemical control, corresponding to a release of 666 ± 279 µM bromide (Figure 3-5, left).

Cell growth was detected with 4,5-dibromo-2-furoic acid and 5-bromo-2-furoic acid. In the first set-up with 4,5-dibromo-2-furoic acid cells of the inoculum were previously cultivated on 1,2,3-trichlorobenzene. Initial cell numbers were 1.7 to 2.4 x 10^6 cells ml^{-1} in three parallel cultures. The final cell numbers after 31 days of incubation were 0.8 to 1.5 x 10^7 cells ml^{-1}, corresponding to a cell growth of 0.4 x 10^{14} cells mol^{-1} released. In the second transfer, initial cell numbers in cultures with 4,5-dibromo-2-furoic acid were 2.5 to 6.6 x 10^6 cells ml^{-1}. In the two cultures with a higher bromide release compared to the chemical control, cell numbers increased to 0.9 and 1.8 x 10^7 cells ml^{-1} after 127 days (Figure 3-5, right), corresponding to a growth yield of 0.1 x 10^{14} cells mol^{-1} halogen released. In the first set-up of cultures with 5-bromo-2-furoic acid initial cell numbers ranged from 1.9 to 2.6 x 10^6 cell ml^{-1} and increased within 31 days to 0.2 to 1.3 x 10^7 cells ml^{-1}, which corresponds to a growth yield of 1 x 10^{14} cells mol-1 halogen released. In the second transfer, cell numbers ranged from 3.5 to 4.3 x 10^6 cells ml^{-1} and increased to 6.2 to 7.4 x 10^7 cells ml^{-1} within 63 days (Figure 3-5, right). Growth yields of 0.95 x 10^{14} cells mol^{-1} halogen released were calculated.

3.1.7 Brominated benzoic acids as electron acceptor

The influence of methoxy and hydroxy substituents onto reductive debromination was investigated with brominated hydroxy- and methoxybenzoic acids. Additionally, growth of strain CBDB1 with brominated benzoic acids was investigated. The transformation of brominated benzoic acids was monitored by measuring the bromide ion concentration in the culture medium *via* ion chromatography. Because both tested brominated benzoic acids contained only one halogen substituent the expected dehalogenation products were 3,5-dimethoxy- or 3,5-dihydroxybenzoic acid. Cultures of strain CBDB1 amended with 4-bromo-3,5-dimethoxybenzoic acid crystals, had a concentration of about 41 ± 11 µM bromide after 24 hours of incubation. In the chemical abiotic control 48 µM bromide was measured after 24 hours of incubation. After 102 days of incubation 57 µM bromide were detected in the chemical abiotic control. In cultures with strain CBDB1, 141 ± 83 µM bromide was detected after 102 days of incubation, corresponding to a release of approximately 100 ± 93 µM bromide. In a second transfer, a concentration of 35 µM bromide after addition of 4-bromo-3,5-dimethoxybenzoic acid crystals and 24 hours of incubation was measured in the abiotic chemical control and 58 ± 15 µM in cultures with strain CBDB1. After 63 days of incubation 24 µM were detected in the chemical abiotic control, while in cultures with strain CBDB1 364 ± 15 µM were detected, corresponding to a release of 305 ± 133 µM bromide (Figure 3-6, left).

In the first set-up of three parallel cultures with 4-bromo-3,5-dihydroxybenzoic acid initial bromide concentrations in the medium after addition of crystals were 11 ± 0.2 µM in cultures with strain CBDB1 and 14 µM in the abiotic chemical control. In the abiotic chemical control, the bromide concentration did not increase after 63 days of incubation, however in cultures with strain CBDB1 bromide concentrations increased to 110 ± 77 µM after 73 days of incubation. In the second transfer, only in two of three parallel cultures with strain CBDB1 and 4-bromo-3,5-dihydroxybenzoic acid, an increase of the bromide concentration was detected. During 63 days of incubation, in one culture the bromide concentration increased from 20 to 95 µM bromide, while in a second culture the bromide concentration increased from 38 to 792 µM (Figure 3-6, left).

In the set-up of cultures with 4-bromo-3,5-dimethoxybenzoic acid with cells previously cultivated on 1,2,3-trichlorbenzene initial cell numbers ranged from 2.5 to 3 x 10^6 cells ml^{-1}. After 31 days no cell growth was detected. After 102 days of incubation 0.8 to 4.2 x 10^7 cells ml^{-1} were observed and a growth yield of 1.5 x 10^{14} cells mol^{-1} halogen released was calculated. In the second transfer with 4-bromo-3,5-dimethoxybenzoic acid growth yields of 1.9 x 10^{14} cells mol^{-1} halogen were calculated, cell numbers increased from 4.0 to 5.6 x 10^5 cells ml^{-1} to 4.3 to 6.3 x 10^7 cells ml^{-1} during 63 days of incubation (Figure 3-6, right).

4-Bromo-3,5-dihydroxybenzoic acid cultures had initial cell numbers of 1.7 to 2.4 x 10^6 cells ml^{-1} and increased after 39 days to 4.1 to 8.1 x 10^6 cells ml^{-1} but decreased slightly after 73 days to 4.1 to 6.3 x 10^6 cells ml^{-1}. Growth yields in the first set-up were 0.3 x 10^{14} cells mol^{-1} halogen released. In the

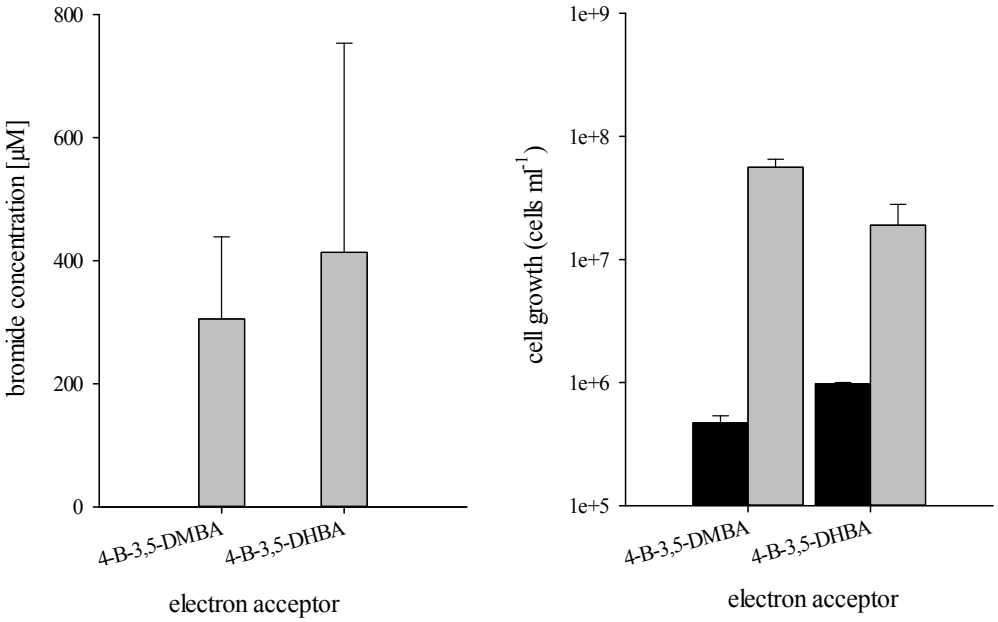

Figure 3-6: Transformation of brominated benzoic acids and concomitant growth of *D. mccartyi* strain CBDB1. Left: Bromide concentration in cultures of strain CBDB1 amended with one of the brominated benzoic acids after 63 days of incubation. The initial bromide concentration was subtracted from the bromide concentration measured after 63 days. Right: Cell numbers of strain CBDB1 after inoculation (black bars) and after 63 days of incubation (grey bars). For 4-bromo-3,5-dimethoxybenzoic acid data show the mean of three parallel cultures ± SD while for 4-bromo-3,5-dihydroxybenzoic acid data show the mean of two parallel cultures ± SD.

second transfer, the initial cell number was 1 x 10^6 ± 1.63 x 10^4 cells ml^{-1} in three parallel cultures. Only in the two cultures with increased bromide concentrations, cell numbers increased after 63 days of incubation. Cell numbers ranging from 1 to 2.8 x 10^7 cells ml^{-1} (Figure 3-6, right). Calculated growth yields from the two active cultures were 0.5 x 10^{14} cells mol^{-1} halogen released.

3.2 Set-up of a photometric microtiter plate-based resting cell activity assay

A photometric microtiter plate-based activity assay was established to allow for a fast screening and identification of new electron acceptors of organohalide-respiring *Dehalococcoidia* and microorganisms independent of analytical methods, which require establishment of specific programs for the detection of individual dehalogenation products. *D. mccartyi* strain CBDB1 was used for this purpose as a model organism.

3.2.1 Material choice for a 96-well microtiter plate for dehalogenase activity measurement

The photometric assay was initially set up in 96-well microtiter polystyrene plates with 200 µl activity assay reaction mix (see also 2.5.1) and 20 or 40 µl of whole resting cell suspension of strain CBDB1 previously concentrated with a rotary evaporator or a filter (see also section 2.4.1). The oxidation of methyl viologen was monitored with a microtiter plate reader at 578 nm for 15 hours after sealing the plate with a transparent plastic film (see also 2.5.3). Additionally the absorbance was measured at 450 nm to correct for the absorbance by condensed water on the sealing plastic film. The negative control contained strain CBDB1 resting cells in cultivation medium and 1,3-dichlorobenzene in the activity assay reaction mix. 1,3-Dichlorobenzene was shown previously not to be transformed by strain CBDB1 (7). The positive control contained strain CBDB1 resting cells in cultivation medium and 1,2,3-trichlorobenzene dissolved in acetone as electron acceptor. Oxidation of methyl viologen in the positive control was higher than in the negative control during the first 100 minutes of measuring time. After approximately 100 minutes however, the oxidation of methyl viologen in the positive control was similar to the oxidation measured in the negative control. GC-FID-based analysis of the activity assay reaction mix containing 1,2,3-trichlorobenzene incubated in wells of the polystyrene microtiter plate without resting cells, revealed a very low electron acceptors concentration in the reaction mix after only one hour of incubation (6 ± 0.4 µM 1,2,3-trichlorobenzene). The very low electron acceptor concentration could explain similar methyl viologen oxidation rates in the positive control as in the negative control after approximately 100 minutes of incubation. Additionally, measured oxidation rates showed high deviations between different wells within the same experimental set-up. The polystyrene plate contained raised rims which did not allow for uniform sealing of all wells by the plastic film allowing for oxygen penetration from the gas phase of the anoxic glove box into wells. This could explain deviations between different rates measured for the same reaction set-ups.

To assure sufficient concentration of electron acceptors during fifteen hours of incubation and uniform sealing of the wells, the activity assay was established in a 96-well microtiter glass plate to minimize hydrophobic interactions between the halogenated compounds and the microtiter plate material. Additionally, a tenfold higher electron acceptor concentration (625 µM) was applied to the reaction mix. The assay was set up similar to the polystyrene plate with 200 µl reaction mix and 20 or 40 µl whole resting cell suspensions per well. The positive control contained strain CBDB1 resting cells and 1,2,3-trichlorobenzene dissolved in acetone as electron acceptor. The negative control contained strain CBDB1 cells and acetone but no electron acceptor. To test for abiotic reactions of the investigated halogenated compounds, chemical controls were set up with each halogenated compound, the activity assay reaction mix but no cells. Furthermore, controls were included containing only the reaction mx without halogenated electron acceptor or cell suspension. The absorbance was measured every 7.5 minutes for at least 700 minutes at 578 nm and 450 nm. In the chemical control without cells the absorbance at 578 nm decreased only slightly from 1.05 to 0.99 within 700 minutes of incubation (Figure 3-7). In the negative control, the absorbance increased from 1.05 to 1.1 within the first 52 minutes of incubation due to the reaction of methyl viologen with additionally introduced titanium(III)citrate with the resting cell inoculum and then decreased continuously to 0.29. This demonstrated that strain CBDB1 resting cells alone oxidized methyl viologen in the absence of an electron acceptor. In the positive control the absorbance decreased with a faster initial rate from 1.06 to 0.1 (Figure 3-7). The activity was calculated in nmol s^{-1} ml^{-1} from the linear part of the slope as indicated in Figure 3-7, when background activity was not subtracted. After subtraction of the background oxidation of methyl viologen the activity was calculated in nkat ml^{-1} or nkat mg^{-1} whole

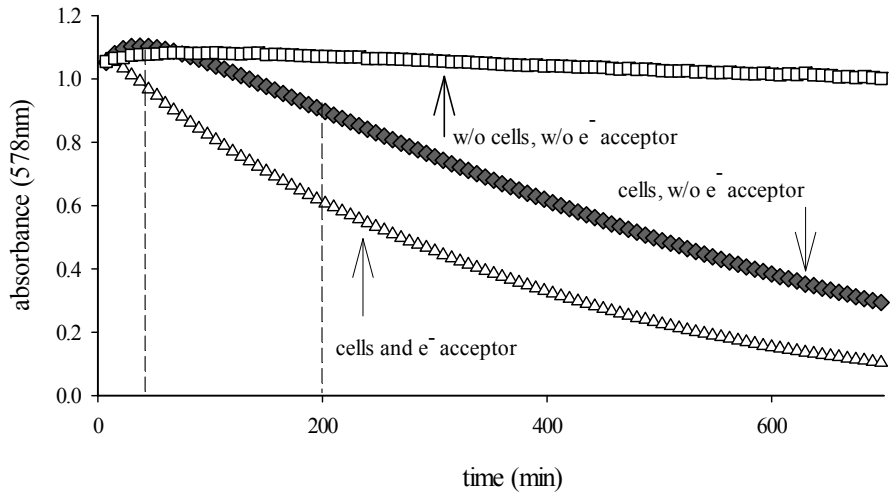

Figure 3-7: Resting cell activity measured at 587 nm in a glass microtiter plate. Methyl viologen oxidation in the chemical control containing the reaction mix, medium without cells and no electron acceptor (□). Methyl viologen oxidation in the negative control containing the reaction mix, resting strain CBDB1 cells in cultivation medium and acetone (◆). Methyl viologen oxidation in the positive control containing the reaction mix, strain CBDB1 cells in cultivation medium and 1,2,3-trichlorobenzene from an acetonic stock solution as electron acceptor (△). The dashed lines indicate the part of the slope which was used for the calculation of strain CBDB1 resting cell activity.

cell protein (see also for more information about background activity the following chapter 3.2.2) using the formula described in Figure 2-4.

3.2.2 Analysis of background oxidation of methyl viologen in the microtiter plate activity assay

The photometric activity assay is based on the measurement of the oxidation of methyl viologen absorbance at 578 nm, reflecting the catalytic activity of reductive dehalogenases transferring electrons from the reduced methyl viologen onto the halogenated compound (Figure 2-2). However, the photometric measurement revealed the oxidation of methyl viologen with resting cells even without halogenated electron acceptor. Potential reasons for this background oxidation were a) halogenated electron acceptors introduced together with the resting cells, b) cultivation medium components, introduced with the resting cells, c) reductive dehalogenases reducing a so far unknown none-halogenated electron acceptor and d) living or dead parts of resting strain CBDB1 cells catalysing the transfer of electrons onto a non-halogenated electron acceptor or parts of the cell acting as electron acceptor.

3.2.2.1 Analysis of a potential electron acceptor carry-over with the inoculum

To investigate whether halogenated electron acceptors from the cultivation medium were carried-over to the activity test via the cell preparation, resting cell suspensions of strain CBDB1 were prepared as described for the activity assay, extracted with hexane and analysed by GC-FID. Resting cell suspension originating from cultures grown with 2-chloro-6-fluorobenzonitrile 1,2,3-trichlorobenzene or 2,3-dichloroanilines were analysed but none of the electron acceptors were detected as traces in the cell suspensions.

3.2.2.2 Medium components and vitamins show low influence on background activity

The influence of medium components on the background activity was tested by comparing the oxidation of methly viologen in resting cell activity assays with i) strain CBDB1 cells in medium with vitamins (see also 2.3.1.3 for detailed descrition of vitamins in the medium), ii) a control with cultivation medium containing vitamins but no cells and iii) a second control with medium prepared without vitamins and without cells. For each set-up the oxidation of methyl viologen was compared between samples containing 1,2,3-trichlorobenzene as halogenated electron acceptor with samples containing acetone and no halogenated electron acceptor. Acetone was chosen because 1,2,3-trichlorobenzene was added to the activity assay reaction mix from an acetonic stock solution, which can introduce oxygen into the test and therefore contribute to methyl viologen oxidation. For a better comparability, the absorbance was calculated as oxidation of methylviologen in nmol s^{-1} ml^{-1} from the linear part of the slope (Figure 3-8, A).

In samples with strain CBDB1 resting cells and 1,2,3-trichlorobenzene the methyl viologen oxidation was about twice as high as it was in samples with resting cells and acetone (Figure 3-8, A). In samples without cells but with medium and vitamins, the methyl viologen oxidation was the same in the set-up with 1,2,3-trichlorobenzene or without. Both corresponded to about one third of the methyl viologen oxidation detected in samples with cells but without 1,2,3-trichlorobenzene, demonstrating that resting cells account for about two third of the detected background methyl viologen oxidation. In the experimental set-up with medium, without vitamins (see for medium components section 2.3.1) and without cells, a similar oxidation of methyl viologen was detected for the set-up with 1,2,3-trichlorobenzene as for the set-up with vitamins, medium but without cells (Figure 3-8, A). This suggests that vitamins such as B_{12} were not involved in abiotic methyl viologen oxidation and 1,2,3-trichlorobenzene reduction. However, even lower methyl viologen oxidation was detected in the set-up with activity assay reaction mix medium without vitamins and without 1,2,3-trichlorobenzene. Therefore, in chemical controls with the halogenated compounds but without resting cell suspension were included inot activity assays.

3.2.2.3 Analysis of the influence of reductive dehalogenase activity on the background methyl viologen oxidation

Previous experiment showed that the resting cell inoculum itself contributed to background oxidation of methyl viologen in the microtiter plate in absence of a halogenated electron acceptor. To investigate whether reductive dehalogeanses are involved in the backgound oxidation of methyl viologen by tranferring electrons onto a non-halogenated, so far not identified electron acceptor, reductive dehalogenase inhibition studies were conducted. For this strain CBDB1 cells were treated with 1 mM propyl iodide as described in section 2.5.5. The inhibtion of reductive dehalogenation was tested in the microtiter plate assay and in a parallel GC-FID-evaluated assay. The GC-FID-evaluated assay was done to prove light reversibility of the inhibition. The production of 1,3-dichlorobenzene from 1,2,3-trichlorobenzene was measured after 5 hours and activities in nmol s^{-1} ml^{-1} were calculated. Samples containing 1,2,3-trichlorobenzene and propyl iodide showed 28% (~12 µM 1,3-dichlorobenzene produced) of the activity compared to samples with 1,2,3-trichlorobenzene without propyl iodide (~45 µM 1,3-dichlorobenzene produced). The activity was restored to about 50% of the original activity after exposure to light (~22 µM 1,3-dichlorobenzene produced). In parallel, cells treated the same way were applied to the microtiter plate activity assay. While the activity of strain CBDB1 resting cells with 1,2,3,4-tetrachlorobenzene was reduced to about 82% of the original activity, the activity of strain CBDB1 resting cells with 1,2,3-trichlorobenzene was reduced to about 68% of the original activity. In contrast, the background oxidation of methyl viologen in negativ controls without halogenated electron acceptor was not reduced by propyl iodide compared to the control without propyl iodid, indicating that the corrinoid-containing reductive dehalogenases did not participate in the background methyl viologen oxidation (Figure 3-8, B). These results were supported

by experiments with ethyl iodide as reductive dehalogenase inhibitor. Samples with 1 mM ethyl iodide showed approximately 50% of the original activity with 1,2,3,4-tetrachlorobenzene and about 80% of the original activity with 1,2,3-trichlorobenzene in the microtiter plate activity assay, however, the background methyl viologen oxidation did not decrease after ethyl iodide treatment.

3.2.2.4 Heat treated cells show decreased background methyl viologen oxidation

By incubating the cells at different temperatures before using them for the activity assay it was shown that heat-labile components (e.g. proteins*)* within the cells are responsible for the background activity. The background activity was around 0.01 nmol s^{-1} ml^{-1} in tests with intact cells but without electron acceptor (Figure 3-8, C, grey bar at 25°C) and a strong activity of reductive dehalogenases was measured when the cells were incubated with 1,2,3-trichlorobenzene as electron acceptor (black bar, 25°C). The reductive dehalogenase activity was destroyed by preincubation at 60°C so that the activity with electron acceptor was the same as the activity without electron acceptor. By preincubating the cells at 80°C also the background activity dropped by about 90% with or without electron acceptor, indicating, the components responsible for the background activity were inactivated at 80°C.

3.2.2.5 Analysis of methyl viologen oxidation in the presence of bacteria with organohalide respiration-independent mode of living

Because the previous tests showed that bacterial cell mass was responsible for the background activity, it was also tested if this was a specific effect of cells of strain CBDB1 or if other cells types which are not depending on organohalide respiration induce the background oxidation of methyl viologen. This was tested by adding *E. coli* cells to the activity test and comparing obtained methyl viologen oxidation rates with the ones obtained from samples with strain CBDB1. *E .coli* K12 strain was grown on LB-medium, harvested and diluted in fresh sterile medium otherwise used for cultivation of strain CBDB1 to obtain a final cell number of 1.6 x 10^7 cells ml^{-1} corresponding to cell numbers of the strain CBDB1 inoculum for activity assays. The *E. coli* K12 sample was then used as an inoculum for the activity assay and measured with 1,2,3-trichlorobenzene and without electron acceptor but acetone. The methyl viologen oxidation in samples with strain CBDB1 but without electron acceptor amounted to about half of the methyl viologen oxidation observed for samples with 1,2,3-trichlorobenzene (Figure 3-8, D). *E. coli* cells induced an oxidation of methyl viologen corresponding to about half of the background methyl viologen oxidation induced by strain CBDB1 (Figure 3-8, D). Together, results suggested that reductive dehalogenases were not involved in the catalysis of background oxidation of methyl viologen but that another heat-labile factor in strain CBDB1 was responsible for the background activity. For all further calculations of activity, the background oxidation of methyl viologen in negative controls with cells but without halogenated

electron acceptor were subtracted from the total methyl viologen oxidation in samples with acceptor but acetone.

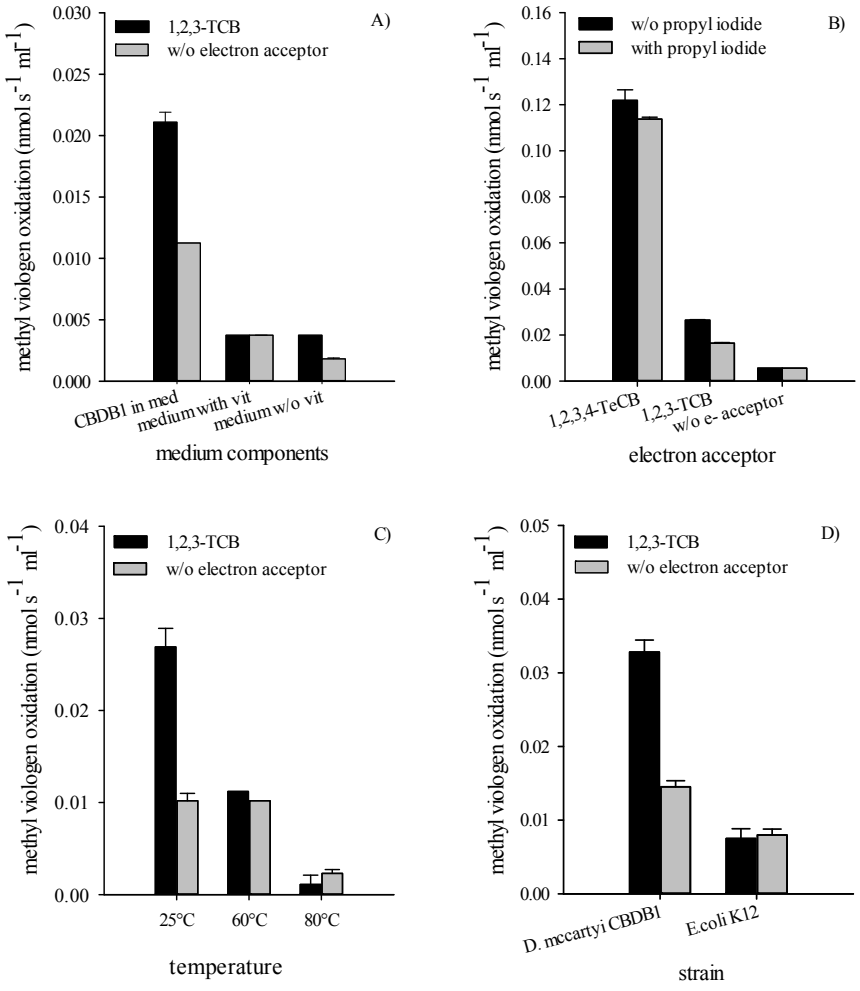

Figure 3-8: Investigation of parameters influencing the background oxidation of methyl viologen (MV) in the photometer-based 96-well microtiter plate activity assay. A) Influence of vitamins and medium components onto MV oxidation. Compared were resting CBDB1 cells with medium and vitamins, medium with vitamins without resting cells and medium without resting cells and without vitamins B) Oxidation of MV in positive controls with cells but without inhibitor and with cells and the corrinoid inhibitor propyl iodide. Shown are results for 1,2,3,4-TeCB, 1,2,3-TCB or without halogenated electron acceptor. C) MV oxidation detected with strain CBDB1 cells exposed to different temperatures in controls with 1,2,3-TCB and in negative controls without electron acceptor D) Oxidation of MV induced by *E. coli* K12 cells compared to strain CBDB1 cells in presence of 1,2,3-TCB and in absence of a halogenated electron acceptor. Abbreviations: MV = methyl viologen, med = medium, vit = vitamins, w/o = without, e- acceptor = electron acceptor.

3.2.1 A total number of 560 cells are required for activity detection in 96-well microtiter plate tests

The photometric activity assay was established to screen for electron acceptors of pure dehalogenating cultures, dehalogenating enrichment cultures, microcosms or cells extracted from sediments. Therefore, the minimum cell number required for detection of activity in the photometric assay was investigated using an actively dehalogenating culture of *Dehalococcoides mccartyi* strain CBDB1 which was grown to 6.2 x 10^6 cells ml^{-1}. Cells were concentrated using a 0.2 µm filter (see section 2.4.2, page 19) to cell numbers of 1.4 x 10^7 cells ml^{-1}. Additionally, a *D. mccartyi* strain CBDB1 culture was tested, which was initially grown on 1,2,3-trichlorobenzene to reach cell numbers of approximately 1.7 x 10^7 cells ml^{-1} but was subsequently not amended with additional doses of 1,2,3-trichlorobenzene. The culture was six months old when used for the assay. Cell numbers after harvesting of cells from the medium were 1.1 x 10^7 cells ml^{-1}. The starvation conditions were investigated to test for the limits of the microtiter plate activity assay to detect organohalide-respiring *Dehalococcoidia* from marine sediments, which possibly reside under starvation conditions in marine sediments. Cell numbers of 1.4 x 10^7, 2.8 x 10^6, 1.4 x 10^6, 2.8 x 10^5, 1.4 x 10^5 and 2.8 x 10^4 cells ml^{-1} were tested. Activity with 1,2,3-trichlorobenzene was still detected when using an inoculum of 20 µl with 2.8 x 10^4 cells ml^{-1} from the actively dehalogenating culture, corresponding to 560 cells per well in 220 µl activity assay reaction mix. For cells from the starvation culture of strain CBDB1, activity was detected at 2.8 x 10^5 cell ml^{-1} corresponding to 5600 cells per well in 220 µl reaction mix. With cell numbers of 1.4 x 10^5 or 2.8 x 10^4 cells ml^{-1} it was not possible to detect activity with this culture, indicating that the age and growth phase of strain CBDB1 cultures contributes to the activity rates measured in activity assays.

3.2.2 Microtiter plate-analysed activities are comparable to GC-FID-analysed activities

Parallel to each photometric measurement, resting cell suspensions of strain CBDB1 were used to set up activity assays in HPLC vials for further analysis by GC-FID as described previously (95, 190). The activities analysed by GC-FID were compared with activities analysed in parallel by the photometer in 96-well microtiter plates. For this, cells were cultivated with 1,2,4-tribromobenzene, harvested and concentrated by rotary evaporation. Subsequently cells were either applied to the microtiter plate activity assay or incubated for 4 h in 1.5 ml HPLC vials with the reaction mix containing either 1,2,3-trichlorobenzene or one of several brominated benzenes. GC-FID analysis of the reaction products showed 1,2,3-trichlorobenzene was dehalogenated to 20.3 µM 1,3-dichlorobenzene within 240 min, corresponding to an activity of 0.016 nkat ml^{-1} cell suspension compared to 0.017 nkat ml^{-1} cell suspension measured in the photometric assay. For brominated benzenes the lowest activity in the GC-based assay was measured for 1,4-dibromobenzene with ~0.01 nkat ml^{-1} and highest activities for 1,2,4-tribromo- and 1,2-dibromobenzene with 0.03 nkat ml^{-1}. In the

photometric assay activities ranged from 0.01 for 1,4-dibromobenzene and 0.04 nkat ml^{-1} for 1,2,4-tribromobenzene. The reaction products of 1,2,4-tribromobenzene detected in the GC-based resting cell activity assay were mainly 1,4-dibromobenzene, in some samples within the defined incubation time a small amount of monobromobenzene was formed. The first dehalogenation product of the three tested dibromobenzenes was monobromobenzene, benzene was not observed. Dehalogenation of monobromobenzene was neither detected in the photometric assay nor in the GC-based assay. When activities were calculated using a value of 30 fg of protein per single cell of strain CBDB1 (personal communication, Anja Kublik, UFZ, Leipzig) activities ranging from 8–38 nkat mg^{-1} protein for the photometric assay and 7–30 nkat mg^{-1} protein for the GC-based assay were obtained (Table 3-1).

Table 3-1: Specific activities in nkat mg $protein^{-1}$ obtained in photometer or GC-based activity assays when assuming 30 fg protein per cell of strain CBDB1. Strain CBDB1 was cultivated previously with 1,2,4-tribromobenzene.

	Photometric assay	***Gas chromatographic assay***	
electron-acceptor for the assay	***Specific activity***	***Specific activity***	***Dehalogenation products***
MBB [a]	0	0	none
1,2-DBB [a]	35.5 ± 2	29.8	MBB
1,3-DBB [a]	16 ± 1	21.0	MBB
1,4-DBB [a]	8 ± 2.5	7.3	MBB
1,2,4-TBB [a]	37.7 ± 6.2	28.3	1,3-DBB
1,2,3-TCB [a]	16.9 ± 0.9	15.8	1,3-DCB

3.3 Analysis of enzyme activity of *D. mccartyi* strain CBDB1

After establishing the microtiter plate resting cell activity assay, the assay was used to investigate activity rates of strain CBDB1 with different halogenated and non-halogenated compounds. Additionally, the influence of the electron acceptor used for cultivation onto activities measured in the resting cell assays was investigated.

3.3.1 Specific activities of resting cells of *D. mccartyi* strain CBDB1 with halogenated compounds

Cells of strain CBDB1 used as an inoculum for the activity assay were grown with 1,2,3-trichlorobenzene or hexachlorobenzene as electron acceptor. Thus, dehalogenation activity was based on reductive dehalogenases expressed during 1,2,3-trichlorobenzene or hexachlorobenzene cultivation. Highest specific activities were measured for 2,4,6-tribromophenol (87.4 nkat mg^{-1}), 2,3-dichlorobenzonitrile (50.6 nkat mg^{-1}) and 2,3-dichlorothiophene (42.1 nkat mg^{-1}) (Table 3-2). The lowest activity was detected for 2,5-dichlorothiophene (4.5 nkat mg^{-1}) (Table 3-2). No activity was measured for 2,3-dichloroaniline, 2,5-, 2,6-dichlorobenzonitrile, 2-chloro-6-fluorobenzonitrile, 4-bromo-3,5-dihydroxybenzoic acid and 5-bromo-2-furoic acid although dehalogenation and growth was observed for all six electron acceptors during cultivation with the same substrates (Table 3-2). For 4,5-

dibromo-2-furoic acid a strong abiotic oxidation of methyl viologen was detected in the chemical control. Therefore, no activity data were determined for 4,5-dibromo-2-furoic acid. Resting cell activity with 2-chloro-6-fluorobenzonitrile, 2,3-dichloroaniline, 4-bromo-3,5-dihydroxybenzoic acid or monobromobenzene was not observed despite the transformation of the respective compounds in cultivation (Table 3-2).

