

Anaerobic transformation of brominated aromatic compounds by *Dehalococcoides mccartyi* strain CBDB1

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Declaration

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This dissertation was carried out at The Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany between October, 2011 and September, 2015 under the supervision of PD Dr. Lorenz Adrian and Prof. Dr. Peter Neubauer. I herewith declare that the results of this dissertation were my own research and I also certify that I wrote all sentences in this dissertation by my own construction.

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Abstract

Brominated flame retardants are widely used compounds for fire safety in our daily life. Many of them have been reported to be toxic to humans and identified as environmental contaminants. *Dehalococcoides mccartyi* strains are well known for their dependence on organohalide respiration as an energy conserving process and have therefore been intensively studied. So far, much less investigations were done on brominated than on chlorinated compounds with *D. mccartyi* strains. *D. mccartyi* strain CBDB1 uses a wide range of halogenated compounds as electron acceptors for organohalide respiration. Most of its electron acceptors *e.g.* chlorinated benzenes, dioxins and polychlorinated biphenyls have the basic chemical structure of aromatic compounds and are bigger molecules than simple halogenated acyclic hydrocarbons such as chlorinated ethenes. From this point, brominated flame retardants share structural similarity with halogenated compounds which were demonstrated to be dehalogenated by *D. mccartyi* strain CBDB1 before. Therefore, in this study, *D. mccartyi* strain CBDB1 was chosen as the model organism and incubated with several brominated organic compounds including brominated flame retardants to investigate reductive debromination.

D. mccartyi strain CBDB1 completely dehalogenated brominated benzenes, tetrabromobisphenol A and bromophenol blue to the non-brominated forms as the final products. Such debromination processes revealed a further dehalogenation extent compared to reductive dechlorination catalyzed by the strain. Neither debromination activities nor cell growth were detected in the cultures of strain CBDB1 incubated with either decabromodiphenyl ether or hexabromocyclododecane. Growth yields of 2.4×10^{13} to 4.6×10^{13} cells mol⁻¹ bromide released were obtained in the cultures of strain CBDB1 incubated with hexabromobenzene or 1,3,5-tribromobenzene as the electron acceptor. Reductive debromination of the two brominated phenols was achieved only when they were supplied at low concentration. Growth yields of 2.7×10^{14} to 3.6×10^{14} cells mol⁻¹ bromide released were obtained in the cultures incubated with bromophenol blue but no cell growth was detected in cultures incubated with tetrabromobisphenol A. This suggests different extents of toxicity are caused by the two brominated phenols. Toxicity tests with bromophenol blue revealed that the debromination reaction and cell growth of strain CBDB1 were continuously delayed with the increase of initial concentrations of bromophenol blue. Resting cell activity assays analyzed by gas chromatography demonstrated that strain CBDB1 debrominated tetrabromobenzenes to tribromobenzenes. With a photometric activity assays, both cultures of strain CBDB1 grown with hexabromobenzene or 1,3,5-tribromobenzene showed higher specific activities on 1,2,4-

tribromobenzene and 1,2-dibromobenzene but lower specific activities on 1,3,5-tribromobenzene and the other tested halogenated benzenes. Results of shotgun proteomics showed that the same dominant reductive dehalogenases were involved in the dehalogenation of brominated benzenes and chlorinated benzenes indicating that these reductive dehalogenases are not strictly substrate-specific. Additionally, several reductive dehalogenase homologous proteins were specifically induced by hexabromobenzene or oligocyclic brominated phenols in cultures of strain CBDB1. This suggests the molecular size and chemical properties of an electron acceptor can influence the expression of reductive dehalogenases. Compound specific isotope analysis revealed identical carbon isotope enrichment factors for 1,2-dibromobenzene and 1,3-dibromobenzene, but significant lower enrichment factors were determined for 1,2,4-tribromobenzene and 1,3,5-tribromobenzene. Identical carbon isotope enrichment factors were determined for live cultures and *in vitro* activity assay with the same electron acceptor indicating the isotope fractionation was not affected by the physiological status of the cells.

Zusammenfassung

Bromierte Flammschutzmittel sind weit verbreitete Verbindungen die in unserem täglichen Leben im Bereich des Brandschutzes eingesetzt werden. Für viele von ihnen wurde berichtet, dass sie für den Menschen giftig sein könnten. Zudem wurden sie häufig als Schadstoffe in der Umwelt identifiziert. *Dehalococcoides mccartyi* Stämme können nur über Organohalid-Atmung Energie konservieren und wurden daher intensiv untersucht. Bisher wurden hauptsächlich Untersuchungen an chlorierten Verbindungen und seltener an bromierten Verbindungen mit *D. mccartyi* Stämmen durchgeführt. *D. mccartyi* Stamm CBDB1 verwendet diverse halogenierte Verbindungen als Elektronenakzeptoren für die Organohalid-Atmung. Die meisten seiner Elektronenakzeptoren, z.B. chlorierte Benzole, Dioxine und polychlorierte Biphenyle sind aromatisch und größer als einfache, halogenierte, acyclische Kohlenwasserstoffe wie z.B. chlorierte Ethene. In dieser Hinsicht haben bromierte Flammschutzmittel eine strukturelle Ähnlichkeit mit chlorierten Verbindungen, die durch *D. mccartyi* Stamm CBDB1 dehalogeniert werden. Daher wurde in dieser Studie *D. mccartyi* Stamm CBDB1 als Modellorganismus ausgewählt und mit mehreren bromierten organischen Verbindungen einschließlich bromierter Flammschutzmittel inkubiert, um die reduktive Debromierung zu untersuchen.

D. mccartyi Stamm CBDB1 debromierte Benzole, Tetrabrombisphenol A und Bromphenolblau vollständig zu nicht-bromierten Endprodukten. Diese Debromierungsprozesse zeigten eine weitreichendere Dehalogenierung verglichen mit den reduktiven Dechlorierungen ähnlicher chlorierter Verbindungen. Weder Debromierungsaktivität noch Zellwachstum wurde in den Kulturen des Stammes CBDB1 detektiert, die entweder mit Decabromdiphenylether oder mit Hexabromcyclododecan inkubiert wurden. In Kulturen mit Hexabrombenzol oder 1,3,5-Tribrombenzol als Elektronenakzeptor wurden Wachstumsausbeuten von $2,4 \times 10^{13}$ bis $4,6 \times 10^{13}$ Zellen Mol^{-1} Bromid erhalten. Eine reduktive Debromierung der beiden bromierten Phenolverbindungen wurde nur dann erreicht, wenn sie in geringen Konzentrationen zugegeben wurden. Wachstumsausbeuten von $2,7 \times 10^{14}$ bis $3,6 \times 10^{14}$ Zellen Mol^{-1} Bromid wurden in den mit Bromphenolblau inkubierten Kulturen erhalten, aber kein Zellwachstum wurde in den mit Tetrabrombisphenol A inkubierten Kulturen nachgewiesen. Dies deutet auf einen unterschiedlichen Grad der Toxizität der beiden bromierten Phenolverbindungen hin. Toxizitätstests mit Bromphenolblau zeigten, dass die Debromierungsreaktion und das Zellwachstum des Stammes CBDB1 kontinuierlich mit dem Anstieg der Anfangskonzentration von Bromphenolblau verzögert wurden. Durch Gaschromatographie analysierte ruhenden Zellaktivitäts-Assays zeigten, dass Stamm CBDB1 Tetrabrombenzole zu Tribrombenzolen

debromierte. In photometrischen Aktivitätsassays zeigten ruhenden Zellen von Stamm CBDB1, die zuvor mit Hexabrombenzol oder 1,3,5-Tribrombenzol kultiviert wurden, höhere spezifische Aktivitäten mit 1,2,4-Tribrombenzol und 1,2-Dibrombenzol als mit 1,3,5-Tribrombenzol und den anderen getesteten halogenierten Benzolen. Ergebnisse der Shotgun-Proteomik zeigten, dass die gleichen dominanten reduktiven Dehalogenasen an der Dehalogenierung von bromierten Benzolen und chlorierten Benzolen beteiligt waren. Dies weist darauf hin, dass diese reduktiven Dehalogenasen nicht streng substratspezifisch sind. Zusätzlich wurden mehrere Proteine, die homolog zu reduktiven Dehalogenasen sind, spezifisch durch Hexabrombenzol oder oligocyclische bromierte Phenole in Kulturen des Stammes CBDB1 induziert. Dies deutet darauf hin, dass die molekulare Größe und die chemischen Eigenschaften eines Elektronenakzeptors die Expression von reduktiven Dehalogenasen beeinflussen können. Die substanzspezifische Isotopenanalyse ergab identische Kohlenstoff-Isotopenanreicherungsfaktoren für 1,2-Dibrombenzol und 1,3-Dibrombenzol, aber es wurden signifikant niedrigere Anreicherungsfaktoren für 1,2,4-Tribrombenzol und 1,3,5-Tribrombenzol bestimmt. Identische Kohlenstoff-Isotopenanreicherungsfaktoren wurden für lebende Kulturen und in vitro-Aktivitätstest mit dem gleichen Elektronenakzeptor bestimmt, was darauf hinweist, dass die Isotopenfraktionierung nicht durch den physiologischen Status der Zellen beeinflusst wurde.

Major Theses

1. Cultivations of *D. mccartyi* strain CBDB1 with several brominated compounds as electron acceptors

- 1.1 Growth with two brominated benzenes: confirmed
- 1.2 Growth with two oligocyclic brominated phenols: confirmed
- 1.3 Growth of strain CBDB1 with deca-BDE and HBCD: failed

2. Identification of the debromination products

- 2.1 Bromine is removed stepwise and benzene is the final product of all bromobenzene congeners
- 2.2 Complete removal of bromide observed for oligocyclic brominated phenols

3. Inhibitory effects on reductive debromination

- 3.1 Accumulation of the debromination products can inhibit the cell growth
- 3.2 Phenols are toxic for strain CBDB1 and the extent of toxicity is related to the hydrophobicity of phenolic compounds

4. RdhA protein expression

- 4.1 Same dominant reductive dehalogenases were expressed in the cultures with either different brominated benzenes congeners or chlorinated benzenes as electron acceptor.
- 4.2 The reductive dehalogenases with the locus tags CbdbA1092 and CbdbA1503 were induced by the two tested oligocyclic brominated phenols.

5. Analysis of resting cell photometric activity assay

Cultures of strain CBDB1 showed varying specific activities on different halogenated benzenes. The chemical properties of an electron acceptor presented a stronger influence on the debromination rate than the set of expressed reductive dehalogenases (i.e. the electron acceptor with which the cells had been grown).

6. Compound specific isotope analysis for cultures with brominated benzenes as electron acceptor

- 6.1 The determined identical carbon isotope enrichment factors indicated the reaction mechanism for the debromination of 1,2- and 1,3-dibromobenzenes is similar.
- 6.2 Carbon isotope fractionation was shown to be mainly affected by the biochemical reaction rather than by the physiological status of the cells.

List of publications and author contributions

Publication 1:

Chao Yang, Anja Kublik, Cindy Weidauer, Bettina Seiwert, Lorenz Adrian. Reductive dehalogenation of oligocyclic phenolic bromoaromatics by *Dehalococcoides mccartyi* strain CBDB1. Environmental Science & Technology. 2015, 49(14): 8497-8505. DOI: 10.1021/acs.est.5b01401.

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Contributions: Chao Yang, Kevin Kuntze and Lorenz Adrian developed the concept of the study. All cultivations of strain CBDB1 were done by Chao Yang. Resting cell activity assay and sample preparations were done by Chao Yang. Bromide analysis on ion chromatography and concentration analysis for brominated benzenes on gas chromatography was done by Chao Yang. RdhA protein expression analysis was done by Chao Yang. Carbon stable isotope analysis on gas chromatography combustion isotope ratio mass spectrometry was done by Kevin Kuntze. The manuscript was written by Chao Yang, Kevin Kuntze and revised by Lorenz Adrian.

Abbreviations

BFRs	<i>Brominated flame retardants</i>
BPB	<i>Bromophenol blue</i>
CbrA	<i>Chlorobenzene reductive dehalogenases</i>
CSIA	<i>compound-specific stable isotope analysis</i>
DBB	<i>Dibromobenzene</i>
DCB	<i>Dichlorobenzene</i>
Deca-BDE	<i>Decabrominated diphenyl ether</i>
emPAI	<i>Exponentially modified protein abundance index</i>
FID	<i>Flame ionization detector</i>
GC	<i>Gas chromatograph</i>
HBB	<i>Hexabromobenzene</i>
HBCD	<i>Hexabromocyclododecane</i>
HCB	<i>Hexachlorobenzene</i>
LC-MS	<i>Liquid chromatograph- mass spectrometry</i>
MBB	<i>Monobromobenzene</i>
Octa-BDE	<i>Octabrominated diphenyl ether</i>
Penta-BDE	<i>Pentabrominated diphenyl ether</i>
PCE	<i>Tetrachloroethene</i>
<i>rdh</i>	<i>Reductive dehalogenase homologous</i>
RdhA	<i>Reductive dehalogenase subunit A</i>
TBB	<i>Tribromobenzene</i>
TBBPA	<i>Tetrabromobisphenol A</i>
TCB	<i>Trichlorobenzene</i>
TCE	<i>Trichloroethene</i>

Table of contents

1	Introduction	1
1.1	Brominated compounds in the environment.....	1
1.1.1	Natural production of organobromine compounds	1
1.1.2	Anthropogenic brominated flame retardants	2
1.2	Toxicity of brominated flame retardants	5
1.3	Microbial reductive dehalogenation	6
1.3.1	Organohalide respiration.....	6
1.3.2	<i>Dehalococcoides mccartyi</i> strain CBDB1	7
1.3.3	Dehalogenation specificity of reductive dehalogenases	8
1.3.4	Progress in microbial reductive debromination	8
1.3.5	Growth of <i>D. mccartyi</i> strains with organohalides as electron acceptor	10
1.4	Compound-specific stable isotope analysis.....	11
1.5	Objective of this work	12
2	Materials and Methods	14
2.1	Chemicals and gases.....	14
2.2	Cultivation of <i>D. mccartyi</i> strain CBDB1	14
2.2.1	Medium preparation.....	14
2.2.2	Different inoculum of cultures.....	15
2.2.3	Brominated benzenes as electron acceptors.....	16
2.2.4	Tetrabromobisphenol A and bromophenol blue as electron acceptors	17
2.2.5	Decabromodiphenyl ether and hexabromocyclododecane as electron acceptors	18
2.3	Analytical methods.....	18
2.3.1	Cell counting.....	18
2.3.2	Bromide analysis	19
2.3.3	Concentration analysis by gas chromatography	20
2.3.4	Concentration analysis by liquid chromatography-mass spectrometry (UPLC-MS)	21
2.4	RdhA Protein Expression Analysis	22
2.4.1	Sample preparation	22

2.4.2	nanoLC MS/MS analysis	22
2.5	Resting cell activity assay	24
2.5.1	Photometric activity assay	24
2.5.2	GC-format activity assay	26
2.6	Compound-specific stable isotope analysis.....	27
3	Results	30
3.1	Growth of strain CBDB1 with brominated compounds	30
3.1.1	Growth with hexabromobenzene	30
3.1.2	Growth with 1,3,5-tribromobenzene.....	31
3.1.3	Growth with bromophenol blue	33
3.1.4	Cultivation using tetrabromobisphenol A as electron acceptor	35
3.1.5	Cultivation using decabromodiphenyl ether and hexabromocyclododecane as electron acceptor.....	37
3.2	Influencing factors and inhibitory effects in the reductive debromination process catalyzed by strain CBDB1	39
3.2.1	Factors influencing cell growth in hexabromobenzene cultures.....	39
3.2.2	Toxicity tests with bromophenol blue	41
3.3	Dehalogenation products of brominated organics by strain CBDB1	44
3.3.1	Debromination products of 1,3,5 -tribromobenzene.....	44
3.3.2	Debromination products of hexabromobenzene	44
3.3.3	Debromination products of bromophenol blue	48
3.3.4	Debromination products of tetrabromobisphenol A	49
3.4	RdhA Protein Expression	52
3.4.1	RdhA protein expression in cultures grown with hexabromobenzene or 1,3,5-tribromobenzene	52
3.4.2	RdhA protein expression in cultures incubated with bromophenol blue or tetrabromobisphenol A	54
3.5	Resting cell photometric activity assay	54
3.6	Effect of dehalogenation of brominated benzenes on the carbon isotope ratio	56
3.6.1	Carbon isotope fractionation in enzymatic assays	56

3.6.2	Carbon isotope fractionation in live cultures	59
4	Discussion	61
4.1	Growth adaption of <i>D. mccartyi</i> strain CBDB1 to brominated compounds.....	61
4.1.1	Growth adaptation to brominated benzenes.....	61
4.1.2	Growth adaption to brominated compounds with oligocyclic structures.....	64
4.1.3	Inhibitory effects on reductive debromination.....	65
4.2	Dehalogenation Patterns of <i>D. mccartyi</i> strain CBDB1	67
4.2.1	Complete removal of bromine in reductive debromination	67
4.2.3	Influence of the chemical property of halogenated compounds on reductive dehalogenation.....	69
4.3	Evaluation on resting cell enzymatic activity assay	70
4.4	Expression of reductive dehalogenases	71
4.5	Potential of applying CSIA to detect reductive debromination.....	73
5	Conclusions	75
	References	77

1 Introduction

1.1 Brominated compounds in the environment

1.1.1 Natural production of organobromine compounds

Brominated compounds are ubiquitous in the environment.^{1,2} Natural production of organohalogens is carried out by an array of enzymes such as haloperoxidases^{3,4} and halogenases^{5,6} and chemical processes (*e.g.* volcanic emissions,⁷ biomass burning⁸ and alkyl halide formation during the oxidation of organic matter by Fe (III)⁹). Among 4,700 natural organohalide compounds that have been identified, more than 2,200 contain bromine and most of them are produced in the marine environment.^{10,11} Although the concentration of bromide in sea water is 65 mg L⁻¹ which is much less than that (19,000 mg L⁻¹) of chloride,¹² inorganic bromide can be converted to organic bromine through enzymatic pathways by marine organisms. Natural organobromines range in structural intricacy from tribromomethane to the highly complex indole alkaloids.¹ Many marine plants, animals (*e.g.*, sponges, bryozoans and nudibranchs), fungi and bacteria are involved in this biotic bromination process. For example, many red and blue-green algae are rich sources of organobromine compounds like manauaealides, lyngbyaloside and grenadadiene;¹³⁻¹⁵ Marine sponges such as *Psammopemma* sp., *Psammaplysilla purpurea*, *Aplysina aerophoba* and *Tedania ignis* can produce bromoindoles and bromophenols;^{1,16-18} Fungus like *Lepista nuda* and *Acorospora gobiensis* produces or contains bromophenols and brominated fatty acids.^{19,20} Marine bacterium *Chromobacterium* sp. was reported to produce bipyrrole and 2,3,4,5-tetrabromopyrrole;²¹ *Xanthomonas juglandis* produced brominated aryl polyene esters.²²

Bromoperoxidases are the ubiquitous enzymes involved in natural bromination which have been discovered in many marine organisms such as algae and bacteria.^{10,23,24} A main function of bromoperoxidase is the biosynthesis of bromoalkanes.²⁵⁻²⁷ Agarwal et al also reported that two flavin-dependent brominases were involved in the synthesis of polybromophenol- and polybromopyrrole-based metabolites, which proved that marine bacteria could be the natural sources of hydroxylated brominated diphenyl ethers.²⁸

On the other hand, in the terrestrial environment where bromide tends to be less abundant than in marine environment, natural bromination could also occur in sediments or during plant growth

and decay through the action of haloperoxidases.²⁹⁻³³ In short, bromine similar to chlorine undergoes biogeochemical cycling in the environment involving natural formation and degradation of organohalides. Because substantive accumulation of organohalides in nature cannot be seen, organisms must also be involved in the biodegradation of both natural and anthropogenic organohalides.^{18,34-40}

1.1.2 Anthropogenic brominated flame retardants

As a main class of anthropogenic halogenated compounds, halogenated flame retardants are widely used in our society for fire safety by being added or applied to combustible materials to increase the fire resistance of the products.⁴¹ Under combustion, chlorinated or brominated flame retardants can interfere with the flame by releasing hydrogen chloride or hydrogen bromide in the vapor phase. The much less active halogen radicals then replace the main chain reaction carriers (*e.g.* H, OH and O) which are essential to maintaining the combustion processes, then the rate of energy production is slowed down and a flame extinguishment is achieved.⁴² Because the carbon-bromine bond is weaker than the carbon-chlorine bond, hydrogen bromide is liberated over a narrower temperature range than hydrogen chloride in the combustion process so that it is more efficient in reaching a high concentration in the flame zone.⁴³ Thus brominated flame retardants (BFRs) have become more popular than chlorinated flame retardants in plastics and textile applications. Mostly used BFRs include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), and hexabromobenzene (HBB) (Figure 1.1).⁴⁴