Table 3-2: Strain CBDB1 resting cell activity. Specific activities were calculated using 30 fg per cell of strain CBDB1.

Electron acceptor	***Sp. act. [nkat mg^{-1}]***	***Cultivation e$^-$ acceptor***	***Activity in cultivation***
2,3-DCA	0	1,2,3-TCB/2,3-DCA	Active
2,4-DCA	0	1,2,3-TCB	Not active
2,6-DCA	0	1,2,3-TCB	Not active
2,3-DCBN	50.6 ± 4.5	1,2,3-TCB	Active (199)
2,5-DCBN	0	1,2,3-TCB	Active (199)
2,6-DCBN	6 ± 8	1,2,3-TCB	Active (199)
2-C-6-FBN	0	1,2,3-TCB/2-C-6-FBN	Active
2,4,6-TBPh	87.4 ± 3.7	1,2,3-TCB	Active
2,4,6-TBPh	27.1 ± 0.5	HCB	Active
2,4-DBPh	41.7 ± 3.9	1,2,3-TCB	Active
2,4-DBPh	18.1 ± 0.5	HCB	Active
2,6-DBPh	14.3 ± 1.9	1,2,3-TCB	Active
2,6-DBPh	24.2 ± 1.4	HCB	Active
4-BPh	1.0 ± 0.2	HCB	Active
4-B-3,5-DMBA	34.0 ± 4.7	1,2,3-TCB	Active
4-B-3,5-DHBA	0	1,2,3-TCB	Active
2,3-DCTh	42.1 ± 1.3	1,2,3-TCB	Active (234)
2,5-DCTh	4.5 ± 1.1	1,2,3-TCB	Active (234)
2-CTh	3.6 ± 0.2	HCB	Active (234)
4,5-DB-2-FA	Abiotic reaction	1,2,3-TCB	Active
5-B-2-FA	0	1,2,3-TCB	Active
2,3-DCPy	0	1,2,3-TCB	n.a.d.
2,6-DCPy	0	1,2,3-TCB	n.a.d.
3-B-2-CPy	30.2 ± 1.7	1,2,3-TCB	Active (234)
PeCPh	21.4 ± 0.8	HCB	Active (119)
2,3,4,5-TeCPh	25.0 ± 0.7	HCB	Active (119)
2,4,6-TCPh	1.2 ± 0.3	HCB	Active (119)
2,4-DCPh	1.7 ± 0.6	HCB	Active (119)
2,6-DCPh	1.9 ± 0.7	HCB	Active (119)
2,3-DCPh	2.5 ± 0.3	HCB	Active (119)
4-BAn	0	1,2,3-TCB	n.a.
4-BIn	0	1,2,3-TCB	n.a.
Non-halogenated e$^-$ acceptor			
humic acids (Sigma Aldrich)	0	1,2,3-TCB	n.a.
anthraquinone	0	1,2,3-TCB	n.a.
2,6-DM-1,4-BQ	0	1,2,3-TCB	n.a.
hydroquinone	0	1,2,3-TCB	n.a.

Abbreviations: n.a.: not analysed; n.a.d. no activity detected.

3.3.2 Attempts to improve sensitivity of the activity assay

To test whether an artificial electron donor with a more negative redox potential could improve resting cell activity in the microtiter plate assay, the activity assay was set up with ethyl viologen as artificial electron donor. Ethyl viologen has a redox potential of -480 mV compared to methyl viologen with -440 mV (190). However, ethyl viologen did also not lead to detectable reduction of 2,3-dichloroaniline, 2-chloro-6-fluorobenzonitrile or monobromobenzene in the activity assay. In addition, activity assays were conducted in HPLC reaction vials which allowed for the extraction of the activity assay reaction mix after incubation and a detection of reaction products by gas chromatography. Resting cells of strain CBDB1 were incubated for up to 60 hours at 30°C with activity assay reaction mix and 2-chloro-6-fluorobenzonitrile, 2,3-dichloroaniline or monobromobenzene. No transformation of monobromobenzene, 2-chloro-6-fluorobenzonitrile or 2,3-dichloroaniline was observed when using gas chromatography for the analysis of reaction products, even after the incubation of cells with the electron acceptor and activity assay reaction mix up to 60 h until extraction.

3.3.3 Analysis of the influence of the electron acceptor used for cultivation onto resting cell activity and reductive dehalogenase expression using brominated benzenes

As shown above, resting cells of strain CBDB1 grown with 1,2,3-trichlorobenzene showed activity with diverse halogenated electron acceptors (Table 3-2). To investigate the influence of the electron acceptor used for cultivation onto activity rates measured in the *in vitro* activity assay in more detail, strain CBDB1 was cultivated with brominated and chlorinated benzenes and activity assays were conducted and obtained rates were compared. Additionally, expression studies were conducted using shotgun proteomics to investigate the induction of specific reductive dehalogenases with different bromobenzene congeners. Brominated benzenes were used in the current study as model compound because brominated compounds have been abundantly detected in marine environments (139) and have relevance by resembling basic structures of brominated flame retardants (66, 191). Additionally, they have the advantage that they do not contain other functional groups and substituents which may introduce another level of complexity due to toxic or inhibitory effects such as for instance observed for compounds with hydroxy substituents (see also 3.1.5).

3.3.3.1 Cross cultivation and activity assays with different brominated benzenes and 1,2,3-trichlorobenzene

Resting cells of *D. mccartyi* strain CBDB1 were tested in in resting cell activity assays with monobromobenzene, 1,2-, 1,3-, 1,4-dibromobenzene and 1,2,4-tribromobenzene after their cultivation with either 1,2,3-trichlorobenzene, monobromobenzene, 1,2-, 1,3-, 1,4-dibromobenzene or 1,2,4-tribromobenzene. All tests were done in triplicates using resting cells from three separate

Table 3-3: Specific activities in nkat mg^{-1} whole cell protein of strain CBDB1 cultivated with different electron acceptors and tested with different electron acceptors in the *in vitro* activity test. All numbers represent means of three cultures tested in three microtiter plate assays with three replica wells each ± standard deviation.

e⁻ acceptor cultivation	***e⁻ acceptor in the activity assay***					
	1,2,3-TCB	MBB	1,2-DBB	1,3-DBB	1,4-DBB	1,2,4-TBB
1,2,3-TCB	36.6 ± 9.1	-5.7 ± 4.7	241 ± 21.5	11.4 ± 8.1	4.5 ± 1.4	219 ± 110
MBB	21.6 ± 8.7	-5.4 ± 4.9	26.1 ± 14.4	7.1 ± 4.0	1.5 ± 4.3	28.4 ± 3.1
1,2-DBB	16.5 ± 9.4	0.0 ± 0.8	23.4 ± 16.5	11.6 ± 7.6	7.9 ± 3.5	22.3 ± 11.9
1,3-DBB	18.5 ± 9.2	0.0 ± 0.6	28.5 ± 14.6	11.2 ± 5.0	7.3 ± 3.8	31.7 ± 9.9
1,4-DBB	17.0 ± 2.0	2.9 ± 3.1	13.5 ± 4.7	8.4 ± 1.4	4.9 ± 2.8	19.3 ± 1.4
1,2,4-TBB	13.4 ± 7.8	1.4 ± 1.9	20.9 ± 17.3	10.9 ± 8.1	7.1 ± 4.6	25.8 ± 15.4

Abbreviations: TCB – trichlorobenzene, MBB – monobromobenzene, DBB – dibromobenzene, TBB – tribromobenzene

cultures, cultivated with the same electron acceptor. In addition each inoculum of strain CBDB1 was tested in triplicates with each electron acceptor in the 96-well microtiter plate activity assays (Table 3-3).

When comparing specific activities of 1,2,3-trichlorobenzene, monobromobenzene, 1,2-, 1,3-, 1,4-dibromobenzene or 1,2,4-tribromobenzene no enhanced specific activities were observed for a specific electron acceptor after the cultivation of the strain CBDB1 inoculum with the same electron acceptor except for 1,2,3-trichlorobenzene (Table 3-3 and Figure 3-9). Cells cultivated with 1,2,3-trichlorobenzene gave even higher activities for 1,2-dibromo- and 1,2,4-tribromobenzene than for 1,2,3-trichlorbenzene. Higher specific activities with 1,2-dibromobenzene or 1,2,4-tribromobenzene than with 1,2,3-trichlorobenzene were observed for all cultivation conditions tested (Table 3-3). In addition, halogenated benzenes with singly flanked halogen substituents such as

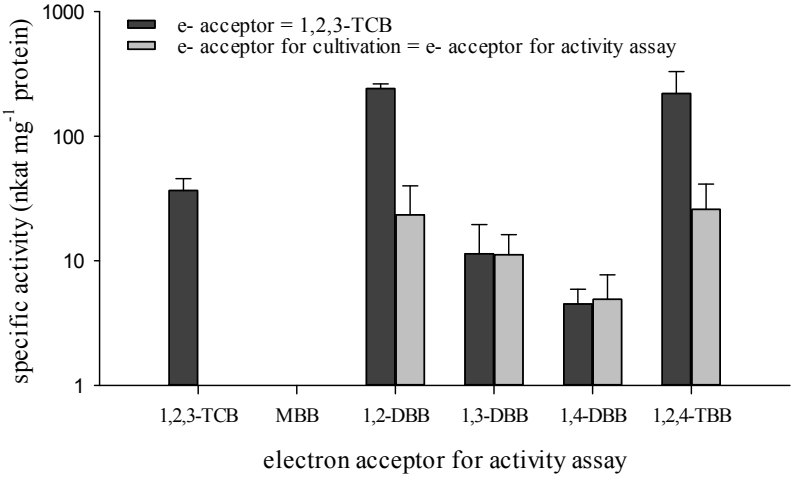

Figure 3-9: Specific activities measured in resting cell activity assays. Resting cells were cultivated prior to activity assays with 1,2,3-trichlorobenzene (black bars) or with the same electron acceptor which was used in the activity assay (grey bars). Shown are the means of three separate 96-well microtiter plate photometer-based activity assay measurements ± SD. Each of the replicas contained a different inoculum of strain CBDB1 grown on the respective electron acceptor. TCB – trichlorobenzene, MBB – monobromobenzene, DBB – dibromobenzene, TBB – tribromobenzene.

1,2,4-tribromobenzene, 1,2-dibromobenzene or 1,2,3-trichlorobenzene gave higher specific activities than halogenated benzenes with isolated halogen substituents, independently from the electron acceptor used for cultivation. For bromobenzenes with isolated bromine substituents secondary bromine substituents in *meta*-position enhanced activity compared to secondary bromine substituents in *para*-position, leading to higher activities with 1,3-dibromobenzene than with 1,4-dibromobenzene (Table 3-3). It was concluded, that chemical properties influenced the activity of resting cells of strain CBDB1 in activity assays with different brominated benzenes more than the electron acceptor used for cultivation. Additionally, it was shown that cultivation on bromobenzenes did not increase activity in the resting cell assays with bromobenzenes. Instead, even higher activities were obtained when cells were previously cultivated with 1,2,3-trichlorobenzene.

3.3.3.2 Expression of reductive dehalogenase after cultivation with brominated benzenes

Specific activities obtained in resting cell assays suggested the same reductive dehalogenases are involved in dehalogenating of bromobenzene congeners or 1,2,3-trichlorobenzene. The expression of reductive dehalogenases in *D. mccartyi* strain CBDB1 cultivated with monobromobenzene, 1,2- 1,3- or 1,4-dibromobenzene or 1,2,4-tribromobenzene was therefore further analysed by shotgun proteomics. In total, seven different reductive dehalogenases were identified in the different cultures. The protein with the highest estimated protein abundance was CbdbA80 when using the emPAI-value (exponentially modified protein abundance index) for an estimation of the relative abundance of reductive dehalogenase proteins (172). CbdbA80 was previously reported to be expressed during cultivation of strain CBDB1 with 1,2,4-trichlorbenzene (104). CbdbA80 was detected in all samples. The 1,2,3-trichlorobenzene dehalogenating protein CbdbA84 (CbrA) (104) was detected only in samples grown with 1,3-dibromobenzene, 1,4-dibromobenzene and 1,2,4-tribromobenzenes but not with 1,2-dibromobenzene or monobromobenzene. CbdbA1455 was expressed in all samples and was according to the emPAI value expressed at the same or at higher levels than CbdbA84.

Table 3-4: Expression of reductive dehalogenases after the cultivation with different bromobenzenes as electron acceptors.

e^--acceptor	***MBB***		***1,2-DBB***		***1,3-DBB***		***1,4-DBB***		***1,2,4-TBB***	
Rdh_{CBDB1}	Score	emPAI	Score	emPAI	Score	emPAI	Score	emPAI	Score	emPAI
CbdbA80	221	0.75	614	2.44	461	1.60	1147	3.82	523	1.75
CbdbA84					306	0.51	127	0.34	379	0.60
CbdbA88			61	0.25						
CbdbA1453			128	0.13	387	0.52			279	0.44
CbdbA1455	85	0.19	121	0.19	314	0.60	254	0.51	293	0.60
CbdbA1618	151	0.21			195	0.28	107	0.13	111	0.13
CbdbA1638	92	0.20			208	0.43				

The score describes the Mascot protein score. It represents a probability value for the identification of a protein; higher protein scores correspond to a more confident match between the experimental detection of ion m/z values and expected m/z values for the respective protein; emPAI – exponentially modified protein abundance index. A higher emPAI value indicates a higher abundance of an Rdh protein. Protein score and emPAI were calculated from ion scores that exceeded the significance threshold (see material and methods).

3.4 Potential for organohalide respiration in deep marine non-contaminated sediments

Diverse reductive dehalogenase homologues have been detected in marine sediments (60, 61), however little is known about the type and spectrum of electron acceptors transformed in marine sediments. Marine microorganisms exposed to natural halogenated compounds could transform a wide range of halogenated compounds from natural and anthropogenic sources and therefore play a significant role for fate prediction of halogenated compounds in the environment.

In this part of the study it was investigated whether dehalogenation activity can be detected in marine sediments from non-contaminated sites using the methyl viologen-based resting cell activity assay, marine sediment microcosms and crude extracts or whole sediment slurries from marine non-contaminated sediment sites. The microcosms used in this study were set up in a parallel project by Camelia Algora (UFZ Leipzig) with non-contaminated sediments from the Chilean continental margin of 4.37–4.42 mbsf (192) using 1,2,3-trichlorobenzene. The microcosms were used to test for the applicability of the microtiter plate resting cell activity assay to identify new electron acceptors of so far uncharacterized dehalogenating microorganisms. Additionally, the dehalogenation activity was further characterized for potentially involved co-factors, oxygen and temperature sensitivity. Microcosms were tested for the presence of strain CBDB1 by PCR to assure that no cross-contamination between the cultures occurred (see also Table 2-12 and Table 2-17). No contamination of strain CBDB1 was detected. In addition, crude sediments and sediment extracts were tested for dehalogenation activity without previous enrichment procedures by cultivation.

3.4.1 Dehalogenation activity with brominated electron acceptors with cells of marine microcosms from the coast off Chile

The potential of marine microcosms from sediments of the coast off Chile to transform brominated compounds in spite of their previous enrichment with 1,2,3-trichlorobenzene was analysed using the microtiter plate resting cell activity assay. Resting cell suspensions of microcosms enriched over four transfers with 1,2,3-trichlorobenzene were tested for activity with brominated aromatic electron acceptors. Activity was detected with all tested brominated compounds. Highest activity was obtained with 2,4,6-tribromophenol and lowest activities with 3-bromo-2,4-dimethoxybenzoic acid (Figure 3-10, left). To test whether the result of the microtiter plate activity assay can be transferred to cultivation, twenty five microcosms from the coast off Chile previously cultivated with 1,2,3-trichlorbenzene during four transfers were amended with an initial concentration of 5 µM 1,2,4-tribromobenzene. 1,2,4-Tribromobenzene was selected despite higher activity detected with 2,4,6-tribromophenols in activity assays, because 2,4,6-tribromophenol was observed to have negative effects on cell growth in cultivation with strain CBDB1 (see also 3.1.5). Only one out of the 25 cultures showed activity with 1,2,4-tribromobenzene. 1,2,4-Tribromobenzene was transformed to 1,3-

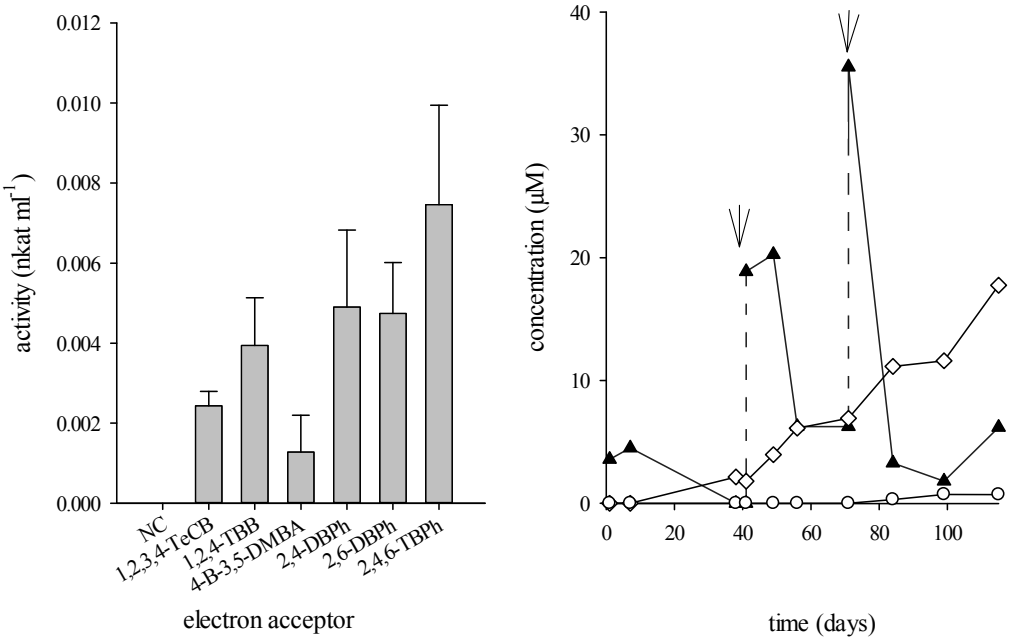

Figure 3-10: Activity of resting cells from marine sediment microcosms with halogenated aromatic compounds. Left: Microcosms enriched with 1,2,3-trichlorobenzene in the microtiter plate activity assay with brominated aromatic electron acceptors, using 1,2,3,4-tetrachlorobenzene as positive control and acetone as negative control. Cells were used directly from culture without concentration procedure and methyl viologen oxidation in the negative control was subtracted from all samples. Right: Cultivation of a marine microcosm with 1,2,4-tribromobenzene (▲) and production of 1,3- and/or 1,4-dibromobenzene (◇) and monobromobenzene (○). Arrows indicate when additional doses of 1,2,4-tribromobenzene were added to the microcosm. Abbreviations: NC – negative control, TeCB – tetrachlorobenzene, TBB – tribromobenzene, 4-B-3,5-DMBA – 4-bromo-3,5-dimethoxybenzoic acid, DBPh – dibromophenol, TBPh – tribromophenol.

and/or 1,4-dibromobenzene. The active microcosms was amended with additional doses of 20 µM 1,2,4-tribromobenzene after 41 days and 35 µM after 71 days. A small amount of monobromobenzene was detected after 84 days of incubation (Figure 3-10, right). The detected loss of brominated benzenes during the cultivation period of 120 days was possibly due to a frequently pierced rubber septa from sample withdrawal during cultivation with 1,2,4-tribromobenzene and during previous cultivation with 1,2,3-trichlorobenzene. Additionally, the headspace was frequently diluted by addition of hydrogen and nitrogen to maintain a pressure of 1.4 bar within the serum bottles after samples withdrawal.

3.4.2 Characterization of dehalogenation activity in marine sediments

To characterize the dehalogenation activity observed in the marine microcosms further, experiments were carried out including oxygen and temperature sensitivity studies and specific inhibition of cobalamin-dependent enzymes using propyl iodide. The tests for characterization of dehalogenation activity were carried out in HPLC reaction vials. This allowed for the application of a larger volume of resting cell suspension and reaction mix and thereby the detection of very small changes of activity rates.

Dehalogenation activity of resting cells from marine sediments exposed to oxygen was not reduced compared to resting cells incubated in the anoxic glove box. To tests this, cells and medium from actively dehalogenating microcosms were removed from the serum bottles and incubated either in the anoxic glove box or outside the glove box under oxic conditions for up to four hours. At time zero and after every hour, triplicates of samples incubated under anoxic conditions and triplicates of samples incubated under oxic conditions were amended with activity assay reaction mix containing approximately 350 µM 1,2,3,4-tetrachlorobenzenes and were incubated for 20 hours at 30°C under anoxic conditions. The chlorinated benzenes were extracted with hexane from the activity assay reaction mix and analysed by GC-FID. 1,2,3,4-Tetrachlorobenzene was transformed to 1,2,4-trichlorobenzene by the resting cell suspensions. Dehalogenation activity decreased with increasing incubation time after extraction from the microcosm serum bottles. Dehalogenation activity decreased at similar rates in samples incubated under oxic or under anoxic conditions (Figure 3-11, left).

Dehalogenation activity in the marine microcosms at different temperatures was investigated. Temperatures of 2°C have been reported for marine sediments (40). For the marine microcosms highest activity of resting cells was observed at 30°C but low dehalogenation activity was also detected at 4°C and at 60°C (Figure 3-11, centre). This was shown by incubating resting cells of the marine microcosms with activity assay reaction mix and 1,2,3,4-tetrachlorobenzene at 4°C, 30°C and 60°C for 2 or 5 hours and analysis of chlorobenzenes by GC-FID.

Dehalogenating activity most likely originated from cobalamin-dependent enzymes such as reductive

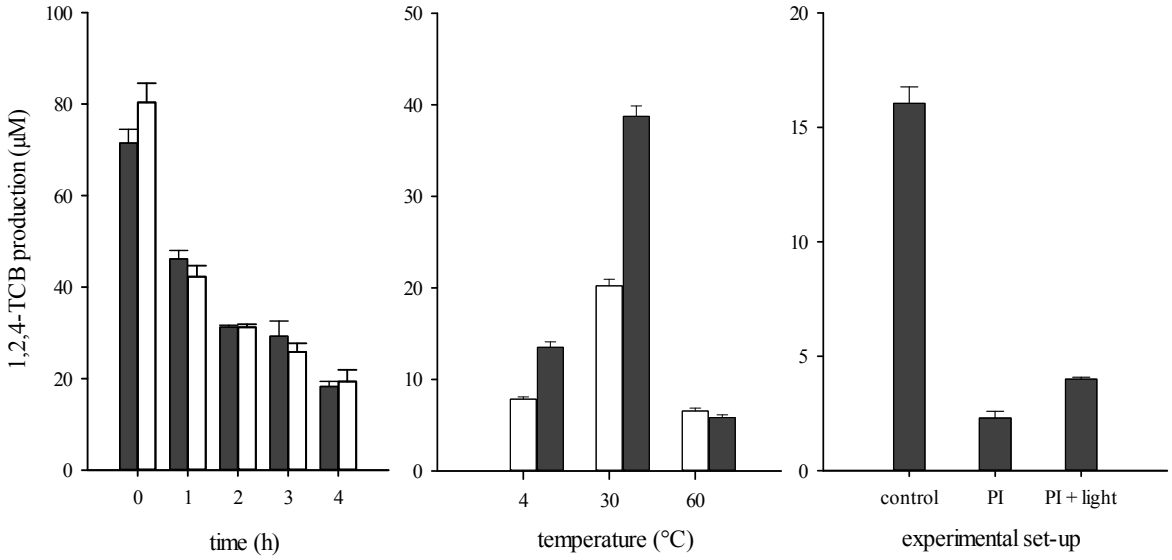

Figure 3-11: Characterization of dehalogenating activity in marine sediments microcosms of the coast off Chile. Left: Effect of oxygen exposure onto dehalogenation activity. 1,2,4-TCB was produced from 1,2,3,4-TeCB by resting cell suspensions incubated for up to five hours under anoxic (dark grey bars) or oxic (light grey bars) conditions. Centre: Temperature dependence of dehalogenation activity. 1,2,4-TCB production from 1,2,3,4-TeCB by resting cell suspensions during incubation for 2 h (light grey bars) or 5 h (dark grey bars) at 4°C, 30°C or 60°C.Right: Specific inhibition of cobalamin-dependent enzymes. 1,2,4-TCB produced from 1,2,3,4-TeCB in the control compared to 1,2,4-TCB production by cells exposed to propyl iodide or propyl iodide and light. Abbreviation: PI – propyl iodide. Shown values are means of triplicates +/- SD.

dehalogenases (93, 94, 105, 109, 110). This was shown by activity assays with resting cells incubated with propyl iodide (see also section 2.5.5), specifically and reversibly inhibiting cobalamin-dependent enzymes. The resting cell suspensions containing 1.5 x 10^7 cells ml^{-1} were incubated with 10 µM propyl iodide dissolved in acetone. Control set-ups contained resting cells and acetone. Another control with resting cells was exposed to a light source after incubation with propyl iodide. Resting cell suspensions were subsequently amended with 800 µl activity assay reaction solution, containing 100 µM 1,2,3,4-tetrachlorobenzene and incubated for 20 h at 30°C in HPLC-vials. The 1,2,4-trichlorobenzene production from 1,2,3,4-tetrachlorobenzene of resting cells incubated with propyl iodide decreased compared to controls with resting cells previously incubated with acetone. The exposure of resting cells incubated with 10 µM propyl iodide to light partially restored activity (Figure 3-11, right).

3.4.3 Dehalogenation activity in *Chloroflexi*-containing sediments

Dehalogenation activity in marine sediments was investigated using *in vitro* activity assays and sediments and crude extracts of marine sediments of the Baffin Bay core 371 and the Bay of Aarhus and tidal flat sediments of the Wadden Sea (see also Table 2-3). Parallel studies in the Microflex research project investigated occurrence and abundance of *Dehalococcoidia* by quantitative PCR. Sediments of the Aarhus Bay and the Baffin Bay core 371 were shown to range from 1 x 10^5 to 1 x 10^7 *Dehalococcoidia* cells g^{-1} sediment through the depth of the 3 or 4 m core, respectively (47). Tidal flat sediments were shown to contain 1 x 10^4 to 1 x 10^5 *Dehalococcoidia* cells g^{-1} sediment in the surface sediments of 1-8 cm (47) (see also Table 2-3). Experiments conducted in the current study showed that a minimum of ~3 x 10^5 cells ml^{-1} of *Dehalococcoidia* strain CBDB1 were required to detect dehalogenation activity in the *in vitro* assays. To obtain a cell number of at least 3 x10^5 *Dehalococcoidia* cells ml^{-1} from sediments, cells of up to 30 g sediments were extracted by Nycodenz and density centrifugation (see also 2.4.4). Obtained cells were subsequently dissolved in 500 µl cultivation medium or physiological NaCl solution and were subsequently added as crude cell suspensions to the activity assay in the microtiter plate or in HPLC-vials. Additionally, sediment slurries of 1 to 10 g sediments from various depths were added directly to the activity assay solutions. Dehalogenation activity was tested with 1,2,3-trichlorobenzene, 1,2,3,4-tetrachlorobenzene, 1,2,4-tribromobenzene or 2,4,6-tribromophenol. Activity assays conducted in HPLC-vials sediments or cell suspension were incubated for up to 72 hours at 30°C and the halogenated compounds were then extracted with hexane for analysis by GC-FID. Dehalogenation activity was neither detected by photometer-based nor by GC-FID-based methods.

3.5 Chemical properties of halogenated aromatic compounds influence reductive dehalogenation patterns

Experiments conducted with strain CBDB1 in the current study and in previous studies demonstrated a wide range of halogenated molecules are transformed by *D. mccartyi* strain CBDB1 (7, 119, 121, 124, 193, 194). The multitude of halogenated compound types and congeners transformed by strain CBDB1 exceeds the number of reductive dehalogenases reported in the genome of strain CBDB1 (26), providing evidence that a single reductive dehalogenase is able to transform more than one single congener. This was supported by *in vitro* activity assays and reductive dehalogenase expression studies in the current study. However, the reasons for this electron acceptor diversity are poorly understood. For the different tested compound classes specific dehalogenation patterns were observed in the current and in previous studies (7, 121) and chemical properties of the halogenated compound seem to have an important influence onto dehalogenation pathways. For instance, isolated bromine substituents were dehalogenated in activity assays by strain CBDB1 cells previously grown with 1,2,3-trichlorobenzene, while isolated chlorine substituents of 1,3-dichlorobenzene are not dehalogenated. In addition, the same was observed for dehalogenating microorganisms from marine sediments which were able to dehalogenate isolated bromine substituents but not chlorine substituents suggesting that the chemical properties of a halogenated electron acceptor are as important as the enzymatic properties of the dehalogenating strain. Therefore, chemical properties of 58 halogenated molecules were evaluated in detail (Table 7-1 to Table 7-16) for their ability to predict experimentally observed dehalogenation pathways of strain CBDB. This was done to gain a deeper understanding of the mechanism of reductive dehalogenation which allows reductive dehalogenases to transform a wide spectrum of halogenated electron acceptors and to better understand which parameters explain the different degree of dehalogenation observed for molecules with different functional groups and halogen types. For all analysed molecules experimental data with strain CBDB1 was available. The findings possibly will allow also for an extrapolation onto properties generally required for halogenated compounds to serve for organohalide-respiring *Dehalococcoidia* as electron acceptor and even for dehalogenation microorganisms from different bacterial classes but will need to be tested and also correlated to enzymatic properties in future studies.

Several chemical descriptors were chosen, which were described previously to depict chemical properties of a molecule such as reactivity or electron density distribution (195). The theoretical descriptors analysed in the current study included HOMO/LUMO, electrophilicity, local electrophilicity, partial charges for the neutral molecule and partial charges of the anionic form of a molecule. The selected chemical descriptors were modelled by Dominik Wondrousch within a cooperation project with the Ecological Chemistry Department of the UFZ. The modelled data were evaluated in the current study for their ability to predict the regioselective removal of halogens during organohalide respiration of strain CBDB1 and to allow for an analysis of the influence of functional

groups onto halogen substituents. In the current study only the chemical descriptors which revealed to successfully predict dehalogenation patterns of strain CBDB1 are shown and discussed.

3.5.1 The most positive halogen atomic net charge predicts regioselective dehalogenation by strain CBDB1

Three different partial charge models, the Mulliken (125, 187), the Hirshfeld (188) and the NBO model (196, 197) were analysed for their ability to predict dehalogenation pathways of halogenated compounds catalysed by strain CBDB1 (Table 7-1 to Table 7-12). For the dehalogenation pathway prediction only electron acceptors with identified reaction pathways were included. Electron acceptors were not included into the analysis, when all halogen substituents are equally likely to be dehalogenated. Examples for such molecules are hexachlorobenzene and dibromobenzenes. Also pentachlorophenol was omitted from the prediction, because the reaction pathways of pentachlorophenol catalysed by strain CBDB1 could not be resolved experimentally (119).

For the prediction of dehalogenation pathways catalysed by strain CBDB1, the data were evaluated according to the following hypotheses: a) the halogenated carbon with the most positive partial charge predicts regioselective dehalogenation, b) the halogenated carbon with the most negative partial charge predicts regioselective dehalogenation, c) the halogen with the most positive partial charge predicts regioselective dehalogenation and d) the halogen with the most negative partial charge predicts regioselective dehalogenation.

Halogenated phenols were evaluated as non-dissociated neutral molecules and also as dissociated phenolate molecules. It is not clear whether *D. mccartyi* strain CBDB1 uses phenols, phenolates, or both as electron acceptor. At 25°C in water, phenol and halogenated phenols are weak acids (examples of pK_a values: phenol 9.99, 4-chlorophenol 9.41, 2-chlorophenol 8.56, 2,3-dichlorophenol 7.44, 2-bromphenol 8.25, 4-bromphenol 9.37), and are therefore ionized only to a small fraction. For the evaluation of partial charges, phenolates have the advantage of symmetry in geometry optimization in contrast to phenols, which contain the non-dissociated hydroxy group. Quantum mechanical methods

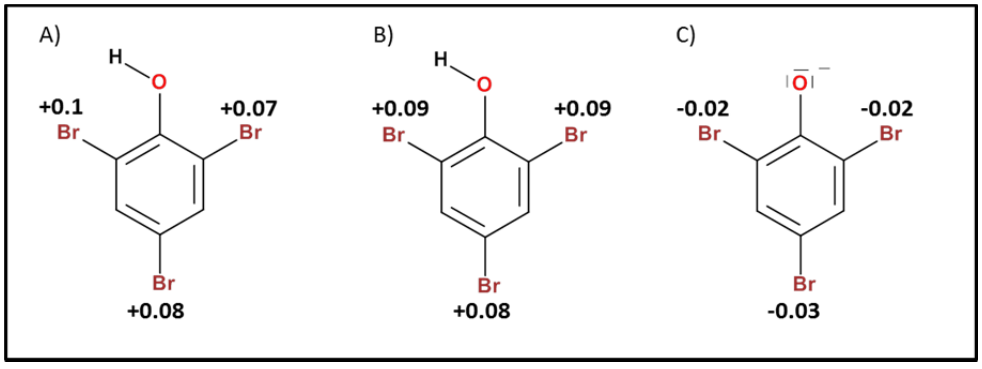

Figure 3-12: Example for the effect of the hydroxy group on the partial charge modelled for halogens for 2,4,6-dibromophenol and 2,4,6-dibromophenolate, shown for NBO halogen atomic net charges. A) 2,4,6-tribromophenol, halogen substituents in *ortho*-position with the modelled partial charge B) 2,4,6-tribromophenol, halogen substituents in *ortho*-position with averaged partial charge C) 2,4,6-tribromophenolate, halogen substituents with the modelled partial charge.

distinguish between conformers that under natural conditions represent the same molecule (for instance 2-bromophenol and "6-bromophenol") depending on the modelled orientation of the hydroxy group and the intramolecular interactions of the hydroxy hydrogen with neighbouring halogens (198). The asymmetry of the modelled hydroxy group influences therefore the modelled partial charges of flanking halogens (Figure 3-12) and thereby the correct prediction of dehalogenation pathways. This effect could be counteracted by averaging the partial charge value of hydroxy group flanking halogens; however this approach can be applied only to symmetrically substituted molecules such as 2,4,6-tribromophenol but not to asymmetric substituted molecules such as 2,3,6-tribromophenol. Therefore, for each model the partial charge data were evaluated taking into account once the dissociated phenolates and once the non-dissociated phenols.

All analysed models gave best prediction for dehalogenation pathways using the most positive halogen atomic net charge as indicator (Table 3-5 A and B). Second best prediction rates were obtained using the most negative atomic net charge of halogen-substituted carbons (Table 3-5 A and B).

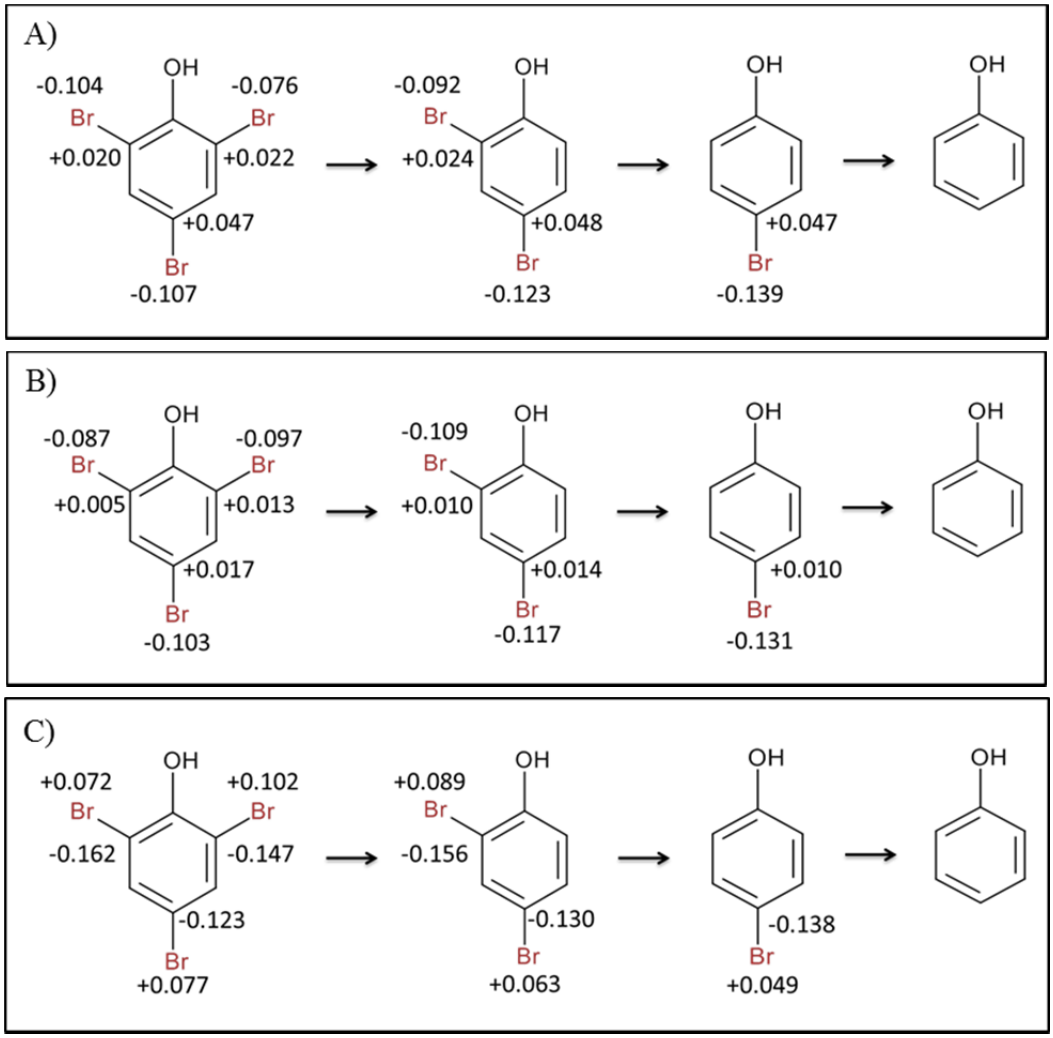

Figure 3-13: Calculations of partial to partial charges to predict the dehalogenation pathway of 2,4,6-tribromophenol by *D. mccartyi* CBDB1 A) Mulliken model, B) Hirshfeld model C) NBO model.

When using the most positive partial halogen charge, the Mulliken partial charge system revealed good prediction rates of dehalogenation pathways catalysed by strain CBDB1 for most molecules except for phenols and 3-bromo-2-chloropyridine (Supplementary Table 7-1 to Table 7-4).

A correct prediction of the dehalogenation pathway of phenols using the most positive halogen partial charge was hampered by the influence of the asymmetric hydroxy group onto the *ortho*-substituted halogens (Table 3-5 A, see also Figure 3-12). Therefore, improved prediction rates were obtained when phenolates instead of phenols were included into analyses (Table 3-5 B, Supplementary Table 7-1 and Table 7-4).

For Hirshfeld partial charges, the prediction was more successful when including phenols instead of phenolates into analyses (Table 3-5 A and B, and Supplementary Table 7-5 to Table 7-8). In addition, Hirshfeld charges did not predict correctly the dehalogenation of 3-bromo-2-chloropyridine when using the halogen with the most positive atomic net charge.

Table 3-5: Rates of successful prediction of dehalogenation products by *D. mccartyi* strain CBDB1 using partial charges. For the evaluation of the prediction rate 27 molecules were used. Phenols were analysed in the non-dissociated (A) and in the dissociated form (B).

A	***Mulliken model***		***Hirshfeld model***		***NBO model***	
Indicator	***Det. TP***	***Pref. TP***	***Det. TP***	***Pref. TP***	***Det. TP***	***Pref. TP***
Most positive C	28%		24%		16%	
Most negative C	68%	68%	80%	76%	76%	72%
Most negative X	20%		8%		24%	
Most positive X	88%	80%	96%	92%	88%	84%
Delta X/C	84%		4%		88%	

B	***Mulliken model***		***Hirshfeld model***		***NBO model***	
Indicator	***Det. TP***	***Pref. TP***	***Det. TP***	***Pref. TP***	***Det. TP***	***Pref. TP***
Most positive C	28%		20%		20%	
Most negative C	88%	88%	64%	60%	92%	84%
Most negative X	8%		20%		8%	
Most positive X	96%	92%	80%	76%	96%	92%
Delta X/C	80%		36%		84%	

The carbon charge refers only to carbon atoms substituted by a halogen. X = halogen atomic net charge, C = carbon atomic net charge. Delta X/C = Prediction rates were calculated using the most pronounced difference between the halogen partial charge and the carbon partial charge. Det. TP = Detected transformation product. Prediction rates were calculated from the number of correct prediction of the exact transformation product detected in cultivation experiments with strain CBDB1; Pref. TP = Preferential transformation product. Prediction rates were calculated from the number of correct predictions of the most abundant transformation product of strain CBDB1 compared to other possible transformation products. Therefore the Pref. TP rate can be the same or smaller than the Det. TP.

For NBO atomic net charges, improved prediction rates were obtained when including phenolates instead of phenols into analyses (Table 3-5 A and B, and Supplementary Table 7-9 to Table 7-12). Improved prediction results were obtained for symmetrically substituted phenols when the net atomic halogen charge was averaged (Figure 3-13). The NBO partial charge model allowed for a correct prediction of the dehalogenation pathway of 3-bromo-2-chloropyridine, as a molecule substituted with bromine and chlorine. Strain CBDB1 debrominated 3-bromo-2-chloropyridine, but it was not able to dechlorinate 3-bromo-2-chloropyridine. The NBO model assigned a more positive partial charge to

bromine than to chlorine, corresponding to a higher electronegativity of chlorine compared to bromine.

3.5.2 Sigma NBO halogen partial charges predict regioselective dehalogenation

The NBO partial charge model allowed for the modelling of the separate sigma and pi contributions to the partial charge of an atom. The NBO sigma and pi partial charges were modelled by Dominik Wondrousch (Department Ecological Chemistry, UFZ Leipzig) (Supplementary Table 7-13 to Table 7-16). Improved prediction rates when using sigma partial charges compared to pi partial charges indicated an increased importance of sigma electron withdrawal from the halogen by negative inductive effects of neighbouring substituents for reductive dehalogenation. This conclusion was drawn from analyses of the halogen sigma and pi partial charges for halogenated heteroaromatic and homoaromatic compounds tested in the current study. For this, sigma-binding electron contributions and pi-binding electron contributions of the halogen with the most positive partial charge were analysed for their ability to reflect the dehalogenation pathways catalysed by strain CBDB1. The halogen with the most positive partial charge was used for this evaluation because it was identified in the current study as the most successful indicator for the dehalogenation pathway prediction of strain CBDB1. The analysis of sigma and pi partial charges revealed different prediction rates for regioselective dehalogenation by strain CBDB1. When using the most positive halogen sigma partial charge 96% and 92% of the experimentally observed dehalogenation pathways were predicted correctly, when including phenolates or phenols into analyses, respectively. The most positive halogen pi partial charge predicted 76% or 60% of regioselective removal of halogens by strain CBDB1, when including phenols or phenolates into analysis, respectively.

3.5.3 Analysis of the influence of different secondary substituents onto the halogen atomic net charges

By evaluating atomic net charges of different atoms within a molecule as an indicator for regioselective removal of halogens during organohalide respiration, the halogen with the most positive atomic net charge was identified as the indicator with highest successful prediction rate. Moreover, improved dehalogenation pathway predictions using sigma partial charges compared to pi partial charges indicated withdrawal of sigma-binding electrons from the halogen as important parameter for dehalogenation. Considering this, the partial charges of halogens flanking non-halogen substituents and the halogen-substituted carbon net charges in *ortho*-position to the non-halogen substituent were compared. Because NBO partial charges allowed for the comparison of sigma and pi contributions to the partial charge, NBO charges were used for this analysis. The non-halogen substituents of 2,6-dichloroanilin, 2,6-dichlorophenol, 2,6-dichlorophenolate and 2,6-dichlorobenzonitrile induced increasingly positive partial charges in the flanking halogen substituents in the order of $-O^-$ < $-NH_2$ < -OH < -CN (Figure 3-14, A). This corresponds to the different degree of reductive dehalogenation of

2,6-dichloroaniline, 2,6-dichlorophenol and 2,6-dichlorobenzonitrile observed experimentally. 2,6-dichloroaniline is not dehalogenated by strain CBDB1 (see also 3.1.1), 2,6-dichlorophenol is dehalogenated only partially and incomplete (119) and 2,6-dichlorobenzonitrile is dehalogenated to a non-halogenated end product (199). An experimental distinction of dehalogenation of phenolates compared to phenols by strain CBDB1 is so far not available. When including 1,2,3-trichlorobenzene into analysis, the halogen atomic net charge places the influence of a halogen substituent in *ortho*-position with an additional halogen substituent in *meta*- and *para*- position in the following order: -O < -NH_2 < -OH < -Cl < -CN.

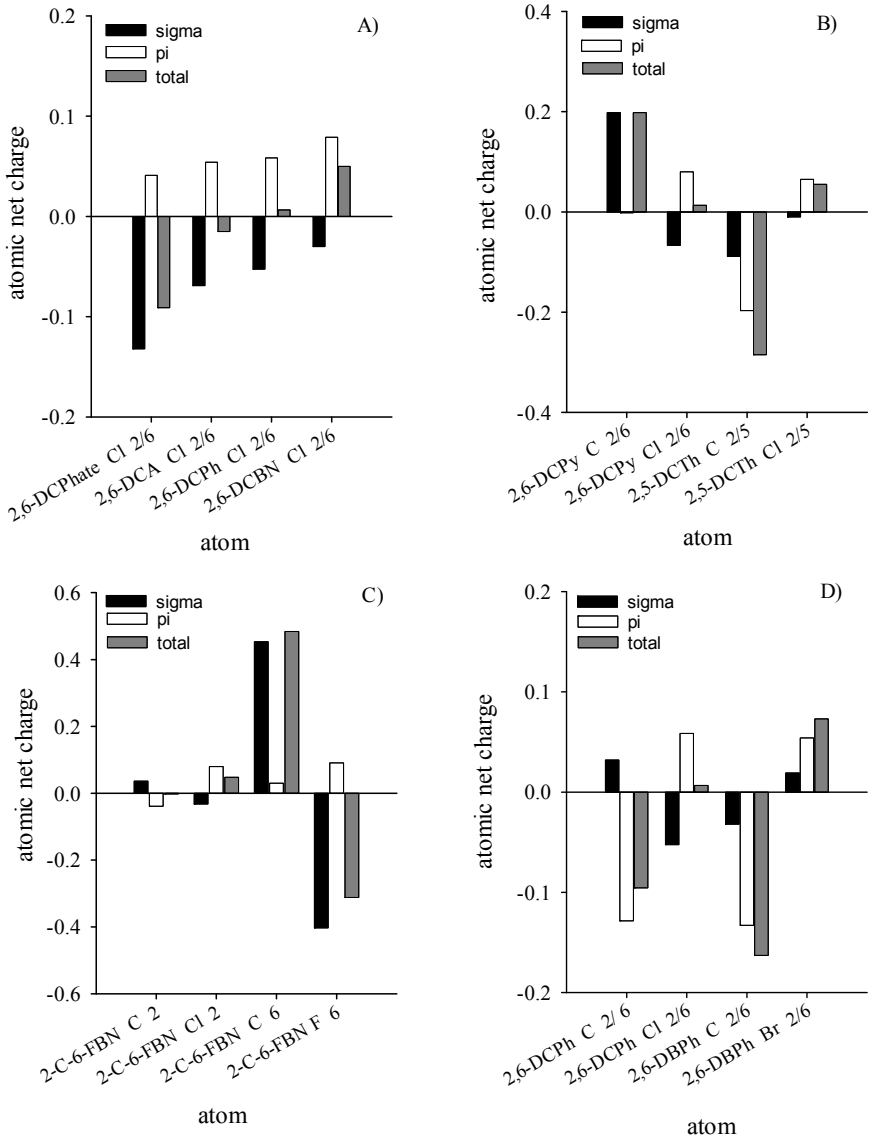

Figure 3-14: Halogen (Q_X) and halogen-substituted carbon (Q_C) atomic net charges and their sigma and pi binding electron contributions of selected molecules. A) Q_X of 2,6-chlorophenolate, -aniline, phenol, and benzonitriles. B) Q_X and Q_C of 2,6-dichloropyridine and 2,5-dichlorothiophene; C) Q_X and Q_C of 2,6-dichlorobenzonitrile and 2-chloro-6-fluorobenzonitrile. D) Q_X and Q_C of 2,6-dichlorophenol and 2,6-dibromophenol.

3.5.4 The influence of chemical properties of heterocycles onto reductive dehalogenation

Halogen and carbon atomic net charges of electron-rich heteroaromatics, such as the five-membered thiophene, were compared with halogen and carbon atomic net charges of the electron-poor pyridine. 2,3- and 2,6-dichloropyridine were not dehalogenated while 2,3- and 2,5-dichlorothiophenes were dehalogenated (see also section 3.1.4 and 3.3.1). When comparing chlorine atoms flanking the heteroatoms of 2,6-dichloropyridine or 2,5-dichlorothiophene a more positive partial charge for halogens of 2,5-dichlorothiophene than for halogens of 2,6-dichloropyridine was observed. Moreover, atomic net charges of the halogen-substituted carbons of pyridine had more positive atomic net charges than halogen-substituted carbons in 2,5-dichlorothiophene (Figure 3-14, B). Furoic acids were not compared with thiophenes and pyridines because all brominated aromatic congeners were debrominated by strain CBDB1 regardless of the molecular structure tested.

3.5.5 The role of the halogen type in reductive dehalogenation

When comparing atomic net charges of fluorine and chlorine in 2-chloro-6-flurobenzonitrile all models assigned fluorine a more negative atomic net charge than chlorine (Figure 3-14, C). The atomic net charge of the fluorine-substituted carbon was more positive than the atomic net charge of the chlorine-substituted carbon. Although for the fluorine-substituted carbon very positive atomic net charges were calculated, no dehalogenation of fluorine was observed underscoring the importance of the halogen charge for reductive dehalogenation compared to the carbon charge. For the halogen partial charge, the sigma partial charge contribution differed more between chlorine and fluorine than the pi partial charge contribution. When comparing chlorine and bromine in 2,6-dibromophenol and2,6-dichlorophenol, more positive partial charges for bromine than for chlorine were modelled. The sigma-binding electrons contributed thereby more to the differences of the total halogen net charge than the pi-binding electrons (Figure 3-14, D). This underscores the importance of withdrawal of sigma binding electrons from the halogen for reductive dehalogenation, i.e. by secondary substituents with negative inductive effects to counteract the negative inductive effect exhibited by the halogen itself.

3.5.6 Correlation of the most positive halogen partial charge with resting cell activity

Partial charge of the most positive halogen substituent were analysed for a correlation with specific activities measured in resting cell activity assays. No correlation was observed when comparing all experimentally tested molecules with each other. However, when only one compounds group with the same halogen type was compared, more positive halogen partial charge values were paralleled with higher activity in resting cell activity assays (Figure 3-15), however no linear correlation was observed.

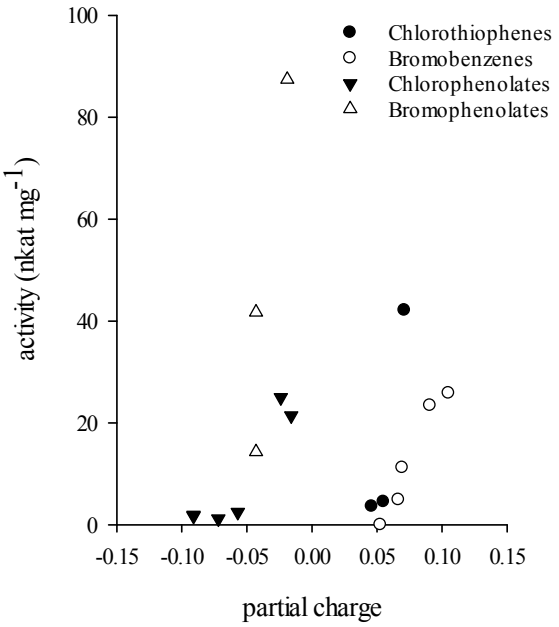

Figure 3-15: Correlation of specific activities of strain CBDB1 with halogen atomic net charges. Shown are the means of triplicate measurements from three different microtiter plate activity assays ± SD. Within each compound class higher activity was observed the more positive atomic net charges were on the halogen atom.

3.6 Non-halogenated compounds as potential respiratory electron acceptors for *Dehalococcoidia*

In this chapter, the investigation of non-halogenated electron acceptors of *Dehalococcoidia* by molecular methods is described. Analysis of the genomes of a single *Dehalococcoidia* cell denominated "DEH-J10" isolated from marine sediments of the Bay of Aarhus (46) and from two single cells "Dsc1" and "Dsc2" from the Peruvian margin (48) revealed the potential of *Dehalococcoidia* subgroups to grow by an organohalide respiration-independent mode of living. In a further genome of a *Dehalococcoidia* single amplified genome denominated "SAG-C11", a gene coding for an RdhA subunit was identified. However, a TAT leader sequence and a gene coding for the putative membrane anchor protein RdhB were missing (personal communication, Dr. Kenneth Wasmund, UFZ, Leipzig) suggesting the RdhA is not a functional, membrane-bound respiratory reductive dehalogenase. Beside the gene coding for RdhA, several genes potentially involved in respiratory processes were identified in the genome, including an operon coding for proteins involved in dissimilatory sulphite reduction (personal communication, Dr. Kenneth Wasmund, UFZ, Leipzig). The region contained *dsrA, dsrB, dsrD, dsrN, dsrC, dsrM* and *dsrK* genes. Downstream of the *dsr*-region, genes were detected which showed highest similarity to genes described in *Dehalogenimonas lykanthroporepellens* involved in siroheme synthesis. Upstream of the *dsr*-region a gene coding for a two-component transcriptional regulator *luxR* was identified. This gene has its most similar homologs

in the *Chloroflexi* species *Dehalogenimonas lykanthroporepellens* BL-DC-9 and *Ktedonobacter racemifer* (Accession number EFH84403) (Figure 3-16).

Dissimilatory sulphite reductase genes were described to occur in a restricted number of bacterial phyla (150) but have never been described to occur in the phylum *Chloroflexi*. In addition, many dissimilatory sulphite reductase sequences of so far uncultured bacterial species remain with unknown phylogeny. Therefore, the diversity and occurrence of dissimilatory sulphite reductase genes linked to the phylum *Chloroflexi* were investigated in the current study. This may contribute to the understanding of phylogenetic diversity of dissimilatory sulphite reductase genes in public databases and the metabolic potential harboured by the class *Dehalococcoidia*. The distribution and diversity of *dsr* genes linked to the class *Dehalococcoidia* was investigated using molecular methods, because previous attempts in the current study to approach this research question by cultivation remained unsuccessful (data not shown).

DNA fragments coding for *dsr* genes and other genes that could provide a phylogenetic link for the determination of the DNA source organism were amplified to link dissimilatory sulphite reductases from marine sediments to *Dehalococcoidia*. The 16S rRNA gene sequence was not encoded in the close genetic environment of *dsr* related genes in SAG-C11. Therefore, a co-amplification of the *dsr* genes and the 16S rRNA gene sequence was not possible. Therefore, genes detected in DEH-SAG-C11 with highest similarity to genes of *Dehalogenimonas lykanthroporepellens* encoded adjacent to *dsr* genes were used as such markers. Another approach was to amplify long-range PCR fragments coding for several *dsr* related genes. This allowed for phylogenetic analysis of several new *dsr* genes and a comparison of their sequence order with the sequence order of the *dsr* coding region of DEH-SAG-C11. For this purpose primers were designed to amplify *dsr* genes with similarity of the *dsr* locus in single cell SAG-C11 in a long-range PCR (Figure 3-16), sediments for DNA extraction were sampled or selected and methods for DNA extraction suitable for long-range PCR approaches were tested.

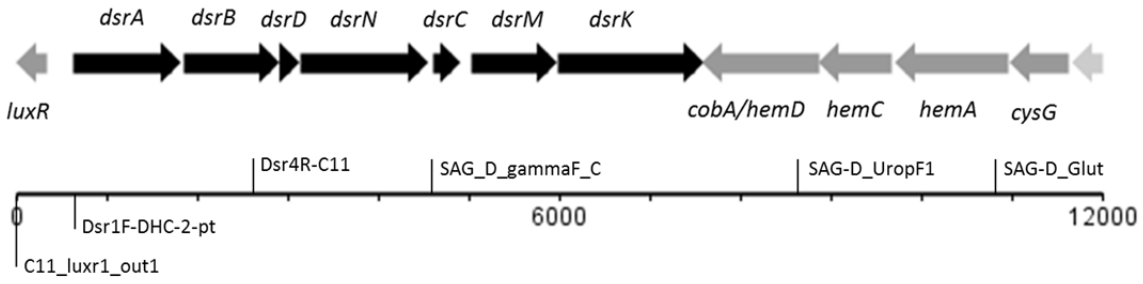

Figure 3-16: Dissimilatory sulphite reductase genes *dsrABDNCMK* (black arrows) and flanking genes with highest similarity to genes occurring in *Dehalogenimonas lykanthroporepellens* BL-DC-9 (dark grey arrows) associated with siroheme synthesis (*cobA/hemD, hemC, hemA* and *cysG*) or transcriptional regulation (*luxR*) detected in *Dehalococcoidia* SAG-C11 and selected primer binding positions.

3.6.1 Selection of sediment cores and cell extraction

Sediments were selected for extraction of cells and DNA to investigate the distribution of *dsr* genes possibly linked to *Dehalococcoidia*. A 3 m sediment core was recovered from the same location of the Aarhus Bay which allowed for the isolation of SAG-C11 to have large quantities of SAG-C11 phylotype-containing sediment for experimental work. At the top of the sediment core sulphate had a concentration of 31.7 mM. The sulphate concentration decreased constantly to ~2 mM at 171 centimetres below sea floor (cmbsf), and remained at this concentration down to 304 cmbsf. The concentration of 2 mM sulphate was attributed to sulphide reoxidation, which might have occurred during sample handling and preparation for shipment of the sediment core. Previous geochemical studies of the same sediment site suggest a sulphate concentration of 0.2 mM from 150 cmbsf downwards (152). To obtain DNA in sufficient quality for long-range PCR applications two approaches were used including a cell extraction by density centrifugation using Nycodenz or by direct DNA extraction using the FastDNA™ Spin Kit for Soil. Nycodenz had the advantage that cells could be separated from soil to subsequently apply DNA isolation methods which have been reported to reduce sheering of DNA (see also section 2.7.1). Additionally, higher amounts of sediments could be extracted compared to the FastDNA™ Spin Kit for Soil, which allows for the extraction of 0.5 g of sediment per provided reaction container.

Cells were extracted from sediments of the Bay of Aarhus from 10–20, 30–36, 66–72, 102–108, 135–141, 168–174, 201–208, 235–341, 268–274, and 301–308 cmbsf using 80% w/v Nycodenz or the FastDNA™ Spin Kit for Soil. Cell extraction from 30 g of sediments of 10–20 cmbsf using Nycodenz yielded 1.9 ± 0.3 to 2.5 ± 0.5 x 10^8 cells ml^{-1}, after resuspending the cell pellet in 500 µl 0.9% NaCl solution. Second extraction steps of the same sediment samples yielded up to 7.8 ± 2.6 x 10^6 cells ml^{-1}.

Additionally, sediments from the Baffin Bay sampled by Camelia Algora during the Polarstern cruise 2010 were included, as a sediment sample from deep-sea sediments (see also Table 2-4 for more details of the sampling sites). Core 371 was selected, due to high copy numbers of *dsrA* genes

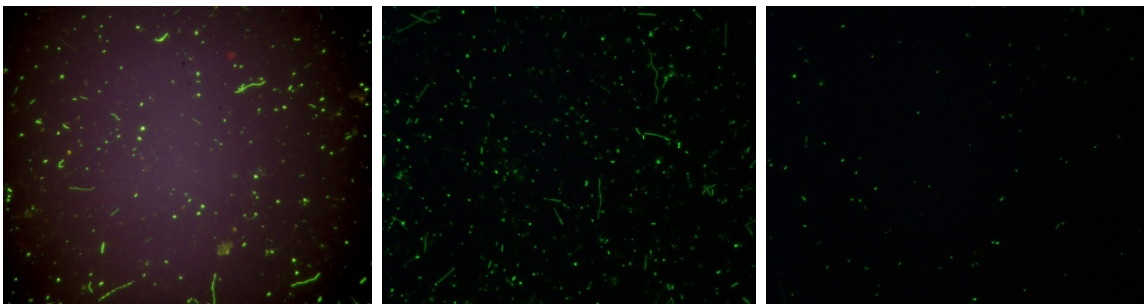

Figure 3-17: Epifluorescence microscopic pictures (185 µm x 148 µm) of cells extracted from 30 g marine sediments with 80% Nycodenz solution resuspended in 500 µl 0.9% NaCl solution and stained with SYBR Green. Left: Cells extracted from tidal flat sediments of 10–20 cmbsf of the Wadden Sea resulting in 1.7 x 10^8 cells ml^{-1}. Middle: Cells extracted from Aarhus sediments of 0–20 cmbsf resulting in 2.5 x 10^8 cells ml^{-1}. Right: cells from Baffin Bay sediments of 280–300 cmbsf resulting in 5.2 x 10^7 cells ml^{-1}.

determined previously (200), a similar distribution of *Chloroflexi* sub-groups in the sulphate zone of Baffin Bay core 371 compared to the core sampled in the Bay of Aarhus and the presence of the SAG-C11 *Dehalococcoidia* subgroup DSC-GIF3-B throughout the sediment core (47). Sediment samples were not available for all depths of the core due to previous experiments in different research projects. Therefore, cells and DNA were extracted only from sediments of 280 and 300 cmbsf. A first extraction of cells from 30 g of sediments from 280 cmbsf using Nycodenz typically yielded 5.2 ± 0.6 x 10^7 cells ml^{-1} when the cell pellet was resuspended in 500 µl 0.9% NaCl solution. A second extraction of cells from the same sediment sample yielded additional 6.1 ± 0.3 x 10^6 cells. The extraction of cells from sediments of 300 cmbsf yielded around 9.5 ± 0.3 x 10^6 cells ml^{-1} and a second extraction of cells from the same sediments yielded 1.3 ± 1.4 x 10^6 cells ml^{-1} (Figure 3-17).

Tidal flat sediments from the Wadden Sea, sampled by Anton Schulte-Fischedick, were included to investigate the presence of dissimilatory sulphite reductase genes linked to *Chloroflexi* in another marine habitat. Previous studies showed the occurrence of *Dehalococcoidia* in these sediments (47). Tidal flat sediments from the Wadden Sea were available from upper layers of 1–20 cmbsf. Cell extractions using Nycodenz yielded between 5.7 ± 0.8 x 10^7 to 1.7 ± 0.4 x 10^8 cells ml^{-1} when resuspending the cell pellet in 500 µl 0.9% NaCl solution and second extractions of cells from the same sediments yielded up to 3.7 ± 1.2 x 10^7 cells ml^{-1}.

Sediment samples from the Peru Margin and the Black Sea were available to investigate dissimilatory sulphite reductase genes linked to *Chloroflexi* (Table 2-3). However, sediments of the Peru Margin and the Black Sea were available only in very low quantities and poor quality. DNA was extracted using the FastDNA™ Spin Kit for Soil.