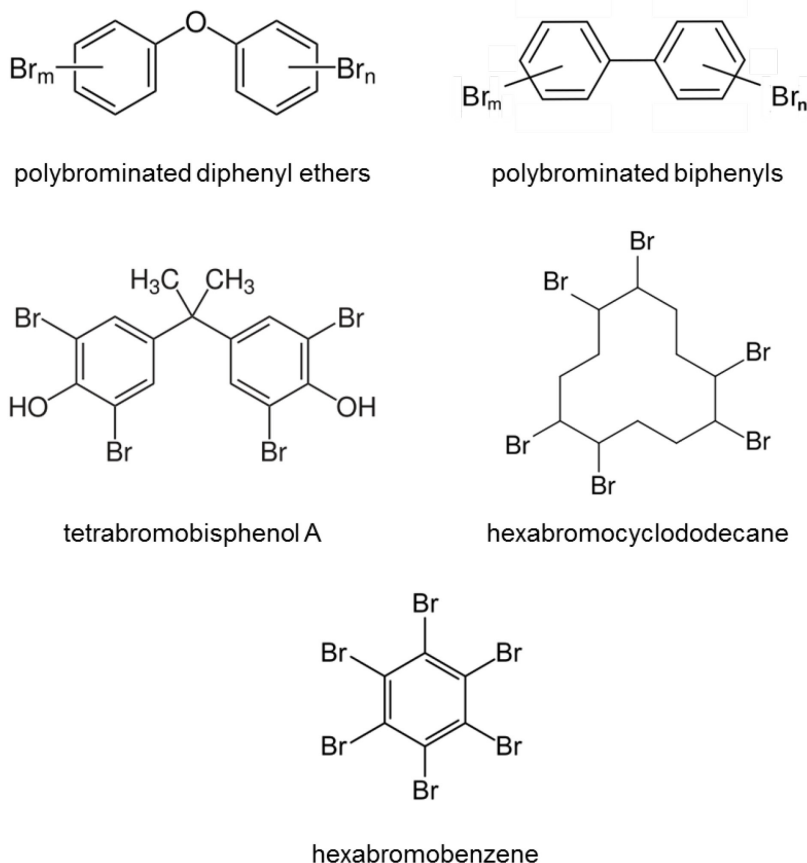


Figure 1.1: Chemical structures of several brominated flame retardants

In general, PBDEs are usually applied in electronic devices (*e.g.* printed circuit boards, cables and plastic covers), furniture, textiles, and construction materials.⁴⁵ Penta-, octa- and deca-brominated diphenyl ethers were the three major technical products.⁴⁶ Penta- and deca-BDE were widely used in European Union (EU), while in North America mainly penta- and octa-BDE were used.⁴⁶⁻⁴⁹ In Asian countries especially in China, deca-BDE was the dominant type produced and used in the market. Furthermore, China is the largest destination of electronic equipment waste from the whole world. By this, technical PBDEs used in other countries were transported to China and released into environment during electronic equipment recycling processes.^{50,51} In the EU, production of penta- and octa-BDE was banned in 2002, and the application of penta- and octa-BDE in electronic devices was banned in July 2006 as defined in the 2002/95/EC directive.⁵² Deca-BDE was stopped to be used in electronics and electrical applications in July 2008 according to the European commission decision 2005/717/EC.⁵³ In North America, the Environmental Protection Agency of the United States issued strict rules to control the production and use of penta- and octa-BDE in 2006,⁵⁴ and announced to cease the production and import of deca-BDE entirely by the end of 2013.⁵⁵ So far, there was no legislation set up regarding PBDEs used in China. PBBs are frequently applied in synthetic

fibers and polymers, in particular to a thermoplastic (acrylonitrile–butadiene–styrene) used in housings for office equipment,⁵⁶ but the production of PBBs was stopped voluntarily by industry over the world since 2000.⁵⁷ Technical HBCD is a mixture of the three main isomers (approximately 10% α -, 10% β - and 80% γ - form of HBCD) often used in polystyrene foam insulation, building and construction industry, and polymer dispersion as textile back-coating on cotton or cotton mixed with synthetic blends.^{41,58} The Japanese Environment Agency has listed HBCD in “chemicals suspected of having endocrine disrupting effects” since 1998.⁵⁹ The Environmental Protection Agency of the United States conducted a risk assessment on HBCD pursuant to the 2010 “Toxic Substances Control Act” action plan.⁶⁰ In the EU, HBCD isomers were included in the list of substances subjected to authorization.⁶¹ In 2013, HBCD received a specific exemption for production and use in expanded polystyrene and extruded polystyrene in buildings by the Stockholm Convention.⁶² TBBPA is used mostly in polymers and electronics (laminate for printed circuit boards) and also as an additive in the manufacturing of acrylonitrile butadiene styrene resins high impact polystyrene, and phenolic resins.⁶³ TBBPA was classified as a persistent bioaccumulative toxic chemical by the Environmental Protection Agency of the United States.⁶⁴ HBB is extensively used as a flame retarding additive in paper, woods, textiles, electronic and plastic materials in Japan and it also can be released from pentabromobenzyl acrylate oligomer.^{65,66} Until now, HBB has not been regulated under any specific regulation for its production or application. In order to protect life from fire accidents, large amounts of BFRs have been produced with the increasing usage of polymeric materials in construction, electronic and computer equipment. The global market demand for BFRs increased from 145,000 tons in 1990 to over 310,000 tons in 2000.^{67,68} In 2011, the worldwide consumption of BFRs was around 390,000 tons which represented 19.7% of the total flame retardants market, other popular used flame retardants included chlorinated, organo-phosphorus, antimony-oxide and aluminum hydroxide forms.⁶⁹

Although BFRs play an important role in the protection of daily products against fire, their properties have initialized concern about their environmental fate, persistence and toxicological tolerability.⁷⁰⁻⁷² Most of the BFRs, such as PBDEs, PBBs and HBB, are additive flame retardants which are mixed together with other components of the polymers being held only due to their hydrophobicity but they are not covalently bound to the polymer. Therefore they can leach out of the products and be taken up by humans or released into the environment. In the environment, these hydrophobic compounds can be adsorbed to particles and transported over long distances.^{73,74} So far, BFRs have been frequently reported to be detected in the environment and bioaccumulated in the food web.^{46,56,75-82} For reactive BFRs like TBBPA which is supposed to be

not easily released into the environment,⁸³ it has also been detected at certain concentrations in soil, sediments, house dusts, plants and human serum.⁸⁴⁻⁸⁷

1.2 Toxicity of brominated flame retardants

Since large amounts of BFRs have been used and distributed into the environment, many researches have evaluated possible adverse effects of BFRs to human health. Birnbaum et al. found that exposure to penta-BDE or octa-BDE congeners could result in histopathological changes and function disruptions in the livers of rats and mice.⁸⁸ More studies on mice demonstrated that thyroid hormone disruption and delayed developmental neurotoxicity could be caused by deca-BDE.⁸⁹⁻⁹¹ Wang et al. reported that a lower concentration of thyroid-stimulating hormone was found in people working in e-waste recycling fields where high concentrations of PBDEs were detected than that in people working away from e-waste.⁹² Akutsu et al. published that the sperm quality in ten Japanese male humans could be influenced by the PBDE concentrations.⁹³ A similar study has found that decrease in women's fecundability was associated with high PBDE concentrations in their blood serum.⁹⁴ Although TBBPA is reported to be the highest-volume BFR produced worldwide which represents about 60% of the total BFR market,⁶³ there are relatively few studies available examining its toxicity. Animal tests showed that prenatal and postnatal exposure to TBBPA can result in lipid metabolic disorders and hepatic or kidney lesions, as well as changes in behavior, locomotion, and hearing.⁹⁵⁻⁹⁷ *In vitro* studies demonstrated that TBBPA can bind to the estrogen receptor and to induce proliferation in human breast cell lines, or induce a dose-dependent formation of reactive oxygen species and calcium levels in human neutrophils.^{98,99} TBBPA is also reported to have the potential to induce the immunosuppressive effects in human natural killer cells.¹⁰⁰ Even less data is available for the toxicity of HBB perhaps due to the fact that HBB is not included under any specific regulation for use or production, nor even regarding maximum tolerance levels in food.¹⁰¹ Tests in rodents and birds revealed that HBB can cause liver oxidative stress or endocrine effects such as acted as an inductor of the microsomal enzyme system; it could also result in higher integrated biomarker response than other BFRs in fish.^{102,103} All in all, the increasing contamination and bioaccumulation of BFRs accompanied with evidence of adverse effects on human health highlight the importance and urgency of avoiding further contaminations and detoxification of contaminated sites.

1.3 Microbial reductive dehalogenation

1.3.1 Organohalide respiration

Organohalide respiration is a microbial respiratory process in which halogenated compounds serve as the electron acceptor and the halogen substituent is removed as a halide in final.¹⁰⁴ Removal of halogens from halogenated compounds including halogenated alkenes, alkanes, aromatic and aliphatic compounds by bacteria was reported happening in both oxic^{105,106} and anoxic¹⁰⁷⁻¹⁰⁹ environments. In contrast to aerobic dehalogenation in which lower halogenated compounds were preferentially dehalogenated,¹¹⁰ higher halogenated compounds were preferentially used as terminal electron acceptor for dehalogenation in anoxic environments.¹¹¹ Moreover, reductive dehalogenation was the most reported dehalogenation process in anoxic environments.¹¹² Remarkably, many of the higher halogenated compounds *e.g.* pesticides, pharmaceuticals and flame retardants are normally resistant to be transformed in oxic environments, making the process of anaerobic reductive dehalogenation important for the detoxification of persistent halogenated compounds in the environment.¹¹³

For the investigation on reductive dehalogenation, over the past decades, several groups of organohalide-respiring bacteria have been identified and studied to dehalogenate a broad spectrum of halogenated compounds. *Proteobacteria* including *Anaeromyxobacter*, *Geobacter* and *Sulfurospirillum* catalyze facultative organohalide respiration and can grow on fermentation.¹¹⁴⁻¹¹⁶ These phyla were mainly reported to dehalogenate halogenated ethenes such as tetrachloroethene and trichloroethene. The *Firmicutes* contain both facultative organohalide-respiring bacteria *Desulfitobacterium*¹¹⁷⁻¹²¹ and obligate organohalide-respiring bacteria *Dehalobacter*.¹²²⁻¹²⁵ These phyla dechlorinate diverse halogenated compounds such as tetra- and tri-chloroethenes, dichloropropanes, dichlorotoluenes, 3-chloro-4-hydroxyphenylacetate and chlorinated phenols. *Dehalococcoides mccartyi* strains from the phylum *Chloroflexi* are a highly specialized group of bacteria only catalyzing obligate organohalide respiration.¹¹⁴ For these strains, the ATP generation for cell growth is coupled to the electron transport during the reductive dehalogenation of halogenated compounds. Most of the *D. mccartyi* strains were enriched in completely synthetic, reduced medium amended with vitamins, fermentable substrates (*e.g.* pyruvate, lactate, and benzoate) or hydrogen as electron donor, and chlorinated compounds as electron acceptors.¹²⁶ Among the pure bacterial isolates, *D. mccartyi* strain BAV1, FL2, GT and VS were reported mainly using chlorinated ethenes as electron acceptor,¹²⁷⁻¹³⁰

whereas *D. mccartyi* strain 195 and CBDB1 were identified to dehalogenate a broader range of halogenated compounds including chlorinated benzenes, chlorinated ethenes, chlorophenols, polychlorinated biphenyls and so on.¹³¹⁻¹³⁴

1.3.2 *Dehalococcoides mccartyi* strain CBDB1

As a typical obligate organohalide-respiring bacterium, *Dehalococcoides mccartyi* strain CBDB1 was isolated from a highly enriched dechlorinating consortium with the sediment of Saale River in Germany. The bacterium is non motile, without a cell wall, disc-shaped with a size ranging from 0.3 to 1 μm in diameter and 0.1 to 0.2 μm in thickness. *D. mccartyi* strain CBDB1 can be routinely cultivated with chlorinated benzenes as electron acceptor, acetate as carbon source, 7 vitamins (vitamin B₁₂ is essential) and hydrogen as electron donor. It cannot oxidize or ferment substrates such as glucose, citrate or succinate for cell growth.¹³¹ In contrast to other organohalide-respiring bacteria using chlorinated alkenes as electron acceptor, strain CBDB1 was first studied and cultivated with chlorinated benzenes which have the basic chemical structure of halogenated aromatics.^{131,135,136} Later on, intensive studies demonstrated that strain CBDB1 could dehalogenate more chlorinated compounds especially including chlorinated aromatics with bigger molecules than chlorinated benzenes.^{132,137-139} Several of these chlorinated aromatics *e.g.*, polychlorinated biphenyls and polychlorinated dibenzodioxins share a similarity in chemical structure and property with some of the brominated flame retardants as mentioned previously. Recent researches extended the electron acceptor range of strain CBDB1 to include not only brominated compounds such as lower brominated benzenes and bromophenols but also halogenated compounds with two types of halogen substituents.^{109,140} These researches also suggested that brominated compounds might be more extensively dehalogenated than chlorinated compounds by strain CBDB1. The broad dehalogenating potential of strain CBDB1 was demonstrated by its 32 reductive dehalogenase homologous (*rdh*) genes (described below) identified by genome sequencing.¹⁴¹ Based on all above, *D. mccartyi* strain CBDB1 has a good research foundation to be chosen as a model organism to investigate the reductive dehalogenation of brominated flame retardants. The investigations can further help to understand the reaction mechanism of reductive dehalogenation, and the research data will benefit the detoxification of resistant brominated compounds in the environment.

1.3.3 Dehalogenation specificity of reductive dehalogenases

Reductive dehalogenases (RDases) are the key enzymes in organohalide respiration. Reductive dehalogenase homologous genes usually are encoded in an operon containing *rdhA*, the gene for the catalytically active enzyme and *rdhB*, the gene encoding a putative membrane-anchoring protein.¹⁴² Genome sequences of organohalide-respiring bacteria revealed the number of *rdhA* genes varies in different strains. The genome of *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 contains 19 *rdhA* genes.¹⁴³ Up to 24 complete *rdhA* genes were found in sequenced *Dehalobacter* genomes.^{122,125} Genomes of *Dehalococcoides mccartyi* strain 195 and strain CBDB1 include 17 and 32 different *rdhA* genes, respectively.^{141,144} However, because RDases are inactive with oxygen and obtaining sufficient biomass is difficult for *e.g.* *Dehalococcoides*, *Dehalogenimonas* and some *Dehalobacter* strains, only a few RDases were biochemically characterized. RDases have broad enzyme specificity towards different organohalides. PceAs identified from *Sulfurospirillum*, *Desulfitobacterium* and *Dehalobacter* were active on tetrachloroethene and trichloroethene but they cannot dechlorinate isomers of dichloroethene.^{118,145,146} CrdA and CprA dehalogenases from *Desulfitobacterium hafniense* and *Desulfitobacterium dehalogenans* dechlorinated many chlorophenols.^{119,147,148} CrdA dehalogenase is only capable of ortho dechlorination, while for different CprA dehalogenases ortho, meta and para dechlorination of chlorophenols were observed. In *D. mccartyi* strains, TceA of *D. mccartyi* strain 195¹⁴² has been shown to dechlorinate trichloroethene to ethene and the strongest activity was observed on trichloroethene. Both VcrA from *D. mccartyi* strain VS and BvcA from *D. mccartyi* strain BAV1 dechlorinated trichloroethene, dichloroethenes and vinyl chloride to ethene, they were also able to transform 1,2-dichloroethane to ethane.^{129,149,150} CbrA from *D. mccartyi* strain CBDB1¹⁵¹ was first found to dechlorinate 1,2,3-trichlorobenzene and 1,2,3,4-tetrachlorobenzene. Later, it was also detected with a high abundance in cultures of strain CBDB1 grown with lower brominated benzenes (*e.g.* 1,2,4-tribromobenzene, 1,3- and 1,4-dibromobenzene).¹⁴⁰

1.3.4 Progress in microbial reductive debromination

Reductive dechlorination was intensively studied, while less research has been done for reductive debromination by organohalide-respiring bacteria. So far, debromination activities detected in cultivation or enzyme extract test catalyzed by organohalide-respiring bacteria showed both similarities and differences with dechlorination processes catalyzed by the same

strain. *D. mccartyi* strain CBDB1 preferentially removes doubly flanked chlorine substituents (e.g. from highly chlorinated benzenes to 1,3,5-trichlorobenzene), but it can also remove singly flanked chlorine substituents from 1,2,4-trichlorobenzene to 1,3-dichlorobenzene and 1,4-dichlorobenzene.¹³⁶ However, strain CBDB1 could not dechlorinate 1,3,5-trichlorobenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene and monochlorobenzene, all of which do not contain chlorine substituents flanked by another halogen substituent. Debromination of 1,2,4-tribromobenzene by strain CBDB1 followed the similar route like chlorinated benzenes to 1,3-dibromobenzene and 1,4-dibromobenzene, on the other hand all dibromobenzenes were proved to be debrominated to benzene via monobromobenzene by strain CBDB1.¹⁴⁰ Therefore, even non-flanked bromine substituents were removed. Tetrachloroethene dehalogenases from *D. hafniense* PCE-S could only dechlorinate trichloroethene to *cis*-dichloroethene, but the same enzymes converted tribromoethene to ethene via *cis*- and *trans*-1,2-dibromoethene, 1,1-dibromoethene and vinyl bromide.¹⁵²

Compared to brominated benzenes or ethenes, brominated flame retardants normally have bigger molecule sizes or additional non-halogen substituents on the molecular structure and some of them have even higher hydrophobicity. In the past years, samples, mixed cultures or isolated strains from sediments, sludge and contaminated sites have been studied for their capacity to transform brominated flame retardants. The low brominated congeners 4-BDE and 4,4'-BDE were found to be transformed to diphenyl ether in a sediment column containing an undefined anaerobic microbial community.¹⁵³ Gerecke et al. reported that within 238 days of incubation 15% of 10 µM deca-BDE was transformed to lower brominated congeners by sewage sludge from Switzerland.¹⁵⁴ He et al.'s group demonstrated the debromination of technical octa-BDE with mixed cultures including *Sulfurospirillum multivorans* and *Dehalococcoides* strains.¹⁵⁵ Later, they published complete debromination of tetra- and penta-BDE by a mixed culture consisting of *Dehalococcoides* and *Desulfovibrio* species.¹⁵⁶ Furthermore, two pure strains, *Acetobacterium* sp. strain AG and *Dehalococcoides* sp. strain DG, were isolated by this group and these strains were able to transform octa- and penta-BDE to less brominated congeners.¹⁵⁷ However, strain DG only debrominated PBDEs in the presence of trichloroethene and so far no pure strain was reported to be able to fully debrominate deca-BDE. TBBPA was found to be degraded anaerobically in river sediments from the Erren River in southern Taiwan, where sulfate-reducing bacteria, methanogen, and eubacteria were involved in the degradation process.¹⁵⁸ Ronen et al. published that 80% of TBBPA concentration in soil was removed by a sequential anaerobic/aerobic process, and the debromination of TBBPA to bisphenol A occurred in the anaerobic process then bisphenol A was mineralized under aerobic conditions.¹⁵⁹ Arbeli et al.

reported an enrichment culture from desert stream sediment in Israel that was able to reductively debrominate TBBPA to bisphenol A, and that sediment soil and gray chalk strongly stimulated the debromination process. Further related studies from the same group showed that microbial debromination of TBBPA in surface sediment was strongly influenced by salinity, temperature, pH and carbon sources. Brominated phenols and other alternative electron acceptors like Fe^{3+} , SO_4^{2-} , NO_3^- could inhibit the debromination of TBBPA.^{160,161} Zhang et al. found that a *Dehalobacter* strain in mixed culture could debrominate TBBPA to bisphenol A where humin was essential for the debromination activity.¹⁶² One pure strain *Comamonas* sp. strain JXS-2-02 isolated from anaerobic sludge could debrominate TBBPA to bisphenol A then into 3,4-dihydroxy mandelic acid and beta resorcylic acid furthermore into acetylbenzoyl and acetophenone via benzoyl-CoA.¹⁶³ Another pure strain reported to transform TBBPA was *Ochrobactrum* sp. strain T isolated from the world's largest e-waste recycling site from Guiyu Town in China. This bacterium simultaneously debrominated and mineralized TBBPA and metabolic intermediates to CO_2 as the final product, however, this biotransformation process was under aerobic conditions.¹⁶⁴ On the other hand, less data was known about the microbial debromination of HBCD and hexabromobenzene. Biotransformation of HBCD was found in anaerobic soils and sediments from several locations in the United States.¹⁶⁵ Hexabromobenzene was detected to be debrominated to penta-, tetra-, and tribromobenzenes in rat, human adipose tissues and river estuary sediments in Japan.¹⁶⁶⁻¹⁶⁸ As for *Dehalococcoides mccartyi* strains, strain CBDB1 and strain 195 showed a substrate range include polychlorinated biphenyls^{134,138} and polychlorinated dibenzodioxins^{134,139} which have similar structure to BFRs. This indicated that *D. mccartyi* strains might have the ability to debrominate brominated flame retardants.

1.3.5 Growth of *D. mccartyi* strains with organohalides as electron acceptor

D. mccartyi strains appear to be highly restricted in their energy conservation. They exclusively use hydrogen as electron donor and halogenated electron acceptor for respiration and cannot gain energy from fermentation. Growth of *D. mccartyi* strains is usually coupled to dehalogenation of organohalides. Under optimized cultivation conditions, similar growth yields ranging from 6.3×10^{13} to 3.1×10^{14} cells per mol halogen released have been observed in *D. mccartyi* strain cultures.^{128,130,133,136,140} This corresponds to the need of 1.9×10^9 to 9.5×10^9 dehalogenation reactions for the production of one cell. Well-grown cultures are essential for the further investigations on dehalogenation processes. So far, *D. mccartyi* strains can be routinely well cultivated with the chlorinated compounds (mainly chlorinated ethenes and benzenes) which

usually were also electron acceptors used for the original enrichment cultures, and optimizations of cultivation are focused on the components of medium. Fewer studies have been done to investigate the toxicity of electron acceptor to the growth of cultures. Jayachandran et al¹³⁶ reported that *D. mccartyi* strain CBDB1 could grow with hexachlorobenzene or pentachlorobenzene applied in crystalline form but not as solutions in hexadecane, while 1,2,3,4-tetrachlorobenzene was toxic to strain CBDB1 if applied in crystalline form, even though strain CBDB1 showed the highest specific dechlorination activities on this compound. The author pointed out the different water solubility of chlorinated benzenes could influence the cultivation of strain CBDB1, but the reason for the inhibitory effect of 1,2,3,4-tetrachlorobenzene was unknown. For the same strain, Adrian et al¹³⁸ showed that high concentrations (20 to 500 $\mu\text{g mL}^{-1}$) of polychlorinated biphenyls were toxic to strain CBDB1, and a much lower concentration of polychlorinated biphenyls per cell was needed to observe the dehalogenation activity. A high initial concentration of 75 μM or above of 2,3,4-trichlorophenol was reported to completely inhibit the growth of strain CBDB1 within the first 13 weeks of incubation, while several doses of 20 to 50 μM feeding allowed the strain to adapt to higher additions of 2,3,4-trichlorophenol without losing dehalogenating ability.¹³⁷ Cooper et al¹⁰⁹ also found that *D. mccartyi* strain CBDB1 dehalogenated a wide range of halogenated aromatics substituted with different types of non-halogen functional groups, and lower growth yields were usually found for electron acceptors containing phenolic groups which might inhibit growth by interfering with the proton gradient across the membrane.

1.4 Compound-specific stable isotope analysis

Isotopes of an element have the same number of protons, but differ in the number of neutrons, and therefore, they have different masses. The quotient between the heavy and the light isotopes is called isotope ratio or isotope signature (*e.g.* $^2\text{H}/^1\text{H}$, $^{13}\text{C}/^{12}\text{C}$, $^{37}\text{Cl}/^{35}\text{Cl}$ $^{81}\text{Br}/^{79}\text{Br}$) and the stable isotope composition of a substance is conventionally expressed in the delta notation (*e.g.* $\delta^{13}\text{C}$) compared to an international reference standard with known isotope composition. The approach of compound-specific stable isotope analysis (CSIA) is based on the observation that during transformation chemical bonds containing heavy isotopes are more stable and cleaved slower than bonds containing lighter isotopes. As a result, the residual fraction of the substrate becomes enriched (more positive $\delta^{13}\text{C}$ values) in the heavier isotopes as a reaction proceeds. In a closed system, the Rayleigh equation can relate changes in isotope composition to changes in concentration. By this, the enrichment factor ϵ is usually calculated to express the extent of

isotope fractionation.^{169,170} If enrichment factors are known, changes in isotope ratio can be used to assess the extent of *in situ* biotransformation of organic compounds, as well as for characterizing types of transformation reactions.^{171,172} In reductive dehalogenation processes, CSIA may provide useful insights in dehalogenation pathways and dehalogenation reaction mechanisms.^{120,173} So far, only two studies have investigated carbon isotope effects during reductive dehalogenation catalyzed by *D. mccartyi* strain CBDB1. Marco-Urrea et al¹³² reported enrichment factors during reductive dehalogenation of chlorinated ethenes. Griebler. et al¹⁷⁴ observed similar carbon isotope effects during dehalogenation of 1,2,3- and 1,2,4-trichlorobenzenes by strain CBDB1. However, isotope effects during the dehalogenation of brominated compounds are poorly investigated not only for *D. mccartyi* strain CBDB1 but also for other microorganisms.¹⁷⁵ Due to this limitation of data on isotope effects during transformation of brominated compounds, it is still unknown if CSIA can be applied to investigate reductive debromination catalyzed by strain CBDB1, and if isotope effects during reductive dehalogenation of brominated compounds have the same extend as chlorinated compounds.

1.5 Objective of this work

The main objective of this work was to study the debromination processes of brominated flame retardants by the model organism *D. mccartyi* strain CBDB1. Therefore the work was divided into aspects as follows:

- (1) To test the dehalogenation ability of *D. mccartyi* strain CBDB1 onto brominated flame retardants, and investigate the growth yields of *D. mccartyi* strain CBDB1 with brominated flame retardants as electron acceptor.
- (2) To assess possible toxic effects associated with tested brominated compounds during reductive debromination.
- (3) To identify the debromination products, rates and pathway and evaluate the extent of debromination versus dechlorination.
- (4) To investigate if specific *rdh* genes and especially the catalytic subunit of reductive dehalogenase homologous proteins (RdhA) are involved in the dehalogenation of brominated flame retardants.

(5) To investigate the specificity of the reductive dehalogenases involved in debromination and if large molecules such as the brominated flame retardants can enter the active center of the enzymes.

(6) To study the carbon isotope fractionation change during reductive debromination for possible application in the future.

For these aspects, in this study, brominated flame retardants including hexabromobenzene, tetrabromobisphenol A, deca-BDE and HBCD together with two additional compounds 1,3,5-tribromobenzene and bromophenol blue were tested as electron acceptors. Hexabromobenzene and 1,3,5-tribromobenzene were selected and first tested mainly because they have similar structures and molecule sizes with chlorinated benzenes. Since *D. mccartyi* strain CBDB1 grows well with hexachlorobenzene as electron acceptor but cannot dechlorinate 1,3,5-trichlorobenzene, testing the two brominated benzene gives a first insight into the debromination compared to dechlorination. Growth adaption of strain CBDB1 to brominated benzenes could support the adaption to brominated compounds with larger molecule sizes further tested. Among the three larger molecule size of brominated flame retardants, tetrabromobisphenol A and deca-BDE are oligocyclic aromatic compounds while HBCD are aliphatic compounds. Such difference in structure might result in difference in reductive debromination when these compounds are applied as electron acceptor for strain CBDB1. In order to gain a deeper understanding of toxic effects caused by phenolic compounds, bromophenol blue was included here because it showed a structural similarity with TBBPA but the two phenolic compounds have different hydrophobicity, and such difference in hydrophobicity might cause a different extent of toxicity to strain CBDB1 during incubation.

2 Materials and Methods

2.1 Chemicals and gases

Tetrabromobisphenol A was obtained from ABCR GmbH & CO. KG (Germany). Bromophenol blue sodium salt was obtained from AppliChem GmbH (Germany). Deca-BDE, HBCD and brominated benzenes (hexabromobenzene, 1,2,3,5- and 1,2,4,5-tetrabromobenzene, 1,2,4- and 1,3,5-tribromobenzene, 1,2-, 1,3- and 1,4-dibromobenzene, and monobromobenzene) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone with a purity of $\geq 99.5\%$ was obtained from Chem solute (Rennigen, Germany). Hexane, dichloromethane and pentane (all with a purity of $\geq 99\%$) were from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Titanium chlorine (15%) was bought from Merck, Darmstadt, Germany. SYBR Green I double strand DNA stain was from Invitrogen Company, Carlsbad, California, USA. Low-melting SeaPlaque® agarose was purchased from Biozym Scientific GmbH, Hessisch Oldendorf, Germany.

All gases were supplied by Air Liquide Deutschland GmbH, Düsseldorf, Germany. Nitrogen and hydrogen were obtained at a purity of 99.999% and carbondioxide at 99.8% (v/v). Trace oxygen in the gases was eliminated by a reduction column (Ochs, Bovenden, Germany) before used for cultures.

2.2 Cultivation of *D. mccartyi* strain CBDB1

2.2.1 Medium preparation

Strain CBDB1 was cultivated in titanium (III) citrate-reduced, carbonate-buffered synthetic medium with hydrogen as electron donor and brominated compounds as electron acceptors, similar as previously described by Jayachandran et al.¹³⁶ At first, 1 M sodium hydrogen carbonate solution was prepared by dissolving NaHCO_3 with anoxic Millipore water and purged with CO_2 in a serum bottle, and then the bottle was sealed with Teflon-lined rubber septa, crimped with aluminum cap and autoclaved. Reducing agent titanium (III) citrate was prepared by the reaction of titanium chlorine and citrate according to Zehnder and Wuhrmann.¹⁷⁶ The stock solution was adjusted to pH 7 by adding sodium carbonate powder and a final concentration of 0.1 M was obtained by adding anoxic water. Before using, an additional 100

μM of Na_2S (correspond to a final concentration of $1\ \mu\text{M}$ in culture) was added to the stock solution of titanium (III) citrate as a sulfur source for bacteria. Synthetic medium was prepared with essential minerals (Table 2.1), trace elements (Table 2.1), selenite-tungstate solution, $0.5\ \text{mg L}^{-1}$ resazurin as redox indicator and $5\ \text{mM}$ acetate as carbon source in Milli-Q Water. Subsequently, the medium was degassed by purging with N_2 for 1 h and dispensed in 30 mL aliquots to serum bottles (max volume of 60 mL) in the anaerobic chamber (Coy-Labs, Grass Lake, Michigan, USA, filled with N_2 and 2% H_2). Bottles were sealed with Teflon-lined butyl rubber septa, crimped with aluminum caps and autoclaved at 121°C for 40 minutes. After autoclaving, 1% (vol/vol) of $1\ \text{M}$ sodium hydrogen carbonate solution (pH buffer) and 1% (vol/vol) of titanium (III) citrate were injected into bottles with a plastic syringe (Braun, Melsungen Germany) connected to a $0.2\ \mu\text{m}$ sterile filter (Minisart®, Sartorius AG, Göttingen, Germany) near a Bunsen flame. Then bottles of mixture were incubated in dark for at least 6 h to ensure that the medium was reduced. Before adding the electron acceptor and inoculation, a mixture of seven vitamins including vitamin B_{12} (Table 2.1) was also added to the medium. For normal transfers, 5% inoculum from a parent culture was used. For special experiment set up, inoculum percentage was changed to obtain a target starting cell density. After inoculation, the headspace of cultures was pressurized with 20% CO_2 /80% N_2 (0.3 bar overpressure) and additional 0.1 bar overpressure of H_2 . All cultivations were set up in replicates and incubated in the dark at 30°C without shaking. Positive controls (inoculum with parents' electron acceptor), negative controls (inoculum without electron acceptor) and abiotic controls (with electron acceptor but without inoculum) were set up in parallel at the same time.

2.2.2 Different inoculum of cultures

Cultures set up with hexabromobenzene, deca-BDE and HBCD as electron acceptors were inoculated from pre-cultivated cultures of strain CBDB1 grown with 1,2,4-tribromobenzene (with a cell density of $3 \times 10^7\ \text{cells mL}^{-1}$). Cultures set up with 1,3,5-tribromobenzene, tetrabromobisphenol A or bromophenol blue as electron acceptors were inoculated from the well grown hexabromobenzene cultures with a cell density more than $1 \times 10^8\ \text{cells mL}^{-1}$. Inoculum from strain CBDB1 well grown with hexachlorobenzene as electron acceptor was also used in the growth adaption experiment using hexabromobenzene as electron acceptor.

Table 2.1: Compounds used for cultivation and their final concentrations in cultures

	Compound	Final Concentration
Minerals	KH_2PO_4	0.2 g/L
	NH_4Cl	0.27 g/L
	NaCl	1 g/L
	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.41 g/L
	KCl	0.52 g/L
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.15 g/L
Trace elements	NTA	12.8 mg/L
	$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$	2 mg/L
	ZnCl_2	0.07 mg/L
	$\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$	0.08 mg/L
	H_3BO_3	0.006 mg/L
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.19 mg/L
	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.002 mg/L
	$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	0.024 mg/L
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.036 mg/L
Vitamins	4-Aminobenzoic acid	20 $\mu\text{g/L}$
	D(+)-Biotin	5 $\mu\text{g/L}$
	Nicotinic acid	50 $\mu\text{g/L}$
	Ca-D(+)-Pantothenate	25 $\mu\text{g/L}$
	Pyridoxine hydrochloride	75 $\mu\text{g/L}$
	Thiamine chloride-di-hydrochloride	50 $\mu\text{g/L}$
	Cyanocobalamin	50 $\mu\text{g/L}$

2.2.3 Brominated benzenes as electron acceptors

Similar to the cultivation of strain CBDB1 with hexachlorobenzene, hexabromobenzene was added to the medium as crystalline form before the medium was autoclaved.¹³⁶ Hexabromobenzene cultures were sealed with butyl rubber septa and aluminum caps. They were covered with aluminum foil to prevent possible photo-degradation of the BFRs. 1,3,5-tribromobenzene was added to the cultures by one of the following two procedures: first in crystalline form like hexabromobenzene and cultures were sealed with butyl rubber stoppers; second from an acetone stock solution of 180 mM to a final concentration of 30 μM in medium and cultures were sealed with Teflon-lined butyl rubber septa. 1,2,4-tribromobenzene was added

from an acetone stock solution of 1.2 M to a final concentration of 20 μM in medium and cultures were sealed with Teflon-lined butyl rubber septa.

Table 2.2: Slow feeding regime for tetrabromobisphenol A and bromophenol blue

Days	electron acceptor added (μM)	
	addition	cumulative concentration
0	0.01	0.01
3	0.01	0.02
6	0.02	0.04
9	0.02	0.06
12	0.04	0.1
15	0.04	0.14
18	0.08	0.22
21	0.08	0.3
24	0.2	0.5
...	0.2 (every 3 days)	
42	0.2	1.7
45	0.5	2.2
...	0.5 (every 3 days)	
90	0.5	11.7
...	0 (no feeding)	
116	0	11.7

“...” means same amount of electron acceptor was added in days between.

2.2.4 Tetrabromobisphenol A and bromophenol blue as electron acceptors

Stock solutions of tetrabromobisphenol A were prepared with pure acetone as solvent. Stock solutions of bromophenol blue (sodium salt) were prepared in anoxic Millipore water and autoclaved. For initial cultivation, tetrabromobisphenol A was added from a stock solution of 300 mM to a final concentration of 10 μM in culture. Bromophenol blue was added to cultures from a stock solution of 1.5 mM to a final concentration of 20 μM . For slow feeding regime experiment, concentrations of tetrabromobisphenol A or bromophenol blue supplemented to cultures were only at low nanomolar concentrations to avoid toxicity. With an anticipated growth rate of 0.33 d^{-1} (one cell division every three days) of strain CBDB1, doses of tetrabromobisphenol A or bromophenol blue were increased and amended every three days until

an accumulated nominal concentration of 11.7 μM had been added (Table 2.2). For toxicity tests, bromophenol blue was added from a 1.5 mM water stock solution to reach initial concentrations of 5, 10, 15, 20 and 30 μM in cultures. To observe a color change during debromination, newly set up cultures with 10 μM of bromophenol blue used 2 mM of L-cysteine as the reducing agent instead of titanium (III) citrate.

2.2.5 Decabromodiphenyl ether and hexabromocyclododecane as electron acceptors

In order to reach an initial concentration at micromolar level in cultures, deca-BDE or HBCD were added from deca-BDE/silica or HBCD/silica aqueous stock suspensions without bringing in too much acetone. The silica stock suspensions were prepared similar as the approach described by Adrian. et al.¹³⁸ In general, 0.3 g of floated silica powder (240 mesh; Fisher Scientific) was weighed out in a serum bottle and closed with Teflon-lined butyl rubber septa. Then a 0.75 mM stock solution (8 mL) of deca-BDE or HBCD dissolved in dichloromethane were injected into the serum bottle and well mixed with silica by slight manual shaking. Afterwards, dichloromethane was slowly evaporated by purging the liquid surface with anoxic sterile N_2 gas under the fume hood. When all dichloromethane was evaporated, the serum bottle was transferred inside the anaerobic chamber and filled with 30 mL of anoxic Millipore water. Later, the suspension was mixed again by manual shaking and autoclaved. The final ratio of deca-BDE / silica / water or HBCD / silica / water was 20 μmol / 1 g / 100 mL. Before using the aqueous stock of deca-BDE or HBCD for feeding of cultures, the stock suspension was shaken again to ensure homogeneous distribution of the silica particles in the suspension. Finally, deca-BDE or HBCD was added to cultures with a plastic syringe to reach a start concentration of 20 μM . For the cultures incubated with crystalline deca-BDE or HBCD as electron acceptor, the same set up approach with hexabromobenzene cultures as previously described were used.¹³⁶

2.3 Analytical methods

2.3.1 Cell counting

Cell numbers were quantified by direct cell counting with an epifluorescence microscope after staining of the cells with SYBR-green I on agarose-coated slides.¹³⁷ SYBR-green I standard was diluted with sterile TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.2) to 1%, aliquoted to Eppendorf

tubes and stored at -20°C before usage. Agarose-coated slides were prepared by completely dissolving 2 g of low-melting SeaPlaque® agarose in 120 mL anoxic Millipore water with stirring and heating. Then 2 mL of the liquefied agarose was transferred to a 76 mm × 26 mm glass slides (Thermo Scientific, Gerhard Menzel GmbH, Germany) in a way that the slide was uniformly covered. Agarose-coated slides were dried under the clean bench before using for cell counting.

For cell counting, 20 µL of a direct culture sample was mixed with 1 µL of SYBR-green I 1% solution in a sterile Eppendorf tube and incubated in dark for 10 minutes. Then, 18 µL of the stained sample was transferred onto a 20 mm × 20 mm cover glass which was then turned over and placed onto an agarose-coated slide. By this step, cells of strain CBDB1 were homogeneously distributed and immobilized within the 20 mm × 20 mm area. For each sample at least 15 random micrographs were taken by a NikonDS-Ri1 digital camera at 400x magnification. Micrographs were taken by the NIS imaging software (Nikon, Tokyo, Japan) and cell numbers were automatically calculated by ImageJ (<http://imagej.nih.gov/ij/>) and self-designed macros as described by Adrian. et al.¹³⁷ The detection limit for the direct cell counting was 1×10^6 cells mL⁻¹.

2.3.2 Bromide analysis

The concentration of bromide ions in cultures was analyzed by a Dionex-120 ion chromatograph equipped with an IonPac AS4A-SC (4 × 250 mm) column. Before measurement by ion chromatography, a volume of 600 µL was sampled from a culture and transferred to a 1.5 mL Eppendorf tube. Then the sample was centrifuged at 20°C at 10,000 g for 10 min to remove cells and debris, and 500 µL of the supernatant were transferred to ion chromatograph plastic vials and closed with filter caps. The eluent used for the ion chromatograph was a solution of 0.7 mM Na₂CO₃ plus 0.7 mM NaHCO₃ in Millipore water at a constant flow rate of 1.0 mL min⁻¹ at room temperature. The whole measurement time was 22 min and the retention time of bromide was 5.4 min. Under this method, the retention time of chloride was at 3.0 min and could be totally eluted before 4.5 min. The sensitivity was down to about 1 µM of bromide and a calibration curve for bromide was done with sodium bromide solution in a range between two and 200 µM.

2.3.3 Concentration analysis by gas chromatography

All brominated benzenes were analyzed by gas chromatography using an Agilent Technologies 7820A GC system (Agilent Technologies, Inc.) equipped with a standard flame ionization detector and a HP-5 (30 m \times 320 μ m \times 0.25 μ m, 5% phenyl methyl siloxan, Agilent Technologies, Inc.) column. Data was monitored at a rate of 50 Hz. Helium served as the carrier gas and nitrogen was used as the makeup gas, both gases were with a purity of 99.9995%. 1,2,4-tribromobenzene, all dibromobenzene congeners, monobromobenzene and benzene were analyzed based on the headspace mode. In detail, 1 mL of culture was sampled and mixed with 1 mL of 1 M NaCl solution in a 10 mL GC headspace vial. Vials were sealed with Teflon-lined butyl rubber septa and crimped with aluminum caps before measurement. In headspace analysis injection was done from the headspace of pre-incubation vials. 1,2,3,5-tetrabromobenzene, 1,2,4,5-tetrabromobenzene and 1,3,5-tribromobenzene were analyzed based on the liquid mode. For this sample preparation, 1 mL culture or reaction mix was transferred to a 2 mL GC vial then 0.3 mL pure hexane was added. Vials were closed with screw caps and the extraction was done on a shaker at a speed of 500 rpm for 1 h. After this, around 80 μ L of the upper layer solvent was transferred to a new GC vial with a micro-insert (0.1 mL, Lab Logistics Group GmbH, Germany) inside for analysis. Both headspace and liquid mode methods were using the following temperature program: initial temperature was 35°C; increased to 55°C at a rate of 2°C min⁻¹; then increased to 250°C at a rate of 15°C min⁻¹, hold 4 min in final. Both inlet and detector temperatures were 250°C, and transfer line temperature was 200°C. Gas flow rate was constant at 1.5 mL min⁻¹. For headspace mode, the temperature for pre-incubation of the vial was 70°C. Calibration curves for headspace mode were done by the samples from crimped serum bottles including 30 mL medium and brominated benzenes with concentrations of 2 to 200 μ M. Calibration curves for liquid mode were done by the injection of different concentrations (2 to 500 μ M) of brominated benzenes dissolved in hexane.

Deca-BDE, HBCD and their debromination products were detected with a gas chromatograph (7890A, Agilent Technologies, Palo, USA) connected to a mass spectrometer (MS) (5975C, electron ionization source, Agilent Technologies, Palo, USA). Sample preparation was also done by hexane extraction: 1 mL of culture was sampled to a glass tube (10 mL) then 0.5 mL hexane was added. Around 400 μ L of extract was transferred to a 2 mL GC vial and closed with the screw cap before analysis. A BPX -5 capillary column (30 m \times 0.25 mm \times 0.25 μ m) (SGE, Darmstadt, Germany) was used for analysis and the temperature program was as follows: initial temperature was 120°C, increased to 300°C at 10°C min⁻¹ and hold for 5 minutes. Injection was

carried out by splitless mode and the inlet temperature was 300°C. The ion source temperature was 280°C and helium was used as carrier gas. Mass spectrometric detection was performed in full scan mode (m/z 50 to 800) and the mass spectra for identification was m/z 79 and 81 to detect Br^- , m/z 487 and 489 to detect BDE-209 and m/z 643 and 645 to detect HBCD.

2.3.4 Concentration analysis by liquid chromatography-mass spectrometry (UPLC-MS)

Concentration analysis for tetrabromobisphenol A, bromophenol blue and their debromination products were done in cooperation with Cindy Weidauer and Bettina Seiwert (Department of Analytics, Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany). In detail, a Waters ACQUITY UPLCTM system connected to Synapt G2STM equipped with an electrospray ionization source (Waters Corp., Milford, USA) and an Acquity-UPLC-HSST3 column (100 × 21 mm, 1.7 μm particle size) were used for analysis. Two mobile phases used were water (A) and acetonitrile (B). Both were prepared together with 5 mM NH_4OAc and 0.05% acetic acid. The follow mobile gradient was applied: 100% A hold for 2 minutes, increased to 95% B in 5 minutes and hold for 1 minute, then decreased to 100% A and hold for 2 minutes. Flow rate was set to 0.6 mL min^{-1} with a column temperature of 60°C and the injection volume was 10 μL . Electrospray ionization (ESI) in negative mode was used for mass spectrometric analysis with the following parameters: source temperature at 140°C, desolvation temperature at 550°C, capillary voltage at 0.7 kV, sampling cone voltage at 35 kV, source offset at 50 kV and desolvation gas flow at 950 L h^{-1} . Mass spectra were recorded from m/z 50 to 1,200 using “resolution mode” on the instrument. Debromination products were identified by their retention time together with the mass spectra of the molecule and of its main fragments. Quantification of TBBPA and bisphenol A was done by the external calibration curve between 0.1 and 10 μM with a detection limit of 0.03 μM . Since the commercial standards of debromination intermediates of tetrabromobisphenol A were unavailable, the peak areas were shown instead of concentrations. Concentrations of BPB and its debromination products were not quantified in this study.