3.6.2 Selection of fragments for analysis

At first, attempts were made to amplify a ~10 kb fragment coding for *dsrABDNCMK* and genes phylogenetically related to genes from *Dehalogenimonas lykanthroporepellens* using specific and degenerated primers targeting a highly conserved region of *dsrA* and SAG-C11 specific region of *hemA*. The forward primer Dsr1F-DHC-2-pt targeting *dsrA* was based on the primer sequence used by Wagner and colleagues (151) and was modified to match the sequence of SAG-C11. It was combined with one of six different reverse primers with different binding positions in *hemA*, different degrees of degeneracy or different 3-prime-end-modifications to avoid degradation of primers by exonuclease activity of the polymerase during long range PCR amplifications (for primer targets and sequences see Table 2-10). However, while it was possible to amplify a ~10 kb fragment using genomic DNA of SAG-C11 as template for polymerase chain reaction, it was not possible to amplify the 10 kb fragment from DNA isolated from marine sediments. Instead, many lower molecular weight fragments were amplified, which were not further investigated. The same was observed during amplification of a ~8 kb fragment coding for *dsrABDNCMK* and *hemD*, which yielded many lower molecular weight fragments but not the targeted 8 kb fragment. The primers C11_luxr1_out1 and Sag_D_gammaF_C

were used for the amplification of a 4.6 kb fragment coding for *luxR* and *dsrABDN* (Figure 3-16). A faint band with a size of 4.6 kb was amplified from DNA isolated from sediments of Aarhus, however, purification and cloning remained unsuccessful. Attempts to improve the amplification of the 4.6 kb fragment by touch-down PCR, additional purification steps of the template DNA used for amplification or addition of different concentrations of DMSO and $MgCl_2$ to the amplification reactions did not enhance the amplification of the band.

Purification and cloning of fragments above 4 kb coding for *dsr* genes and a phylogenetic marker gene remained unsuccessful, therefore, attempts to amplify a smaller 2.5 kb fragment were carried out using primers LuxR_out1 and DsrR4_C11 without any degeneracy (Table 2-10). These primers target *dsrB* and the transcriptional regulator sequence, which in SAG-C11 has highest similarity with *luxR* of *Dehalogenimonas lykanthroporepellens* strain BL-DC-9. Amplification and cloning of a 2.5 kb fragment amplified from shallow sediments of 10–20 cmbsf yielded twenty-two clones harbouring a 2.5 kb insert coding for *dsrB* and a putative transcriptional regulator of the *arsR* family (*luxR*) (Table 7-17). Most sequences were highly similar to *dsrB* and *luxR* of SAG-C11, however, several sequences differed from *drsAB* and *luxR* genes of SAG-C11 (see for further discussion section 3.6.3). The amplification of the 2.5 kb fragment coding for *dsrAB* and *luxR* using degenerated primers (Table 2-10) or the amplification of the 2.5 kb fragment from deeper sedimentary levels with specific primers remained unsuccessful. Generally, the amplification of fragments of above 1 kb coding for *dsr* genes and marker genes with highest similarities to *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 from DNA isolated from marine sediments was difficult. Primers with low degeneracy amplified sequences with very high sequence similarity to *dsrAB* genes of SAG-C11, and thus did not fulfil their designated purpose for the analysis of sequence diversity of functional genes in the class *Dehalococcoidia*. Primers with higher degeneracy amplified besides DNA fragments with the targeted size a broad range of smaller fragments. Even after purification and cloning of fragments with the targeted size, sequencing revealed a variety of different functional genes that were not targeted (data no shown).

Different primer binding positions and primers with different degree of degeneracy were tested to amplify *dsr* genes with higher diversity. The primer combinations Dsr1F-DHC-2-pt1 and SAG_D_gammaF_A, Dsr1F-DHC-2-pt2 and SAG_D_gammaF_B and Dsr1F-DHC-2-pt3 and SAG_D_gammaF_C or Dsr1F-DHC-2-pt2 and SAG_D_gammaF_C all based upon *dsr* sequences identified in SAG-C11, target a fragment of ~3.9 kb (see also Table 2-10 and Figure 3-16). These primers used in different combinations amplified many unspecific fragments of ~0.5 to ~6 kb and a fragment of the targeted size of 3.9 kb, when using DNA isolated from sediment samples of Aarhus, the Baffin Bay or the Wadden Sea. Purification and cloning of the 3.9 kb fragment amplified from sediments of Aarhus from 10-20, 135-141, 168-174 and 268-274 cmbsf, from the Baffin Bay from 280–300 cmbsf and from tidal flat sediments from the Wadden Sea of 10–20 cmbsf, allowed for the

identification of twenty-six clones coding for *dsrA* and *dsrN* (Supplementary Table 7-18). No fragments corresponding to a size of 3.9 kb were amplified from sediments of Peru and the Black Sea.

Obtained sequences will be submitted to NCBI sequence read archive (for SRA number see Supplement).

3.6.3 Linking dissimilatory sulphite reductase genes with *Dehalococcoidia*

Dissimilatory sulphite reductase genes amplified from marine sediments in the current study were investigated for their phylogenetic origin and potential relatedness to *Dehalococcoidia.* BlastX searches with *luxR* and *dsr* sequences derived from marine sediments were carried out using the NCBI nr database supplemented with the amino acid sequences of SAG-C11. Phylogenetic trees were constructed with amino acid sequences of predicted open reading frames of *dsrA*, *dsrB* and *dsrN* amplified in the current study and with amino acid sequences retrieved from the NCBI nr database. Additionally, nucleotide and amino acid sequences were compared in global alignments for their similarity to sequences detected in SAG-C11 (see also 2.8).

In the previous chapter the identification of twenty-two clones harbouring a 2.5 kb insert coding for LuxR and DsrAB was described (Supplementary Table 7-17). BlastX searches of the LuxR sequences derived from marine sediments revealed highest scores (and lowest e-values) for the LuxR transcriptional regulator of *Dehalococcoidia* SAG-C11 confirming the specificity of the used primers for *Dehalococcoidia*-related *luxR* genes. The next best hits belonged to the *Chloroflexi* strains *Ktedonobacter racemifer* (DSM 44963) and *Dehalogenimonas lykanthroporepellens* BL-DC-9 or to the *Firmicutes* strain *Dehalobacter* DCA (Supplementary Table 7-17). The high sequence diversity of *luxR* transcriptional regulators in different bacterial groups did not allow for the construction of reliable phylogenetic trees. Global alignments of the approximately 112 amino acid counting LuxR sequences derived from sediments of the Aarhus Bay gave 93% and 100% sequence similarity to LuxR of SAG-C11 (Supplementary Table 7-17). Sequence similarities of the global alignments of *luxR* nucleotide sequences were between 96% and 100%. Amino acid sequence similarities to LuxR of *Ktedonobacter racemifer* (DSM 44963) and *Dehalogenimonas lykanthroporepellens* BL-DC-9 or to the *Firmicutes* strain *Dehalobacter* DCA were between 47% and 55% in local alignments (Supplementary Table 7-17).

DsrB amino acid sequences amplified with the same fragment coding for LuxR derived from sediments of the Aarhus Bay had highest BlastX scores and lowest e-values for DsrB of *Chloroflexi* SAG-C11. All analysed DsrB sequences amplified on one fragment with *luxR* clustered with DsrB sequence of SAG-C11 in phylogenetic analysis using the Neighbor Joining algorithm (Figure 3-18). The DsrB sequence of clone AA169 was not included into phylogenetic analysis because the obtained sequence was very short and the luxR sequences could not be analysed for this clone (see also Table 7-17).

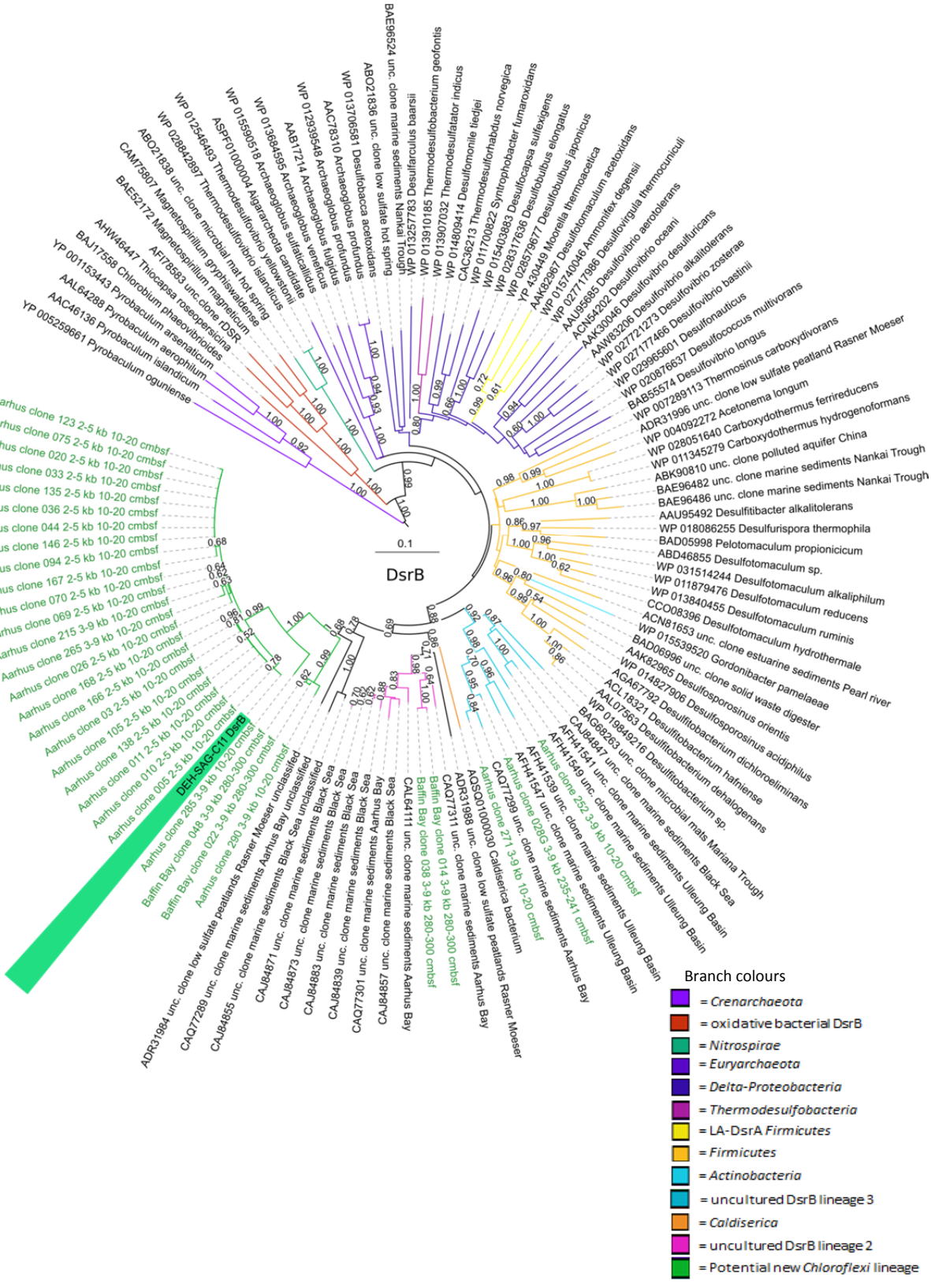

Figure 3-18: Phylogenetic tree based on a global alignment of ~295 DsrB amino acid sequences using the Neighbor Joining method implemented in Mega 6.0 with 1000 bootstrap tests and pairwise deletion, evolutionary distance were computed with the p-distance method. Sequences amplified in the current study are marked with a green caption. DsrB of SAG-C11 is highlighted with a green background color. Numbers show bootstrap values above 0.5.

Phylogenetic analysis by the Minimum Evolution, or the Maximum Likelihood algorithm showed likewise to the Neighbor Joining algorithm, clustering of the DsrB sequences with DsrB sequences of SAG-C11. When comparing sediment derived DsrB sequences of 70 to 238 amino acids with DsrB of SAG-C11 in global alignments, sequence similarities of 97% to 100% were obtained (Supplementary Table 7-17). Nucleotide sequences similarities were between 95% and 100% when compared to *dsrB* of SAG-C11 (Supplementary Table 7-17).

Additionally, nineteen clones harbouring a 3.9 kb insert derived from sediments of the Bay of Aarhus and coding for *dsrA* and *dsrN* were analysed (Supplementary Table 7-18). BlastX analysis of the obtained *dsrA* sequences revealed highest scores and lowest e-values for DsrA of *Dehalococcoidia* SAG-C11 or uncultured sulphate reducers (Supplementary Table 7-18). Phylogenetic analysis of the DsrA sequences from Aarhus sediments showed ten DsrA sequences encoded on the 3.9 kb fragment, clustered with DsrA of SAG-C11 (Figure 3-19). Short DsrA sequences of clones AA238 and AA139 were not included into phylogenetic analysis. DsrA sequences of 87 to 386 amino acids revealed 83% and 99% sequence similarities when compared to DsrA of SAG-C11 in global alignments. The respective nucleotide sequence similarities ranged from 76% and 98% (Supplementary Table 7-18). All sequences amplified from sediment layer below 10–25 cm and two DsrA sequences from shallow sediments of Aarhus (clones Aarhus clone 271 and Aarhus clone 252), affiliated with sequences previously termed ‘uncultured DsrAB lineage 3’ (201) (Figure 3-19) shown to become more prevalent with increasing depth and lower sulphate concentrations in sediments (152). DsrA sequences amplified from deeper sedimentary levels had 75% to 81% amino acid sequence similarity in global alignments with DsrA of C11 (87 to 386 amino acids), and nucleotide similarity was between 72% and 75%.

Although several DsrA amino acid sequences did not cluster with DsrA of SAG-C11, their DsrN sequences amplified within the same fragment coding for DsrA, gave highest scores and lowest e-values for DsrN of SAG-C11 in BlastX searches. In addition, DsrN sequences formed a stable cluster with DsrN of SAG-C11 in phylogenetic analysis together with sequences of the phylum *Firmicutes* but not with DsrN homologous cobyrinic acid a,c diamid synthase sequences of the phylum *Chloroflexi* using the Neighbor Joining algorithm (Figure 3-20). This was confirmed by using the Maximum Likelihood or Minimum Evolution algorithm (data not shown). Sequence similarities of DsrN amino acid sequences ranged from 50% to 95% when compared to DsrN of *Dehalococcoidia* SAG-C11 in global alignments using sequences of 58 to 485 amino acids (see Supplementary Table 7-18), corresponding nucleotide sequence similarities to *dsrN* of SAG-C11 ranged from 59% and 96%. BlastX analysis of the *dsrA* sequence derived from tidal flat sediments of the Wadden Sea gave highest scores and lowest e-values for DsrA of SAG-C11. The DsrA and the DsrN sequences affiliated with the respective genes of SAG-C11 in phylogenetic analysis (Figure 3-19 and Figure 3-20). The 285 amino acid DsrA sequence showed 100% similarity with DsrA of SAG-C11 in global alignments and the respective nucleotide sequence 98%. The DsrN amino acid and nucleotide sequences both

gave 97% similarity to DsrN and *dsrN* of SAG-C11, respectively. DsrA sequences derived from Baffin Bay sediments gave highest scores and lowest e-values for DsrA of SAG-C11 or uncultured sulphate reducer (Table 7-18).

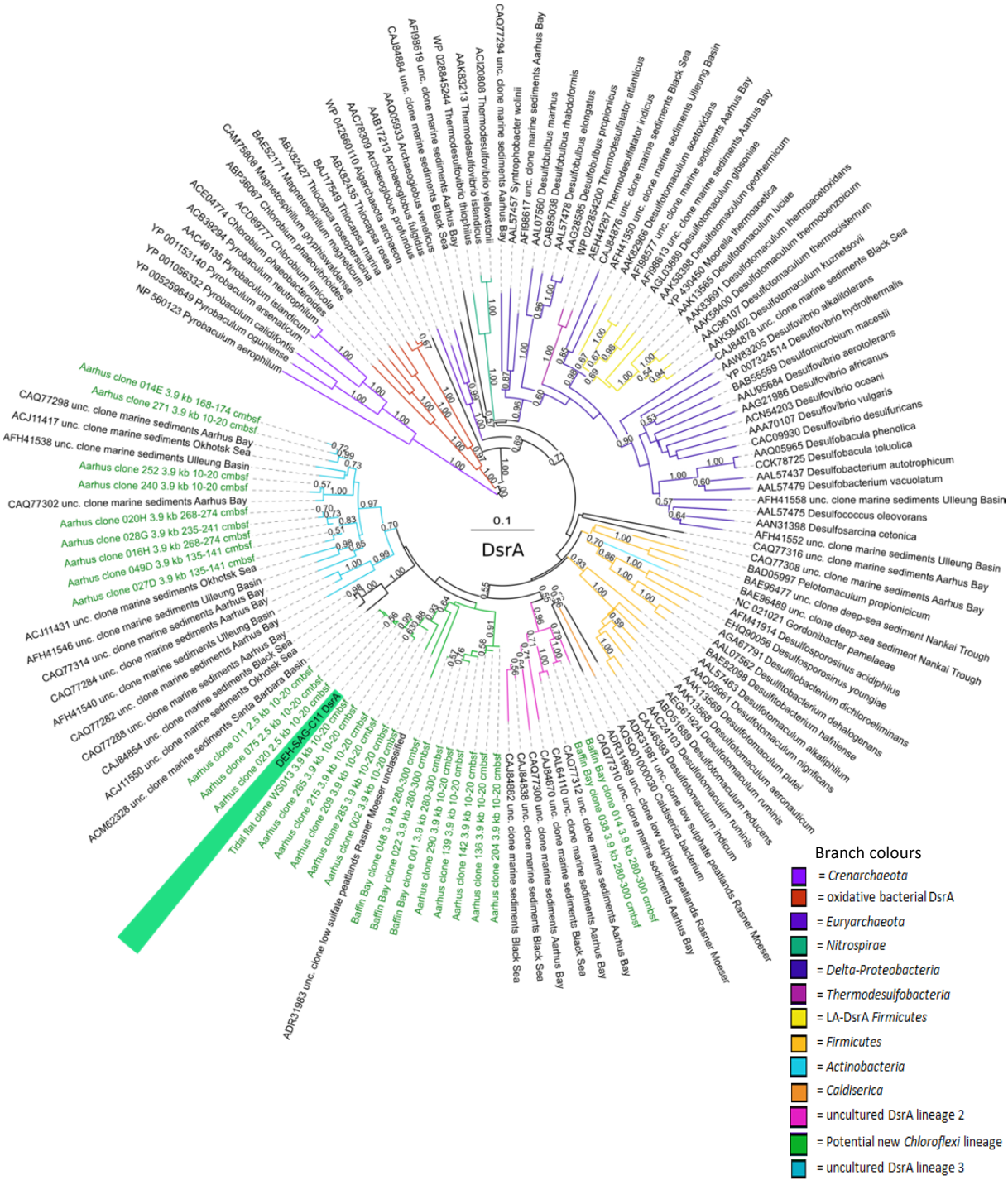

Figure 3-19: Phylogenetic tree based on a global alignment of DsrA sequences of ~259 amino acids, using the Neighbor-joining method implemented in Mega 6.0 with 1000 bootstrap tests and pairwise deletion, evolutionary distance were computed with the p-distance method. Sequences amplified in the current study are marked with a green caption. DsrA of SAG-C11 is highlighted with a green background color. Numbers show bootstrap values above 0.5.

Amino acid sequences similarities of DsrA sequences derived from sediments of the Baffin Bay were between 81% to 85% to DsrA of *Dehalococcoidia* SAG-C11 in global alignments and corresponding nucleotide sequence similarities ranged from 74% to 78%. Three of the obtained DsrA and DsrB sequences clustered with DsrA or DsrB sequences of SAG-C11 in phylogenetic analysis (Figure 3-19 and Figure 3-18).

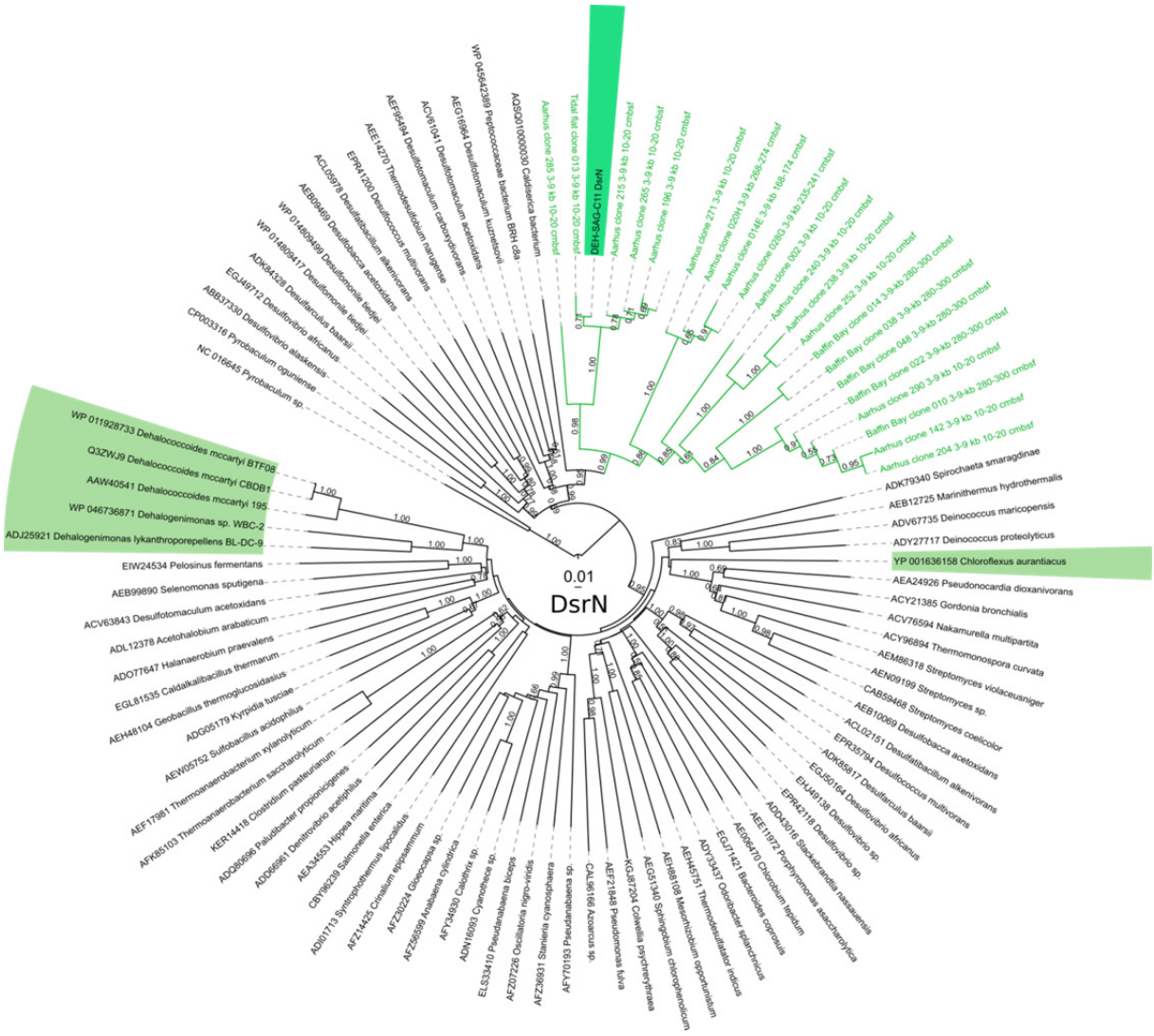

Figure 3-20: Phylogenetic tree based on DsrN sequences and DsrN homologs (cobyrinic acid a,c diamid synthase) of ~450 amino acids using the Neighbor joining method implemented in Mega 6.0 with 1000 bootstrap tests and pairwise deletion, evolutionary distance were computed with the p-distance method. Sequences amplified in the current study are marked with green captions. Green branches indicate the 'SAG-C11-DsrN cluster'. DsrN or cobyrinic acid a,c diamid synthase sequences of classified members of the phylum *Chloroflexi* are highlighted in green. Numbers show bootstrap values above 0.5.

BlastX analysis of *dsrN* sequences gave highest scores and lowest e-values for DsrN of *Dehalococcoidia*-C11, and clustered with DsrN of SAG-C11 suggesting that the *dsr* sequences most likely belong to *Chloroflexi*. Two of the DsrA sequences obtained from sediments of the Baffin Bay affiliated with sequences of the 'uncultured DsrAB lineage 2' (201). Their respective DsrN sequences affiliated with DsrN of SAG-C11 which clustered with DsrN sequences of members of the *Firmicutes*

or DsrN homologous cobyrinic acid a,c diamid synthase sequences of other *Chloroflexi* strains including strains of the class *Dehalococcoidia* (Figure 3-20).

3.6.3.1 Comparison of the nucleotide sequence similarity of sequences obtained from amplifications with *dsr* nucleotide sequences of SAG-C11 and of *Caldiserica*

The 2.5 kb inserts of the three clones AA011, AA020 and AA075 were sequenced using internal primers (Table 2-20). The gene sequence was *luxR-dsrA-dsrB* as observed in *Dehalococcoidia* SAG-C11 (Figure 3-16). Alignments of the three fragments amplified from sediments revealed 98–100% nucleotide sequence similarities with *Dehalococcoidia* SAG-C11 after global alignments. The DsrA sequences gave highest BlastX scores and lowest e-values for DsrA of *Dehalococcoidia* SAG-C11. DsrA sequences clustered similar as observed for the respective *dsrB* and *dsrN* sequences with *dsr* gene sequences of SAG-C11 (Figure 3-19).

Six clones containing a 3.9 kb fragment were sequenced by gene walking using internal primers. All fragments showed the same *dsr* gene organization as observed in SAG-C11: *dsrA-dsrB-dsrD-dsrN*-dsrC (Figure 3-16). The same *dsr* operon organization was observed in *Caldiserica* sp. (AQSQ01000030) (202). In addition, DsrA and DsrB sequences of *Caldiserica* closely affiliated with DsrAB sequences of genes amplified in the current study and SAG-C11. Therefore, the *dsrABDN* gene sequences derived from marine sediments of the Bay of Aarhus in the current study were compared with the *dsrABDN* gene sequence of SAG-C11 and the *dsrABDN* gene sequence of *Caldiserica* in global alignments using the p-distance method implement in Mega 6.0 (Table 3-6). Sequences amplified from sediments had 66% to 97% nucleotide sequence similarities with the *dsrABDN* sequence of SAG-C11 and 60% to 62% nucleotide sequence similarity with the *dsrABDN* sequence of *Caldiserica*. All analysed sequences showed higher overall similarities to the *dsrABDN* nucleotide sequence of SAG-C11 than to *dsrABDN* nucleotide sequence of *Caldiserica*.

Table 3-6: Nucleotide sequence similarities of selected clone inserts compared with *dsr* genes of *Dehalococcoidia* SAG-C11 and *dsr* genes of *Caldiserica.*

clone	***dsrABDN nucleotide sequence similarities SAG-C11***	***dsrABDN nucleotide sequence similarities Caldiserica***
290	74%	62%
285	84%	62%
271	66%	60%
265	97%	61%
252	68%	61%
215	96%	61%

3.6.3.2 Inferring species level from *dsrAB* sequences

Selected *dsrAB* sequences amplified in the current study were compared with the *dsrAB* gene sequence of *Dehalococcoidia* SAG-C11 to gain analyse phylogenetic diversity of *dsrAB* sequences potentially linked to *Chloroflexi* (Table 3-7). The *dsrAB* sequences derived from primer binding

position dsr1F and dsr4R have been used previously to assess species diversity by relating 16S rRNA genes sequences diversity to *dsrAB* gene sequences diversity. For this purpose, an approximately 1.9 kb *dsrAB* gene stretch was used (150, 203). A nucleotide sequence similarity of 76% suggests another genus level within the class *Dehalococcoidia* harbours genes for dissimilatory sulphite reduction.

An additional marker is the average GC content of the *dsrAB* sequence compared to the genome. The SAG-C11 GC content is 47.8% and its respective *dsrAB* sequence 48.6%. All analysed *dsrAB* sequences amplified in the current study showed a similar GC content as the genome of SAG-C11.

Table 3-7: Comparison of selected 1761 nucleotide *dsrAB* sequences amplified in the current study with primers modified according to primers dsr1F und dsr4R with the *dsrAB* sequence of SAG-C11. Similarities were calculated with p-distance method, pairwise deletion and 1000 bootstraps.

Clone	***dsrAB nucleotide sequence similarity to C11***	***GC content***
Aarhus_290	75.7%	48.6%
Aarhus_285	86.5%	49.7%
Aarhus_271	70.1%	48.1%
Aarhus_265	97%	48.6%
Aarhus_252	71%	47.5%
Aarhus_215	96.9%	48.4%
Aarhus_075	98.3%	48.7%
Aarhus_020	98.9%	48.6%
Aarhus_011	99.9%	48.6%
BaffinBay_048	75.8%	49.2%
BaffinBay_038	72.8%	47.6%
BaffinBay_022	75.8%	49.0%
BaffinBay_014	72.6%	47.6%

4 Discussion

Dehalococcoidia belong to one of the most widespread and abundant bacterial groups in anoxic marine sediments (34, 40-51, 204). All cultivated and isolated members of the class *Dehalococcoidia* depend on organohalide respiration. However, recent studies of single amplified *Dehalococcoidia* genomes and pan genomes provided evidence that the class *Dehalococcoidia* is more versatile and that not all members depend on reductive dehalogenation (46, 48, 62). Nevertheless, the detection of 16S rRNA and *rdhA* gene sequences in marine sediments affiliating with sequences of organohalide-respiring *Dehalococcoidia,* suggest that *Dehalococcoidia* subgroups depending on organohalide respiration occur in marine sediments. Most studies conducted with cultivated *Dehalococcoidia* focused on the investigation of dehalogenation of xenobiotics. It thus remained poorly understood which natural halogenated electron acceptors could allow for growth and energy conservation in marine non-contaminated sediments. In addition, different dehalogenation patterns and substrate spectra were observed for organohalide-respiring *Dehalococcoidia* but little was known about chemical properties of an electron acceptor onto reductive dehalogenation. *D. mccartyi* strain CBDB1 was used as a model organism and cultivated with different halogenated electron acceptors to gain a deeper understanding of chemical parameters required of a compound to serve as electron acceptor for organohalide respiration by *Dehalococcoidia*. The tested compounds contained different halogens, substituents and heteroatoms. This may allow for the identification of potential natural electron acceptors of *Dehalococcoidia* and a further insight into the role of organohalide respiring *Dehalococcoidia* in transformation of diverse halogenated xenobiotics. A special focus was placed on brominated aromatic compounds, since they have been reported to occur naturally in marine environments (128, 132, 133, 136-139), but are also introduced into the environment from anthropogenic sources, i.e. as flame retardants (66, 191). Additional to chemical parameters the role of enzymatic properties of a strain for its dehalogenation spectra and patterns was investigated for brominated benzenes using activity assays and shotgun proteomics. Molecular methods were applied to investigate potential electron acceptors of so far uncultivated and non-dehalogenating members of the class *Dehalococcoidia* in marine sediments. Together this research contributes to the further understanding of the respiratory modes of *Dehalococcoidia* and the reasons for their widespread distribution and abundance in marine sediments. Additionally, this research provides insights into the mechanisms of reductive dehalogenation and may contribute to an improved fate prediction of halogenated compounds from natural and anthropogenic sources in the environment.

4.1 Strategies to investigate electron acceptors used by bacteria of the class *Dehalococcoidia*

Different strategies were developed and applied in the current work to gain insight into the electron acceptor diversity of *Dehalococcoidia* which may elucidate why members of the class *Dehalococcoidia* occur abundantly in marine non-contaminated sediment sites. These strategies included i) the cultivation of *Dehalococcoides mccartyi* model strain CBDB1 ii) resting cell activity assays to screen and measure transformation rates of electron acceptors of organohalide-respiring *Dehalococcoidia* and marine dehalogenating microcosms iii) the analysis of reductive dehalogenase expression in *D. mccartyi* model strain CBDB1 after cultivation with different electron acceptors iv) the characterization of chemical properties of electron acceptors tested with model strain CBDB1, v) the analysis of the diversity and distribution of functional genes in *Dehalococcoidia* associated with respiratory functions *via* molecular tools. These strategies are discussed briefly in this chapter while the results from the different strategies will be discussed in later chapters.

4.1.1 Cultivation of the model organism *D. mccartyi* strain CBDB1

One strategy selected was the cultivation of *D. mccartyi* strain CBDB1 with different potential electron acceptors as model organism for *Dehalococcoidia*. In marine sediments 16S rRNA gene sequences were detected affiliating with cultivated *D. mccartyi* strains (40). Additionally, reductive dehalogenase homologues genes that were highly similar to reductive dehalogenase sequences from strain CBDB1 were reported in marine sediments (60, 61). These findings suggested the presence of organohalide-respiring *Dehalococcoidia* in marine non-contaminated sediments related to cultivated *D. mccartyi* strains such as strain CBDB1 isolated from terrestrial sites. The usage of strain CBDB1 as model organism allowed for the investigation of growth and transformation of halogenated electron acceptors in fully synthetic medium, under defined conditions as described previously for *D. mccartyi* strains and various chlorinated electron acceptors (7-16). Attempts for enrichment and cultivation of *Dehalococcoidia* from marine sites were conducted in a parallel project by Camelia Algora. Also in the current study, cultivation attempts of marine *Dehalococcoidia* were carried out but remained unsuccessful (data not shown). Previous studies focused on the transformation of halogenated contaminants from anthropogenic sources by *D. mccartyi* strains (10, 12, 104, 118, 205), in the current study the electron acceptors for cultivation were however chosen based on their functional groups and halogen substituents and not primarily based on their potential as contaminant. This allowed for an investigation of the basic parameters that determine whether a compound is used as an electron acceptor by a *D. mccartyi* strain or not. Results may allow for an extrapolation onto other organohalide-respiring *Dehalococcoidia* and naturally occurring halogenated compounds (131, 139, 206) to estimate their potential to promote growth of organohalide-respiring *Dehalococcoidia* in

marine sediments and eventually may allow for cultivation and enrichment of *Dehalococcoidia* from marine sediments.

4.1.2 Activity measurements in a microtiter plate-based assay for identification of new halogenated electron acceptors

D. mccartyi strain CBDB1 was also used as a model organism to establish a resting-cell activity assay in a 96-well microtiter plate format, enabling fast screening of halogenated compounds towards their use as electron acceptor by organohalide-respiring bacteria, including organohalide-respiring *Dehalococcoidia*. Reductive dehalogenase activity assays using viologens as artificial electron donor were shown to be a sensitive tool to investigate the presence of reductive dehalogenases independent of molecular tools and have been used previously to investigate activity with different electron acceptors of *D. mccartyi* strains (95, 105, 111, 190). In the current study resting cells of *D. mccartyi* strain CBDB1 were used as catalysts to measure the transformation rate of halogenated compounds while other studies used a similar assay to characterize crude extracts of *D. mccartyi* cells or purified reductive dehalogenases (95, 105, 111, 190). The assay is believed to operate with whole cells because the catalysing enzymes are located on the outer side of the outer membrane and are therefore accessible by viologens as electron donor and halogenated electron acceptors (29). The 96-well microtiter plate format assay established in this study allowed for quick screening of multiple electron acceptors in parallel, including several control set-ups, while using only small amounts of resting cell suspension. The evaluation of the activity assay with a photometer in comparison with the evaluation by chromatography-dependent methods did not require establishing of different analytical methods for each investigated electron acceptor and allowed testing of undefined and/or complex molecules such as for instance humic acids. Additionally, photometry allowed monitoring of reductive dehalogenation in real-time (94). It allowed therefore for a quick screening and rate determination of electron acceptors for organohalide-respiring bacteria.

Parallel activity assays, evaluated by analysis of reaction products with GC-FID, yielded similar results when using the same culture and electron acceptors for comparison, demonstrating that the 96-well microtiter plate-based activity assay is suitable for the screening of new electron acceptors. However, photometer and GC-FID evaluated activity assays only assess the activity of already expressed proteins, not of those that are encoded in the genome but which are not expressed under the cultivation conditions used for the preparation of resting cells. This might lead to differences in the results obtained from cultivation and *in vitro* activity tests, which was observed for several compounds despite their transformation in culture by strain CBDB1. Such results could indicate that specific reductive dehalogenases were not expressed under the cultivation conditions used for preparation of the resting cells for the activity assay. However, when cells were previously cultivated with the same electron acceptor which was subsequently used in activity assays, no improved activity rates for electron acceptors tested was observed suggesting, the absence of measurable activity was not related

to reductive dehalogenase expression at least for those compounds tested in the current study (see also 4.2.3). Instead, the failure to detect activity in resting cell activity assays with monobromobenzene, 2-chloro-6-fluorobenzonitrile or 4-bromo-3,5-dihydroxybenzoic acid, despite observed activity in cultures, might have been rather related to the lower sensitivity of the activity test compared to cultivation. Attempts to increase the sensitivity of the activity test by using a higher amount of cells, an electron donor with lower redox potential such as ethyl viologen (190, 207) or increasing the incubation time, did not allow detection of activity. This suggests that the test in its current form does not work with certain reductive dehalogenases. This could be due to incompatibility of the used electron donor with the dehalogenase, pH, ionic strength, temperature or other conditions. Some reactions might be extremely slow and not suitable for *in vitro* assays.

In the microtiter plate assay a background decolourization, i.e. slow and continuous oxidation of the reduced methyl viologen, was measured in the presence of resting cells, even if no halogenated electron acceptor was added. Inhibition studies conducted with alkyl iodides specifically inhibiting cobalamin-dependent enzymes (94, 111) did not show a decrease of background methyl viologen oxidation, indicating that reductive dehalogenases or other corrinoid-containing enzymes were not responsible for this slow oxidation of methyl viologen (Figure 3-8). Medium components such as vitamins contributed only to a low amount to the measured background oxidation of methyl viologen. Tests with cells exposed to more than 80°C did not show background oxidation of methyl viologen indicating that heat sensitive cell components induced the background methyl viologen oxidation. Together the data suggested that a protein was responsible for oxidation of methyl viologen. A possible reason for the observed background activity is the presence of hydrogenase activity. Protons could serve as alternative electron sink for electrons provided by reduced methyl viologen at low redox potential (standard redox potential MV^{2+}/MV^{-+} -446 mV) (207, 208). The transfer of electrons by methyl viologen and hydrogenases could drive the production of hydrogen ($\Delta E^{o'}$ H^+/H_2 -414 mV) as it was described in previous studies (209-212). Findings from literature about reactions that could participate in methyl viologen reactions together with the conducted experiments in the current study suggested that reductive dehalogenases were not involved in the transfer of electrons to a so far unknown electron acceptor. Therefore, the background activity measured in negative controls was subtracted from samples containing organohalogens to obtain the reductive dehalogenase activity and transformation rates of halogenated compounds by resting cells used as catalyst.

The established 96-well microtiter plate activity assay was used to investigate new electron acceptors of *D. mccartyi* strain CBDB1. The transformation of halogenated compounds in culture with *D. mccartyi* strain CBDB1 suggests that a variety of halogenated compounds can principally serve as electron acceptor for organohalide-respiring *Dehalococcoidia*, the reaction itself also depends on the presence of appropriate enzymes in the studied environment. Tidal flat sediments, marine sediments and marine deep-sea sediments (depths greater than 500 m) and dehalogenating sediment microcosms were studied for reductive dehalogenation activity using the 96-well microtiter plate-based assay or

the GC-FID-evaluated assay but no activity was detected. For a successful detection of dehalogenation activity, the encoding genes must be present in the sediments but additionally the enzymes must be expressed. The failure to detect dehalogenation activity directly in sediments underscores the need for previous enrichment by cultivation. However, while cultivation and enrichment with halogenated electron acceptors allows for a more sensitive detection of potential reductive dehalogenation activity in marine sediments, enzymes might be induced during cultivation which were not expressed under the natural conditions in the sediment environment. Possibly, a higher biomass of cells directly extracted from freshly sampled sediments will allow in the future to use the activity assay as a strategy to detect dehalogenation activity in crude extracts of sediments without prior enrichment with halogenated compounds by cultivation.

4.1.3 Analysis of reductive dehalogenase expression as a strategy to gain insights into enzyme specificity

Cultivated species of the class *Dehalococcoidia* typically contain several reductive dehalogenase homologues (29) but only for few reductive dehalogenases it is known which electron acceptors induce their transcription (103, 106, 107). Therefore, also electron acceptors, which may induce reductive dehalogenases of marine organohalide-respiring microorganisms, remain unknown. Shotgun proteomics of proteins extracted from marine sediments could be used to investigate natural reductive dehalogenation activity in marine sediments. However, metagenomic data for comparison would be required and the separation of sufficient amounts of proteins in high quality for proteomic measurements seems unrealistic at present for sediments in which dehalogenation activity cannot be detected by activity assays. Therefore, in the current study reductive dehalogenase activity after induction with specific electron acceptors was analysed using activity assays with resting cells of the model organism strain CBDB1. Additionally, shotgun proteomics with cells of strain CBDB1 grown with different electron acceptors was used to gain insights into potential functions of the diverse reductive dehalogenases in members of the class *Dehalococcoidia*. Using shotgun proteomics for a prediction of potential functions was used previously for an uncharacterized *Dehalococcoides*-containing mixed culture (106). In the current study shotgun proteomics and activity assays were mainly used to understand the importance of different halogen types onto the expression of reductive dehalogenases using different bromobenzene congeners. This contributed to the understanding of potential electron acceptors of reductive dehalogenases in marine sediments (see also chapter 4.2.3, page 93).

4.1.4 Evaluation of density functional theory-based data as a strategy to elucidate chemical parameters influencing reductive dehalogenation

Thousands of halogenated molecules occur in the environment (125, 139, 206). However, due to their diversity and often low concentration as individual compounds, testing of the multitude of detected compounds with organohalide-respiring *Dehalococcoidia* for reductive dehalogenation by cultivation or activity assays is difficult: Often, identified natural compounds are commercially not available and their isolation from natural environments in sufficient amounts for cultivation or activity assays is challenging. The identification of chemical properties, which influence reductive dehalogenation, could allow for a classification of halogenated compounds with respect to their potential to serve as electron acceptor for organohalide-respiring *Dehalococcoidia* independent of cultivation or activity assays (see also 4.3). This could contribute to a deeper understanding of the ecological niche of *Dehalococcoidia* and a better fate prediction of halogenated compounds from natural or/and anthropogenic sources in the environment. *In silica* modelling of a molecule's chemical properties could provide such a tool. For this, chemical parameters of halogenated electron acceptors were modelled using density functional theory, a method previously described to model electronic structures (213). Several parameters were evaluated for their ability to predict transformation pathways catalysed by reductive dehalogenases of strain CBDB1. Partial charges revealed to serve best as a predictor of dehalogenation pathways which is in agreement with previous studies using Mulliken partial charges to predict dehalogenation pathways of chlorobenzenes, dioxins and chlorophenols catalysed by strain CBDB1 (195). In the current study, the Mulliken model was extended to a more diverse set of electron acceptors, including molecules with diverse functional groups and different types of halogens occurring in natural halogenated electron acceptors. By extending the Mulliken model to a broader set of halogenated electron acceptors, several molecules were identified whose transformation pathways were not predicted correctly. The Mulliken model is the oldest method for partial charge modelling and was described to have a strong dependence on the basis sets (187, 214). Moreover, the Mulliken model does not take into account differences in electronegativity of different atoms, which can be especially important when comparing different halogen substituents with different electronegativity (215). Therefore, in the current study, the Hirshfeld (188) and the NBO (197) partial charge models were added for the evaluation of transformation pathways. The Weinhold-Reed Natural Population Analysis to obtain natural bond orbital (NBO) partial charges is considered as an improved procedure compared to the Mulliken analysis (196, 214). Both models depend on basis functions that represent wave functions (214). The Hirshfeld model is less basis set-dependent and can be calculated directly via density functional theory using a hypothetical promolecule (188, 215). By using several partial charge models, a verification of observed trends was achieved, while minimizing potential errors. All tested models indicated that the observed regioselective removal of halogens is governed by the most positive halogen partial charge

with up to 96% correct prediction rates for the transformation pathways of strain CBDB1. However, each model showed inconsistency with specific molecule types which could be related to the different approaches used for calculating the charge distribution i.e. for Hirshfeld the portioning of electron densities and the use of a promolecule and for Mulliken and NBO the analysis of wave functions (214). For instance, modelling of partial charges revealed that not all partial charge models are suitable to predict the dehalogenation pathways compounds with both, bromine and chlorine substituents. Mulliken and Hirshfeld charges calculated with the Gaussian 09 revision C.0149 program and density functional theory level B3LYP/6-31G(d,p) yielded generally more positive partial charges for chlorine substituents than for bromine substituents when comparing brominated and chlorinated congeners. Both models assigned to bromine atoms a more negative charge than to chlorine atoms, thus not corresponding to a higher electronegativity of chlorine compared to bromine (216). Although Mulliken and Hirshfeld charges predicted correctly the transformation pathway when the molecule was substituted with only one type of halogen, both models did not predict correctly a more extensive dehalogenation of brominated benzenes or phenols than of chlorinated benzenes or phenols when using the most positive halogen charge as predictor for the removal of halogens. This trend was reflected in the prediction of the dehalogenation of 2-chloro-3-bromopyridine to 2-chloropyridine. Only the NBO model was suitable for a comparison of electron acceptors substituted with both bromine and chlorine.

The variance found in the different models emphasizes the need for several charge models applied in parallel to allow for a verification of obtained values. Additionally, the influence of the basis-sets used for calculation of the partial charge will need to be investigated, however, this was beyond the scope of the current study and will need to be investigated in future studies. Also with the current approach, it was not possible to define an absolute threshold for the positive charge on a halogen substituent to predict dehalogenation. For this, an even broader data set of different electron acceptors might have to be evaluated. However, the usage of modelled chemical descriptors allowed for the description of chemical properties which influence reductive dehalogenation catalysed by strain CBDB1 beyond classical chemical concepts such as inductive and mesomeric effects (see for further discussion section 4.3). In addition, this approach may harbour the potential to elucidate chemical properties influencing reductive dehalogenation of different organohalide-respiring bacterial strains, possibly also from marine sites.

4.1.5 Analysis of *Dehalococcoidia* genes associated with respiratory functions in marine sediments

Another strategy to investigate potential electron acceptors of marine *Dehalococcoidia* is the analysis of the presence of functional genes associated with respiratory functions in *Dehalococcoidia.* This analysis was only possible due to the recent development of single cell genome sequencing technologies because only these allowed the design of specific primers and a correlation of functional

genes with phylogenetic marker genes. The information retrieved from the genomic content of *Dehalococcoidia* single-cells was used as a basis in the current study to investigate the potential of *Dehalococcoidia* to use non-halogenated electron acceptors. A gene with highest similarities to a reductive dehalogenase subunit A was identified in one of the single cells (personal communication Dr. Kenneth Wasmund, UFZ, Leipzig) however it was not associated with a TAT leader sequence or a gene coding for the membrane anchor RdhB. Therefore, its function in a respiratory process as known from other organohalide-respiring *Dehalococcoidia* strains seemed unlikely. Instead, several other genes associated with respiratory functions were identified in the *Dehalococcoidia* genomes. Dissimilatory sulphite reduction was described so far only in a restricted number of bacterial phyla and many *dsr* sequences remain with unknown phylogeny. Therefore, *dsr* genes were selected for further investigation in the current study by a targeted amplification of *dsr* genes by PCR because it provides insight into the reasons for *Dehalococcoidia* diversity in marine sediments but also into the general ecology of marine sediments. As a source DNA had to be isolated from marine sediments in sufficient quality to allow for the amplification of functional genes. In this study fragments of up to 3.9 kb were amplified to allow for a detailed analysis of phylogeny and gene order of the amplified sequences. Methods had to be chosen which avoided the sheering of DNA into small fragments. For the isolation of DNA from marine sediments different methods were tested which ranged from commercially available purification kits to methods using density centrifugation to isolate cells from sediments and subsequent DNA extraction procedures as were described previously (166, 167, 217). While DNA purification kits have the advantage of a standardized protocol they have the disadvantage that only 0.5 g of sediment can be applied to the extraction procedure or many extractions have to be done in parallel. Density centrifugation with Nycodenz was used in the current study beside commercially available kits, as it allowed for the extraction of cells from larger amounts of sediments, which was important especially for sediments with lower cell numbers and decreasing amounts of extractable DNA. However, Holmsgaard and colleagues showed that microbial populations extracted from soil using density centrifugation with Nycodenz had significantly lower biodiversity than the microbial populations in the soil obtained with direct DNA extraction methods (217) suggesting that also in the current study an important fraction of diversity within functional genes of *Dehalococcoidia* might have been overlooked. Indeed, only few sequences coding for the targeted genes were obtained after screening of hundreds of clones with a correct insert size (see for further discussion 4.3.6), however, this approach allowed first insights into the functional gene diversity of the non-dehalogenating community of the class *Dehalococcoidia*.

4.2 The electron acceptor diversity of *D. mccartyi* strain CBDB1 and marine dehalogenating microorganisms

In the current study, reductive dehalogenation of brominated aromatic compounds that resemble naturally occurring brominated compounds (132, 135, 218) was investigated with strain CBDB1 and marine dehalogenating consortia. In addition, several chlorinated and one fluorinated aromatic compound with different functional groups have been tested with strain CBDB1 to account for the structural diversity of natural halogenated compounds and halogenated contaminants from anthropogenic sources and to further elucidate the role of *Dehalococcoidia* and marine dehalogenating microorganisms in the fate prediction of halogenated compounds in anaerobic sediment environments. *Dehalococcoidia* model organism *D. mccartyi* strain CBDB1 dehalogenated brominated benzenes, phenols, pyridines, furoic acids and benzoic acids and was able to grow during their dehalogenation. Strain CBDB1 furthermore dehalogenated chlorinated anilines, thiophenes and benzonitriles in cultivation and/or in activity assays. These findings demonstrate the diversity of electron acceptors which potentially can support growth of organohalide-respiring *Dehalococcoidia* in sediment environments and could explain the occurrence of reductive dehalogenase homologous and 16S rRNA gene sequences related with organohalide-respiring representatives of the class *Dehalococcoidia* in non-contaminated sediment sites. Reductive dehalogenation of brominated aromatic compounds was furthermore observed when testing marine microcosms suggesting that the bacterial community in marine sediments participates in the natural halogen cycle and the transformation of halogenated xenobiotics in the environment.

4.2.1 Growth of *D. mccartyi* strain CBDB1 with halogenated electron acceptors

Strain CBDB1 was cultivated with different halogenated aromatic compounds to study the electron acceptors diversity that may sustain growth of organohalide-respiring *Dehalococcoidia*. Growth of strain CBDB1 was shown with 2,3-dichloroanilines and 2-chloro-6-fluorobenzonitrile and brominated phenols, brominated furoic acids and brominated benzoic acids. Several of the tested halogenated compounds, including two dichloroanilines and both tested dichloropyridines did not allow for growth of strain CBDB1, demonstrating that functional groups, the type and the degree of halogenation influence reductive dehalogenation and thereby growth of *D. mccartyi* strain CBDB1.

Obtained growth yields of strain CBDB1 cultivated with 2,3-dichloroaniline and 2-chloro-6-fluorobenzonitrile are well in agreement with growth yields obtained in previous studies with strain CBDB1 cultivated with chlorinated phenols, ethenes and benzenes (7, 10, 11, 119). No formation of 2-chlorobenzonitrile was observed and 2-fluorobenzonitrile was not transformed by strain CBDB1 suggesting that growth of strain CBDB1 with 2-chloro-6-fluorobenzonitrile was supported by dechlorination and not by defluorination. Bacterial growth *via* defluorination was demonstrated with monofluoroacetate (219). However, monofluoroacetate served as carbon source and not as respiratory

electron acceptor for the aerobic *Moraxella* strain (220). Growth with chlorinated anilines was reported for instance with 4- and 3-chloroaniline when used as a carbon sources (221, 222).

All brominated compounds tested in the current study allowed for growth of strain CBDB1. Brominated compounds were reported to be especially abundant in marine environments (139) but little information about growth of cultivated members of the *Dehalococcoidia* with brominated aromatics was so far available. Previous studies observed growth of *D. mccartyi* strain BAV1 with the aliphatic vinyl bromide (10) however, growth yields were not reported. For a *D. mccartyi*- and *Desulfovibrio*-containing co-culture grown with the aromatic polybrominated diphenylether as electron acceptor, growth yields of 2×10^{14} cells mol^{-1} halogen released were estimated for the *D. mccartyi* sub-population (15). In comparison to that, growth yields ranging from 0.1×10^{14} to 1.8×10^{14} cells mol^{-1} halogen released were obtained in the current work for strain CBDB1 cultivated with brominated electron acceptors. Growth yields were lower with compounds that either reacted abiotically and/or contained phenolic functional groups such as phenols or hydroxybenzoic acid than with compounds which were stable or which contained no hydroxy functional groups (Figure 4-1). Possibly, the biotically-catalysed release of bromide was overestimated for cultures containing strain CBDB1 or strain CBDB1 did not completely couple the biotic release of bromine to growth. Brominated electron acceptors containing hydroxy groups showed lower growth yields compared to electron acceptors containing methoxy or carboxy groups. For instance 4-bromo-3,5-dihydroxybenzoic acid yielded 0.4×10^{14} cells mol^{-1} halogen released while 4-bromo-3,5-dimethoxybenzoic acid yielded 1.8×10^{14} cells mol^{-1} halogen released suggesting inhibitory effects of the phenolic compounds onto growth of strain CBDB1. Moreover, growth of strain CBDB1 with bromophenols was achieved only after initial amendment of low dosages of bromophenols and a sequential addition of increasing doses of bromophenols along with continuing growth of strain CBDB1 (see section 3.1.5). A similar effect was observed during cultivation of strain CBDB1 with chlorinated phenols (119). Boyle and colleagues reported lower growth yields of *Desulfovibrio* strain TBP-1 when grown with 2,4,6-tribromophenol compared to sulphate as terminal electron acceptor in spite of sulphate being the energetically less favourable electron acceptor (141). Phenols were described previously to interfere with the membrane proton gradient and the generation of ATP (223-225) and pentachlorophenol was used as respiratory inhibitor (226). Toxic or inhibitory effects of the hydroxy group may therefore explain lower growth yields of strain CBDB1 (Figure 4-1). Another possibility is that different electron acceptors allow for different extent of proton translocation, which needs to be studied further by biochemical methods.

Many reported brominated compounds from marine environments produced by algae, hemichordates and sponges as chemical defence mechanisms against bacterial growth contain phenolic groups and have been shown to inhibit microbial activity (227, 228) and could therefore counteract growth of *Dehalococcoidia* in marine sediments. Interestingly, in *Dehalococcoidia* pan-genomes RBG-1351 and RBG-2, the genetic potential to degrade hydroxylated aromatics *via*

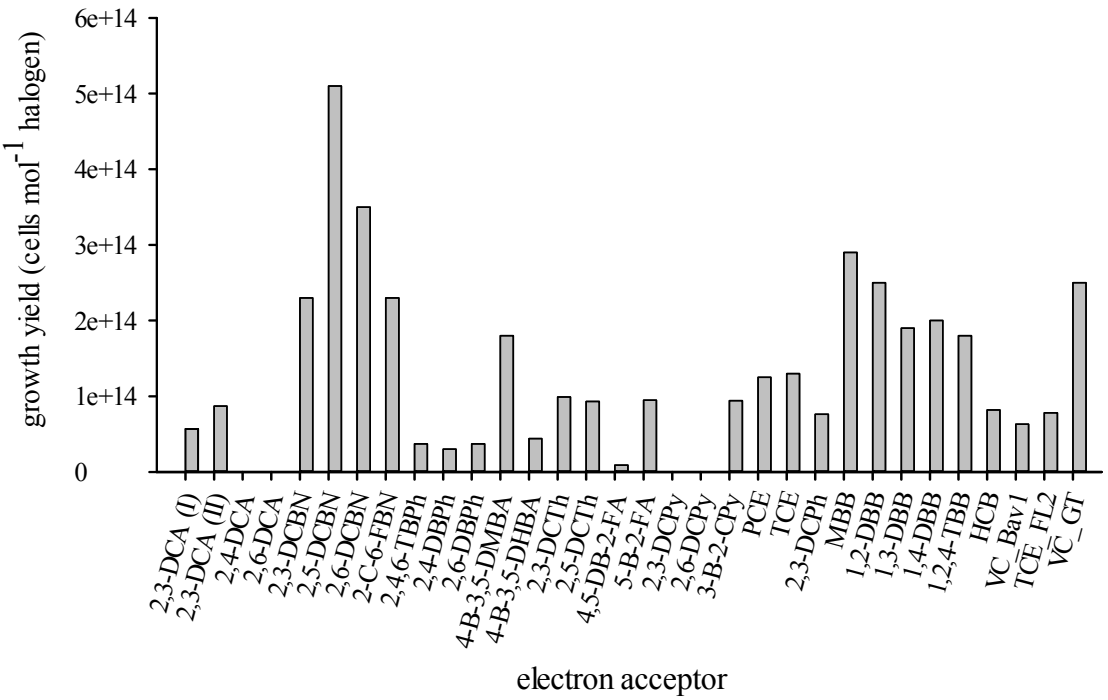

Figure 4-1: Comparison of growth yields obtained with strain CBDB1 (all values but the last three), BAV1, FL2 and GT (indicated in the sample name) cultivated with different halogenated electron acceptors.

pyrogallol hydroxytransferases were detected (144). In DEH-SAG-C11 several genes for degradation of phenolic compounds have been detected (personal communication, Dr. Kenneth Wasmund, UFZ, Leipzig). Therefore, phenolic compounds could serve also as carbon source for several *Dehalococcoidia* subgroups. Generally, brominated compounds were shown in this study to support growth of *D. mccartyi* strain CBDB1 even in presence of phenolic groups and could therefore potentially sustain growth of organohalide-respiring *Dehalococcoidia* in marine sediments. In addition, for several brominated compounds growth yields similar to those observed for *D. mccartyi* strain CBDB1 cultivated with chlorinated phenols (119), tetra- and trichloroethene (194) and for *D. mccartyi* strains BAV1, FL2 and GT, grown with different chlorinated ethenes (10-12) were obtained indicating that brominated electron acceptors are equally well suited as chlorinated electron acceptors to support growth of organohalide-respiring *Dehalococcoidia* (Figure 4-1). The ability of *D. mccartyi* strains to grow with a broad variety of brominated and chlorinated electron acceptors underscores the important role of *Dehalococcoidia* species in the natural halogen cycle and in fate prediction of halogenated compounds in the environment.

4.2.2 Dehalogenation pathways of halogenated electron acceptors catalysed by strain CBDB1

Dehalogenation pathways catalysed by strain CBDB1 were investigated by cultivation and/or by GC-FID evaluated activity assays. Results of the current study demonstrated that dehalogenation pathways catalysed by strain CBDB1 follow distinct rules similar as previously reported for

chlorinated benzenes, phenols and dioxins (7, 119, 121). However, results of the current study demonstrated that these rules become are complex for electron acceptors containing different halogen types and functional groups.

Halogens of chlorinated benzenes have been previously classified as doubly flanked, singly flanked or isolated when they have two, one or no neighbouring chlorine substituent, respectively (122). In the current study, these terms are extended as follows: halogen atoms flanked by hydrogen-substituted carbon atoms in both *ortho* positions to the halogen are referred to as isolated. Halogens flanked in o*rtho* position by a hydrogen-substituted carbon and one functional group different to a hydrogen-substituted carbon including heteroatoms, are referred to as singly flanked. Halogen substituents flanked in both *ortho* positions by a functional group different to a hydrogen-substituted carbon are referred to as doubly-flanked.

The removal of isolated chlorine substituents by strain CBDB1 was not observed in the current study. This is in agreement with previous studies using chlorinated phenols, benzenes, biphenyls or dioxins as electron acceptor for strain CBDB1 (7, 118, 119, 124). The removal of isolated chlorine substituents was reported previously for aliphatic compounds and *D. mccartyi* species (8, 10, 12), but not for aromatic compounds so far. However, in mixed cultures also the dehalogenation of isolated substituents was shown but it remains to be elucidated whether this process is catalysed by reductive dehalogenases and in metabolic or co-metabolic processes (229, 230).

Singly flanked chlorines were dehalogenated for some but not all of the compounds tested. For 2,6-dichlorobenzonitrile, for instance, the singly flanked chlorine was dehalogenated whereas the singly flanked chlorine of 2,6-dichloroanilines were not removed indicating an enhancing effect of the cyano group onto reductive dehalogenation. In addition, the singly flanked fluorine of 2-chloro-6-fluorobenzonitrile was not removed whereas the singly flanked chlorine atom was dehalogenated indicating a preference of chlorine over fluorine during reductive dehalogenation by strain CBDB1. A preferential dehalogenation of doubly flanked chlorines over singly flanked chlorines was observed for most of the tested compounds including for instance the removal of the doubly flanked halogen of 2,3-dichloroaniline and -benzonitrile (199). Dehalogenation of anilines was reported in methanogenic groundwater slurries (231) and for chlorinated benzonitriles in soil samples incubated under aerobic conditions (232, 233). The removal of the doubly flanked chlorine of 2,3-dichloroaniline or -benzonitrile in cultivation or activity assays is in agreement with previous studies showing strain CBDB1 dehalogenated the doubly flanked halogens of 2,3-dichlorophenol or 1,2,3-trichlorobenzene (7, 119). A preferential removal of singly flanked chlorine substituents compared to doubly flanked chlorine substituents was observed for *D. mccartyi* strain 195 and 1,2,3-trichlorobenzene, however only in presence of another halogenated electron acceptor (205).

However, exceptions of dehalogenation patterns of strain CBDB1 were observed. The doubly flanked chlorine of 2,3-dichloropyridine or 3-bromo-2-chloropyridine was not removed. Instead, the singly flanked bromine of 3-bromo-2-chloropyridine was dehalogenated (234) indicating a preferential

dehalogenation of bromine over chlorine for reductive dehalogenation by strain CBDB1. In addition, this demonstrates that a prediction of dehalogenation pathways based on dehalogenation patterns observed with other molecule types is not sufficient but needs to be combined with chemical properties of the electron acceptor.

When comparing dehalogenation pathways of brominated and chlorinated congeners in more detail, similar dehalogenation pathways were observed, however, brominated electron acceptors were always dehalogenated to a further extent than their chlorinated equivalents. The dehalogenation pathway of 2,4,6-tribromophenol and 2,4-dibromophenol for instance were similar to those observed for 2,4,6-trichlorophenol and 2,4-dichlorophenol (119). For all congeners the halogen substituents in *ortho*-position to the hydroxy group were preferred over the isolated halogen substituent in *para*-position. However, 2,4-dichlorophenol was converted only slowly and incompletely and 4-chlorophenol was not dehalogenated (119) whereas 2,4-dibromophenol was fully dehalogenated to phenol *via* 4-monobromophenol. A similar result was obtained for 1,2,4-tribromobenzene and 1,2,4-trichlorobenzene. Both compounds were dehalogenated to the 1,3- and 1,4-dihalogenated benzenes (7, 193). However, while the isolated chlorine substituents of 1,3- and 1,4-dichlorobenzene were not dehalogenated by strain CBDB1, the isolated bromine substituents of 1,3- and 1,4-dibromobenzene were removed, yielding monobromobenzene and subsequently benzene (7, 193). Debromination was reported for brominated biphenyl and *D. mccartyi* strains BAV1 and 195 (143). However, debromination was incomplete and occurred only in presence of a chlorinated aliphatic compound. The complete removal of all bromine substituents of an aromatic electron acceptor during reductive dehalogenation was shown for polybrominated diphenylethers by a co-culture of *D. mccartyi* and *Desulfovibrio* strains (15). However, a participation of the *Desulfovibrio* strain in debromination could not be excluded since *Desulfovibrio* species were described to dehalogenate bromo- and iodophenols (141, 142). For aliphatic compounds debromination of vinyl bromide by *D. mccartyi* strain BAV1 was observed in culture, yielding ethene as non-halogenated end product (10) and by the purified TCE reductive dehalogenase of *D. mccartyi* strain 195 dehalogenating various brominated alkanes and alkenes.