2.4 RdhA Protein Expression Analysis

2.4.1 Sample preparation

Expression of RdhA proteins in cultures of strain CBDB1 cultivated with hexabromobenzene (sixth transfer), 1,3,5-tribromobenzene (first and third transfers), tetrabromobisphenol A (first transfer) and bromophenol blue (first and third transfers) were analyzed by shotgun proteomics, i.e. extraction of all proteins and concurrent analysis by nanoLC-MS/MS. At first, 30 mL of each culture was harvested first by filtration through a 1.2 μm cellulose acetate filter (Minisart®, Sartorius AG, Göttingen, Germany) then by centrifugation (Eppendorf centrifuge, 5804R) at 6,000 g and 16°C for 60 min under anoxic conditions. The supernatant was removed and the cell pellet was suspended in 30 μL of 50 mM ammonium bicarbonate (pH 7.8). Re-suspended cells were disrupted by three repeated cycles of freezing and thawing as described previously.¹⁷⁷ Obtained protein lysates were reduced by adding 60 mM dithiothreitol and incubated on a thermomixer (Eppendorf, Comfort 5355, 1.5 mL) at 30°C for 1 h with a speed of 400 rpm, alkylated by adding 120 mM iodoacetamide at 30°C for 1 h in the dark, and digested with 0.6 μg trypsin (Proteomic Sequencing Grade, Promega) at 37°C overnight. Digestion was stopped by adding 100% formic acid to a final concentration of 0.1% and peptide solution was obtained by centrifugation (Eppendorf centrifuge, 5415R) for 20 min at 13,000 g. Tryptic peptides were desalted using ZipTip-C18 material (Merck Millipore, Darmstadt, Germany) and the solvents were evaporated by a centrifugal vacuum concentrator (Christ, lyophilization devices, RVC 2-25 CD plus, Germany) as described previously.¹⁷⁷ Samples were stored at -20°C and resuspended in 15 μL of 0.1% formic acid before nanoLC MS/MS analysis.

2.4.2 nanoLC MS/MS analysis

Resuspended peptides were analyzed by an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoUPLC system (Dionex Ultimate 3000 RSLCnano System, Thermo Fisher Scientific). Samples were first loaded on a trap column (Acclaim PepMap100 C₁₈, 75 μm \times 2 cm, film thickness 3 μm , Thermo Scientific) in the nanoUPLC system to wash off the contaminating salts for 6 min. 96% eluents A and 4% B (Table 2.3) were used at a flow rate of 5 $\mu\text{L min}^{-1}$. Afterwards, peptides were eluted onto the analytical column (Acclaim PepMap100 C₁₈, 75 μm \times 25 cm, film thickness 3 μm , Thermo Scientific) and separated by reversed phase chromatography using a constant flow rate of 300 nL

min⁻¹ at 40°C. For this, a linear gradient of eluent B (Table 2.3) was applied and peptide separation was achieved within 120 min.

Table 2.3: Parameters applied in nanoLC MS/MS analysis

Parameter	Setting
Injection volume	5 µL
Oven temperature	40°C
Eluent A	0.1% formic acid in filtered H ₂ O
Eluent B	80% acetonitrile, 20% double distilled H ₂ O and 0.008% formic acid
Flow rate	0.3 µL/min
Gradient	4% eluent B from 0 - 6 min
	55% eluent B from 7 - 126 min
	90% eluent B from 127 - 131 min
	4% eluent B from 132 – 143 min

The separated peptides were analyzed by an Orbitrap Fusion mass spectrometer equipped with a nano-electrospray ion source (TriVersa NanoMate, Advion). The Orbitrap Fusion was operated in a data-dependent top speed mode with a maximum cycle time of 3 s. Precursor scans at a resolution of 120,000 between m/z 350 and 2,000 were acquired in the Orbitrap. Scans were done with an automatic gain control (AGC) target of 4×10^5 and a maximum ion injection time of 50 ms. The most abundant precursor ions (intensity $\geq 5 \times 10^4$) with an assigned monoisotopic m/z and a charge state of two to seven were selected for MS/MS analysis and isolated by the quadrupole of the machine (1.6 m/z isolation window). Precursor Fragmentation of the precursor ions was done by higher-energy collisional dissociation (HCD) with a normalized collision energy of 30%. The fragment ions were scanned by the Orbitrap mass analyzer at a resolution of 60,000 (AGC target 5×10^4 , maximum ion injection time 100 ms). Obtained LC–MS/MS data were further evaluated with Proteome Discoverer v1.4.1.14 (Thermo Scientific). Identification of peptides was performed using the Mascot database search engine as described previously.¹⁴⁰ Data were searched against the UniProt *D. mccartyi* strain CBDB1 database using the following parameters: cleavage enzyme was trypsin, maximum of two missed cleavages were allowed, mass tolerance of precursor and fragment were 10 ppm and 0.05 Da, respectively. Carbamidomethylation on cysteine was included as static. Oxidation on methionine was set as dynamic modification. Proteins were considered to be expressed when at least two peptides were identified at a false discovery rate (FDR, Target Decoy PSM Validator) of 0.05. For the analyzed triplicate cultures with each electron acceptor, Proteins were ranked when they were expressed in

at least two replicates according to their mean emPAI values (exponentially modified protein abundance index).¹⁷⁸

2.5 Resting cell activity assay

Reductive dehalogenase activity of strain CBDB1 on brominated benzenes was analyzed based on an *in vitro* assay which used reduced methyl viologen as the artificial electron donor and whole non-disrupted resting cells as the catalyst (Figure 2.1). In this study, two different assay setups were applied to measure the dehalogenase activity of strain CBDB1. The first was a photometric activity assay in which a microtiter plate reader (Synergy HT, BioTek, Bad Friedrichshall, Germany) was used and activity was measured photometrically by following the oxidation of methyl viologen at 578 nm. The second was an assay based on gas chromatography measurement in which concentration changes of the substrate and debromination products was used to quantify the dehalogenation activity.

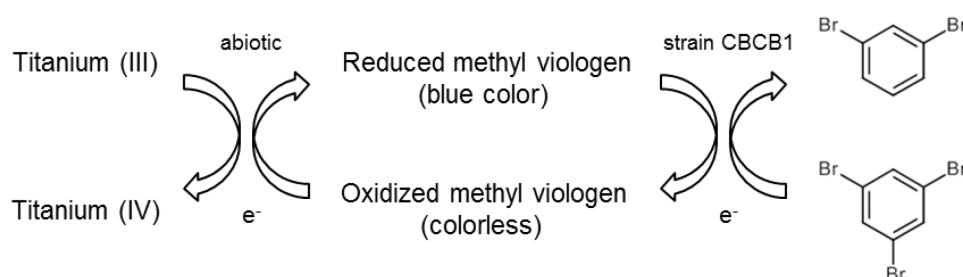


Figure 2.1: Principle of the viologen-based resting cell enzymatic activity assay

2.5.1 Photometric activity assay

The photometric activity assay was carried out in the anaerobic tent and the measurement was based on the absorbance change of the reaction mix at an OD of 578 nm which is the absorption maximum for methyl viologen. For all the compounds tested, 2 mL of reaction mix containing 125 mM potassium acetate buffer (pH 5.8), 1.25 mM methyl viologen and 500 μ M of electron acceptor were pipetted in glass vials. Approximately 2 mM titanium citrate was added afterwards to adjust the final absorbance between 1.5 and 4.0 at 578 nm. 200 μ L of each reaction mix was transferred into each well of a glass microtiter plate. Wells on the microtiter plate were designed to have tested substrates, abiotic controls (test substrate without cell suspensions), positive controls (reaction mix with cell suspensions and substrate which showed activity before) and

negative controls (reaction mix with cell suspensions and only solvent of substrate) in replicates (Figure 2.2).

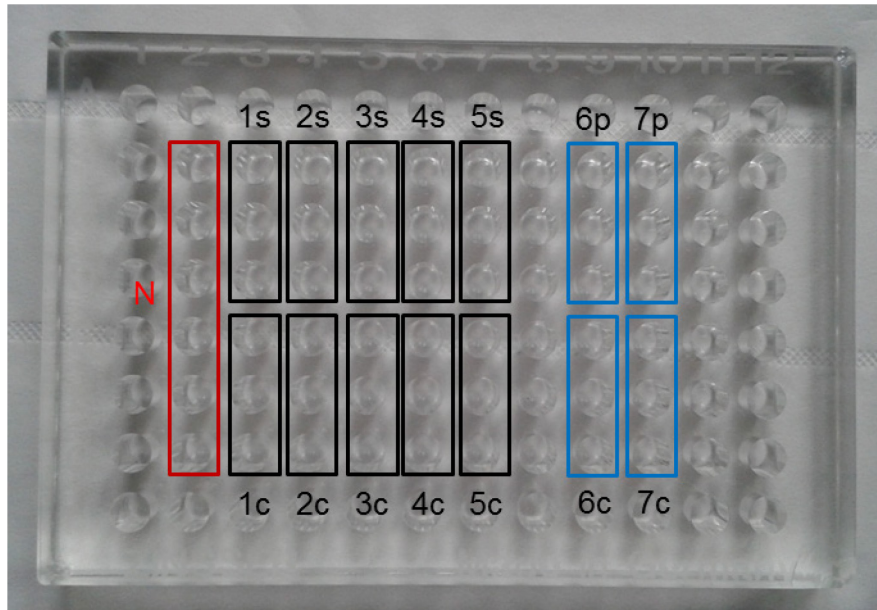


Figure 2.2: Sample design of the enzymatic activity assay in a 96 wells glass microtiter plate. Labels mean: (from 1s to 5s) wells filled with reaction mix for the tested substrates in triplicate, (from 1c to 5c) abiotic controls of the tested substrates without bacterium in triplicate, (6p and 7p) positive controls in triplicate with substrates which showed activity before, (6c and 7c) abiotic controls of the substrates chosen for positive controls, (N) wells filled with negative controls in six replicates with strain CBDB1 but without an electron acceptor.

The reaction was started by adding 30 μL of cell suspensions from hexabromobenzene or 1,3,5-tribromobenzene dehalogenating cultures with a cell number in the culture of at least 2.0×10^7 cells mL^{-1} . The microtiter plate was sealed with a transparent plastic film (VWR, Dresden, Germany) to protect the samples from oxygen contamination and evaporation during the assay process. Absorbance was monitored for 3 hours at 30°C by the microtiter plate reader placed in the anaerobic tent with a measurement interval of 7.5 min. Enzyme activity was calculated from the linear slope of the absorbance curve (Figure 2.3) and by using the following formula:

$$SA_{\text{Sample}} = \frac{(\Delta E_{\text{Substrate}} - \Delta E_{\text{Blank}}) \times V_{\text{Total}} \times 16.67}{\epsilon_{\text{MV}} \times d \times V_{\text{Sample}} \times P}$$

SA_{sample} means the specific activity calculated for measured sample (nkat mg^{-1}), $\Delta E_{\text{substrate}}$ is the decrease of A_{578} measured in a sample with electron acceptor and cells (min^{-1}), ΔE_{blank} means the decrease of A_{578} measured in negative controls without electron acceptor but with cells (min^{-1}), V_{total} means the combined volume of reaction mix and resting cells added (mL), V_{sample} means the volume of resting cells added (mL), ϵ_{MV} is the extinction co-efficient of methyl viologen

($9.78 \text{ cm}^{-1} \text{ mM}^{-1}$), d means the pathway of light through the sample (cm), P means the protein amount in the sample (mg), and it is calculated by number of injected cells \times the protein content per cell (30 fg/cell). Here, the protein determination value of 30 fg protein per cell for strain CBDB1 was used according to the newest data of Anja Kublik, UFZ Leipzig, Germany (unpublished).

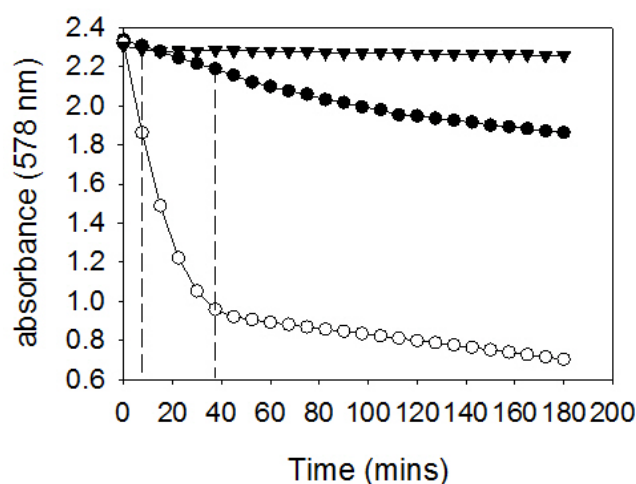


Figure 2.3: Absorbance of the photometric activity assay measured at 587 nm in a 96 wells glass microtiter plate. Symbols mean: (○) Methyl viologen oxidation in samples containing the reaction mix, resting cells of strain CBDB1 and 1,2-dibromobenzene as electron acceptor, (●) Methyl viologen oxidation in negative controls containing the reaction mix, resting cells of strain CBDB1 and acetone, (▼) Methyl viologen oxidation in abiotic controls containing the reaction mix, 1,2-dibromobenzene and medium. The dashed lines mean that the part of the slope in between was used for the calculation of specific activity.

2.5.2 GC-format activity assay

Debromination products of 1,2,3,5- and 1,2,4,5-tetrabromobenzene were identified by the activity assay based on gas chromatograph. In this type of activity assay, 1 mL of reaction mix was prepared in a 2 mL GC vial containing 1.25 mM of methyl viologen, 2 mM of titanium citrate, 125 mM of potassium acetate (pH 5.8) and 100 μM of 1,2,3,5- or 1,2,4,5-tetrabromobenzene. The reaction was initiated by adding 150 μL non-disrupted whole cell suspensions of HBB cultures with a cell number of $2 \times 10^8 \text{ cells mL}^{-1}$ and vials were sealed with screw caps and Teflon-lined butyl rubber septa). Abiotic controls without electron acceptor in replicates were set up at the same time. After 0, 30, 60, 90 and 120 minutes of incubation inside the anaerobic tent, one sample from the replicates was taken out and the reaction was stopped by

adding 150 μL of Na_2SO_4 (saturated solution around 1.4 M, the pH value was adjusted to 1 by adding H_2SO_4) and well mixed with air quickly to lower the pH value below 3 in the sample. By this, the dehalogenation activity of resting cells was ensured to be stopped either by low pH value or oxygen. Subsequently, brominated benzenes in the mixture were extracted with 0.2 mL hexane and analyzed by GC-FID following the procedure described before.

2.6 Compound-specific stable isotope analysis

CSIA was applied during reductive debromination of 1,2,4- and 1,3,5-tribromobenzene, 1,2- and 1,3-dibromobenzene by *D. mccartyi* strain CBDB1. Samples were prepared based on the procedure of enzyme activity assay. At first, 1 mL of reaction mix was set up in glass vials containing 1.25 mM of methyl viologen, 2 mM of titanium citrate, 125 mM of potassium acetate (pH 5.8) and 300 μM of electron acceptor in replicates. Cell suspensions used were from well-grown hexabromobenzene cultures with a cell number of 1×10^8 cells mL^{-1} . Due to different reaction speed, the volumes of cell suspensions added to different electron acceptors were 30 μL (1,2,4-tribromobenzene), 30 μL (1,3,5-tribromobenzene), 15 μL (1,2-dibromobenzene) and 150 μL (1,3-dibromobenzene). Abiotic controls without cell suspension and negative controls without electron acceptor were also set up at the same time. All samples were incubated in the anaerobic tent to avoid oxygen contaminations. After reactions were initiated, every 15 minutes (from 0 to 300 minutes) one sample from the replicates was stopped by adding 150 μL of Na_2SO_4 (saturated solution around 1.4 M, the pH value was adjusted to 1 by adding H_2SO_4) same as described previously. Stopped samples were transported to the 4°C cooling room quickly. Subsequently, 150 μL of pentane was added to the samples and an extraction was carried out on a shaker at a speed of 500 rpm for 1 h. Concentrations of electron acceptors and their products were detected by GC-FID for brominated benzenes as described before.

Stable carbon isotope ratios of brominated benzenes and their debromination products were analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS, Thermo GC Trace 1310 coupled with Thermo-Finnigan MAT 253 IRMS, Bremen, Germany). Three to five μL of the pentane extract were injected into the gas chromatograph with a split of 1:1 using a split/splitless injector at 250°C. For chromatographic separation, a Zebron ZB-1 capillary column (60 m \times 0.32 mm \times 1 μm film thickness, Phenomenex) was used with the following temperature program: 40°C hold for 5 min, increase of 5°C min^{-1} to 80°C, increase of 20°C min^{-1} to 230°C and hold for 10 min, then followed by an increase of 20°C min^{-1} to 320°C

and hold for 15 min. The carbon isotope ratios were expressed in the delta notation ($\delta^{13}\text{C}$) relative to the international standard Vienna Pee Dee Belemnite (V-PDB) according to the following equation.¹⁷⁹

$$\delta^{13}\text{C}_{\text{sample}} = \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1$$

R_{sample} and R_{standard} were the $^{13}\text{C}/^{12}\text{C}$ ratios of the sample and the standard. $\delta^{13}\text{C}$ -values were reported in parts per thousand (‰). All samples were measured at least three times and the typical uncertainty of analysis was <0.5 ‰.

For the description of stable isotope fractionation of biochemical reactions the Rayleigh equation can be applied in its most general form.¹⁷⁰

$$\frac{R_t}{R_0} = \left(\frac{\frac{C_t}{C_0}}{\frac{R_t + 1}{R_0 + 1}} \right)^\epsilon$$

Where R_t , R_0 and C_t , C_0 are the stable isotope ratios and the concentrations of a compound at a given point in time (t) and at the beginning of a transformation reaction (0), respectively. The isotope enrichment factor ϵ correlates the changes in stable isotope ratios (R_t/R_0) with the changes in the concentrations (C_t/C_0).

For stable carbon isotope ratios ($R=^{13}\text{C}/^{12}\text{C}$) with natural abundance, the assumption $R+1 \approx 1$ is valid and the simplified version of the Rayleigh equation can be used for the assessment of stable carbon isotope fractionation of biodegradation processes:

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0} \right)^\epsilon$$

According to the equation for calculating the $\delta^{13}\text{C}$ -values:

$$\frac{\delta_t^{13}\text{C} + 1}{\delta_0^{13}\text{C} + 1} = \frac{\left(\frac{R_t}{R_{\text{standard}}} \right)}{\left(\frac{R_0}{R_{\text{standard}}} \right)} = \frac{R_t}{R_0}$$

The carbon isotope enrichment factor (ϵ) was determined from the logarithmic form of the Rayleigh equation,

$$\ln \left(\frac{(\delta_t^{13}\text{C} + 1)}{(\delta_0^{13}\text{C} + 1)} \right) = \varepsilon_c \ln \left(\frac{C_t}{C_0} \right)$$

Plotting $\ln(C_t/C_0)$ versus $\ln[(\delta_t^{13}\text{C}+1)/(\delta_0^{13}\text{C}+1)]$ gives ε_c from the slope of the linear regression ($m = \varepsilon_c$). Since carbon isotope enrichment factors are typically small, ε_c values were reported in parts per thousand (‰). The error of the isotope enrichment factors is reported as 95% confidence interval determined by a regression curve analysis.¹⁸⁰

3 Results

3.1 Growth of strain CBDB1 with brominated compounds

3.1.1 Growth with hexabromobenzene

D. mccartyi strain CBDB1 cultivated with crystalline hexabromobenzene was first inoculated from cultures grown with 1,2,4-tribromobenzene and set up with an initial cell density of 1×10^6 cells mL⁻¹. Growth of strain CBDB1 was quantified by direct cell counting and debromination activity was quantified by bromide analysis. In the first cultures incubated with hexabromobenzene, a constant bromide release was detected after inoculation and bromide concentration reached 440.7 ± 113.4 μ M on day 77 (Figure 3.1). A slight increase of the bromide concentration was also found in abiotic controls within the incubation time (69.9 ± 3.8 μ M on day 77), indicating that hexabromobenzene was abiotically debrominated either due to photolytic cleavage or by reacting with the reducing agent. The cell density of first transfer cultures with hexabromobenzene increased to 1.6×10^7 cells mL⁻¹ until day 41 which corresponded to a growth yield of 7.6×10^{13} cells mol⁻¹ of bromide released in this time period.

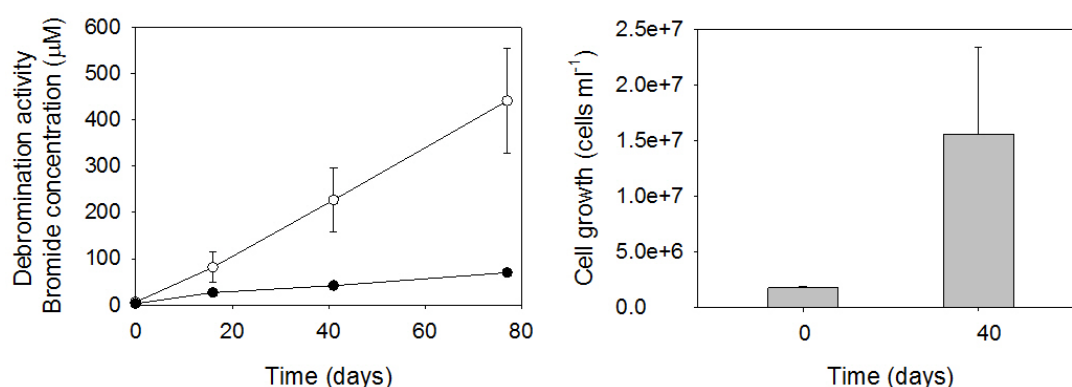


Figure 3.1: The first transfer cultures of *D. mccartyi* strain CBDB1 with hexabromobenzene as electron acceptor. Left: the measured bromide concentration in the cultures of strain CBDB1 within 77 days of incubation. Symbol means: (○) cultures incubated with hexabromobenzene, (●) abiotic controls without inoculation. Right: (gray bar) means the cell density of strain CBDB1 in active cultures at the start of inoculation (1×10^6 cells mL⁻¹) and after 40 days of incubation. Shown are means of triplicate cultures \pm SD.

In the third transfer of cultures with hexabromobenzene as an electron acceptor, similar results were observed in bromide release and cells growth (Figure 3.2). Bromide concentration increased fast after inoculation which reached $481.8 \pm 138.1 \mu\text{M}$ on day 41 and $1022.5 \pm 282.8 \mu\text{M}$ on day 67 in grown cultures. Abiotic bromide release was again detected in controls which reached $55.5 \pm 1.1 \mu\text{M}$ on day 67. In cultures, along with the bromide release, the cell density increased to $8.5 \times 10^6 \text{ cells mL}^{-1}$ after 27 days incubation then continuously went up to $2.4 \times 10^7 \text{ cells mL}^{-1}$ until day 67. Growth yields before day 27 and afterward in the third transfer cultures were 4.2×10^{13} and $0.2 \times 10^{13} \text{ cells mol}^{-1}$ of bromide released, respectively. In total, a growth yield of $2.4 \times 10^{13} \text{ cells mol}^{-1}$ of bromide released was determined for the whole incubation time. Growth of strain CBDB1 using hexabromobenzene as electron acceptor without losing debromination ability was confirmed by up to seven consecutive transfers. The cell density of these hexabromobenzene cultures reached the $1 \times 10^8 \text{ cells mL}^{-1}$ and further up to $5 \times 10^8 \text{ cells mL}^{-1}$ after 3 and 6 months incubation, respectively.