Together, the more extensive dehalogenation of brominated compared to chlorinated aromatics by strain CBDB1 indicates that organohalide-respiring *Dehalococcoidia* may have a more diverse range of brominated electron acceptors than anticipated, especially for compounds with few or isolated halogen substituents such as described to occur in marine sediments (see also Figure 1-2) (128, 131-136, 139, 235). Findings of the current study suggest that organohalide-respiring *Dehalococcoidia* in marine sediments may use brominated natural compounds with a low degree of halogenation as electron acceptors for respiration. In addition, dehalogenation of compounds with a low degree of halogenation as described for natural compounds has been shown in the current study to be facilitated or inhibited by heteroatoms and functional groups also occurring in natural organohalogens (131).

4.2.3 Specific activities of strain CBDB1 with halogenated electron acceptors

The rates at which halogenated compounds are transformed by *D. mccartyi* strain CBDB1 were investigated in activity assays with resting cells, using methyl viologen as artificial electron donor. Specific activities with brominated electron acceptors were lower than specific activities with their chlorinated congeners. For instance, 1,2,4-tribromobenzene gave a specific activity of 25.8 ± 15.4 nkat mg^{-1} protein, when previously cultivated with 1,2,4-tribromobenzene. Higher specific activities of 219.4 ± 110 nkat mg^{-1} protein were obtained when cells were previously cultivated with 1,2,3-trichlorobenzene while the specific activity for 1,2,3-trichlorobenzene was 36 ± 9 demonstrating that observed higher specific activities with 1,2-dibromo- and 1,2,4-tribromobenzene were not related with the previous cultivation with a brominated congener. For 1,2,4-trichlorobenzene a specific activity of only 0.3 nkat mg^{-1} protein was observed in a previous study (111). When comparing strain CBDB1 cells cultivated with the same electron acceptor, 1,2,4-tribromobenzene and 1,2-dibromobenzene constantly gave higher specific activities than 1,2,3-trichlorobenzene and were dehalogenated to a further extent than 1,2,3-trichlorobenzene. This is in agreement with previous studies using the purified trichloroethene reductive dehalogenase of *D. mccartyi* strain 195 (95). 1,2-Dibromoethane was reduced to ethene at a specific activity of 500 nkat mg^{-1} protein while 1,2-dichloroethane was transformed at 125 nkat mg^{-1} protein. Vinyl bromide and vinyl chloride were transformed to ethene at specific activities of 3 and 0.6 nkat mg^{-1}, respectively (95). The finding that brominated electron acceptors were transformed at higher rates compared to chlorinated electron acceptors in the current study and in previous studies with brominated aliphatic electron acceptors (95) is in accordance with observations of abiotic dehalogenation reactions, in which the aryl-bromine bond was shown to be weaker than the aryl-chlorine bond (236, 237). In addition, when correlating specific activities of brominated benzenes and phenols or chlorinated thiophenes and phenols with partial charges, more positive partial charges of the halogen were paralleled with increased specific activity rates when examining only one compound class. However, when the different compound classes were compared no consistent trend was observed. Enhanced activity rates for compounds with more positive halogen partial charges was observed for chlorobenzenes in previous studies (195). Enhanced dehalogenation activity with brominated compounds compared to chlorinated compounds could be related with different chemical properties of the halogen species such as electronegativity and size. Higher transformation rates observed with brominated aromatic and aliphatic electron acceptors compared to chlorinated compounds suggest that the ability to transform brominated electron acceptors could be advantageous for *Dehalococcoidia* in marine sediment environments in which reactions rates might be generally slower due to low temperatures (40).

4.2.4 Activity assays and shotgun proteomics show the same reductive dehalogenases transform several halogenated compounds

In the current study it was investigated whether the same enzymes are involved in debromination or dechlorination of halogenated aromatic compounds and could therefore explain the broad electron acceptor range of strain CBDB1. In addition, marine organohalide-respiring *Dehalococcoidia* could occupy a broader niche in non-contaminated marine sediments if the same reductive dehalogenases are able to transform aromatic compounds with different types of halogen substituents. Specific activities obtained in the microtiter plate assay after cultivation of *D. mccartyi* strain CBDB1 with trichlorobenzene or brominated benzenes suggested the same reductive dehalogenase enzymes are involved in dehalogenation of brominated and chlorinated benzenes. In addition brominated phenols, hydroxy- and methoxybenzoic acids and chlorinated thiophenes and benzonitriles were dehalogenated by reductive dehalogenases expressed during cultivation with 1,2,3-trichlorobenzene or hexachlorobenzene. This is in good accordance with previous studies conducted with halogenated aliphatic compounds and the purified TCE reductive dehalogenase of *D. mccartyi* strain 195 (95). However, in previous transcription and expression studies with different *D. mccartyi* strains, a simultaneous expression of several reductive dehalogenases when cultivated on one single electron acceptor was observed (102, 103, 107, 108, 238). Therefore, reductive dehalogenase expression was investigated in the current study by shotgun proteomics with strain CBDB1 cells grown with brominate benzenes to test whether the same reductive dehalogenases previously shown to transform chlorinated benzenes (104) can be identified after cultivation with brominated benzenes. A simultaneous expression of four to six reductive dehalogenases was observed. The most abundant reductive dehalogenase expressed after cultivation with bromobenzenes was CbdbA80 according to obtained emPAI values. The emPAI value allows for an estimation of the relative abundance of a protein (172). Indeed, CbdbA80 was previously shown to be expressed in strain CBDB1 after cultivation with 1,2,3- and 1,2,4-trichlorobenzene and after cultivation with 2,3-dichlorophenol (104, 106). The reductive dehalogenase CbrA encoded by *cbrA* (previously referred to as *cbdbA84*) was detected in the current study after cultivation with 1,3-dibromobenzene, 1,4-dibromobenzene or 1,2,4-tribromobenzene. Previous studies using the purified CbrA enzyme demonstrated dehalogenation of 1,2,3-trichlorobenzene to 1,3-dichlorobenzene (104). The expression of CbrA in cultures grown with brominated benzenes as electron acceptors suggests CbrA is involved in dehalogenation of brominated and chlorinated benzenes. Interestingly, the *cbrA* gene sequence branched with the reductive dehalogenases subseafloor cluster I (60) suggesting that reductive dehalogenase detected in marine non-contaminated sediments may have similar functions (106) and may transform brominated and chlorinated aromatic compounds. Reductive dehalogenase enzymes with a diverse electron acceptor spectrum could allow organohalide-respiring *Dehalococcoidia* to

exploit diverse natural organohalogens and broaden thereby the niche of organohalide-respiring *Dehalococcoidia* in marine sediments.

4.2.5 Dehalogenation activity in marine sediments and sediment microcosms with brominated compounds

Previous studies showed the presence of reductive dehalogenase homologous genes (*rdh*-genes) (60, 61) in marine non-contaminated sediments. Furthermore, tetrachloroethene dehalogenation activity was reported in sediment microcosms with tidal flat sediments of the North Sea containing *Chloroflexi* (52) suggesting that organohalide-respiring *Dehalococcoidia* subpopulations could be detected in marine sediments with the activity assay and dehalogenation rates could be determined. In addition *D. mccartyi* strain MB and 'Dehalobium chloroercia' were isolated from estuarine sites (14, 22). However, when applying sediments or isolated cells directly retrieved from different marine sediments to the 96-well microtiter plate-based assay or to the GC-FID-evaluated assay, no dehalogenation activity was detected, despite the previous detection of *Dehalococcoidia* in the used samples (47). This suggested that either reductive dehalogenase genes were not expressed, cells were inactive, dead or that not all *Dehalococcoidia* cells detected via quantitative PCR depend on reductive dehalogenation, as recent studies indicated (46, 48, 144). Another possibility is the expression of many different reductive dehalogenase for different electron acceptors at low levels. Transformation rates could be therefore below the detection limit of the activity assay.

In contrary to activity assays with crude marine sediments or cell extracts directly retrieved from the environment, activity was detected with cells from marine sediment microcosms previously enriched on 1,2,3-trichlorobenzene (provided by Camelia Algora, UFZ Leipzig) with the 96-well microtiter plate assay. Activity studies revealed *in vitro* activity with different brominated compounds indicating that reductive dehalogenases of marine dehalogenating microorganisms induced by 1,2,3-trichlorobenzene catalyse debromination of different aromatic compounds, similar as observed for *D. mccartyi* strain CBDB1. Previous studies showed transformation of 2,4,6-tribromophenol in microcosms with pristine deep-sea sediments (60) and several studies showed dehalogenation of brominated compounds in enrichment cultures from estuarine sediments by bacterial clades mostly related to the delta-*Proteobacteria* (140, 141). However, no biochemical tests were conducted in these studies to investigate whether corrinoid-dependent enzymes such as reductive dehalogenases were involved in dehalogenation. In the current study inhibition studies with resting cells of marine microcosms were conducted as previously described for *Dehalococcoides* spp. (105, 111). The reduction of dehalogenating activity after exposure to propyl iodide and a slightly increased dehalogenation activity after exposure to light suggested the involvement of a corrinoid-dependent enzyme. Most of the characterized reductive dehalogenases have been shown to contain corrinoid as the active cofactor (93, 94, 105, 109, 110), except for the 3-chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei* containing iron in the catalytic centre (112).

Reductive dehalogenation activity was also observed when samples were incubated at 4°C suggesting that these enzymes may function also at low temperatures in deeper marine sediments. The dehalogenation activity of resting cells exposed to oxygen did not decrease significantly in comparison to cells incubated under anoxic conditions. In contrary to this, for *D. mccartyi* strain CBDB1 for instance an increased stability of reductive dehalogenation activity was observed when stored under anoxic conditions (170).

Marine sediment microcosms previously enriched with 1,2,3-trichlorobenzene were amended with 1,2,4-tribromobenzene to test for dehalogenation in culture and confirm results obtained by *in vitro* activity assays. 1,2,4-tribromobenzene was dehalogenated to a further extent than 1,2,3-trichlorobenzene. The same was observed for *D. mccartyi* strain CBDB1 (7, 193) suggesting that cobalamin-dependent reductive dehalogenases generally have a broader dehalogenation potential for brominated electron acceptors than for chlorinated electron acceptors when comparing compounds with similar molecular structures. Lower debromination activity was reported for *D. mccartyi* strain BAV1 and 195 with brominated biphenyls than for chlorinated aliphatic compounds (143) however, this might be related to the different size and molecular structures of the molecules underscoring the importance of chemical and structural properties of the electron acceptor itself for reductive dehalogenation.

Together the findings show that microorganisms performing reductive dehalogenation of brominated aromatics occur in marine pristine sediments. The dehalogenation of various brominated compounds in activity assays and in culture suggests that different organobromines are suitable natural electron acceptors for organohalide-respiring microorganisms in marine non-contaminated sediment sites. Organohalide respiration with organobromines may therefore explain the diversity and activity of reductive dehalogenase in marine sediments detected by molecular methods in previous studies (60, 61) and by biochemical and cultivation-dependent methods in the current study in non-contaminated sediments. In addition, the findings of the current study shows that marine sediments harbour the capacity for transformation of halogenated aromatic compounds from natural but also anthropogenic sources and may therefore play an important role in the natural halogen cycle.

4.3 Chemical properties of the halogenated electron acceptor influence reductive dehalogenation

A multitude of halogenated compounds has been described to occur in the environment containing different types of functional groups and halogens (139, 206, 235). Additionally, it was estimated that 15–20% of newly discovered marine natural products were organohalogens (235). However, it is not clear which organohalogens could serve as electron acceptors for organohalide-respiring *Dehalococcoidia*. Each *Dehalococcoidia* species and even each strain shows specific substrate spectra (7-19). Therefore, the enzymes harboured by *Dehalococcoidia* species play a crucial role in its

substrate range. However, results of the current study together with results of previous studies (119, 121, 124, 193) demonstrated that *D. mccartyi* strain CBDB1 transformed more different halogenated compounds than reductive dehalogenases are encoded in the genome indicating a single reductive dehalogenase transforms more than one halogenated compound. Additionally, the same reductive dehalogenases were shown to be involved in the current study in the dehalogenation of brominated or chlorinated benzenes. However, dehalogenation patterns and activities were seemingly influenced by chemical properties of the electron acceptor itself as observed for instance for brominated and chlorinated benzenes for which *in vitro* assays revealed that brominated benzenes were dehalogenated to a further extent regardless of the electron acceptor used for the induction of reductive dehalogenase expression in cultivation. To investigate potential reasons for this degree of unspecificity, chemical properties of halogenated compounds tested in the current and in previous studies were evaluated in more detail and correlated with observations made in cultivation and in biochemical experiments. The evaluation of experimental results of the current study combined with results of previous studies revealed that different substituents and functional groups strongly influence the regioselective dehalogenation by *Dehalococcoidia* model strain CBDB1 and should be considered together with enzymatic properties of the dehalogenating strain for the fate prediction of halogenated compounds from natural and anthropogenic sources in anaerobic environments. The inclusion of chemical descriptors allowed for a systematic classification of the chemical effects which govern reductive dehalogenation in strain CBDB1. While the conclusions are based on experiments with *D. mccartyi* strain CBDB1, the findings allow for some speculative extrapolation onto cobalamin-dependent reductive dehalogenase enzymes in general – provided similar rules apply to all cobalamin-dependent respiratory reductive dehalogenases.

4.3.1 The influence of secondary substituents onto reductive dehalogenation

Natural halogenated molecules contain a broad diversity of secondary halogen and non-halogen substituents. Chlorinated compounds containing cyano groups have been detected in cyanobacteria, sponges and fungi (235), chlorinated or brominated organohalogens containing hydroxy groups are ubiquitously present and amino groups have been reported to occur naturally in form of halogenated amino acids (235). In addition, amino, hydroxy and cyano groups are present in some of the most commonly detected halogenated contaminants from anthropogenic sources. Dehalogenation pathways of strain CBDB1 discussed in section 4.2.2, revealed different influence of substituents onto reductive dehalogenation. Analyses of results obtained in the current study combined with results of previous studies suggested an enhancing effect of secondary substituents in *ortho*- position to the halogen onto reductive dehalogenation in the order -CN > -X > -OH > -NH_2. For instance, whereas 1,2-dichlorobenzene was not dehalogenated by strain CBDB1 (7), 2-chlorobenzonitrile was dehalogenated to the non-halogenated benzonitrile (199) suggesting the cyano group of benzonitriles enhanced reductive dehalogenation more than a secondary halogen substituent in *ortho* position.

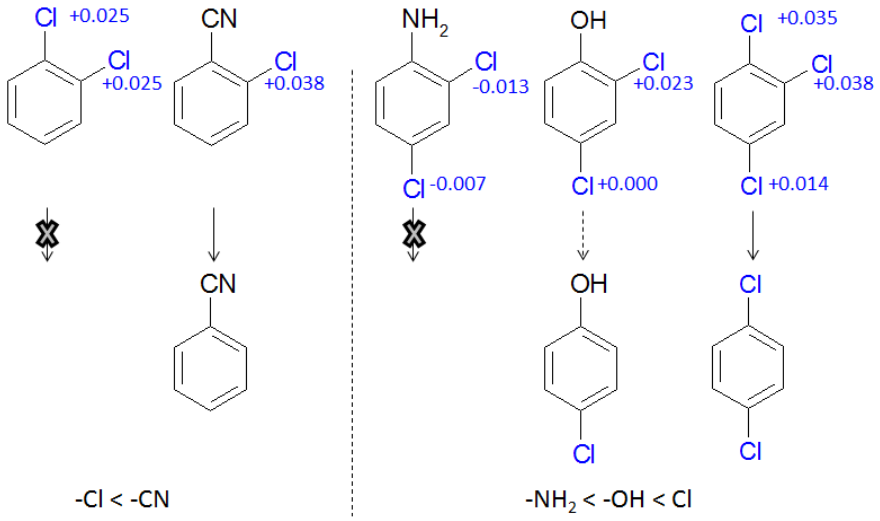

Figure 4-2: The influence of functional groups onto reductive dehalogenation when comparing halogenated benzonitriles, anilines, benzenes and phenols. 1,2-Chlorobenzene was not transformed by strain CBDB1 (7) while 2-chlorobenzonitrile was dehalogenated (199), 2,4-Dichloroanilines were not dehalogenated while 2,4-dichlorphenol only partially (119) and 1,2,4-trichlorobenzene was dehalogenated to 1,4- and 1,3-dichlorobenzene (7).

The halogen singly flanked by another halogen in 1,2,4-trichlorobenzene (7) was removed, whereas the halogen singly flanked by the hydroxy group in 2,4-dichlorophenol was removed only partially (119) and the singly flanked chlorine of 2,4-dichloroaniline was not removed at all. This suggests that secondary halogen substituents enhance reductive dehalogenation more than the hydroxy or amino groups of phenols and anilines and hydroxy substituents enhance reductive dehalogenation more than amino substituents (119). This trend was also reflected in the partial charges of halogens flanking the functional groups in *ortho*-position (Figure 4-2). Increasingly positive partial charges for the halogen in *ortho*- position and the halogenated carbon were observed suggesting substituents withdrawing electron density from the halogen and the halogenated carbon atom enhanced reductive dehalogenation. In addition, this observation was corroborated by a correlation of the Hammett substituent constants (239) with dehalogenation patterns observed for strain CBDB1 suggesting substituents with an electron withdrawing effect enhance reductive dehalogenation. The Hammett constants serve for *meta*- and *para*- secondary substituents, steric interference with *ortho*- secondary substituents do not allow for a linear correlation. However, the results of this study combined with previous studies suggest, the Hammett constituents might serve as a rule of thumb for the effect of a substituent onto reductive dehalogenation in *ortho* position when no DFT modelled electron density distribution data are available. Using the Hammett constants, also indications about the dissociation state of the hydroxy group of halophenols during reductive dehalogenation can be gained. The incomplete or slow dehalogenation of several dichlorophenols could be explained in part by a dissociated hydroxy group, which according to the Hammett substituent constant has even weaker electron withdrawing properties than the amino group of anilines and would therefore exhibit only a

weak enhancing effect onto reductive dehalogenation. However, also toxic effects of phenols may explain this observation (225).

Together, results of the current study combined with findings of previous studies indicate, substituents with a negative inductive effect withdrawing electrons from the halogen and the halogen-substituted carbon enhance reductive dehalogenation. The stronger the negative inductive effect of the substituent, the more likely was the removal of halogen secondary substituents.

4.3.2 Chemical properties of heterocycles influence reductive dehalogenation

The influence of heteroatoms onto reductive dehalogenation was investigated with furoic acids, thiophenes and pyridines. In natural systems oxygen, nitrogen and sulphur heteroatoms occur in histidin, NADH, purine and pyrimidine bases, pyranose and furanose carbohydrates and many of the ubiquitous enzymatic cofactors. Halogenated congeners of these molecules have been reported to occur in the organic material of sediments (235). In addition, many halogenated pesticides used at present are heterocyclic compounds. However, little is known about how a heterocyclic structure influences reductive dehalogenation and thus how stable such halogenated heterocyclic compounds are in anaerobic environments towards microbial degradation. In the current study, different dehalogenation pathways observed for different types of heterocycles indicated heteroatoms influence reductive dehalogenation pathways. While both tested chlorinated thiophenes were dehalogenated in culture (234) and in activity assays, both tested chlorinated pyridines were not dehalogenated suggesting the sulphur heteroatom in the five-membered heterocycle enhanced reductive dehalogenation more than nitrogen in the six-membered heterocycle. Thiophenes are heteroaromatic electron acceptors with electron-rich carbons because the free electron pair of the sulphur heteroatom participates in the aromatic system of the five-membered ring. In pyridines, the nitrogen atom has three hybridized electrons of which it donates one electron to the pi electron system but its free electron pair does not participate in the six-membered aromatic system (240). Therefore, the nitrogen heteroatom of pyridines exhibits no positive mesomeric effect and pyridines are considered as electron-poor heterocycles with electron-deficient carbons (240). These properties were reflected in the modelled partial charges of the two types of heteroaromatic electron acceptors (Figure 4-3). More negative partial charges were observed for carbon atoms flanking the sulphur atom in thiophenes than for carbon atoms flanking the nitrogen atom in pyridines. The chlorine-substituted carbon atoms of 2,3- and 2,6-dichloropyridine exhibited the most positive partial charge of all molecules tested. This would suggest enhanced reductive dehalogenation according to previous mechanistic models of reductive dehalogenation, considering an electron transfer onto the positively polarized halogen-substituted carbon atom (90, 115, 241-245). However, the contrary was observed: the electron-rich thiophenes with halogen-substituted carbons with a more negative partial charge than for instance carbon atoms of pyridines were dehalogenated suggesting a positive mesomeric effect is beneficial or at least not counteracting reductive dehalogenation.

The oxygen heteroatom of brominated furoic acids did not inhibit dehalogenation, however it is difficult to estimate whether the oxygen heteroatom enhanced dehalogenation, based on the data of the current study. All tested molecules containing an oxygen heteroatom were brominated and so far, all bromine substituents tested in the current study were dehalogenated by strain CBDB1, regardless of the chemical structure of the molecule. Previous studies demonstrated however a positive influence of the oxygen heteroatom of chlorinated dibenzo-*p*-dioxins onto dehalogenation catalysed by *D. mccartyi* strain CBDB1 (121). Strain CBDB1 dehalogenated preferentially the chlorine atom doubly flanked by chlorine and an oxygen heteroatom instead of chlorines doubly flanked by two chlorines. This may have significant implications for the toxicity of a compound. For instance, due to the positive influence of the oxygen heteroatom of dibenzo-*p*-dioxins, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin was dehalogenated by strain CBDB1 to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin which is the most toxic halogenated dioxin known (121). However, the oxygen heteroatom in dibenzo-*p*-dioxin is not part of the halogenated ring and could be therefore considered as methoxy substituent flanking the chlorine substituent. Further research is therefore required to elucidate the influence of heteroatoms in different ring structures. Results of the current study suggest that the influence of a heteroatom onto reductive dehalogenation requires consideration of the molecule's overall structure as much as type and degree of halogenation.

4.3.3 The role of the halogen type in reductive dehalogenation

Natural halogenated compounds include chlorine-, bromine-, iodine-, and fluorine-containing organic compounds (235) and iodated, brominated, chlorinated and fluorinated compounds are introduced into the environment in form of contrasting agents, flame retardants, pesticides and antibiotics into the environment. Results of this study demonstrate not all halogen substituents are equally well suitable for transformation *via* reductive dehalogenation. When comparing brominated,

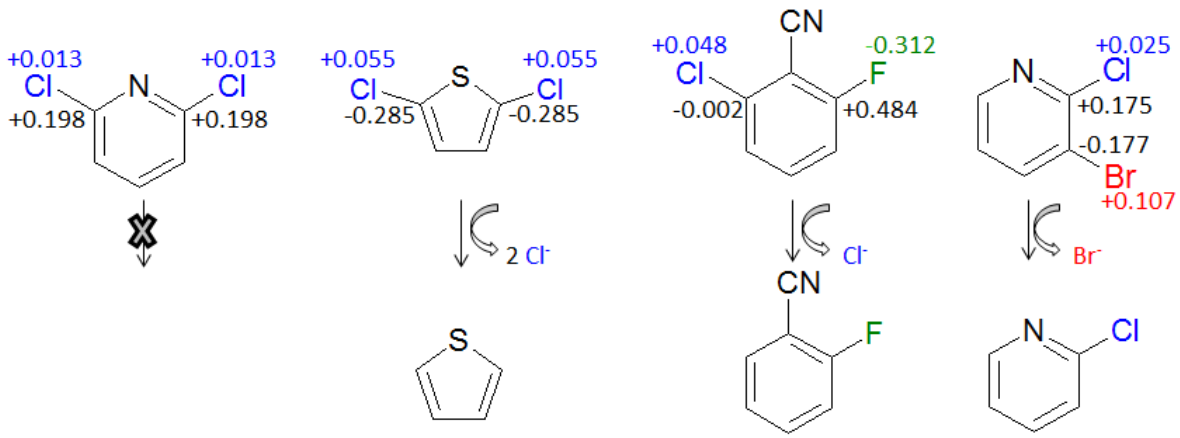

Figure 4-3: Dehalogenation reactions and partial charges of halogenated aromtics. Chlorine partial charges are indicated in blue, fluorine partial charges in green, bromine partial charges in red and partial charges of the halogen-substituted carbons in black.

chlorinated and fluorinated molecules tested in this study, and specifically molecules substituted with two different halogen types, such as 2-chloro-6-fluorobenzonitrile and 3-bromo-2-chloropyridine, an enhanced removal of bromine compared to chlorine was observed, which was also reflected in the NBO partial charges (Figure 4-3). Fluorine was not removed even though it was flanked in 2-chloro-6-fluorobenzonitrile by the functional group with the strongest electron withdrawing properties of all substituents tested so far with strain CBDB1. The enhanced removal of bromine substituents compared to chlorine substituents supports the observation made with non-halogen substituents and heteroatoms that the most relevant parameter for reductive dehalogenation is the withdrawal of electron density from the halogen and not from the carbon. Otherwise, fluorine should allow for the most extensive dehalogenation, while bromine should be removed to a lesser extent than chlorine.

This can be explained by the chemical properties of different halogen substituents. Halogens exhibit a negative inductive effect (-I-effect) by withdrawing electron density from neighbouring atoms with lower electronegativity and a positive mesomeric effect (+M-effect) by donating a free electron pair participating in the aromatic system in the order I < Br < Cl < F (i.e. bromine has a weaker negative inductive effect than chlorine but a stronger positive mesomeric effect) (246, 247). This means that fluorine will induce a more positive partial charge on the halogenated carbon than for instance bromine. Results of the current study demonstrated the less electronegative a halogen was, the more likely was its dehalogenation by strain CBDB1. A weaker aryl-bromine bond compared to the aryl-chlorine bond (236, 237) could additionally facilitate reductive dehalogenation.

For some molecules tested in the current study, abiotic dehalogenation of bromine was observed and lower growth yield of strain CBDB1 were obtained suggesting an optimum range for the strength of the aryl-halogen bond is required to allow for energy generation via reductive dehalogenation. This could be especially true for iodinated compounds with an even weaker carbon-iodine bond compared to the carbon-bromine bond. Therefore, abiotic dehalogenation under reduced conditions such as observed for brominated aromatic compounds could compete with biotic dehalogenation reactions by organohalide-respiring *Dehalococcoidia* species.

4.3.4 Chemical properties governing dehalogenation pathways of strain CBDB1 suggest halogen–cobalamin interaction during reductive dehalogenation

Previous studies suggested that reductive dehalogenation reactions are nucleophilic substitutions in which the C-X bond cleavage is initiated by a $B_{12}Co^{I}$ attack at the carbon site and in which an intermediate Co–C bond is formed (90, 115, 241-245). The findings of this study however indicate that the site of interaction is rather the halogen than the carbon for the following reasons:

The aromatic pyridine heterocycle is considered as an electron-poor ring-system suggesting enhanced transfer of electrons on carbon flanking nitrogen. Thus a nucleophilic attack of cobalamin on the halogen-substituted carbon for 2,3-dichloropyridine or 2,6-dichloropyridine should be facilitated. However, the contrary was observed. The electron-poor pyridine was not dehalogenated while the

electron-rich heterocycle thiophene was dehalogenated. Furthermore, if halogens were removed by a nucleophilic attack onto the substituted carbon atom the reactivity of halogens for a nucleophilic attack (S_NAr) should increase with increased C_{Ar}–X bond dissociation energy in the order F > Cl > Br > I (248), due to an increasing electron deficiency at the aromatic halogen-substituted carbon as site of nucleophilic attack. However the opposite reactivity in the order Br > Cl > F during reductive dehalogenation was observed in the current study. Instead, the reactivity order correlated with reduction potentials reported for monohalogenated benzenes yielding the order Br > Cl > F with Br having the least negative reduction potential (249). In addition, the regioselective dehalogenation catalysed by strain CBDB1 was predicted by the most positive partial charge of the halogen and not by the most positive partial charge of a halogen-substituted carbon.

Therefore, instead of an electron transfer to the halogen-substituted carbon in a nucleophilic substitution reaction, a single electron transfer (250) onto the halogen may be considered. Co^I and other transition metals with a singlet d^8 configuration were shown to act as Lewis bases, and thus can form hydrogen bonds with an electron accepting compound (251-254). A halogen could function as such an electron accepting Lewis acid, due to a region with positive electrostatic potential. This region with positive electrostatic potential is called σ-hole (255). It could interact with the lone electron pair of a Lewis base (256-258). Therefore, the halogen and the Co^I could potentially interact as Lewis base and Lewis acid, respectively (personal communication, Prof. Dr. Schüürmann, Department for Ecological Chemistry, UFZ, Leipzig).

The σ-hole is progressively larger and more positive for iodine > bromine > chlorine. Additionally the presence of the σ-hole depends on the environment of the halogen atom in the molecule and requires electron density withdrawing atoms in the molecule (255). The improved prediction rates of dehalogenation pathways catalysed by strain CBDB1 using sigma partial charges compared to pi partial charges indirectly support the hypothesis of the involvement of the apical σ-hole of halogen substituents of aryl compounds. Interestingly the σ-hole does not occur in fluorine (255) suggesting that the absence of this region prevents reductive dehalogenation of fluorinated molecules. However, this will require further studies with diverse fluorinated aromatic compounds.

It remains to be elucidated why electron-poor pyridines are not reductively dehalogenated. Electron-poor pyridines should be more reactive to the formerly proposed S_NAr reaction mechanism due to a more positively polarized halogen-substituted carbon but also to the newly proposed single electron transfer because the molecule exhibits an overall larger electron affinity. Nevertheless, the reduced reactivity of pyridines towards reductive dehalogenation emphasise the importance of the carbon–halogen bond polarization for reductive dehalogenation.

The direct interaction of the cobalamin cofactor with the halogen is supported by recent descriptions of the crystal structure of two reductive dehalogenases. Electron paramagnetic resonance measurements and modelling with a reductive dehalogenase from the aerobic strain *Nitratireductor*

pacificus strain pht-3B indicated a direct cobalamin-halogen interaction (117). Additionally, for the reductive dehalogenase of *Sulfurospirillum multivorans* a very small pocket containing the active site was reported (259), which suggests that an electron transfer onto the halogen would be facilitated compared to the transfer onto the shielded carbon atom. For the electron acceptor range of *Dehalococcoidia* in marine sediments a cobalamin–halogen interaction could allow for the usage of a broad range of different halogenated molecules with different structures. However, a cobalamin–halogen interaction implies that organohalide-respiring *Dehalococcoidia* cannot evade organohalogens for respiration with the encoded set of reductive dehalogenases and suggests that organohalide-respiring *Dehalococcoidia* cannot substitute halogenated compounds by compounds with halogen-like functional groups such as for instance pseudohalogens. Previous studies conducted in this research group corroborate this hypothesis by showing that several tested non-halogenated compounds did not allow for growth of strain CBDB1 (260).

4.3.5 Implication for other reductively dehalogenating microorganisms

While findings of the current study suggest a halogen–cobalamin interaction for reductive dehalogenation catalysed by strain CBDB1, it remains to be elucidated to which extent these rules apply to different dehalogenating strains. Among the *D. mccartyi* strains different dehalogenation patterns can be observed, and even more distinct patterns have been reported for different *Dehalococcoidia* species and other reductively dehalogenating microorganisms. The prediction of dehalogenation pathways by using the most positive halogen partial charge served for strain CBDB1 but will need to be evaluated for different dehalogenating strains with different dehalogenation patterns. Other strains may require different chemical parameters for a successful description and prediction of their dehalogenation patterns and a correlation of the chemical descriptors with substrate spectra of purified reductive dehalogenases will be required.