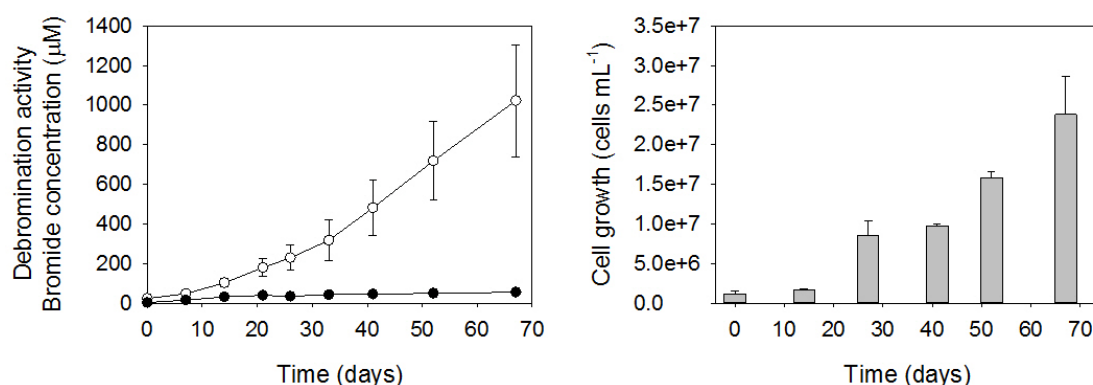


Figure 3.2: The third transfer cultures of *D. mccartyi* strain CBDB1 with hexabromobenzene as electron acceptor. Left: the measured bromide concentration in the cultures of strain CBDB1 within 67 days of incubation. Symbol means: (○) cultures incubated with hexabromobenzene, (●) abiotic controls without inoculation. Right: (gray bar) means the cell density of strain CBDB1 in the active cultures within 67 days of incubation, the starting cell density was $1 \times 10^6 \text{ cells mL}^{-1}$. Shown are means of triplicate cultures \pm SD.

3.1.2 Growth with 1,3,5-tribromobenzene

The first transfer of *D. mccartyi* strain CBDB1 cultures with 1,3,5-tribromobenzene as electron acceptor were inoculated from hexabromobenzene debrominating cultures with a cell density around $1 \times 10^8 \text{ cells mL}^{-1}$ in triplicates. A starting cell density of $5 \times 10^6 \text{ cells mL}^{-1}$ was applied and 1,3,5-tribromobenzene was amended from an acetone stock solution (5 μL of 180 mM) to

reach a final concentration of 30 μM . GC-FID measurements detected debromination products from day 6 on and approximately 120 μM of benzene was formed after 54 days of cultivation (Figure 3.3). In abiotic controls with 1,3,5-tribromobenzene but without inoculation, no debromination products were detected. Along with three times additional feeding of 30 μM 1,3,5-tribromobenzene, the cell density of active cultures increased constantly to 1.4×10^7 cells mL^{-1} after 49 days and finally reached 3.6×10^7 cells mL^{-1} after 54 days. This corresponded to cell growth yields of 3.7×10^{13} and 1.7×10^{14} cells mol^{-1} of bromide released between day 0 to 49 and day 49 to 54, respectively.

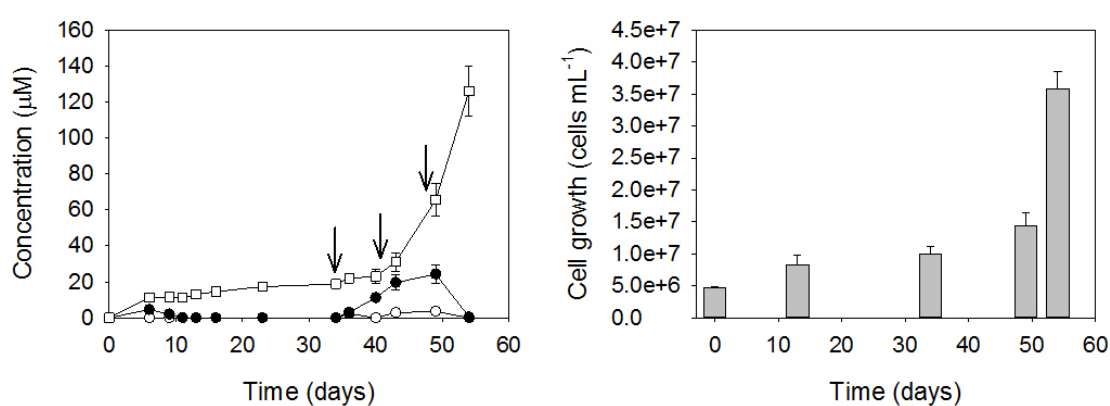


Figure 3.3: The first transfer cultures of *D. mccartyi* strain CBDB1 with 1,3,5-tribromobenzene as electron acceptor. Left: concentrations of debromination products in the cultures within 54 days of incubation. Symbol means: (\circ) 1,3-dibromobenzene, (\bullet) monobromobenzene, (\square) benzene. The concentration of 1,3,5-tribromobenzene could not be determined because it was added above its solubility and was present as crystals in the medium. Arrows mean additional 30 μM of 1,3,5-tribromobenzene was added to the cultures. Right: (gray bar) means the cell density of strain CBDB1 in the active cultures. Shown are means of triplicate cultures \pm SD.

In order to get higher cell densities of cultures but without bringing in too much volume of acetone from 1,3,5-tribromobenzene stock solution, further transfers of *D. mccartyi* strain CBDB1 were cultivated with crystalline 1,3,5-tribromobenzene in triplicates following a similar cultivation procedure as with hexabromobenzene. For this, bromide concentration was measured to identify the debromination activity. In the third transfer of 1,3,5-tribromobenzene cultures, a bromide release of 491 ± 96.9 μM was observed after 59 days of incubation. No increase of bromide concentration was detected both in abiotic controls and negative controls (Figure 3.4). The initial cell density in 1,3,5-tribromobenzene third transfer cultures was 2.7×10^6 cells mL^{-1} . After 39 days of incubation a cell density of 1.9×10^7 cells mL^{-1} was detected and it finally reached 2.1×10^7 cells mL^{-1} after 49 days. This corresponded to a growth yield of 4.6×10^{13}

cells mol⁻¹ of bromide released in total. Strain CBDB1 was transferred more than four times with 1,3,5-tribromobenzene as the sole electron acceptor without losing dehalogenating activity. Furthermore, cell densities of the third and fourth transfers of 1,3,5-tribromobenzene cultures reached 1×10^8 cells mL⁻¹ after 3 months of cultivation.

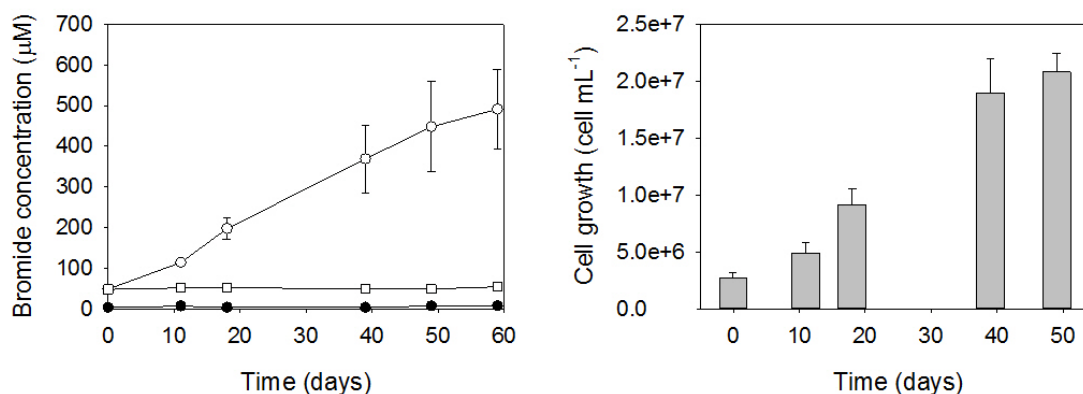


Figure 3.4: The third transfer cultures of *D. mccartyi* strain CBDB1 with 1,3,5-tribromobenzene as electron acceptor. Left: the measured bromide concentration in cultures within 59 days of incubation. Symbol means: (○) cultures of strain CBDB1 incubated with 1,3,5-tribromobenzene, (□) negative controls with inoculation but without electron acceptor, (●) abiotic controls with 1,3,5-tribromobenzene but without inoculation. Right: (gray bar) means the cell density of strain CBDB1 in the active cultures. Shown are means of triplicate cultures \pm SD.

3.1.3 Growth with bromophenol blue

D. mccartyi strain CBDB1 was first cultivated with 20 μM of bromophenol blue with a starting cell density of 2×10^6 cells mL⁻¹ and inoculated from well-grown hexabromobenzene cultures. There was a slight increase of cell density to $3.7 \pm 0.2 \times 10^6$ cells mL⁻¹ after 45 days of incubation (Figure 3.5). The cell density reached a final value of 4.6×10^6 cells mL⁻¹ on day 80 accompanied by approximately 5 μM of bromide release in total. This corresponds to a growth yield of 3.63×10^{14} cells mol⁻¹ of bromide released. No bromide formation was detected in the abiotic controls containing bromophenol blue but without inoculum (data not shown).

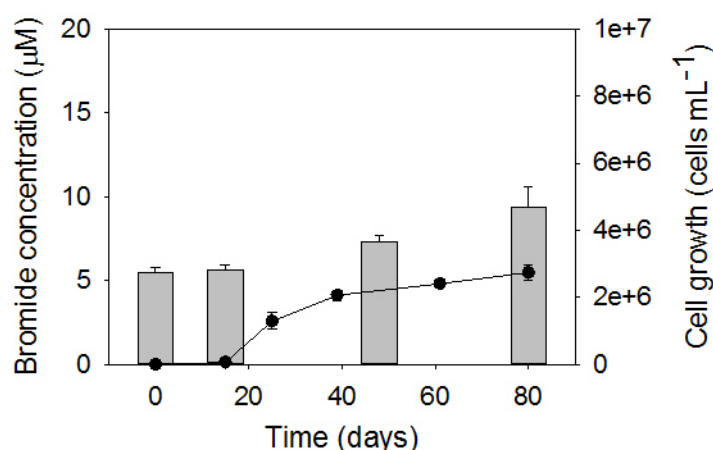


Figure 3.5: The first test cultures of *D. mccartyi* strain CBDB1 with 20 μM of bromophenol blue as electron acceptor. (●) means the measured bromide concentration in cultures of strain CBDB1 during incubation time. (Gray bar) shows the cell density of strain CBDB1 in cultures within 80 days of incubation. Shown are means of triplicate cultures ± SD.

Because the growth and debromination in this first experiment was very low and to investigate if strain CBDB1 was able to grow by debrominating bromophenol blue, new cultures were set up in triplicates with a much lower initial concentration of bromophenol blue in the culture. The rationale was that the low growth in the first cultivation could have been due to toxic effects of high initial bromophenol blue concentrations. These second cultures were inoculated with a starting cell density of 6×10^6 cells mL⁻¹ again from well-grown hexabromobenzene cultures. Bromophenol blue was supplemented only at low nanomolar concentrations (see details of the feeding regime in section 2). Under this feeding regime, debromination activity was found in bromophenol blue cultures (Figure 3.6). Continuous bromide release was detected and the concentration reached 26.2 μM after 75 days of incubation, and it went up to 45.1 μM on day 116 even after feeding was stopped on day 100. Only low bromide concentrations (< 2 μM) were released in abiotic controls. The final concentration ratio of released bromide molecules per supplemented bromophenol blue molecule in active cultures was 3.83 ± 0.56 , indicating that strain CBDB1 cleaved off all four bromine substituents from bromophenol blue. The cell density in bromophenol blue cultures slowly increased to 1.8×10^7 cells mL⁻¹ after 75 days of incubation and stayed above 10^7 cells mL⁻¹ after the feeding was stopped on day 100. This corresponds to a growth yield of 2.7×10^{14} cells mol⁻¹ of bromide released in the first 75 days of cultivation. Debromination of the parent compounds was confirmed by repeating the experiment three times.

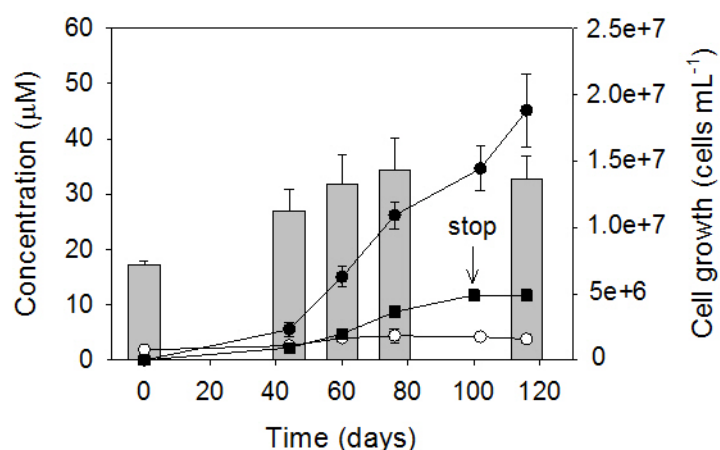


Figure 3.6: The first transfer cultures of *D. mccartyi* strain CBDB1 with bromophenol blue as electron acceptor under the slow feeding regime. Symbol means: (●) the measured bromide concentration in cultures, (■) the calculated total concentration of bromophenol blue added, (○) the measured bromide concentration in abiotic controls without inoculation. Arrow means the slow feeding regime was stopped on day 100. (Gray bar) means the cell density of strain CBDB1 in cultures within 116 days of incubation. Shown are means of triplicate cultures \pm SD.

3.1.4 Cultivation using tetrabromobisphenol A as electron acceptor

The first cultures of strain CBDB1 with tetrabromobisphenol A as electron acceptor were inoculated from well-grown hexabromobenzene cultures to get a starting cell density of 2×10^6 cells mL⁻¹. Tetrabromobisphenol A from an acetone stock solution (1 μ L of 300 mM) was added to the cultures to reach an initial concentration of 10 μ M. After 78 days of incubation, only little bromide ($<0.3\mu$ M) was released in tetrabromobisphenol A cultures (Figure 3.7), suggesting that no microbial debromination occurred. The cell density dropped below 1×10^6 cells mL⁻¹ after 40 days of incubation. There was no bromide detection in the abiotic controls containing tetrabromobisphenol A but without inoculation. In positive controls, strain CBDB1 grew well with crystalline hexabromobenzene.

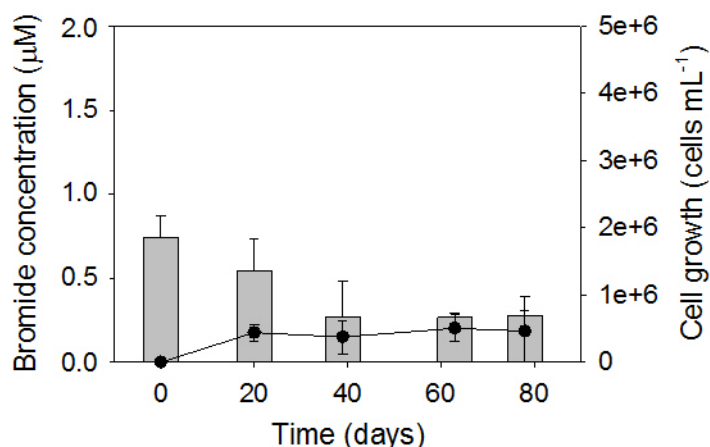


Figure 3.7: The first test cultures of *D. mccartyi* strain CBDB1 with 10 μM of tetrabromobisphenol A as electron acceptor. (●) means the measured bromide concentration in cultures of strain CBDB1 during the incubation time. (Gray bar) means the cell density of strain CBDB1 in cultures within 78 days of incubation. Shown are means of triplicate cultures \pm SD.

Similar to bromophenol blue, the slow-feeding regime was also applied to new tetrabromobisphenol A cultures to test if there was debromination activity when tetrabromobisphenol A was added at very low concentrations. For this, cultures were inoculated from hexabromobenzene cultures to get a starting cell density of around 6×10^6 cells mL^{-1} . Additional tetrabromobisphenol A was supplemented at low nanomolar concentration each time to avoid possible toxicity. After 60 days of incubation approximately 18 μM of bromide had been released in the tetrabromobisphenol A cultures, and this bromide concentration increased constantly and reached 42 ± 3.3 μM on day 118. Very little bromide was released in the abiotic controls where slow-feeding regime of tetrabromobisphenol A was applied but without inoculation (Figure 3.8). In tetrabromobisphenol A debrominating cultures, the final concentration ratio of released bromide anions per supplemented tetrabromobisphenol A molecule was 3.77 ± 0.29 , indicating that tetrabromobisphenol A was fully debrominated to bisphenol A. However, the cell density in tetrabromobisphenol A cultures decreased slowly but continuously in the first 90 days of incubation and then sharply dropped after slow-feeding was stopped on day 100. On the other hand, the cell density of negative controls without electron acceptor decreased below 1×10^6 cells mL^{-1} after 80 days.

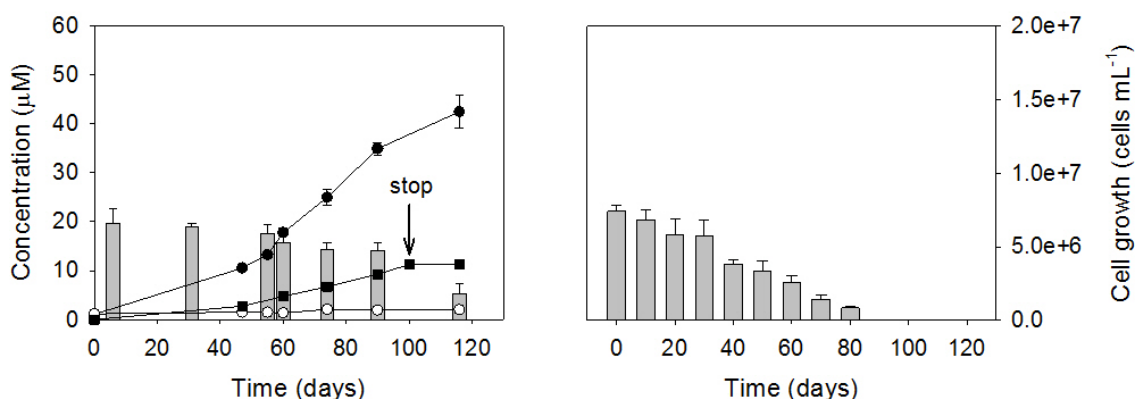


Figure 3.8: Reductive debromination and growth of strain CBDB1 during the slow feeding of tetrabromobisphenol A (left) and of negative controls without an electron acceptor (right). Inocula were from cultures growing with hexabromobenzene. Symbol means: (●) the measured bromide concentration in cultures, (■) the calculated total concentration of tetrabromobisphenol A added, (○) the measured bromide concentration in abiotic controls without inoculation. Arrow means the slow feeding regime was stopped on day 100. (Gray bar) means the cell density of strain CBDB1 in cultures and negative controls during the incubation time. Shown are means of triplicate cultures \pm SD.

3.1.5 Cultivation using decabromodiphenyl ether and hexabromocyclododecane as electron acceptor

Initially, the cultures of *D. mccartyi* strain CBDB1 using either decabromodiphenyl ether or hexabromocyclododecane as the electron acceptor were inoculated from 1,2,4-TBB debrominating cultures. Decabromodiphenyl ether or hexabromocyclododecane was added from a silica stock suspension to reach a final concentration of 20 μM in medium and the starting cell density was 2×10^6 cells mL^{-1} . After 86 days of incubation, the concentration of hexabromocyclododecane decreased to 19 ± 0.2 μM and the concentration of hexabromocyclododecane decreased to 18 ± 2.8 μM . However, no debromination products were detected in either of the two cultures (Figure 3.9). The cell density in decabromodiphenyl ether cultures and hexabromocyclododecane cultures decreased from 2.2×10^6 cells mL^{-1} to 1.6×10^6 cells mL^{-1} and from 2.6×10^6 cells mL^{-1} to 1.3×10^6 cells mL^{-1} after 65 days of incubation, respectively.

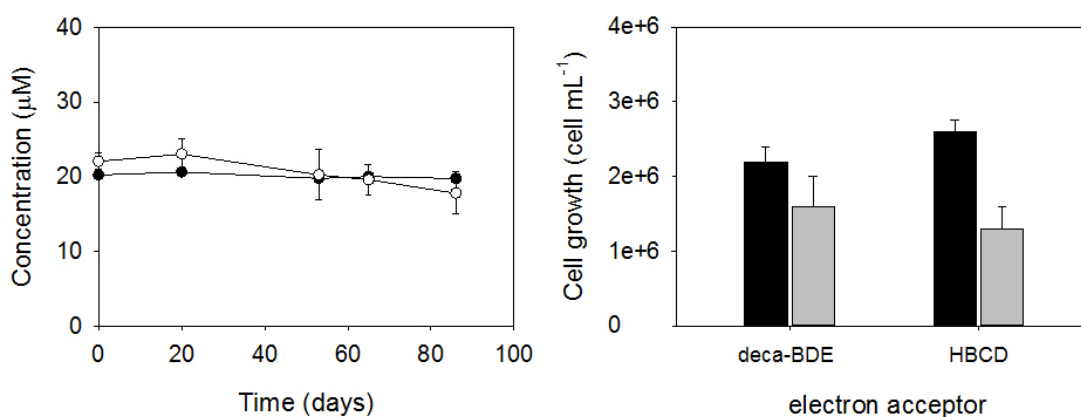


Figure 3.9: Cultures of strain CBDB1 incubated with decabromodiphenyl ether (deca-BDE) or hexabromocyclododecane (HBCD) as the electron acceptor. Left: the measured bromide concentration in cultures incubated with either deca-BDE (●) or HBCD (○). Right: the cell density of strain CBDB1 in cultures with one of the two compounds after inoculation (black bars) and after 65 days of incubation (gray bars). Shown are means of triplicate cultures ± SD.