However, rules identified for strain CBDB1 may allow a deeper understanding of reactivity patters of dehalogenating strains despite of variations observed for dehalogenation patterns of different bacterial strains. *D. mccartyi* strain DCMB5 for instance shows similar dehalogenation patterns as strain CBDB1 for chlorinated benzenes and dibenzo-*p*-dioxins (261). Like strain CBDB1, strain DCMB5 preferentially dehalogenated the chlorine atom of 1,2,4-trichloro-dibenzo-*p*-dioxin flanked by two electron withdrawing groups suggesting rules identified for strain CBDB1 account also for other *D. mccartyi* strains performing hydrogenolytic dehalogenation. Additionally, strain DCMB5 did not dehalogenate the isolated chlorine substituent of 2-monochlorodibenzo-*p*-dioxin or 1,4-dichlorobenzene but the chlorine substituent of 1-monochlorodibenzo-*p*-dioxin yielding a non-halogenated reaction product (261), demonstrating the importance of electron density withdrawal from the halogen by flanking substituents or heteroatoms for reductive dehalogenation. For *D. mccartyi* strain 195, a dehalogenation of singly flanked chlorine substituents was reported for 1,2,3,4-tetrachlorobenzene or 1,2,3-trichlorobenzene. However, also strain 195 seems to require

functional groups withdrawing electron density from the halogen. For instance, strain 195 dehalogenated 2,3,4,6-tetrachlorobiphenyl to 2,4,6-trichlorobiphenyl, preferentially removing doubly flanked chlorine substituents similar to strain CBDB1, while 2,4,6-trichlorobiphenyl was not dehalogenated by strain 195 (205). Also, strain 195 was not able to dehalogenate isolated chlorine substituents. Therefore, dehalogenation patterns of different *D. mccartyi* strains suggest, that generally functional groups withdrawing electron density from the halogen enhance reductive dehalogenation, although the presence of different reductive dehalogenases in the genome of DCMB5 and 195 may lead to different dehalogenation patterns. It remains therefore to be elucidated to which extent the enzymatic properties of reductive dehalogenases compared to the chemical properties of the electron acceptor influence the dehalogenation pattern of organohalide-respiring microorganisms.

4.3.6 Potential applications and usage of the findings for fate prediction of halogenated compounds in the environment

The importance of substituent influence on the potential for reductive dehalogenation has practical impact. Continuously new halogenated compounds are developed for which an experimental-based assessment of degradation products is time and cost intensive. The analysis of chemical parameters that influence reductive dehalogenation could help to better understand and predict the fate of halogenated compounds in anoxic environments and improve remediation strategies building upon organohalide-respiring microorganisms.

Halogenated aromatic compounds such as chlorpyrifos methyl, bromacil, chlorothalonil, triclocarban or triclosan, atrazin, bromoxynil, dichlobenil, and polybrominated diphenylethers contain diverse non-halogen functional groups and/or heteroatoms and are currently used as pesticides, wood protectants, flame retardants, disinfectants or pharmaceuticals. The functional groups investigated in the current study are present in many of these halogenated compounds and a better understanding of their transformation and degradation pathways in anaerobic environments will allow for improved fate prediction. For instance, imidacloprid is one of the most commonly used neonicotinoid pesticides used at present (262) and is suspected to be involved in the currently observed bee dying (263). The six membered heterocycle of imidacloprid contains a nitrogen heteroatom and a chlorine substituent similar to the chlorinated pyridines tested in the current study. According to the findings of the current study using chlorinated pyridines, imidacloprid is likely not dehalogenated by *D. mccartyi* strain CBDB1. If these findings are extrapolated onto other dehalogenating strains it can be speculated that such compounds may accumulate in anaerobic environments such as for instance aquifers. The neonicotinoids clothianidin and thiamethoxam however, are chlorinated five-membered sulphur- and nitrogen-containing heterocycles. According to the results of the current study a dehalogenation by *D. mccartyi* stains can be predicted. However, further research is required to establish an experimentally acquired basis for such prediction systems. If the approach of this study will be extended onto more dehalogenating strains and diverse halogenated compounds, recommendations for

the usage or treatment of specific halogenated chemicals could be made. In addition, potential toxic effects of functional groups, as observed in the current study for compounds containing hydroxy substituents, will need to be included into such prediction systems to take into account adverse side effects of the halogenated chemical itself onto the dehalogenating bacterial community. For instance, the pharmaceutical oxyclozanid or the disinfectant triclosan could exhibit growth-inhibiting effects onto dehalogenating microorganisms such as strain CBDB1 due to the hydroxy substituents. This could diminish the success of bioremediation strategies using reductively dehalogenating bacteria.

In addition, some *D. mccartyi* strains were described to produce the very toxic vinyl chloride during reductive dehalogenation (8, 11). These examples demonstrate that remediation strategies building upon reductive dehalogenation by *D. mccartyi* strains need careful evaluation according to the contaminants present in the soil and the expected reaction products. For this, a prediction system based on chemical descriptors correlated with dehalogenation patterns of particular dehalogenating strains can be highly valuable for a first *in silica* assessment of potential reaction products. In addition, such prediction systems may allow improved fate prediction of halogenated contaminants by naturally occurring dehalogenating microorganisms when combined with bio molecular or mass spectrometry tools, allowing for the identification of strains and reductive dehalogenase genes or even proteins in contaminated sites.

4.4 Non-halogenated electron acceptors of *Dehalococcoidia* in marine sediments

Genome annotation of two *Dehalococcoidia* single-cells (46, 48) and *Dehalococcoidia* metagenomes (144) revealed metabolic features, which allow for an organohalide respiration-independent mode of living. In *Dehalococcoidia* SAG-C11 from sediments of the Aarhus Bay a gene coding for a reductive dehalogenase subunit A was identified however a TAT leader sequence and a gene coding for the membrane anchor RdhB were not detected suggesting the RdhA is not involved in respiratory functions in SAG-C11. However, genes coding for dissimilatory sulphite reductase subunits were identified (personal communication, Dr. Kenneth Wasmund, UFZ, Leipzig). The reductive type of dissimilatory sulphite reductases have been described in the bacterial phyla *Proteobacteria* (*Delta-Proteobacteria*)*, Firmicutes, Nitrospirae, Thermodesulfobacteria*, *Caldiserica* and *Actinobacteria,* and the archaeal phyla *Euryarchaeota* and *Crenarchaeota* and the candidate division *Aigarchaeota* (146-149, 202) but never in the phylum *Chloroflexi*. In addition, phylogenetic analysis of environmental *dsrAB* sequences revealed many lineages without any cultivated representatives suggesting that several dissimilatory sulphite reducing microorganisms have not yet been identified (150, 201). Furthermore, sediment sites in which high sulphate reduction activity was described did not reveal high *dsrA* gene copy numbers or 16S rRNA genes of sulphate reducing prokaryotes (49, 163) suggesting that currently available primers do not amplify full *dsr* gene diversity. Primers used in the current study allowed for the amplification of *dsrA*, *dsrAB* or *dsrN*

sequences which clustered together with the dissimilatory sulphite reductase genes of *Dehalococcoidia* SAG-C11 (Figure 3-18 and Figure 3-19) indicating the phylum *Chloroflexi* contributes to *dsr* gene diversity and suggesting that dissimilatory sulphite reduction occurs in a broader variety of prokaryotes than previously recognized.

The occurrence and diversity of dissimilatory sulphite reductase genes related to *Chloroflexi* was investigated by molecular tools because previous attempts to enrich *Chloroflexi* by cultivation in the current and in previous studies remained unsuccessful. For the molecular investigation different sediments were chosen in which the *Dehalococcoidia* subgroup of SAG-C11 was detected. SAG-C11 belongs to the DSC-GIF3-B subgroup and was shown to represent about 4% of *Dehalococcoidia* 16S rRNA gene sequences in the sediment core from 10 to 300 cmbsf at the Aarhus Bay site (47). The DSC-GIF3-B subgroup was also described in the sediment core 371 of the Baffin Bay (47). It was suggested that similar biogeochemical conditions at the two sites might explain the similar distribution (47). Sediments from the core of the Bay of Aarhus and core 371 from the Baffin Bay exhibited similar TOC ranges through the depths of the cores (47, 200). Most of the analysed DsrA or DsrB sequences obtained in the current study from sediments of the Aarhus Bay, the Baffin Bay and the DsrA sequence obtained from the Wadden Sea, formed stable cluster with DsrA or DsrB sequences of *Dehalococcoidia* SAG-C11. The occurrence of the DSC-GIF3-B subgroup in different sediment sites (47) suggests the potential for sulphite reduction by members of the class *Dehalococcoidia* could be even more widespread in different sediment sites and could be investigated by primers developed in the current study.

Several *dsr* sequences amplified in the current study derived from sediments of the Bay of Aarhus affiliated with environmental sequences previously termed ‘uncultured DrsAB lineage 3’ (150). In addition, two of the amplified sequences from the Baffin Bay affiliated with DsrA or DsrB sequences previously termed ‘uncultured DsrAB lineage 2’. ‘Uncultured *dsrAB* lineage 2’ contains *dsrAB* sequences derived from sediments of 500 cmbsf with very low sulphate concentrations (152). For these sequences, it remains to be elucidated whether they belong to the phylum *Chloroflexi* or to a so far unidentified bacterial division.

Beside phylogenetic analysis of the *dsr* genes by different treeing methods, additional evidence for linking dissimilatory sulphite reductase genes amplified from marine sediments with *Dehalococcoidia* was gained by the analysis of *luxR* genes co-amplified with *dsrAB*. In SAG-C11 *dsr* genes were flanked on both sides by genes previously described in *Dehalogenimonas* (Figure 3-16 page 69), including the *luxR* gene and genes involved in siroheme synthesis, required for bacterial sulphur metabolism (159). All obtained LuxR amino acid sequences amplified on one fragment with *dsrAB* had highest sequence similarity to LuxR of SAG-C11 suggesting *dsrAB* sequences amplified with *luxR* belong to *Dehalococcoidia*.

Additionally, a fragment coding for *dsrABDN* was amplified. Because this fragment did not code for any marker genes previously identified in *Dehalogenimonas* an additional link between

dissimilatory sulphite reductase genes amplified from sediments and dissimilatory sulphite reductase genes detected in *Dehalococcoidia* SAG-C11 was provided by analysis of the dissimilatory sulphite reductase gene order within the amplified fragment. Different organization of the dissimilatory sulphite reductase genes within different sulphate reducing Bacteria or Archaea have been described previously (156). For instance, in *Thermodesulforhabdus norvegica* dissimilatory sulphite reductase genes follow the order *dsrD-dsrA-dsrB-dsrN-dsrC* (264), whereas in *Desulfotomaculum acetoxidans* the order is *dsrC-dsrN-dsrA-dsrB-dsrD*, in *Syntrophobacter fumaroxidans dsrA-dsrB-dsrN-dsrC*. In *Archaeoglobus fulgidus* a so far uncharacterized archaeal protein succeeds *dsrA* and *dsrB* and *drsD*. Sequencing of several fragments amplified from marine sediments with internal primers in the current study revealed the same organization of *dsr* genes as detected in DEH-SAG-C11, however *dsr* gene organization was also similar to the *dsr* gene organization in *Caldiserica*. Both show a *dsr* gene order of *dsrA-dsrB-dsrD-dsrN-dsrC-dsrM-dsrK* (202) of which *dsrA* to *dsrC* were amplified within the 3.9 kb fragment (Figure 3-16) from marine sediments in the current study. The genes coding for DsrJOP, detected in all genomes of sulphate reducing organisms so far analysed were not identified in the genome of SAG-C11 and the Gram-positive bacteria, *Caldivirga maquilingensis* (156, 265) and *Caldiserica* (202). Moreover, DsrA, DsrB and DsrN sequences of *Caldiserica* sp. affiliated closely in phylogenetic analysis with the DsrA, DsrB or DsrN sequences amplified from sediments in the current study. Direct comparison of *dsr* genes sequences amplified from sediments with *dsr* sequences of *Caldiserica* and DEH-SAG-C11 revealed higher sequence identities for *dsr* genes of SAG-C11 than for *dsr* genes of *Caldiserica,* suggesting dissimilatory sulphite reductase genes amplified from sediments origin from *Dehalococcoidia*. In addition, the GC content of *dsrAB* fragments amplified in the current study is highly similar to the one of the SAG-C11 genome whereas the GC content is more than 10% different to the GC content of the genome of *Caldiserica.* Interestingly, *dsrAB* genes of *Caldiserica* showed also high GC content divergence to their own genome with a GC content of the genome of 35% compared to the GC content of *Caldiserica dsrAB* of 44.1%. *Caldiserica* sp. may have acquired *dsr* genes *via* a horizontal gene transfer (150). Possibly, *Caldiserica* sp. could have acquired its *dsrAB* sequence from a *Chloroflexi*-related ancestor or both species acquired *dsr* genes by horizontal gene transfer from a *Firmicutes* related ancestor.

The comparison of *dsrAB* sequences amplified in the current study with *dsrAB* sequences of SAG-C11 suggested, at least another species and possibly another genus level within the class *Dehalococcoidia* harbours the potential for dissimilatory sulphite reduction. This was deduced from correlation of *dsrAB* sequence identities with 16S rRNA identities as described previously (150, 203). 16S rRNA and DsrAB phylogenies have been described as largely congruent, except for several distinct lateral gene transfers (150) and evolutionary relationships may be inferred to a certain extent based on the *dsrAB* gene sequences, similar to the ones inferred from 16S rRNA sequences (266, 267). Therefore, *dsrAB* sequences have been used as phylogenetic marker to investigate sulphate reducer diversity (151, 157, 158, 268-270). A 16S rRNA gene sequence similarity of less than 99% was

proposed as threshold for delineating species level operational taxonomic units (266). Based on this a *dsrAB* sequence identity of less than 90% was recommended as a threshold which will likely represent two different species (150). Analysed *dsrAB* sequences amplified in the current study revealed 70% to 100% sequence identity with *dsrAB* of SAG-C11 suggesting *dsr* genes of another *Dehalococcoidia* species were amplified in the current study. In addition, 71% to 87% *dsrAB* gene identity has been related to 86.5% to 94.5 % 16S rRNA identity (150). Yarza and colleagues suggested that a 16S rRNA sequence identity of 94.5% or lower provide strong evidence for two distinct genera (267). Therefore it seems likely that *dsr* sequences of another *Dehalococcoidia* genus were amplified in the current study.

Together the amplification of diverse dissimilatory sulphite reductase genes related with *Chloroflexi* indicated that the potential for sulphite respiration is not restricted to one species within the class *Dehalococcoidia,* phylum *Chloroflexi.* The findings provide further evidence that the class *Dehalococcoidia* is metabolically versatile and contribute to a deeper understanding of the ubiquitous distribution of *Dehalococcoidia* in marine sediments and the stratification of *Dehalococcoidia* according to bio geo chemical gradients in diverse sediment environments (47).

4.5 Implication for *Dehalococcoidia* from marine sediments

The broad range of halogenated electron acceptors of strain CBDB1 identified in the current and in previous studies (7, 118, 119, 121, 124, 193) exceed the number of reductive dehalogenases present in the genome of strain CBDB1 (26, 29), indicating that each reductive dehalogenase catalyses the dehalogenation of various halogenated molecules. The interaction of the cobalamin cofactor with the halogen substituent instead of the halogen-substituted carbon might allow for a less specific interaction of the reductive dehalogenase with the substrate. A broad spectrum of halogenated compounds as electron acceptors for organohalide-respiring *Dehalococcoidia* suggests that a multitude of natural organohalogens in the environment could serve as natural halogenated electron acceptor for respiration and growth of *Dehalococcoidia* even if the individual halogenated compounds are present in very low quantities. Haloorganic soil humic acids (271, 272) were detected for instance in humic forest lake sediments (273) and peatlands (274). In addition, a huge diversity of brominated and chlorinated compounds produced by marine and terrestrial organisms (235) and even bacteria (275) were described to contain similar or the same chemical structures as electron acceptors tested in the current study. Halogenated electron acceptors introduced into sediments by precipitation and decay of dead marine organisms or by living organisms releasing brominated compounds into the sediments (227) could allow for organohalide respiration at low rates and continuous metabolic activity of *Dehalococcoidia.* Together, these compounds could provide an ecological niche for *Dehalococcoidia* subgroups depending on organohalide respiration. Members of the subgroup “Ord-DEH” to which all so far isolated *D. mccartyi* strains belong was shown to occur in marine sediments mostly in upper

sedimentary levels, except for sediments from the Peru margin where this group was detected up to 30 mbsf (47). Site-specific variation might be also due to different sediment types, which accumulated over geological time scales. An investigation of the organohalogen content of these sites could elucidate this question. However, the detected reductive dehalogenase diversity in marine sediments (60, 61) does not necessarily indicate diversity of *Dehalococcoidia* species as even one single strain can harbour many reductive dehalogenase genes (26, 29). Therefore, the investigation of *rdhA* genes and would need to be combined with analysis of 16S rRNA genes or single cell approaches to learn more about the diversity of organohalide-respiring *Dehalococcoidia* in marine sediments.

The analysis of metabolic features of *Dehalococcoidia* derived from marine sediments in previous studies revealed *Dehalococcoidia* are metabolically versatile (46, 48, 144) and the findings of the current study demonstrated that the potential for organohalide-independent respiration is more widespread in the class *Dehalococcoidia*. Possibly, the enrichment of *Dehalococcoidia* with an organohalide respiration-independent mode of living remained so far unsuccessful because strongly selecting cultivation conditions have not been identified so far. The organohalide-respiring mode of living of cultivated members of the class *Dehalococcoidia* provided the possibility to use organohalogens as a selecting parameter for enrichment and isolation. Because isolated *Dehalococcoidia* strains grow slowly and to only low cell densities, an enrichment of their marine relatives with strategies using more common and less toxic electron acceptors may be extremely difficult. Faster growing bacterial species might outcompete *Dehalococcoidia*. Possibly cultivation of single cells could provide a tool to cultivate *Dehalococcoidia* with an organohalide respiration-independent mode of living (276, 277).

5 Conclusion

Dehalococcoidia have been shown in the current study to dehalogenate diverse halogenated electron acceptors containing different halogen and non-halogen substituents and heteroatoms. The number of identified electron acceptors exceeds the number of reductive dehalogenase homologues in the genome of strain CBDB1 indicating single reductive dehalogenases transform several halogenated substrates each. A low substrate specificity of reductive dehalogenases leading to a versatile electron acceptor range could be explained in part by the cobalamin–halogen interaction proposed in the current study. The diverse range of halogenated electron acceptors transformed by *Dehalococcoidia* – shown in the current study for *D. mccartyi* strain CBDB1 – would allow organohalide-respiring *Dehalococcoidia* to occupy a broader niche in natural environments than anticipated. Growth with brominated electron acceptors shown for the model strain CBDB1 in the current study suggests brominated compounds could serve in marine sediments to sustain growth of *Dehalococcoidia* depending on organohalide respiration. For *Dehalococcoidia* growing independently from organohalide respiration, sulphite respiration could contribute to the broad diversity and stratification of *Dehalococcoidia* along geochemical gradients. Functional genes associated with sulphite respiration were linked to *Dehalococcoidia* in the current study and were shown to be diverse and distributed in various sediment environments. Together the diverse electron acceptor range and modes of respiration catalysed by *Dehalococcoidia* described in the current study allow for a better understanding of the ubiquitous and abundant occurrence of *Dehalococcoidia* in marine sediments. In addition, the findings of the current study may allow for enhanced fate prediction of halogenated compounds from natural and anthropogenic sources in anaerobic sediment environments.

6 Outlook

To further explore growth of *Dehalococcoidia* with natural halogenated compounds, the cultivation of strain CBDB1 may be expanded in the future onto halogenated molecules with more complex ring structures. In addition, the expression of enzymes during cultivation with complex structures and with compounds containing different functional groups may reveal further reasons for the reductive dehalogenase diversity harboured in genomes of *D. mccartyi* strains. The role of the reductive dehalogenase diversity will be also clarified by recent successful heterologous expressions of functional reductive dehalogenases (99), which will allow for a further electron acceptor characterization of single purified reductive dehalogenases. These findings can be combined with the chemical descriptors identified in the current study to verify and compare the influence of enzymatic vs. chemical properties of the electron acceptor. A special focus could be placed onto different heteroaromatics with the same heteroatom integrated in different ring systems, such as for instance electron-poor pyridine and electron-rich pyrrol, to elucidate further the mechanism governing reductive dehalogenation. The halogenation of large and complex ring structures such as humic acids by haloperoxidases and subsequent cultivation of strain CBDB1 may provide further information about the significance of a halogen–cobalamin interaction for the usage of large and complex aromatic organohalogen structures.

Additionally, the influence of functional groups onto dehalogenation pathways but also growth should be studied regarding toxic and inhibitory effects as observed in the current and in previous studies for the hydroxy group. Other functional groups may exhibit toxic effects similar to the hydroxy group of phenols. To investigate toxic or inhibitory effects further, *D. mccartyi* strain CBDB1 could be monitored for changes on the mRNA or protein expression level using for instance halogenated and non-halogenated anisols and phenols in parallel cultivation experiments. In addition, an evaluation of toxic effects of obtained dehalogenation products onto the dehalogenating strain could be investigated by addition of these reaction products to cultures together with a non-toxic halogenated electron acceptor and comparison of growth and dehalogenation rates. Furthermore, ecotoxicological assays with the transformation products of reductive dehalogenation by *Dehalococcoides* strains using prokaryotic and eukaryotic model systems could allow for a better understanding of the benefit of dehalogenation reactions for a reduction of toxicity of halogenated contaminants.

Also the combination of various strains with specific functions permitting the mineralization of the tested halogenated compounds could be an interesting field of investigation.

For the activity assay a more extensive investigation of the influence of the electron acceptor used during cultivation onto activity measured in the *in vitro* assay and a standardization of the cell growth status at the moment of cell harvest could help to standardize the activity assay further. At present measured activities are related to whole cell proteins, however this does not necessarily reflect the amount of reductive dehalogenases expressed at the moment of cell harvest and the activity of the

enzymes according to the growth phase of the microbial inoculum. This introduces variations in measured activities, even though not relative but absolut. However, the usage of single purified reductive dehalogenases will permit a quick improvement and further standardization of the activity assay.

Furthermore, the partial charge model developed in the current study will need to be verified for its stability towards changes of the basis sets and for an even broader electron acceptor range. Here a special focus should be placed on the identification of a threshold predicting whether a compound is dehalogenated or not by a specific strain. Possibly, such identification is only possible for one substance class and not for many different. Therefore, the investigation of dehalogenation pathways of different highly halogenated compounds could provide further experimental data as a basis for the evaluation of chemical descriptors. When purified reductive dehalogenases are available also a comparison of dehalogenation rates in activity assays with partial charges will lead to a better understanding of the importance of partial charge distributions onto dehalogenation. A broad set of different dehalogenating strains of the species *D. mccartyi*, other dehalogenating members of the class *Dehalococcoidia* and different other dehalogenating microorganism will require further studies using different chemical descriptors to identify which parameters influence reductive dehalogenation for other strains with different dehalogenation patterns. This may lead to different mechanistic models in other microorganisms.

For *Dehalococcoidia* from marine sediments the newly gained information about their versatile metabolism offers the possibility to set up long-term cultivation experiments designed for specific *Dehalococcoidia* subgroups. For *Dehalococcoidia* subgroups that are not depending on organohalide respiration, cultivation set-ups using the genomic information from single amplified genomes of *Dehalococcoidia* could be conducted and dissimilatory sulphite reductase activity could be tested by using sulphate, sulphite or organosulphonates as electron acceptors. For organohalide-respiring *Dehalococcoidia* brominated compounds could be used for enrichment, as they may allow organohalide-respiring *Dehalococcoidia* to grow even if reductive dehalogenases with less reduction potential than those of strain CBDB1 are encoded in their genomes.

An additional interesting field could be the investigation of the role of cytoplasmic reductive dehalogenase subunit A, which has been detected in SAG-C11 but also in *Dehalogenimonas* strains (20). Both strains possess LuxR transcriptional regulator, which have been suggested to be inhibited for instance by halogenated furanones produced by marine algae (132). The half-life of the protein LuxR was shown to be reduced 100-fold by halogenated furanones (132). An investigation of cytoplasmic reductive dehalogenase homologs for their potential role in detoxification of molecules, or in providing aromatic compounds for degradation pathways could elucidate the role of reductive dehalogenase likely active in the cytoplasm of members of the *Dehalococcoidia.*

Table 7-1: Mulliken Q_C and Q_X values for homo- and heteroaromatic compounds tested experimentally in this study. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1.

	C2		*C3*		*C4*		*C5*		*C6*	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
2,3-DCA	**-0.130**	**0.005**	-0.099	0.005						
2,4-DCA	-0.104	-0.020			-0.101	-0.025				
2,6-DCA	-0.133	-0.023							-0.133	-0.023
2,3-DCBN	**-0.104**	**0.079**	-0.100	0.036						
2,5-DCBN	**-0.107**	**0.051**					-0.106	0.013		
2,6-DCBN	**-0.102**	**0.052**							**-0.102**	**0.052**
2-CBN	**-0.109**	**0.039**								
2-C-6-FBN	**-0.107**	**0.050**							0.362	-0.261
2,4,6-TBPh	**0.020**	**-0.104**			0.047	-0.107			**0.022**	**-0.076**
2,4,6-TBPhate	**-0.051**	**-0.218**			0.01	-0.236			-0.05	-0.218
2,4-DBPh	**0.024**	**-0.092**			0.048	-0.123				
2,4-DBPhate	**-0.052**	**-0.243**			0.008	-0.259				
2,6-DBPh	**0.020**	**-0.093**							**0.022**	**-0.120**
2,6-DBPhate	-0.048	-0.243							-0.048	-0.243
2-BPh	**0.023**	**-0.110**								
2-BPhate	-0.050	-0.272								
4-BPh					**0.047**	**-0.139**				
4-BPhate					0.007	-0.284				
4-B-3,5-DMBA					**-0.044**	**-0.077**				
4-B-3,5-DHBA					**-0.020**	**-0.093**				
2,3-DCTh	**-0.305**	**0.056**	-0.043	0.025						
2,5-DCTh	**-0.307**	**0.037**					**-0.307**	**0.037**		
2-CTh	**-0.299**	**0.025**								
4,5-DB-2-FA					0.007	-0.074	0.293	-0.029		
5-B-2-FA							0.296	-0.051		
2,3-DCPy	0.106	0.031	-0.085	0.031						
2,6-DCPy	0.092	0.013							0.092	0.013
3-B-2-CPy	0.083	0.033	**0.066**	**-0.079**						

Table 7-2: Mulliken Q_C and Q_X values for selected chloro- and bromobenzenes. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1.

	C1		C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
HCB	**-0.076**	**0.076**	**-0.076**	**0.076**	**-0.076**	**0.076**	-0.076	**0.076**	**-0.076**	**0.076**	**-0.076**	**0.076**
PCB	-0.083	0.044	**-0.078**	**0.070**	**-0.079**	**0.071**	**-0.078**	**0.070**	-0.083	0.044		
1,2,3,5-TeCB	-0.086	0.038	**-0.081**	**0.063**	-0.086	0.038			-0.092	0.013		
1,2,4,5-TeCB	**-0.086**	**0.037**	**-0.086**	**0.037**			**-0.086**	**0.037**	**-0.086**	**0.037**		
1,2,3-TCB	-0.086	0.024	**-0.082**	**0.054**	-0.086	0.024						
1,2,4-TCB	**-0.088**	**0.026**	**-0.087**	**0.030**			-0.093	0.001				
1,2,4-TBB	**0.044**	**-0.083**	**0.041**	**-0.078**			0.053	-0.105				
1,3,5-TCB	-0.095	0.006			-0.095	0.006			-0.095	0.006		
1,2-DCB	-0.088	0.014	-0.088	0.014								
1,2-DBB	**0.042**	**-0.094**	**0.042**	**-0.094**								
1,3-DCB	-0.094	-0.010			-0.094	-0.010						
1,3-DBB	**0.049**	**-0.114**			**0.049**	**-0.114**						
1,4-DCB	-0.094	-0.013					-0.094	-0.013				
1,4-DBB	**0.054**	**-0.119**					**0.054**	**-0.119**				
MCB	-0.093	-0.027										
MBB	**0.053**	**-0.133**										

Table 7-3: Mulliken Q_C and Q_X values for chlorophenols. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1. Partial charges were given in italics when the dehalogenation of chlorophenols was slowly and incompletely.

	C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
PeCPh	**-0.131**	**0.069**	**-0.088**	**0.068**	**-0.084**	**0.063**	**-0.088**	**0.068**	**-0.125**	**0.037**
2,3,4,5-TeCPh	**-0.125**	**0.059**	**-0.090**	**0.061**	**-0.085**	**0.055**	-0.095	0.032		
2,3,4,6-TeCPh	**-0.125**	**0.063**	**-0.091**	**0.061**	-0.089	0.030			-0.138	0.005
2,3,5,6-TeCPh	**-0.137**	**0.030**	-0.095	0.036			-0.095	0.035	**-0.128**	**0.061**
2,3,4-TCPh	**-0.139**	**0.019**	**-0.089**	**0.053**	-0.094	0.018				
2,3,5-TCPh	**-0.142**	**0.021**	-0.095	0.029			-0.102	0.002		
2,3,6-TCPh	**-0.127**	**0.053**	-0.097	0.023					-0.143	-0.008
2,4,5-TCPh	**-0.142**	**-0.005**			**-0.092**	**0.022**	**-0.096**	**0.027**		
2,4,6-TCPh	**-0.139**	**-0.002**			-0.097	-0.002			**-0.130**	**0.029**
3,4,5-TCPh			-0.095	0.024	**-0.089**	**0.048**	-0.096	0.027		
2,3-DCPh	**-0.143**	**0.010**	-0.095	0.014						
2,4-DCPh	*-0.131*	*0.015*			-0.097	-0.017				
2,5-DCPh	*-0.134*	*0.010*					-0.103	-0.014		
2,6-DCPh	-0.133	0.013							-0.141	*-0.018*
3,4-DCPh			*-0.096*	*0.017*	*-0.095*	*0.008*				
3,5-DCPh			-0.102	-0.009			-0.103	-0.007		
2-CPh	-0.134	-0.003								
3-CPh			-0.101	-0.024						
4-CPh					-0.100	-0.032				

Table 7-4: Mulliken Q_C and Q_X values for chlorophenolates. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1. Partial charges were given in italics when the dehalogenation of chlorophenolates was slowly and incompletely.

	C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
PeCPhate	**-0.1868**	**-0.0585**	**-0.0689**	**-0.0396**	**-0.1197**	**-0.0446**	**-0.0689**	**-0.0396**	**-0.1868**	**-0.0585**
2,3,4,5-TeCPhate	**-0.1890**	**-0.0732**	**-0.0709**	**-0.0500**	**-0.0729**	**-0.0550**	-0.0729	-0.0878		
2,3,4,6-TeCPhate	**-0.1928**	**-0.0686**	**-0.0711**	**-0.0511**	-0.1241	-0.0871			-0.1868	-0.1078
2,3,5,6-TeCPhate	**-0.1883**	**-0.0708**	-0.0744	-0.0845			-0.0744	-0.0845	**-0.1883**	**-0.0708**
2,3,4-TCPhate	**-0.1945**	**-0.0873**	**-0.0712**	**-0.0645**	**-0.1289**	**-0.1054**				
2,3,5-TCPhate	**-0.1908**	**-0.0857**	-0.0762	-0.0962			-0.0787	-0.1346		
2,3,6-TCPhate	**-0.1921**	**-0.0827**	-0.0739	-0.1027					-0.1890	-0.1268
2,4,5-TCPhate	**-0.1882**	**-0.1252**			**-0.1289**	**-0.0987**	**-0.0746**	**-0.1011**		
2,4,6-TCPhate	**-0.1921**	**-0.1193**			-0.1301	-0.1307			**-0.1921**	**-0.1193**
3,4,5-TCPhate			-0.0747	-0.1009	**-0.1302**	**-0.0658**	-0.0747	-0.1009		
2,3-DCPhate	**-0.1953**	**-0.1027**	-0.0723	-0.1196						
2,4-DCPhate	*-0.1931*	*-0.1417*			-0.1330	-0.1516				
2,5-DCPhate	*-0.1903*	*-0.1449*					-0.0754	-0.1566		
2,6-DCPhate	*-0.1912*	*-0.1424*							*-0.1912*	*-0.1424*
3,4-DCPhate			*-0.0735*	*-0.1186*	*-0.1348*	*-0.1180*				
3,5-DCPhate			-0.0797	-0.1495			-0.0797	-0.1495		
2-CPhate	-0.1923	-0.1672								
3-CPhate			-0.0719	-0.1789						
4-CPhate					-0.1361	-0.1748				

Table 7-5: Hirshfeld QC and QX values for homo- and heteroaromatic compounds tested experimentally in this study. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1.