For a new set up of cultures of strain CBDB1 with either decabromodiphenyl ether or hexabromocyclododecane as electron acceptor, decabromodiphenyl ether or hexabromocyclododecane were supplied as crystalline form similar to the approach for hexabromobenzene cultures described previously. The inoculum of strain CBDB1 was from well-grown hexabromobenzene debrominating cultures and the starting cell densities in both cultures were around 5×10^6 cells mL⁻¹. For these cultures, no increase in bromide concentration was detected in both cultures after 90 days of incubation. There was also no cell growth during the whole incubation, indicating strain CBDB1 might not be able to use either decabromodiphenyl ether or hexabromocyclododecane for reductive dehalogenation. No debromination activity for both compounds by strain CBDB1 was confirmed after repeating all experiments three times.

3.2 Influencing factors and inhibitory effects in the reductive debromination process catalyzed by strain CBDB1

3.2.1 Factors influencing cell growth in hexabromobenzene cultures

(1) Cultures using different inocula

In this test, two different parent cultures were used for a new transfer with hexabromobenzene as an electron acceptor to test the importance of adaptation to an electron acceptor. One parent culture was previous grown with hexabromobenzene, and the other was previous grown with hexachlorobenzene as electron acceptor. New transfer cultures were set up in triplicates with crystalline hexabromobenzene using the same cultivation approach described previously. The starting cell density in both cultures was 2.0×10^6 cells mL⁻¹. After 46 days of incubation, the cell density of the cultures inoculated from hexabromobenzene cultures reached 9.6×10^6 cells mL⁻¹ while the cell density of the cultures inoculated from hexachlorobenzene cultures was still around 2.7×10^6 cells mL⁻¹ (Figure 3.10).

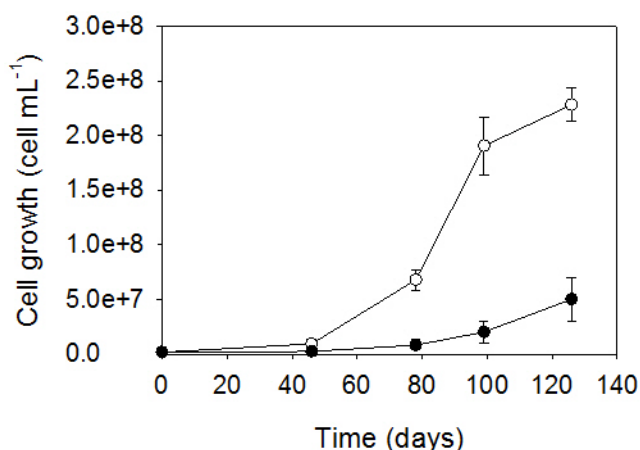


Figure 3.10: Cell growth of *D. mccartyi* strain CBDB1 with hexabromobenzene as electron acceptor. Two different inocula were used: (○) inoculated from cultures of strain CBDB1 pre-grown with hexabromobenzene, (●) inoculated from cultures of strain CBDB1 pre-grown with hexachlorobenzene. Shown are means of triplicate cultures \pm SD.

The growth rate of hexachlorobenzene-inoculated cultures was slower than the rate of hexabromobenzene inoculated cultures, and this difference became larger with the cultivation continued. The cell density of hexabromobenzene-inoculated cultures reached 6.8×10^7 cells mL⁻¹ on day 78 and finally went up to 2.3×10^8 cells mL⁻¹ after 126 days of incubation. On the other hand, the cell density of hexachlorobenzene inoculated cultures slowly reached 5.0×10^7

cells mL⁻¹ after 126 days of incubation, and this value reached 1×10^8 cells mL⁻¹ after another month of incubation time (data not shown).

(2) Cultures using different sealing septa for cultivation

In order to investigate if the accumulated debromination products of hexabromobenzene have influence on the growth of *D. mccartyi* strain CBDB1, a new test was set up by using two different sealing septa for cultivation during the transfers of hexabromobenzene cultures. One type were Teflon-lined rubber septa which did not adsorb organic compounds, and the other type were butyl rubber septa which adsorb hydrophobic organic compounds and can therefore take up products from the headspace of a vial. The expectation was that dehalogenation products from the crystalline hexabromobenzene would be removed by the butyl rubber septa. The parent cultures used in the test were previously well-grown with crystalline hexabromobenzene (the fourth transfer cultures, with butyl rubber septa). Similar as the parent cultures, the cell density of hexabromobenzene cultures sealed with butyl rubber septa grew from 2.1×10^7 cells mL⁻¹ to 3.7×10^7 cells mL⁻¹ after 60 days of incubation, and this number constantly increased to 1.8×10^8 cells mL⁻¹ on day 120 (Figure 3.11).

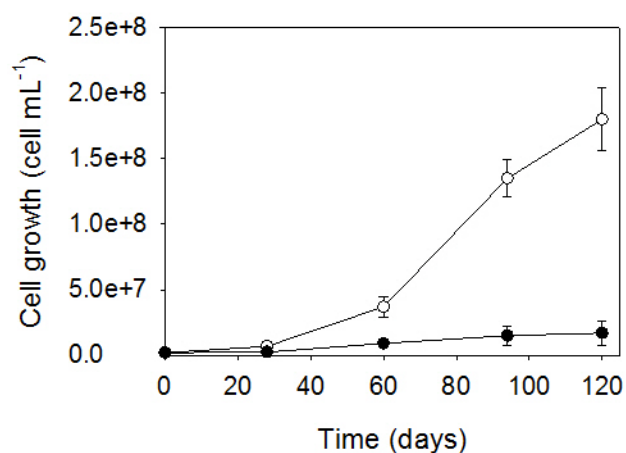


Figure 3.11: Cultivation of *D. mccartyi* strain CBDB1 with crystalline hexabromobenzene in flasks sealed with either Teflon-lined rubber septa (●) or with butyl rubber septa (○). Shown are means of triplicate cultures \pm SD.

On the other hand, the cell density of hexabromobenzene cultures sealed with Teflon-lined rubber septa increased slowly from 2.0×10^6 cells mL⁻¹ to 9.1×10^6 cells mL⁻¹ after 60 days incubation, and this number only went to 1.7×10^7 cells mL⁻¹ until day 120. Finally, the cell density of cultures sealed with Teflon-lined rubber septa kept constant around 2.0×10^7 cells

mL^{-1} even after 4 months of incubation while the cell density of cultures sealed with butyl rubber septa had already went up to more than $1.0 \times 10^8 \text{ cells mL}^{-1}$.

3.2.2 Toxicity tests with bromophenol blue

(1) Test on the initial concentration of bromophenol blue

To investigate the observed inhibition of *D. mccartyi* strain CBDB1 by bromophenol blue in more detail, increasing initial concentrations (5, 10, 15, 20 and 30 μM) of bromophenol blue were added to medium bottles and new cultures were inoculated with the same starting cell density of $6 \times 10^6 \text{ cells mL}^{-1}$ from strain CBDB1 grown with hexabromobenzene. Generally, cultures with higher bromophenol blue concentrations started the debromination process later than cultures with lower concentrations exhibiting the toxic effect of bromophenol blue (Figure 3.12). In cultures with initial bromophenol blue concentrations of 5 μM or 10 μM , bromide was detectable after 15 days of incubation. However, more time was needed until bromide concentrations could be detected in cultures set up with initial 15, 20 or 30 μM of BPB. For the tested cultures with five different bromophenol blue concentrations, the time when half of the total bromide was released was 22, 22, 33, 48 and 60 days, respectively. There was no clear concentration threshold above which debromination was completely inhibited. Overall, the cell densities in all cultures did not increase significantly during the whole incubation time (Figure 3.12), but clear correlations of cell growth and bromophenol blue debromination were identified. In the first 15 days of incubation when almost no debromination activity was detected in any of the cultures, cell densities decreased constantly at all bromophenol blue concentrations. Subsequently, a steep increase of cell density was found for all bromophenol blue concentrations cultures when debromination was started. The starting time of this steep increase was earlier for the cultures with low concentrations of bromophenol blue and later for those cultures with higher concentrations of bromophenol blue. Most evidently, this growth trend can be seen in the cultures with 30 μM bromophenol blue in which the cell density decreased slowly until day 52 then debromination and growth quickly started. In fact, the cell densities exactly followed the order of the initial concentrations of bromophenol blue where the highest cell density was found after 75 days in the cultures which had the highest initial concentration of bromophenol blue.

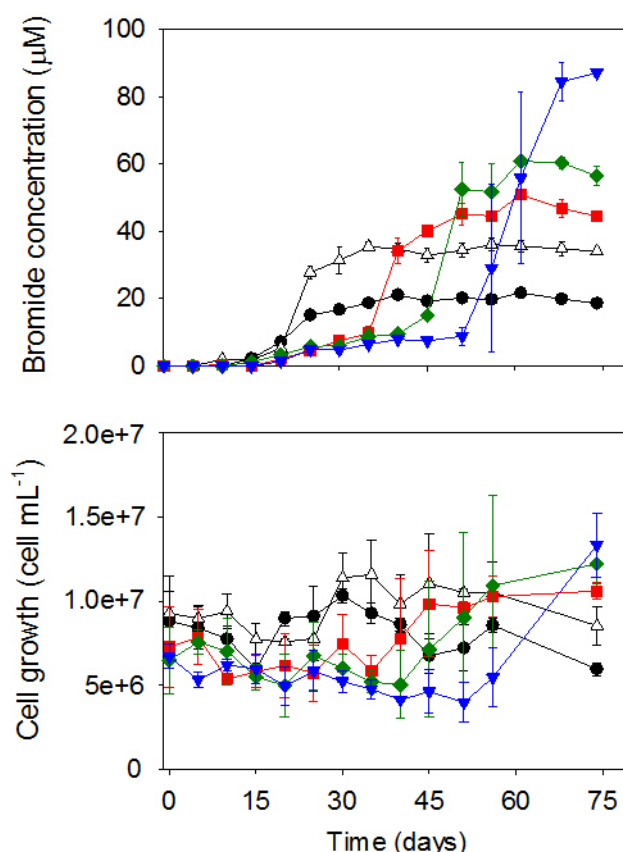


Figure 3.12: Toxicity of bromophenol blue to cultures of *D. mccartyi* strain CBDB1. Bromide concentration (top panel) and cell densities (bottom panel) in the cultures were measured within the incubation time. Cultures were inoculated from cultures pre-grown with hexabromobenzene and amended with different initial concentrations of bromophenol blue: 5 μM (●, black line); 10 μM (Δ , black line); 15 μM (■, red line); 20 μM (◆, green line); and 30 μM (∇ , blue line). Shown are means of triplicate cultures \pm SD.

(2) Test of the effect of the initial cell density on debromination of bromophenol blue

Based on the results of the toxicity tests, a fixed initial concentration of 10 μM bromophenol blue was applied in a second series of cultures. Three different starting cell densities 1×10^6 , 5×10^6 and 1×10^7 cells mL^{-1} (inoculated from hexabromobenzene cultures) were applied to investigate the influence of initial cell density onto bromophenol blue debromination and growth of strain CBDB1. With the higher initial cell density, faster debromination was initiated as evidenced by bromide release (Figure 3.13). Bromide was detectable in the cultures with initial cell density of 5×10^6 cells mL^{-1} around three weeks after inoculation. In the cultures with doubled initial cell density (1×10^7 cells mL^{-1}), the detection time of bromide was advanced to one week. During the whole incubation time no significant growth was detected in all cultures

amended with 10 μM of bromophenol blue (Figure 3.13). However, in the cultures with the highest initial cell density a growth of 4×10^6 cells mL^{-1} was identified during the strongest bromide release from day 8 to day 22.

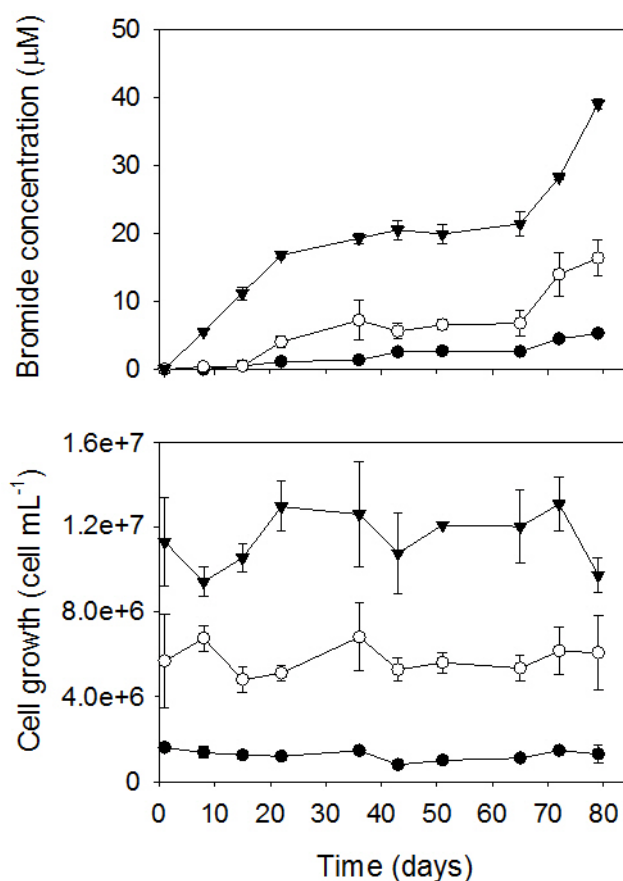


Figure 3.13: Effect of different initial cell densities to the cultures of *D. mccartyi* strain CBDB1 incubated with bromophenol blue. Bromide concentration (top panel) and cell densities (bottom panel) in the cultures were measured within the incubation time. Cultures were set up with an initial 10 μM of bromophenol blue and inoculated from cultures pre-grown with hexabromobenzene. Symbols mean starting cell densities: 1×10^6 (\bullet), 5×10^6 (\circ), and 1×10^7 cell mL^{-1} (\blacktriangledown). Shown are means of triplicate cultures \pm SD.

3.3 Dehalogenation products of brominated organics by strain CBDB1

3.3.1 Debromination products of 1,3,5 -tribromobenzene

D. mccartyi strain CBDB1 reductively debrominated 1,3,5-tribromobenzene to benzene via 1,3-dibromobenzene and monobromobenzene (Figure 3.3). Benzene was detected with a concentration of 11.3 μM on day 6 after inoculation, and it slowly accumulated to 14.6 μM before additional 1,3,5-tribromobenzene was supplied to the cultures. Finally, after three additional feedings of 30 μM 1,3,5-tribromobenzene, the benzene concentration reached 126.1 μM on day 54. 1,3-dibromobenzene hardly accumulated during the whole incubation time. Only trace concentrations of 1,3-dibromobenzene could be detected shortly after new 1,3,5-tribromobenzene was supplied, such as 2.4 μM on day 36 and 3.6 μM on day 49. This indicated that 1,3-dibromobenzene was quickly formed and then further debrominated by strain CBDB1. Monobromobenzene was detected at low concentrations (around 3 μM) within the first 9 days after inoculation. When new 1,3,5-tribromobenzene was added to the cultures, especially if the interval time of feeding was less than 8 days, accumulation of monobromobenzene was detected, showing the highest concentration of 24.3 μM on day 49. The concentration of monobromobenzene finally dropped to 0.5 μM after 54 days of incubation. Due to low water solubility, the concentration of 1,3,5-tribromobenzene was not analyzed for these cultures.

3.3.2 Debromination products of hexabromobenzene

For identifying the debromination products of hexabromobenzene by *D. mccartyi* strain CBDB1, sample extracts from different time points of the cultures grown with hexabromobenzene were initially analyzed on a GC-MS instrument. However, due to the detection limitation of the GC-MS instrument in our lab and the lack of commercial standards of pentabromobenzene, 1,2,3,4-tetrabromobenzene and 1,2,3-tribromobenzene, only one tribromobenzene congener (on the basis of its m/z value), monobromobenzene and benzene were identified as the debromination products of hexabromobenzene. The detected tribromobenzene congener had the same retention time with 1,3,5-tribromobenzene but it could also be 1,2,3-tribromobenzene. As the results shown before, the debromination of 1,3,5-tribromobenzene by strain CBDB1 has been demonstrated. Reductive dehalogenation of 1,2,4-tribromobenzene by strain CBDB1 was also evidenced by the previous research¹⁴⁰ It is still not clear whether other brominated benzenes are intermediates from the debromination of hexabromobenzene. In order to get a further

understanding about the whole debromination pathway of hexabromobenzene by strain CBDB1, here we tested the debromination products of 1,2,3,5-tetrabromobenzene and 1,2,4,5-tetrabromobenzene using the GC-format resting cell activity test (see section 2.5.2). All the reaction mix of the activity assay set-up in multiple replicates and debromination activity in the activity tests were stopped after different incubation times within the first 20 min after inoculation in order to identify the initial debromination product.

For the activity assay with 1,2,4,5-tetrabromobenzene as electron acceptor, after 20 min of reaction, the concentration of 1,2,4,5-tetrabromobenzene in the hexane extract decreased from 378.4 μM to 310.0 μM . In the meantime, the concentration of 1,3/1,4-dibromobenzene (the two congeners could not be differentiated due to identical retention times) increased from 1.2 μM to 8.4 μM (Figure 3.14). 1,2,4-tribromobenzene is the only possible debromination product of 1,2,4,5-tetrabromobenzene. However, there was no formation of 1,2,4-tribromobenzene detected in any samples during the whole reaction time. No debromination products were found in abiotic controls without cell suspension. The concentrations of brominated benzenes in negative controls without electron acceptor were all below the detection limit. It was highly possible that in the test 1,2,4,5-tetrabromobenzene was first dehalogenated to 1,2,4-tribromobenzene by strain CBDB1, then 1,2,4-tribromobenzene was quickly further transformed to 1,3-dibromobenzene and 1,4-dibromobenzene thereby no accumulation of 1,2,4-tribromobenzene was detected.

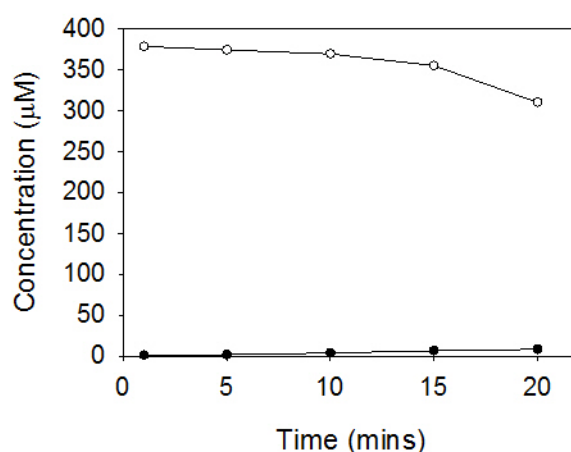


Figure 3.14: Reductive debromination of 1,2,4,5-tetrabromobenzene by strain CBDB1 in the activity assay. Concentrations of the substrate and debromination products at different stop points were measured. Symbols mean: (○) 1,2,4,5-tetrabromobenzene, (●) 1,3/1,4-dibromobenzene which could not be differentiated due to identical retention times.

For the activity assay with 1,2,3,5-tetrabromobenzene as electron acceptor, after 13 min of incubation, the concentration of 1,2,3,5-tetrabromobenzene in the hexane extract decreased from 434.0 μM to 330.8 μM and the concentration of 1,3,5-tribromobenzene increased from 8.1 μM to 22.0 μM (Figure 3.15). This demonstrated that *D. mccartyi* strain CBDB1 debrominated 1,2,3,5-tetrabromobenzene to 1,3,5-tribromobenzene in the activity assay. 1,3/1,4-dibromobenzene was quickly formed with a concentration of 1.9 μM after 1 min of reaction and it finally accumulated to 13.2 μM at 13 min. Similar as the test of 1,2,4,5-tetrabromobenzene no accumulation of 1,2,4-tribromobenzene was detected during the whole reaction time and none of debromination products were found in both controls. However, different to 1,2,4,5-tetrabromobenzene, the possible one step debromination products of 1,2,3,5-tetrabromobenzene are 1,3,5-, 1,2,4- and 1,2,3-tribromobenzene. In order to test if the production of 1,3-dibromobenzene was from 1,3,5-tribromobenzene under such conditions, another activity assay with 1,3,5-tribromobenzene as electron acceptor was performed. In this assay, the initial concentration of 1,3,5-tribromobenzene in the reaction mix was only added at 4.0 μM (corresponding to 20.0 μM in the 200 μL hexane extract) and five stop time points were selected within 13 min. No debromination products were found in the extracts of all stopped samples indicating 1,3,5-tribromobenzene could not be transformed to 1,3-dibromobenzene in the activity assay. Therefore, the results indicated that 1,2,3,5-tetrabromobenzene was also debrominated to 1,2,4-tribromobenzene by strain CBDB1 then quickly debrominated to 1,3/1,4-dibromobenzene. Due to the unavailable standard of 1,2,3-tribromobenzene, our results could not exclude that 1,2,3,5-tetrabromobenzene was also debrominated to 1,2,3-tribromobenzene and then further to 1,3-dibromobenzene.

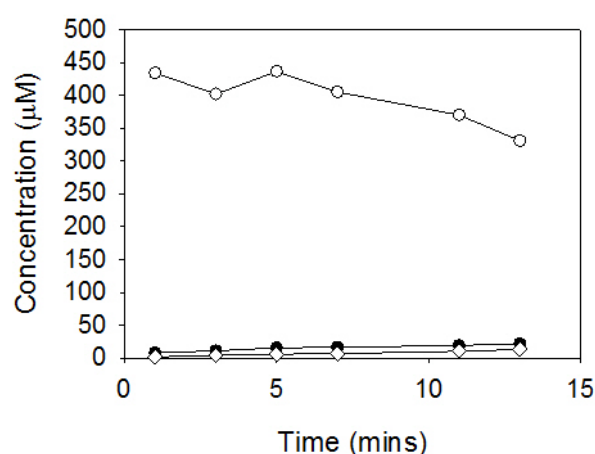


Figure 3.15: Reductive debromination of 1,2,3,5-tetrabromobenzene by strain CBDB1 in the activity assay. Concentrations of the substrate and debromination products at different stop points were measured. Symbols mean: (○) 1,2,3,5-tetrabromobenzene, (●) 1,3,5-tribromobenzene, (◇) 1,3/1,4-dibromobenzene which could not be differentiated due to identical retention times.