	C2		C3		C4		C5		C6	
	Qc	QX	Qc	QX	Qc	QX	Qc	QX	Qc	QX
2,3-DCA	**0.008**	**-0.068**	0.029	-0.072						
2,4-DCA	-0.060	-0.078			0.020	-0.091				
2,6-DCA	0.016	-0.080							0.016	-0.080
2,3-DCBN	**0.043**	**-0.029**	0.036	-0.050						
2,5-DCBN	**0.047**	**-0.042**					0.040	-0.061		
2,6-DCBN	**0.051**	**-0.040**							**0.051**	**-0.040**
2-CBN	**0.048**	**-0.050**								
2-C-6-FBN	**0.051**	**-0.041**							0.104	-0.067
2,4,6-TBPh	**0.005**	**-0.087**			0.017	-0.103			**0.013**	-0.097
2,4,6-TBPhate										
2,4-DBPh	**0.010**	**-0.109**			0.014	-0.117				
2,4-DBPhate										
2,6-DBPh	**0.002**	**-0.111**							**0.009**	**-0.101**
2,6-DBPhate										
2-BPh	**-0.026**	**-0.125**								
2-BPhate										
4-BPh					**-0.039**	**-0.131**				
4-BPhate										
4-B-3,5-DMBA					**-0.003**	**-0.120**				
4-B-3,5-DHBA					**-0.012**	**-0.096**				
2,3-DCTh	**-0.009**	**-0.041**	0.006	-0.060						
2,5-DCTh	**-0.001**	**-0.051**					**-0.001**	**-0.051**		
2-CTh	**-0.003**	**-0.061**								
4,5-DB-2-FA					-0.006	-0.058	0.095	-0.013		
5-B-2-FA							0.085	**-0.038**		
2,3-DCPy	0.102	-0.066	0.030	-0.057						
2,6-DCPy	0.113	-0.071							0.113	-0.071
3-B-2-CPy	0.115	-0.047	**0.015**	**-0.096**						

Table 7-6: Hirshfeld Q_C and Q_X values for selected chloro- and bromobenzenes. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1.

	C1		C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
HCB	**0.029**	**-0.029**	**0.029**	**-0.029**	**0.029**	**-0.029**	**0.029**	**-0.029**	**0.029**	**-0.029**	**0.029**	**-0.029**
PCB	0.035	-0.043	**0.027**	**-0.034**	**0.032**	**-0.032**	**0.027**	**-0.034**	0.035	-0.043		
1,2,3,5-TeCB	0.039	-0.047	**0.025**	**-0.040**	0.039	-0.047			0.041	-0.059		
1,2,4,5-TeCB	**0.034**	**-0.049**	**0.034**	**-0.049**			**0.034**	**-0.049**	**0.034**	**-0.049**		
1,2,3-TCB	0.034	-0.059	**0.027**	**-0.046**	0.034	-0.059						
1,2,4-TCB	**0.030**	**-0.059**	**0.036**	**-0.055**			0.039	-0.069				
1,2,4-TBB	**0.029**	**-0.079**	**0.033**	**-0.075**			0.026	-0.100				
1,3,5-TCB	0.045	-0.064			0.045	-0.064			0.045	-0.064		
1,2-DCB	0.031	-0.067	0.031	-0.067								
1,2-DBB	**0.029**	**-0.089**	**0.029**	**-0.089**								
1,3-DCB	0.040	-0.077			0.040	-0.077						
1,3-DBB	**0.028**	**-0.108**			**0.028**	**-0.108**						
1,4-DCB	0.035	-0.080					0.035	-0.080				
1,4-DBB	**0.024**	**-0.112**					**0.024**	**-0.112**				
MCB	0.035	-0.091										
MBB	**0.024**	**-0.125**										

Table 7-7: Hirshfeld Q_C and Q_X values for chlorophenols. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1. Partial charges were given in italics when the dehalogenation of chlorophenols was slowly and incompletely.

	C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
PeCPh	**0.007**	**-0.039**	**0.029**	**-0.033**	**0.017**	**-0.041**	**0.027**	**-0.033**	**0.018**	**-0.030**
2,3,4,5-TeCPh	**0.016**	**-0.047**	**0.031**	**-0.038**	**0.015**	**-0.047**	0.032	-0.052		
2,3,4,6-TeCPh	**0.020**	**-0.043**	**0.028**	**-0.039**	0.024	-0.056			0.014	-0.047
2,3,5,6-TeCPh	**0.005**	**-0.036**	0.032	-0.048			0.035	-0.048	**0.016**	**-0.045**
2,3,4-TCPh	**0.007**	**-0.044**	**0.027**	-0.044	0.020	-0.065				
2,3,5-TCPh	**0.003**	**-0.044**	0.035	-0.053			0.041	-0.067		
2,3,6-TCPh	**0.018**	**-0.050**	0.032	-0.058					0.010	-0.057
2,4,5-TCPh	**0.012**	**-0.056**			**0.021**	**-0.062**	**0.034**	**-0.056**		
2,4,6-TCPh	**0.017**	**-0.052**			0.029	-0.072			**0.027**	**-0.059**
3,4,5-TCPh			0.035	-0.057	**0.013**	**-0.053**	0.037	-0.056		
2,3-DCPh	**0.004**	**-0.052**	0.031	-0.065						
2,4-DCPh	*0.023*	*-0.070*			0.025	-0.084				
2,5-DCPh	*0.018*	*-0.074*					0.035	-0.080		
2,6-DCPh	*0.022*	*-0.071*							*0.012*	*-0.064*
3,4-DCPh			*0.034*	*-0.064*	*0.017*	*-0.074*				
3,5-DCPh			0.040	-0.075			0.042	-0.074		
2-CPh	0.018	-0.084								
3-CPh			0.038	-0.087						
4-CPh					0.021	-0.096				

Table 7-8: Hirshfeld Q_C and Q_X values for chlorophenolates. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1. Partial charges were given in italics when the dehalogenation of chlorophenolates was slowly and incompletely.

	C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
PeCPhate	**-0.0196**	**-0.1307**	**0.0041**	**-0.1143**	**-0.0326**	**-0.1258**	**0.0041**	**-0.1143**	**-0.0196**	**-0.1307**
2,3,4,5-TeCPhate	**-0.0223**	**-0.1418**	**0.0050**	**-0.1218**	**-0.0368**	**-0.1345**	0.0066	-0.1423		
2,3,4,6-TeCPhate	**-0.0193**	**-0.1381**	**0.0014**	**-0.1238**	-0.0292	-0.1475			-0.0145	-0.1579
2,3,5,6-TeCPhate	**-0.0233**	**-0.1400**	0.0070	-0.1385			0.0070	-0.1385	**-0.0233**	**-0.1400**
2,3,4-TCPhate	**-0.0240**	**-0.1524**	**0.0011**	**-0.1338**	-0.0354	-0.1623				
2,3,5-TCPhate	**-0.0265**	**-0.1512**	0.0077	-0.1476			0.0094	-0.1702		
2,3,6-TCPhate	**-0.0230**	**-0.1489**	0.0030	-0.1537					-0.0201	-0.1733
2,4,5-TCPhate	**-0.0175**	**-0.1712**			**-0.0338**	**-0.1572**	**0.0036**	**-0.1533**		
2,4,6-TCPhate	-0.0142	-0.1669			-0.0261	-0.1725			-0.0142	-0.1669
3,4,5-TCPhate			0.0072	-0.1522	**-0.0416**	**-0.1435**	0.0072	-0.1522		
2,3-DCPhate	**-0.0284**	**-0.1642**	0.0023	-0.1664						
2,4-DCPhate	*-0.0193*	*-0.1841*			-0.0325	-0.1894				
2,5-DCPhate	*-0.0236*	*-0.1872*					0.0054	-0.1882		
2,6-DCPhate	*-0.0201*	*-0.1850*							*-0.0201*	*-0.1850*
3,4-DCPhate			*0.0028*	*-0.1669*	*-0.0407*	*-0.1729*				
3,5-DCPhate			0.0099	-0.1819			0.0099	-0.1819		
2-CPhate	-0.0257	-0.2040								
3-CPhate			0.0045	-0.2054						
4-CPhate					-0.0396	-0.2079				

Table 7-9: NBO Q_C and Q_X values for homo- and heteroaromatic compounds tested experimentally in this study. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1.

	C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
2,3-DCA	**-0.110**	**0.008**	-0.049	0.019						
2,4-DCA	-0.074	-0.013			-0.069	-0.007				
2,6-DCA	-0.075	-0.015							-0.075	-0.015
2,3-DCBN	**-0.035**	**0.070**	-0.067	0.043						
2,5-DCBN	**-0.017**	**0.047**					-0.052	0.023		
2,6-DCBN	**-0.001**	**0.050**							**-0.001**	**0.050**
2-CBN	**-0.010**	**0.038**								
2-C-6-FBN	**-0.002**	**0.048**							**0.484**	**-0.312**
2,4,6-TBrPh	**-0.162**	**0.072**			-0.123	0.077			-0.147	0.102
2,4,6-TBrPhate	**-0.185**	**-0.019**			-0.170	-0.034			**-0.185**	**-0.019**
2,4-DBPh	**-0.156**	**0.089**			-0.130	0.063				
2,4-DBrPhate	**-0.191**	**-0.043**			-0.177	-0.055				
2,6-DBrPh	**-0.169**	**0.057**							**-0.156**	**0.088**
2,6-DBrPhate	**-0.191**	**-0.043**							**-0.191**	**-0.043**
2-BrPh	**-0.165**	**0.073**								
2-BrPhate	**-0.196**	**-0.070**								
4-BrPh					**-0.138**	**0.049**				
4-BPhate					**-0.185**	**-0.078**				
4-Br-3,5-DMBA					**-0.182**	**0.106**				
4-Br-3,5-DHBA					**-0.221**	**0.083**				
2,3-DCTh	**-0.305**	**0.071**	-0.124	0.037						
2,5-DCTh	**-0.285**	**0.055**					**-0.285**	**0.055**		
2-CTh	**-0.287**	**0.046**								
4,5-DBr-2-FA					-0.249	0.127	0.182	0.161		
5-B-2-FA							0.187	0.139		
2,3-DCPy	0.174	0.026	-0.106	0.039						
2,6-DCPy	0.198	0.013							0.198	0.013
3-Br-2-CPy	0.175	0.025	**-0.177**	**0.107**						

Table 7-10: NBO Q_C and Q_X values for selected chloro- and bromobenzenes. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1.

	C1		C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
HCB	**-0.077**	**0.077**	**-0.077**	**0.077**	**-0.077**	**0.077**	**-0.077**	**0.077**	**-0.077**	**0.077**	**-0.077**	**0.077**
PCB	-0.054	0.051	**-0.087**	**0.071**	**-0.070**	**0.073**	**-0.087**	**0.071**	-0.054	0.051		
1,2,3,5-TeCB	-0.047	0.047	**-0.098**	**0.065**	-0.047	0.047			-0.033	0.025		
1,2,4,5-TeCB	**-0.065**	**0.045**	**-0.065**	**0.045**			**-0.065**	**0.045**	**-0.065**	**0.045**		
1,2,3-TCB	-0.056	0.035	**-0.092**	**0.058**	-0.056	0.035						
1,2,4-TCB	**-0.074**	**0.035**	**-0.058**	**0.038**			-0.041	0.014				
1,2,4-TBB	**-0.142**	**0.102**	**-0.127**	**0.105**			-0.108	0.079				
1,3,5-TCB	-0.025	0.020			-0.025	0.020			-0.025	0.020		
1,2-DCB	-0.068	0.026	-0.068	0.026								
1,2-DBB	**-0.136**	**0.091**	**-0.136**	**0.091**								
1,3-DCB	-0.033	0.006			-0.033	0.006						
1,3-DBB	**-0.101**	**0.070**			**-0.101**	**0.070**						
1,4-DCB	-0.050	0.003					-0.050	0.003				
1,4-DBB	**-0.116**	**0.067**					**-0.116**	**0.067**				
MCB	-0.042	-0.009										
MBB	**-0.109**	**0.053**										

Table 7-11: NBO Q_C and Q_X values for chlorophenols. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1. Partial charges were given in italics when the dehalogenation of chlorophenols was slowly and incompletely.

	C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
PeCPh	**-0.123**	**0.067**	**-0.071**	**0.072**	**-0.097**	**0.065**	**-0.074**	**0.070**	**-0.106**	**0.036**
2,3,4,5-TeCPh	**-0.114**	**0.058**	**-0.066**	**0.067**	**-0.107**	**0.058**	-0.047	0.041		
2,3,4,6-TeCPh	**-0.100**	**0.063**	**-0.081**	**0.066**	-0.075	0.039			-0.102	0.010
2,3,5,6-TeCPh	**-0.134**	**0.029**	-0.052	0.044			-0.048	0.045	**-0.116**	**0.060**
2,3,4-TCPh	**-0.126**	**0.022**	**-0.078**	**0.057**	-0.084	0.029				
2,3,5-TCPh	**-0.143**	**0.022**	-0.045	0.038			-0.024	0.017		
2,3,6-TCPh	**-0.111**	**0.054**	-0.057	0.035					-0.111	-0.001
2,4,5-TCPh	**-0.110**	**0.001**			**-0.085**	**0.032**	**-0.057**	**0.037**		
2,4,6-TCPh	**-0.095**	**0.004**			-0.053	0.013			**-0.077**	**0.035**
3,4,5-TCPh			-0.040	0.036	**-0.118**	**0.051**	-0.041	0.038		
2,3-DCPh	**-0.138**	**0.013**	-0.054	0.026						
2,4-DCPh	*-0.086*	*0.023*			-0.061	0.000				
2,5-DCPh	*-0.102*	*0.019*					-0.032	0.003		
2,6-DCPh	*-0.087*	*0.022*							*-0.104*	*-0.009*
3,4-DCPh			*-0.052*	*0.028*	*-0.095*	*0.020*				
3,5-DCPh			-0.017	0.008			-0.018	0.009		
2-CPh	-0.096	0.008								
3-CPh			-0.027	-0.005						
4-CPh					-0.070	-0.013				

Table 7-12: NBO Q_C and Q_X values for chlorophenolates. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1. Partial charges were given in italics when the dehalogenation of chlorophenolates was slowly and incompletely.

	C2		C3		C4		C5		C6	
	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X	Q_C	Q_X
PeCPhate	**-0.157**	**-0.022**	**-0.075**	**-0.016**	**-0.159**	**-0.019**	**-0.075**	**-0.016**	**-0.157**	**-0.021**
2,3,4,5-TeCPhate	**-0.163**	**-0.034**	**-0.071**	**-0.024**	**-0.173**	**-0.028**	-0.046	-0.056		
2,3,4,6-TeCPhate	**-0.155**	**-0.029**	**-0.085**	**-0.026**	-0.133	-0.053			-0.129	-0.063
2,3,5,6-TeCPhate	**-0.169**	**-0.031**	-0.046	-0.052			-0.046	-0.052	**-0.169**	**-0.031**
2,3,4-TCPhate	**-0.162**	**-0.045**	**-0.081**	**-0.037**	-0.144	-0.068				
2,3,5-TCPhate	**-0.176**	**-0.044**	-0.041	-0.062			-0.015	-0.095		
2,3,6-TCPhate	**-0.166**	**-0.041**	-0.053	-0.068					-0.138	-0.078
2,4,5-TCPhate	**-0.134**	**-0.078**			**-0.148**	**-0.062**	**-0.056**	**-0.068**		
2,4,6-TCPhate	**-0.125**	**-0.072**			-0.107	-0.089			**-0.125**	**-0.072**
3,4,5-TCPhate			-0.040	-0.067	**-0.189**	**-0.036**	-0.040	-0.067		
2,3-DCPhate	**-0.174**	**-0.057**	-0.047	-0.082						
2,4-DCPhate	*-0.131*	*-0.091*			-0.116	-0.106				
2,5-DCPhate	*-0.144*	*-0.094*					-0.020	-0.115		
2,6-DCPhate	*-0.132*	*-0.091*							*-0.132*	*-0.091*
3,4-DCPhate			*-0.050*	*-0.083*	*-0.159*	*-0.078*				
3,5-DCPhate			-0.008	-0.108			-0.008	-0.108		
2-CPhate	-0.139	-0.112								
3-CPhate			-0.012	-0.134						
4-CPhate					-0.125	-0.124				

Table 7-13: NBO σ and π Q_X values of homo- and heteroaromatic compounds tested experimentally in this study. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1.

	C2		C3		C4		C5		C6	
	σ	π	σ	π	σ	π	σ	π	σ	π
2,3-DCA	**-0.052**	**0.059**	-0.049	0.068						
2,4-DCA	-0.068	0.055			-0.063	0.056				
2,6-DCA	-0.069	0.054							-0.069	0.054
2,3-DCBN	**-0.014**	**0.084**	-0.032	0.074						
2,5-DCBN	**-0.029**	**0.080**					-0.047	0.077		
2,6-DCBN	**-0.030**	**0.079**							**-0.030**	**0.079**
2-CBN	**-0.039**	**0.077**								
2-C-6-FBN	**-0.032**	**0.080**							-0.403	0.091
2,4,6-TBPh	**0.022**	**0.049**			0.021	0.055			**0.038**	**0.063**
2,4,6-TBPhate										
2,4-DBPh	**0.027**	**0.061**			0.010	0.053				
2,4-DBPhate										
2,6-DBPh	**0.027**	**0.060**							**0.010**	**0.047**
2,6-DBPhate										
2-BPh	**0.015**	**0.057**								
2-BPhate	**-0.104**	**0.033**								
4-BPh					**-0.002**	**0.050**				
4-BPhate					**-0.102**	**0.022**				
4-B-3,5-DMBA					**0.036**	**0.069**				
4-B-3,5-DHBA					**0.029**	**0.053**				
2,3-DCTh	**0.003**	**0.068**	-0.029	0.065						
2,5-DCTh	**-0.010**	**0.065**					**-0.010**	**0.065**		
2-CTh	**-0.018**	**0.063**								
4,5-DB-2-FA					0.081	0.079	0.068	0.059		
5-B-2-FA							**0.067**	**0.072**		
2,3-DCPy	-0.058	0.083	-0.035	0.074						
2,6-DCPy	-0.067	0.080							-0.067	0.080
3-B-2-CPy	-0.058	0.083	**0.037**	**0.068**						

Table 7-14: NBO σ and π Q_X values for selected chloro- and bromobenzenes. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1.

	C1		C2		C3		C4		C5		C6	
	σ	π	σ	π	σ	π	σ	π	σ	π	σ	π
HCB	**-0.006**	**0.082**	**-0.006**	**0.082**	**-0.006**	**0.082**	**-0.006**	**0.082**	**-0.006**	**0.082**	**-0.006**	**0.082**
PCB	-0.505	0.555	**-0.529**	**0.599**	**0.041**	**0.032**	**-0.529**	**0.599**	-0.505	0.555		
1,2,3,5-TeCB	-0.139	0.185	**-0.714**	**0.778**	-0.139	0.185			-0.735	0.759		
1,2,4,5-TeCB	**-0.513**	**0.557**	**-0.513**	**0.557**			**-0.513**	**0.557**	**-0.513**	**0.557**		
1,2,3-TCB	-0.571	0.605	**0.027**	**0.031**	-0.571	0.605						
1,2,4-TCB	**-0.037**	**0.071**	**-0.035**	**0.073**			-0.053	0.067				
1,2,4-TBB	**0.034**	**0.066**	**0.037**	**0.067**			0.017	0.061				
1,3,5-TCB	-0.050	0.069			-0.050	0.069			-0.050	0.069		
1,2-DCB	-0.045	0.070	-0.045	0.070								
1,2-DBB	**0.026**	**0.064**	**0.026**	**0.064**								
1,3-DCB	-0.200	0.206			-0.200	0.206						
1,3-DBB	**0.009**	**0.060**			**0.009**	**0.060**						
1,4-DCB	-0.743	0.746					-0.743	0.746				
1,4-DBB	**0.007**	**0.059**					**0.007**	**0.059**				
MCB	-0.747	0.738										
MBB	**-0.005**	**0.056**										

Table 7-15: NBO σ and π Q_X values for chlorophenols. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1. Partial charges were given in italics when the dehalogenation of chlorophenols was slowly and incompletely.

	C2		C3		C4		C5		C6	
	σ	π	σ	π	σ	π	σ	π	σ	π
PeCPh	**-0.024**	**0.060**	**-0.010**	**0.079**	**-0.009**	**0.073**	**-0.010**	**0.081**	**-0.009**	**0.076**
2,3,4,5-TeCPh	**-0.016**	**0.074**	**-0.014**	**0.081**	**-0.014**	**0.072**	-0.032	0.073		
2,3,4,6-TeCPh	**-0.013**	**0.075**	**-0.015**	**0.080**	-0.028	0.067			-0.046	0.055
2,3,5,6-TeCPh	**-0.029**	**0.058**	-0.030	0.073			-0.030	0.075	**-0.014**	**0.074**
2,3,4-TCPh	**-0.036**	**0.058**	**-0.020**	**0.077**	-0.036	0.065				
2,3,5-TCPh	**-0.035**	**0.057**	-0.034	0.073			-0.052	0.068		
2,3,6-TCPh	**-0.019**	**0.073**	-0.038	0.072					-0.054	0.053
2,4,5-TCPh	**-0.053**	**0.054**			**-0.034**	**0.065**	**-0.036**	**0.072**		
2,4,6-TCPh	**-0.051**	**0.055**			-0.049	0.061			**-0.034**	**0.069**
3,4,5-TCPh			-0.037	0.072	**-0.019**	**0.070**	-0.036	0.073		
2,3-DCPh	**-0.043**	**0.056**	-0.044	0.070						
2,4-DCPh	*-0.043*	*0.066*			-0.059	0.059				
2,5-DCPh	*-0.045*	*0.064*					-0.062	0.064		
2,6-DCPh	*-0.061*	*0.052*							*-0.044*	*0.065*
3,4-DCPh			*-0.043*	*0.071*	*-0.043*	*0.063*				
3,5-DCPh			-0.059	0.066			-0.058	0.067		
2-CPh	-0.054	0.062								
3-CPh			-0.069	0.064						
4-CPh					-0.069	0.056				

Table 7-16: NBO σ and π Q_X values for chlorophenolates. Indicated in bold are positions from which halogen substituents were removed by strain CBDB1. Partial charges were given in italics when the dehalogenation of chlorophenols was slowly and incompletely.

	C2		C3		C4		C5		C6	
	σ	π	σ	π	σ	π	σ	π	σ	π
PeCPhate	**-0.076**	**0.054**	**-0.072**	**0.056**	**-0.062**	**0.043**	**-0.076**	**0.054**	**-0.072**	**0.056**
2,3,4,5-TeCPhate	**-0.087**	**0.052**	**-0.079**	**0.055**	**-0.069**	**0.041**	-0.105	0.048		
2,3,4,6-TeCPhate	**-0.082**	**0.053**	**-0.081**	**0.055**	-0.091	0.037			-0.109	0.046
2,3,5,6-TeCPhate	**-0.083**	**0.051**	-0.102	0.050			-0.102	0.050	**-0.083**	**0.051**
2,3,4-TCPhate	**-0.095**	**0.050**	**-0.090**	**0.053**	-0.103	0.035				
2,3,5-TCPhate	**-0.094**	**0.050**	-0.110	0.048			-0.138	0.042		
2,3,6-TCPhate	**-0.091**	**0.050**	**-0.115**	**0.047**					-0.122	0.043
2,4,5-TCPhate	**-0.122**	**0.044**			**-0.098**	**0.036**	**-0.115**	**0.047**		
2,4,6-TCPhate	**-0.116**	**0.045**			-0.122	0.033			**-0.116**	**0.045**
3,4,5-TCPhate			-0.114	0.047	**-0.075**	**0.039**	-0.114	0.047		
2,3-DCPhate	**-0.105**	**0.047**	-0.127	0.045						
2,4-DCPhate	*-0.133*	*0.042*			-0.136	0.030				
2,5-DCPhate	*-0.135*	*0.041*					-0.155	0.040		
2,6-DCPhate	*-0.132*	*0.041*							*-0.132*	*0.041*
3,4-DCPhate			*-0.128*	*0.045*	*-0.111*	*0.033*				
3,5-DCPhate			-0.149	0.041			-0.149	0.041		
2-CPhate	-0.151	0.038								
3-CPhate			-0.172	0.038						
4-CPhate					-0.153	0.028				

Table 7-17: Results of a BlastX search using the nucleotide sequences of predicted open reading frames of the 2.5 kb fragments amplified in the current study against the NCBI nr database supplemented with amino acid sequences of SAG-C11 and similarities obtained in local or global alignments with amino acid and nucleotide sequences of SAG-C11 genes with genes amplified in the current study.

clone	LuxR	Similarity (global alignment)		LuxR	Similarity (local alignment)	DsrB	Similarity (global alignment)	
	1st hit	Amino acid sequence (112 amino acids compared)	Nucleotide sequence (corresponding to amino acid sequence)	2nd hit	Amino acid sequence	1st hit	Amino acid sequence (no. of amino acids compared)	Nucleotide sequence (corresponding to amino acid sequence)
3	SAG-C11	100%	100%	*Ktedonobacter racemifer*	48%	SAG-C11	100% (158)	100%
5	SAG-C11	100%	100%	*Ktedonobacter racemifer*	48%	SAG-C11	100% (238)	100%
10	SAG-C11	100%	100%	*Ktedonobacter racemifer*	48%	SAG-C11	100% (238)	100%
11	SAG-C11	97%	99%	*Ktedonobacter racemifer*	47%	SAG-C11	100% (238)	100%
20	SAG-C11	94%	96%	*Ktedonobacter racemifer*	47%	SAG-C11	98% (238)	98%
26	SAG-C11	95%	97%	*Ktedonobacter racemifer*	48%	SAG-C11	98% (100)	97%
33	SAG-C11	95%	96%	*Dehalobacter* strain DCA	55%	SAG-C11	98% (232)	97%
36	SAG-C11	95%	96%	*Dehalobacter* strain DCA	55%	SAG-C11	98% (194)	97%
44	SAG-C11	94%	97%	*Dehalobacter* strain DCA	53%	SAG-C11	98% (197)	97%
69	SAG-C11	s.n.e.	s.n.e.	s.n.e.	s.n.e.	SAG-C11	97% (223)	95%
70	SAG-C11	s.n.e.	s.n.e.	s.n.e.	s.n.e.	SAG-C11	97% (224)	95%
75	SAG-C11	93%	96%	*Dehalobacter* strain DCA	53%	SAG-C11	98% (238)	97%
94	SAG-C11	95%	96%	*Dehalobacter* strain DCA	55%	SAG-C11	98% (201)	97%
105	SAG-C11	100%	100%	*Ktedonobacter racemifer*	48%	SAG-C11	100% (204)	100%
123	SAG-C11	95%	96%	*Dehalobacter* strain DCA	55%	SAG-C11	98% (204)	97%
135	SAG-C11	95%	96%	*Dehalobacter* strain DCA	55%	SAG-C11	98% (213)	97%
138	SAG-C11	100%	100%	*Ktedonobacter racemifer*	48%	SAG-C11	100% (206)	100%
146	SAG-C11	95%	96%	*Dehalobacter* strain DCA	55%	SAG-C11	98% (204)	97%
166	SAG-C11	100%	100%	*Ktedonobacter racemifer*	48%	SAG-C11	100% (167)	100%
167	SAG-C11	94%	97%	*Dehalobacter* strain DCA	53%	SAG-C11	98% (164)	96%
168	SAG-C11	95%	96%	*Dehalobacter* strain DCA	55%	SAG-C11	97% (147)	96%
169	SAG-C11	s.n.e.	s.n.e.	s.n.e.	s.n.e.	SAG-C11	98% (70)	96%

s.n.e.= blast searches revealed targeted sequence match but the sequence was not evaluated due to poor quality

Table 7-18: Results of a BlastX search with *dsrA* and *dsrN* nucleotide sequences amplified in the current study against the NCBI nr database supplemented with amino acid sequences of SAG-C11 together with similarities of amino acid and nucleotide sequence of *dsr* genes amplified in the current study with DsrA/*dsrA* and DsrN/*dsrN* sequences of SAG-C11.

clone	DsrA/*dsrA*			DsrN/*dsrN*		
	1st BlastX hit (corresponding nucleotide accession no.)	Sequence similarity to DsrA/ *dsrA* of SAG-C11 (global alignment)		1st BlastX hit	Sequence similarity to DsrN/ *dsrN* of SAG-C11 (global alignment)	
		Amino acid sequence (no. of amino acids)	Nucleotide sequence (corresponding to amino acid sequence)		Amino acid sequence (no. of amino acids)	Nucleotide sequence (corresponding to amino acid sequence)
2	SAG-C11	88% (257)	77%	SAG-C11	59% (302)	64%
136	Unc. AB645401	90% (107)	79%	SAG-C11	Not available	Not available
139	Unc. AB645401	88% (83)	78%	SAG-C11	65% (56)	69%
142	Unc. AB645382	89% (142)	80%	SAG-C11	61% (68)	71%
204	SAG-C11	90% (248)	80%	SAG-C11	62% (128)	70%
209	SAG-C11	99% (154)	98%	SAG-C11	94% (210)	95%
215	SAG-C11	99% (352)	98%	SAG-C11	94% (475)	95%
238	Not analysed	87% (14)	85%	SAG-C11	51% (112)	64%
240	Unc. FM179971	78% (238)	75%	SAG-C11	62% (183)	66%
252	Unc. FM179971	75% (348)	72%	SAG-C11	58% (476)	62%
265	SAG-C11	99% (352)	98%	SAG-C11	95% (475)	96%
271	Unc. FM179969	77% (339)	72%	SAG-C11	56% (484)	59%
285	SAG-C11	90% (346)	88%	SAG-C11	76% (371)	78%
290	SAG-C11	84% (343)	76%	SAG-C11	65% (476)	70%
AA027D	Unc FM179971	77% (339)	73%	SAG-C11	s.n.e	s.n.e
AA049D	Unc. FJ403866	82% (55)	78%	SAG-C11	s.n.e	s.n.e
AA014E	Unc. FJ403979	78% (248)	73%	SAG-C11	50% (125)	59%
AA028G	Unc. FJ403979	83% (209)	72%	SAG-C11	55% (253)	61%
AA016H	Unc. FJ403928	81% (182)	75%	SAG-C11	Not available	Not available
AA020H	Unc. FJ403928	79% (208)	74%	SAG-C11	59% (168)	63%
BB1	SAG-C11	85% (293)	78%	SAG-C11	62% (266)	69%
BB14	Unc. FM179975	81% (302)	75%	SAG-C11	60% (276)	65%
BB22	SAG-C11	84% (348)	76%	SAG-C11	68% (219)	72%
BB38	Unc. FM179975	81% (308)	74%	SAG-C11	64% (222)	64%
BB48	SAG-C11	86% (317)	78%	SAG-C11	69% (205)	72%
WS13	SAG-C11	100% (216)	98%	SAG-C11	97% (316)	97%

s.n.e= blast searches revealed targeted sequence match but the sequence was not evaluated due to poor quality

Table 7-19: Accession numbers of dissimilatory sulphite reductase sequences obtained in this study

Clone	*Accession number*
Aarhus_Bay_dsr_clone_AA001_dsrA	KU561069
Aarhus_Bay_dsr_clone_AA142_dsrA	KU561070
Aarhus_Bay_dsr_clone_AA209_dsrA	KU561071
Aarhus_Bay_dsr_clone_AA215_dsrA	KU561072
Aarhus_Bay_dsr_clone_AA285_dsrA	KU561073
Baffin_Bay_dsr_clone_BB022_dsrA	KU561074
Baffin_Bay_dsr_clone_BB048_dsrA	KU561075
Wadden_Sea_dsr_clone_WS013_dsrA	KU561076
Aarhus_Bay_dsr_clone_AA240_dsrA	KU561077
Aarhus_Bay_dsr_clone_AA252_dsrA	KU561078
Aarhus_Bay_dsr_clone_AA271_dsrA	KU561079
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Sequences obtained with internal primers

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