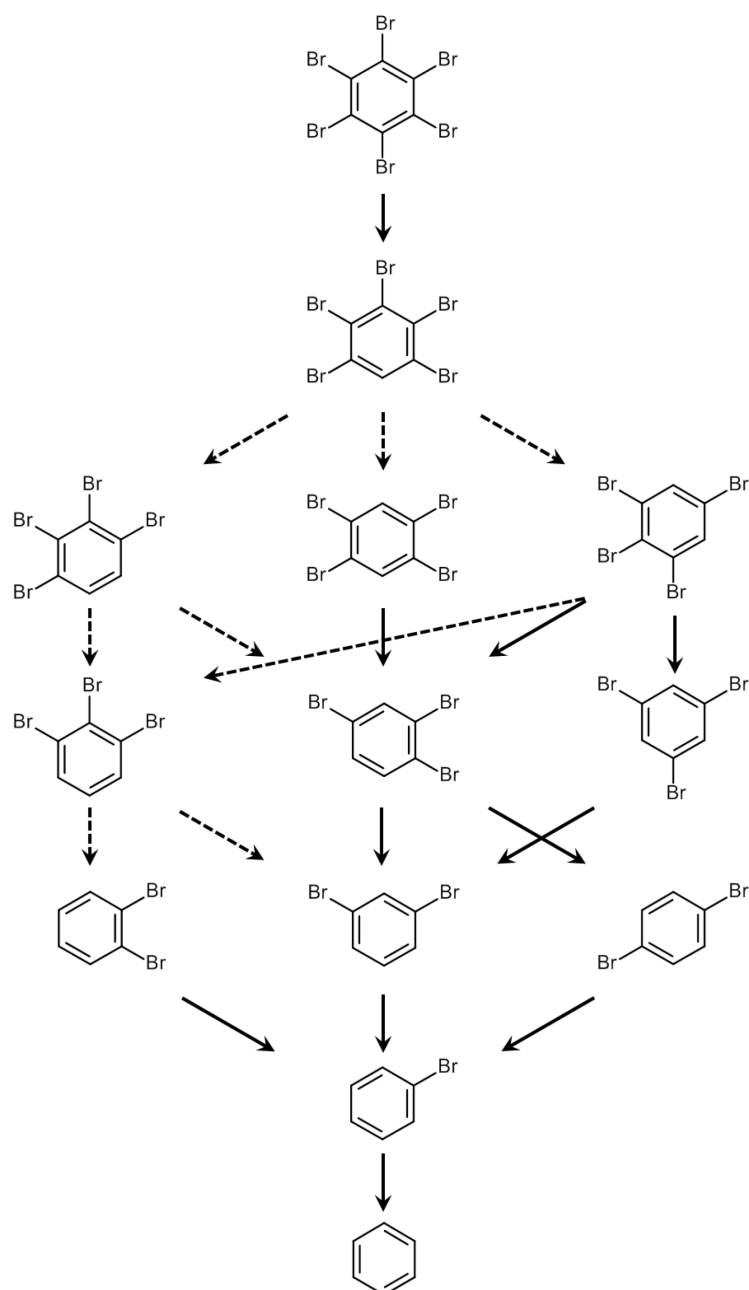


Figure 3.16: Debromination pathways of hexabromobenzene by *D. mccartyi* strain CBDB1. Arrows with solid line are the pathways certified by this study and results from Wagner, et al.¹⁴⁰ Arrows with dotted line show unidentified pathways.

Debromination products of 1,2,4,5-tetrabromobenzene and 1,2,3,5-tetrabromobenzene from the activity assay were confirmed by repeating the experiments three times. Together with the published data for the debromination products of 1,2,4-tribromobenzene and 1,2-dibromobenzene,¹⁴⁰ the current knowledge about the debromination pathway of hexabromobenzene by *D. mccartyi* strain CBDB1 is shown in figure 3.16. The only final debromination product of hexabromobenzene by strain CBDB1 from all possible pathways is benzene.

3.3.3 Debromination products of bromophenol blue

Debromination products of bromophenol blue by *D. mccartyi* strain CBDB1 were identified by ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOF-MS). For this, cultures were incubated in triplicates with an initial bromophenol blue concentration of 10 μ M and no additional feeding was applied. Strain CBDB1 reductively dehalogenated bromophenol blue to the non-halogenated congener (phenol red) by stepwise removing all four bromine substituents as shown by bromide measurements (Figure 3.17).

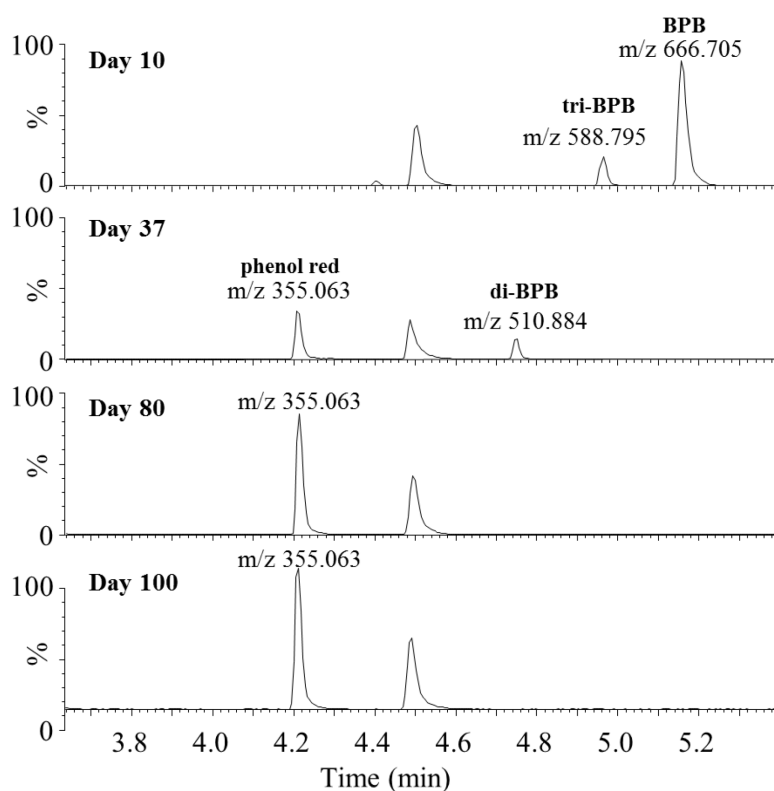


Figure 3.17: Base peak chromatogram of debromination products from bromophenol blue (BPB) in the cultures of strain CBDB1 analyzed by UPLC-MS. The proposed formula for tribrominated congener (tri-BPB [M-H]) is $C_{19}H_{12}O_5SBr_3$, that for dibrominated congener (di-BPB [M-H]) is $C_{19}H_{13}O_5SBr_2$, and that for phenol red [M-H] is $C_{19}H_{15}O_5S$. The monobrominated congener was not detected. The peak at a retention time of 4.54 min was an unknown compound and was also detected in the negative controls without an electron acceptor.

After 10 days of incubation, the tribrominated congener was first detected. Subsequently, dibrominated congeners were identified at day 37 when none of the two higher-brominated congeners were left. From day 37, phenol red was also detected and the concentration increased constantly over time until no brominated congeners were left. The monobrominated congener was never detected during the whole incubation time. bromophenol blue and phenol red are

widely used pH indicators and they have blue and yellow colors in water solution around pH 7, respectively.¹⁸¹ In accordance with that, the color of the bromophenol blue debrominating cultures (in which 2 mM of L-cysteine was used as the reducing agent instead of titanium (III) citrate) turned from blue via rose red to yellow during the incubation time, whereas the color in abiotic controls without inoculum stayed blue (Figure 3.18). The pH values in both the debrominating cultures and the abiotic controls stayed between 6.8 and 7.0 during the whole incubation time, which confirmed that the color change was due to debromination and not due to pH changes.

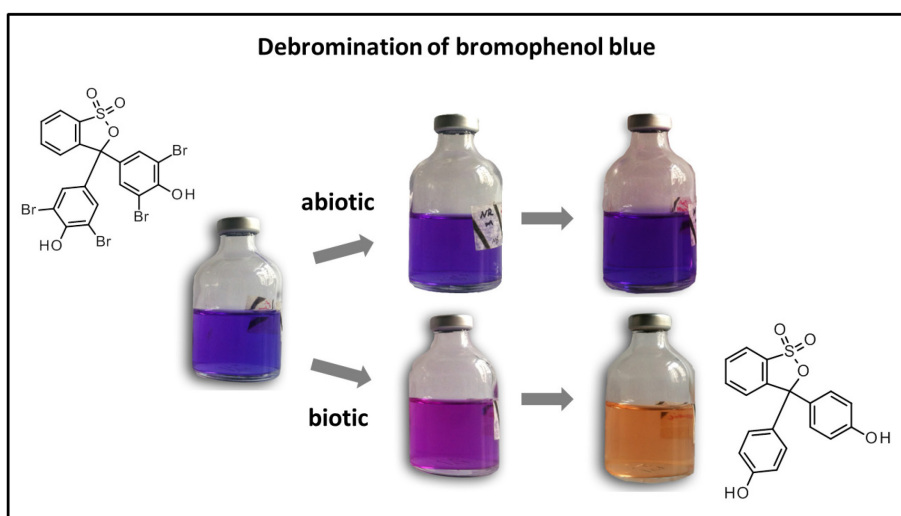


Figure 3.18: Color change of the medium in reductive debromination of bromophenol blue by strain CBDB1. 2 mM of L-cysteine instead of titanium (III) citrate was used as the reducing agent in the cultures in order to observe the color change.

3.3.4 Debromination products of tetrabromobisphenol A

As with bromophenol blue, debromination products of tetrabromobisphenol A were identified by UPLC-TOF-MS. For this, cultures of strain CBDB1 were incubated in triplicates with tetrabromobisphenol A under the slow feeding regime and samples were taken and extracted during the incubation. Strain CBDB1 reductively dehalogenated tetrabromobisphenol A via tribromobisphenol A, dibromobisphenol A, and monobromobisphenol A to bisphenol A as the final product (Figure 3.19).

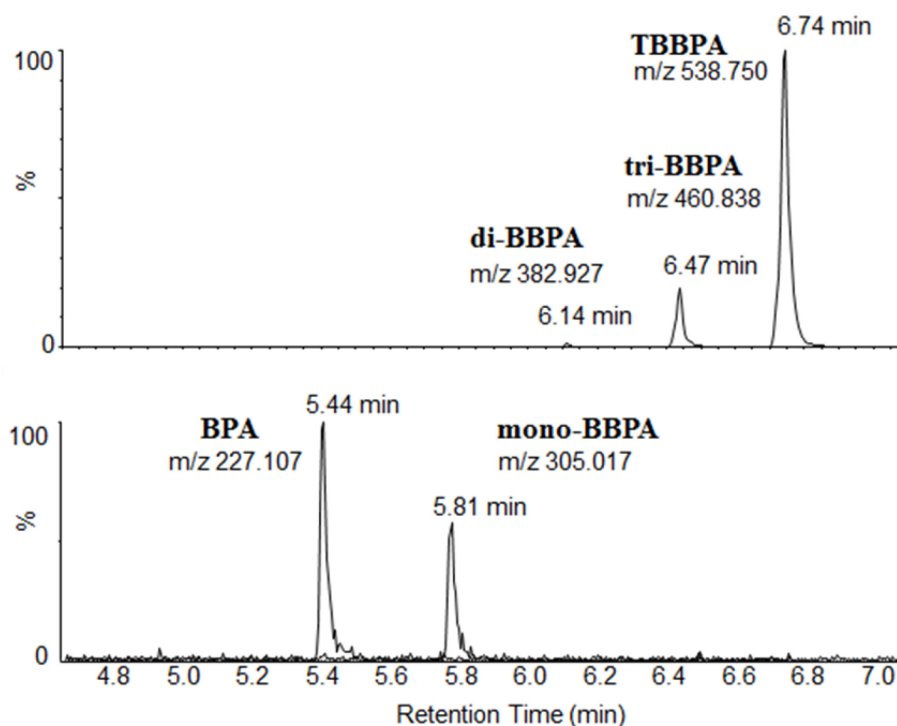


Figure 3.19: Base peak chromatogram of debromination products from tetrabromobisphenol A (TBBPA) in the cultures of strain CBDB1 after 4 days (top panel) and 22 days (bottom panel) of incubation. The proposed formula for tribrominated congener (tri-BBPA [M–H]) is $C_{15}H_{12}O_2Br_3$, that for dibrominated congener (di-BBPA [M–H]) is $C_{15}H_{13}O_2Br_2$, for monobrominated congener (mono-BBPA[M–H]) is $C_{15}H_{14}O_2Br$, and that for bisphenol A (BPA [M–H]) is $C_{15}H_{15}O_2$.

Quantitative results showed that tetrabromobisphenol A was dehalogenated throughout the whole cultivation (Figure 3.20). Tetrabromobisphenol A was only detectable in the first 12 days of incubation, with a maximum concentration of 1.38 μM on day 12, after which tetrabromobisphenol A did not further accumulate. Tribromobisphenol A was detected from day 2 in traces and reached maximum area counts (corresponding to an estimated concentration of 0.21 μM) on day 12; afterwards, the area counts dropped below the detection limit. Similar results were found for di- and monobromobisphenol A. The final debromination product bisphenol A accumulated from day 12 on and reached 13.7 μM after 51 days of cultivation. In abiotic controls without inoculum, concentrations of tetrabromobisphenol A increased parallel with the slow feeding regime and no debrominated products were detected over time. The mass loss of tetrabromobisphenol A in cultures compared to that in abiotic controls could be due to the adsorption of the hydrophobic tetrabromobisphenol A to cell membranes of strain CBDB1.

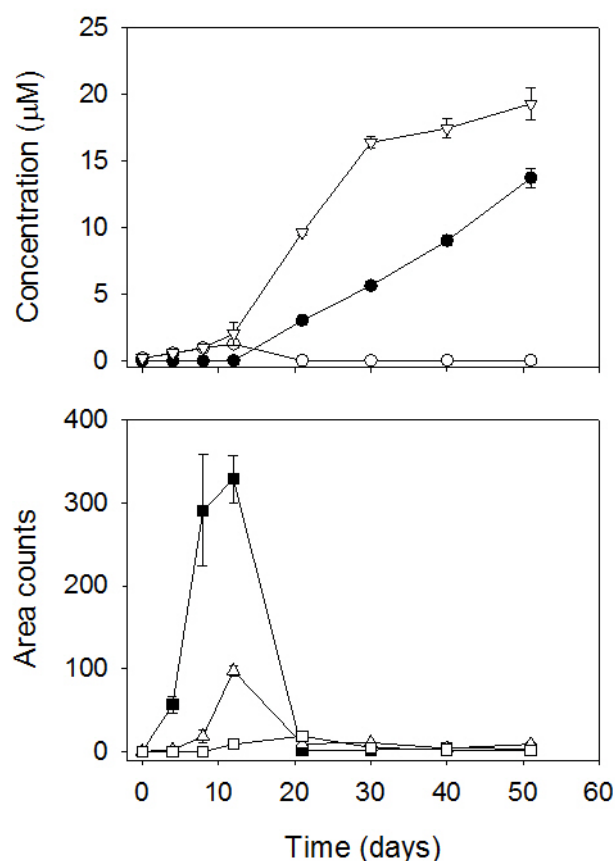


Figure 3.20: Quantification of tetrabromobisphenol A and its debromination products in the cultures of strain CBDB1 under the slow feeding regime. (top panel) Measured concentration of tetrabromobisphenol A (○) and bisphenol A (●) in active cultures, and accumulated tetrabromobisphenol A (▽) in abiotic controls without inoculation. (bottom panel) Area counts of intermediates detected in active cultures by UPLC-MS. Symbols mean: tribromobisphenol A (■), dibromobisphenol A (Δ), and monobromobisphenol A (□). Concentrations of the three compounds were not quantified due to unavailable commercial standards. Shown are means of triplicate cultures \pm SD.

3.4 RdhA Protein Expression

3.4.1 RdhA protein expression in cultures grown with hexabromobenzene or 1,3,5-tribromobenzene

The expression of specific reductive dehalogenases in *D. mccartyi* strain CBDB1 cultivated with hexabromobenzene or 1,3,5-tribromobenzene were investigated by using shotgun proteomics. In sum, seven distinct RdhA proteins were identified in the cultures incubated with these two brominated benzenes (Table 3.1). CbdbA80 and CbrA (CbdbA84, the trichlorobenzene dehalogenase¹⁵¹) were the two most abundant RdhA proteins according to the emPAI value for the estimation of the relative abundance of reductive dehalogenase proteins.¹⁷⁸ CbdbA1453 was expressed in both the sixth transfer of hexabromobenzene cultures and the first transfer of 1,3,5-tribromobenzene cultures but not in the second transfer of 1,3,5-tribromobenzene cultures. CbdbA187 was only expressed in cultures grown with hexabromobenzene and CbdbA1588 was only expressed in the first transfer of 1,3,5-tribromobenzene cultures.

Table 3.1: RdhA proteins identified in cultures of *D. mccartyi* strain CBDB1 incubated with hexabromobenzene, 1,3,5-tribromobenzene, 1,2,4-tribromobenzene, tetrabromobisphenol A, or bromophenol blue as electron acceptors.

e ⁻ acceptor	hexabromobenzene		1,3,5-tribromobenzene				1,2,4-tribromobenzene ^R		tetrabromobisphenol A		bromophenol blue			
	6th transfer		1st transfer		2nd transfer		5th transfer		1st transfer		1st transfer		3rd transfer	
Rdh _{CBDB1}	Score	emPAI	Score	emPAI	Score	emPAI	Score	emPAI	Score	emPAI	Score	emPAI	Score	emPAI
CbdbA80	421	1.40	866	3.07	426	0.73	523	1.75	337	1.46	870	3.63	447	1.71
CbdbA84 (CbrA)	561	1.96	796	2.79	456	1.39	379	0.6	433	1.97	612	2.45	nd	nd
CbdbA1618	37.5	0.1	86	0.15	44	0.08	111	0.13	114	0.32	201	0.65	71	0.15
CbdbA1455	32	0.12	55	0.12	34	0.08	293	0.6	82	0.29	173	0.76	61	0.31
CbdbA1453	43	0.06	124	0.29	nd	nd	279	0.44	154	0.26	90	0.12	nd	nd
CbdbA1638	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	87	0.22	nd	nd
CbdbA1588	nd	nd	68	0.18	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CbdbA187	113	0.25	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CbdbA1092	nd	nd	nd	nd	nd	nd	nd	nd	327	1.17	349	1.29	231	1.02
CbdbA1503	nd	nd	nd	nd	nd	nd	nd	nd	204	0.36	339	0.73	428	1.47

^R means data was from Wagner. et al.¹⁴⁰ 1st, 2nd, 3rd, 5th, and 6th were the respective generation transfer cultures. Proteins were ranked when they were expressed in at least two replicates out of the measured triplicate cultures according to their mean emPAI values.

3.4.2 RdhA protein expression in cultures incubated with bromophenol blue or tetrabromobisphenol A

Compared with brominated benzenes, bromophenol blue and tetrabromobisphenol A have more aromatic rings and additional hydroxyl substituents. To investigate if these differences in structure induces specific reductive dehalogenases, the expression of RdhA proteins in cultures of *D. mccartyi* strain CBDB1 grown with bromophenol blue and tetrabromobisphenol A were also analyzed by shotgun proteomics. For both brominated phenolic compounds, a set of seven to eight distinct RdhA proteins were identified in at least two cultures of the first generation triplicate (Table 3.1). Similar to cultures grown with hexabromobenzene or 1,3,5-tribromobenzene, CbdbA80 and CbrA (Cbr84) had the highest estimated RdhA protein abundance in all analyzed cultures incubated either with bromophenol blue or tetrabromobisphenol A according to the emPAI value. Notably, two specific RdhA proteins (CbdbA1092 and CbdbA1503) were expressed during cultivation with bromophenol blue or tetrabromobisphenol A but were not detected in the cultures grown with hexabromobenzene, 1,3,5- or 1,2,4-tribromobenzene. To check if such expression profiles of RdhA proteins remained stable during cultivation, we again analyzed the third transfer of cultures with bromophenol blue as the sole electron acceptor. In the third-transfer cultures, only six of the originally identified RdhA proteins in the first generation cultures remained expressed, indicating that the protein expression profile of the first generation cultures might have been still influenced from the inoculum grown with hexabromobenzene. CbdbA80 was still expressed as the most abundant RdhA protein in cultures grown with bromophenol blue, whereas CbdbA84 (CbrA) was almost undetectable in all tested third-transfer cultures. The relative estimated protein abundance of CbdbA1503 was similarly high as that of CbdbA80, while CbdbA1092 was present as the third most abundant RdhA protein.

3.5 Resting cell photometric activity assay

The photometric microtiter plate-based activity assay allowed a fast comparison of activity rates of *D. mccartyi* strain CBDB1 with different brominated benzenes as electron acceptors. The calculated activity values for different electron acceptors tested on the same culture could help to explain the specific expression of reductive dehalogenases, they also

give useful information on selecting the appropriate electron acceptor for large cultivations in the future application. Here, the photometric activity assay was set up in a 96 well glass microtiter plate (see details in section 2.5.1). Resting cells of strain CBDB1 used for this activity assay were grown with hexabromobenzene or 1,3,5-tribromobenzene and cell density in both cultures was around 2.0×10^8 cells mL⁻¹. Thus, specific activities in the assay were based on the expression of reductive dehalogenases in the cultures incubated with the two brominated benzenes. Monobromobenzene, 1,2-, 1,3-, 1,4-dibromobenzene and 1,2,4-, 1,3,5-tribromobenzene were tested as electron acceptor in this assay. 1,2,3- and 1,2,4- trichlorobenzene were included as positive controls. With each electron acceptor the test was done in triplicates.

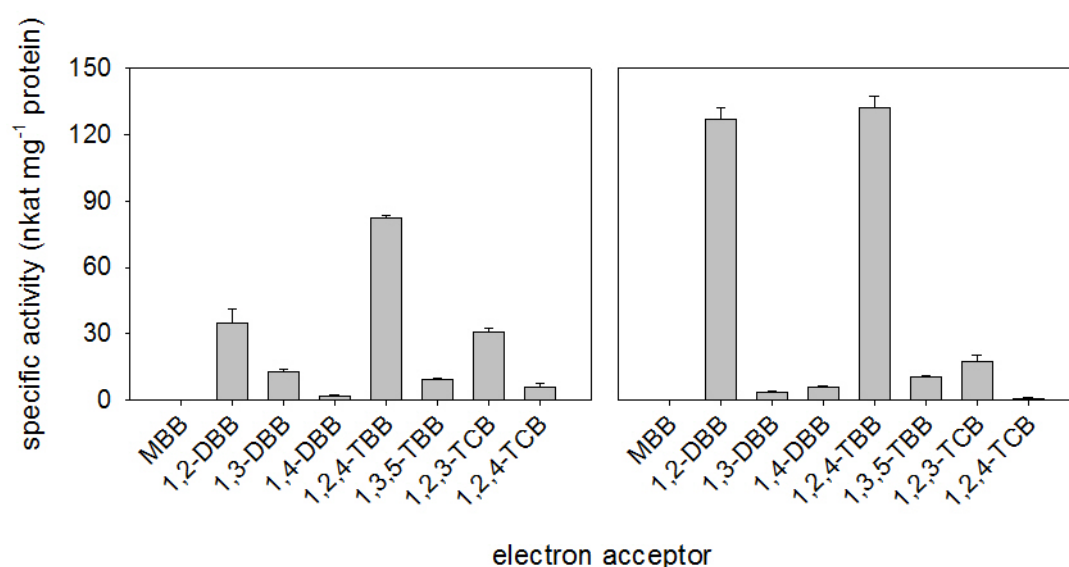


Figure 3.21 Specific activities with halogenated benzenes (gray bars) measured in the photometric activity assay. Resting cells of strain CBDB1 were pre-grown with either hexabromobenzene (left) or 1,3,5-tribromobenzene (right). Shown are the means of triplicate measurements for each electron acceptor in the 96-well microtiter plate photometer-based activity assay \pm SD. Abbreviations: MBB (monobromobenzene), DBB (dibromobenzene), TBB (tribromobenzene), TCB (trichlorobenzene).

For both cultures tested, higher specific activities were detected for 1,2,4-tribromobenzene and 1,2-dibromobenzene than for all the other electron acceptors. No activity was detected with monobromobenzene although the debromination of monobromobenzene was evidenced in the cultures grown with 1,3,5-tribromobenzene (Figure 3.21). Cultures grown with hexabromobenzene showed a similar activity with 1,2,3-trichlorobenzene and 1,2-dibromobenzene, but a much lower activity with 1,2,4-trichlorobenzene. Cultures grown with 1,3,5-tribromobenzene gave a high activity for 1,2-dibromobenzene which was almost

the same as for 1,2,4-tribromobenzene. Very low specific activity was observed for 1,3,5-tribromobenzene and 1,3-dibromobenzene although the two compounds were the initial electron acceptor and debromination product during cultivation. This culture also showed nearly no activity for 1,2,4-trichlorobenzene compared to 1,2,3-trichlorobenzene. The results indicated that specific activities detected in the assay were independent from the electron acceptor used for cultivation. The chemical properties of halogenated compounds such as the position of halogen substituents might have influence on the enzymatic activity of the resting cells of strain CBDB1.

3.6 Effect of dehalogenation of brominated benzenes on the carbon isotope ratio

In order to get further insights into the reaction mechanism during reductive debromination and also to calculate carbon isotope enrichment factors for future quantification of biotransformation of brominated compounds at field sites, carbon isotope analysis was applied during reductive debromination of brominated benzenes by *D. mccartyi* strain CBDB1 both in vitro (enzymatic assay) and in vivo (live cultures). Here, 1,2-, 1,3-, and 1,4-dibromobenzene as well as 1,2,4-, and 1,3,5-tribromobenzene were selected as electron acceptors. Hexabromobenzene and brominated flame retardants with big molecules were not chosen in this test. The reasons were: i) brominated benzenes have the basic structure of brominated aromatic compounds especially of the brominated flame retardants; ii) previous tests revealed the ability of strain CBDB1 to use lower brominated benzenes as terminal electron acceptors and have diverse activities in resting cells enzymatic assay; iii) the enrichment factor in the debromination of hexabromobenzene or other tested brominated flame retardants by strain CBDB1 could not be measured or quantified by current methods due to their strong hydrophobicity. The selected lower brominated benzenes have better solubility in acetone or water compared to hexabromobenzene and other brominated flame retardants, which would give a much better sensitivity of measurement on GC-IRMS.

3.6.1 Carbon isotope fractionation in enzymatic assays

As shown above in section 3.5, different *in vitro* activities were observed for brominated benzenes from cultures of *D. mccartyi* strain CBDB1 cultivated with different electron

acceptors (either hexabromobenzene or 1,3,5-tribromobenzene). Here, resting cells of strain CBDB1 grown with hexabromobenzene were used in the activity assay to investigate carbon isotope fractionation patterns during reductive debromination of brominated benzenes. For the tested dibromobenzenes, the debromination of 1,2-dibromobenzene and 1,3-dibromobenzene was accompanied by a change in isotope composition of the residual substrate towards more positive $\delta^{13}\text{C}$ -values (left panels in Figure 3.22 and Figure 3.23). The carbon isotope composition of 1,2-dibromobenzene showed a change in carbon isotope ratio ($\delta^{13}\text{C}$) from $-26.9 \pm 0.3\text{‰}$ to $-11.4 \pm 0.5\text{‰}$ with a removal of 94.5% after 65 min. During reductive debromination of 1,3-dibromobenzene, carbon isotope ratio increased from $-26.0 \pm 0.1\text{‰}$ to $-13.1 \pm 0.5\text{‰}$ when 89% was dehalogenated after 180 min. Based on the Rayleigh equation, similar carbon isotope enrichment factors of $\epsilon_{\text{C}} = -5.84 \pm 0.4\text{‰}$ and $-5.9 \pm 1.1\text{‰}$ were determined for 1,2- and 1,3-dibromobenzenes in the enzymatic assay, respectively (right panels in Figure 3.22 and Figure 3.23). Because the debromination of 1,4-dibromobenzene in this activity assay could not go further than 34%, no clear change of carbon isotope composition for 1,4-dibromobenzene was observed (data not shown).

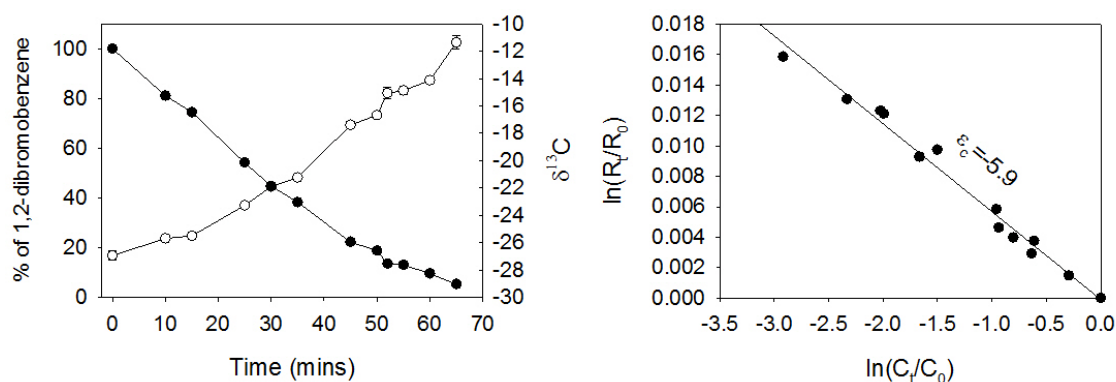


Figure 3.22: Carbon isotope fractionation of 1,2-dibromobenzene in enzymatic assays by resting cells of *D. mccartyi* strain CBDB1. Left panel: Change in relative amount of initial concentration (●) and carbon isotope composition (○). Bars indicate standard deviations based on triplicate injection of one sample. Right panel: double logarithmic representation of the data based on Rayleigh equation to determine the enrichment factor.

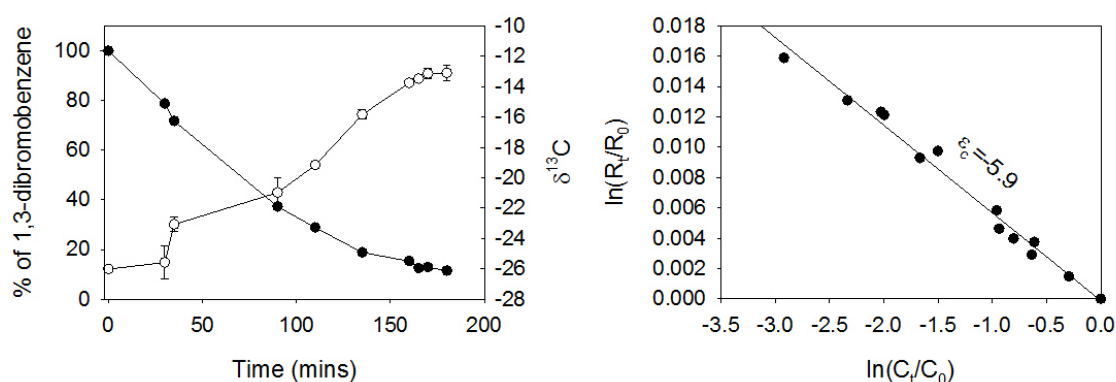


Figure 3.23: Carbon isotope fractionation of 1,3-dibromobenzene in enzymatic assays by resting cells of *D. mccartyi* strain CBDB1. Left panel: Change in relative amount of initial concentration (●) and carbon isotope composition (○). Bars indicate standard deviations based on triplicate injection of one sample. Right panel: double logarithmic representation of the data based on Rayleigh equation to determine the enrichment factor.

For the tested tribromobenzenes, 1,3,5-tribromobenzene was only slightly enriched in ^{13}C with a change in $\delta^{13}\text{C}$ from $-25.8 \pm 0.2\text{‰}$ to $-24.1 \pm 0.4\text{‰}$ when 84.5% of the initial 1,3,5-tribromobenzene was dehalogenated after 420 min (Figure 3.24). The carbon isotope composition of 1,2,4-tribromobenzene changed from $-26.7 \pm 0.1\text{‰}$ to $-24.9 \pm 0.5\text{‰}$ with a removal of 79.9% after 30 min. However, when 92.1% of 1,2,4-tribromobenzene was dehalogenated after 50 min, the carbon isotope composition recovered back to $-26.0 \pm 0.9\text{‰}$ (data was not plotted in figures). This recovery could be due to the interference from the undissolved 1,2,4-tribromobenzene (correspond to 400 μM in the pentane extract) supplied at the beginning of the activity assay. Calculated carbon isotope enrichment factors determined for 1,3,5- and 1,2,4-tribromobenzene (calculated with the data for the first 30 min of incubation time) were with $\epsilon_c = -0.9 \pm 0.3\text{‰}$ and $-1.2 \pm 1.0\text{‰}$ which were rather low but still significant. Neither debromination nor isotope fractionation of brominated benzenes were observed in abiotic controls without resting cells.

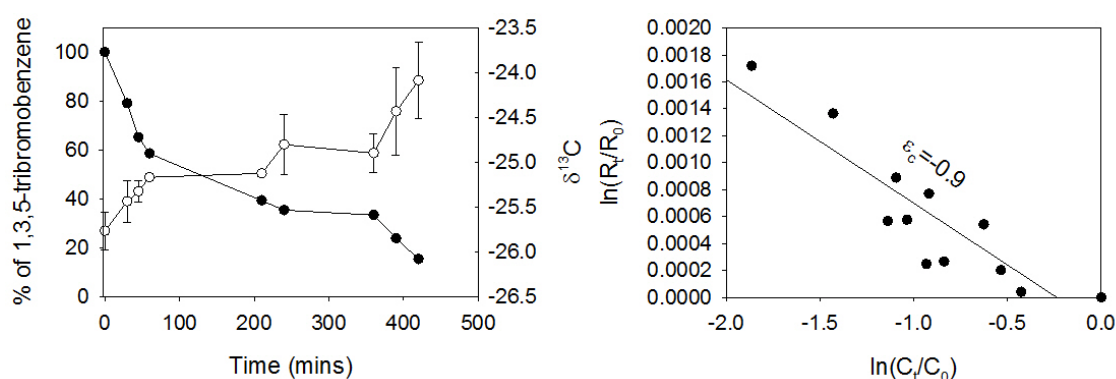


Figure 3.24: Carbon isotope fractionation of 1,3,5-tribromobenzene in enzymatic assays by resting cells of *D. mccartyi* strain CBDB1. Left panel: Change in relative amount of initial concentration (●) and carbon isotope composition (○). Bars indicate standard deviations based on triplicate injection of one sample. Right panel: double logarithmic representation of the data based on Rayleigh equation to determine the enrichment factor.

3.6.2 Carbon isotope fractionation in live cultures

To investigate if the carbon isotope fractionation patterns are different in comparison to those measured in the in vitro enzymatic activity assay, 1,3-dibromobenzene and 1,2,4-tribromobenzene were used as electron acceptors for the cultivation of *D. mccartyi* strain CBDB1. Cultures grown with the two brominated benzenes were set up in replicates with controls. After concentration analysis by GC-FID, the corresponding bottle of culture was sacrificed as described above and brominated substrate and the debromination products were extracted by the addition of pentane (see section 2.6). The carbon isotope enrichment factor determined during the reductive debromination of 1,3-dibromobenzene dehalogenating cultures was with $\epsilon_c = -5.6 \pm 1.0\%$ identical to ϵ_c calculated in the enzymatic activity assay (Figure 3.25). For the cultures grown with 1,2,4-tribromobenzene, there was nearly no change in the carbon isotope composition of 1,2,4-tribromobenzene from the sacrificed cultures (data not shown). Even though cultures were exactly sacrificed when different proportion of 1,2,4-tribromobenzene was debrominated, GC-C-IRMS analysis was failed to observe an increase in $\delta^{13}\text{C}$ -values of the residual 1,2,4-tribromobenzene in all samples. Thus, no carbon isotope enrichment factor could be determined in these cultures. The reason could be due to that the concentration measured by GC-FID only presented the concentration dissolved in the medium, while the sample for

GC-C-IRMS analysis were the extraction from the whole sacrificed cultures including the undissolved trace of 1,2,4-tribromobenzene.

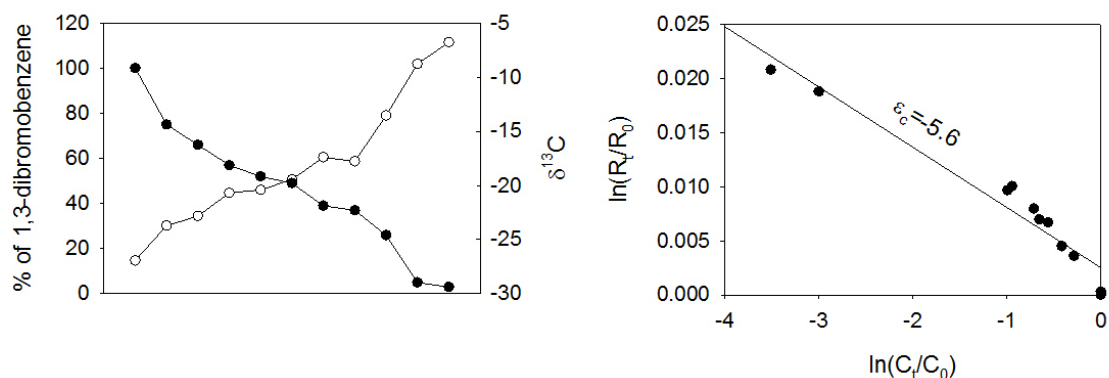


Figure 3.25 Carbon isotope fractionation of 1,3-dibromobenzene in live cultures of *D. mccartyi* strain CBDB1. Left panel: Change in relative amount of initial concentration (●) and carbon isotope composition (○). Bars indicate standard deviations based on triplicate injection of one sample. Right panel: double logarithmic representation of the data based on Rayleigh equation to determine the enrichment factor.

4 Discussion

4.1 Growth adaption of *D. mccartyi* strain CBDB1 to brominated compounds

Reductive dehalogenation catalyzed by anaerobic bacteria exhibits a promising way to eliminate organic halogenated pollutants from the environment. Before large scale application for industry or contaminated field sites are being done, extensive laboratory study for selected microorganisms is essential to obtain optimized cultivation parameters, to understand reaction mechanisms and to identify transformation products. *D. mccartyi* strain CBDB1 has been shown to dehalogenate many halogenated compounds and demonstrated to use a broad range of electron acceptors. For the newly tested brominated compounds, the first step was to investigate if strain CBDB1 can catalyze debromination and if it gains energy for growth. Additionally, strain CBDB1 was routinely cultivated and transferred with chlorinated benzenes as electron acceptors. The current cultivation parameters are optimized based on chlorinated benzenes, and the tested brominated compounds have different chemical properties than chlorinated benzenes. Thus it was necessary to identify if these cultivation parameters were suitable for the newly tested brominated compounds, and to investigate the influence of chemical properties of brominated compounds onto the debromination process and the growth of strain CBDB1.

4.1.1 Growth adaptation to brominated benzenes

In this study, reductive debromination was detected for cultures of *D. mccartyi* strain CBDB1 with hexabromobenzene or 1,3,5-tribromobenzene as electron acceptor. For both cultures, cell growth was coupled with the release of bromide ions indicating that strain CBDB1 had been adapted to the new electron acceptors. Together with the reported well-grown cultures of strain CBDB1 with crystalline hexachlorobenzene, the successful cultivation of strain CBDB1 with the two brominated benzenes in this study showed that feeding with an electron acceptor in crystalline form might be a suitable approach for halogenated compounds which have very low solubility both in water and certain organic solvents (e.g. acetone in this study). Compared to the addition of electron acceptor from acetone stock solutions, the advantages of this feeding with electrons acceptors in crystalline form are: i) because the compound has a low solubility in water, the actual

concentration of dissolved compound was low which would reduce the risk of toxicity to strain CBDB1, ii) it allows a continuous supply of electron acceptor from the crystals to strain CBDB1 which is helpful to obtain a high cell density culture, iii) it avoids to bring in organic solvent necessary to dissolve the electron acceptor to reach a certain concentration in the cultures. Successful cultivation of hexabromobenzene cultures inoculated from strain CBDB1 grown with hexachlorobenzene confirmed that strain CBDB1 could directly adapt from chlorinated benzene to brominated benzene but with a longer incubation time. The reason of a longer incubation time could be the necessity to express specific reductive dehalogenases induced by hexabromobenzene (will be discussed in section 4.2.3). Remarkably, cell densities in two brominated benzene cultures could reach the same level as in the cultures grown with hexachlorobenzene without exchanging the headspace of cultures to remove toxic volatile products. The debromination process of brominated benzenes cultures can be quickly detected by bromide analysis in the study because the background of bromide ions is low. In contrast, monitoring the change of chloride concentration is difficult in cultures with chlorinated benzenes because of the high concentration of background chloride in the medium. From these points, our results suggest that hexabromobenzene and 1,3,5-tribromobenzene are more convenient than chlorinated benzenes to be used as electron acceptor for routine cultivation or large scale cultivation of strain CBDB1.

Observed growth yields for *D. mccartyi* strain CBDB1 grown with hexabromobenzene (2.4×10^{13} cells mol⁻¹ of bromide released) or 1,3,5-tribromobenzene (4.6×10^{13} cells mol⁻¹ of bromide released) were similar to growth yields reported for strain CBDB1 grown with hexachlorobenzene (2.0×10^{13} cells mol⁻¹ of chloride released) or pentachlorobenzene (3.0×10^{13} cells mol⁻¹ of chloride released),¹³⁶ but lower than growth yields for strain CBDB1 grown with 1,2,4-tribromobenzene (1.8×10^{14} cells mol⁻¹ of bromide released), dibromobenzenes (from 1.9×10^{14} to 2.5×10^{14} cells mol⁻¹ of bromide released) or monobromobenzene (2.9×10^{14} cells mol⁻¹ of bromide released).¹⁴⁰ Growth yields for hexabromobenzene and 1,3,5-tribromobenzene were also lower than growth yields (from 6.3×10^{13} to 3.1×10^{14} cells mol⁻¹ of halogen released) detected in several other pure *D. mccartyi* strains (e.g. strain BAV1, 195, FL2 and GT).^{127,128,130,133} These results revealed growth yields were lower for *D. mccartyi* strains especially strain CBDB1 grown with highly halogenated compounds than growth yields for *D. mccartyi* strains grown with lower halogenated compounds. In fact, higher halogenated benzenes and 1,3,5-tribromobenzenes have stronger hydrophobicity than lower brominated benzenes and

chlorinated ethenes. Meanwhile, different halogenated counterparts e.g. hexabromobenzene vs. hexachlorobenzene did not result in a distinct difference in growth yields of strain CBDB1, indicating that the chemical property of electron acceptor showed a strong impact on the growth yields and that each single dehalogenation step is coupled to energy conservation.

On the other hand, the calculated growth yields only reflect the ability of per molar halogen to support the growth of *D. mccartyi* strains but not include the information of incubation time of cultures. In this study, the specific dehalogenation rates (with the average cell density for calculation) of hexabromobenzene and 1,3,5-tribromobenzene cultures were 1.1×10^{-9} $\mu\text{mol bromide day}^{-1} \text{ cell}^{-1}$ and 7.7×10^{-10} $\mu\text{mol bromide day}^{-1} \text{ cell}^{-1}$, respectively. Both two values were about 10 times of specific dehalogenation rates (from 6.2×10^{-11} to 9.6×10^{-11} $\mu\text{mol bromide day}^{-1} \text{ cell}^{-1}$) determined in cultures of strain CBDB1 grown with 1,2,4-tribromobenzene, dibromobenzenes and monobromobenzene.¹⁴⁰ This indicated that cells of strain CBDB1 grown with the two tested brominated benzenes were very fast in removing bromine even though the two cultures had lower growth yields. In the reported cultures grown with 1,2,4-tribromobenzene, dibromobenzenes and monobromobenzene, electron acceptors were added with a fixed concentration from acetone stock solutions and amended when the parent compound were consumed. Therefore, such cultures were repeatedly exposed to starvation before further electron acceptors were added and this starvation might have impacted the maintenance of a high activity status of the cultures. In this study, the continuous supply of electron acceptor from crystalline form feeding of hexabromobenzene and 1,3,5-tribromobenzene avoided such a problem and sustained the activity of cells for reductive debromination. However, the crystalline form feeding approach was not suitable for 1,2,4-tribromobenzene, dibromobenzenes and monobromobenzene due to their high water solubility which led to toxic concentrations in cultures with strain CBDB1. Our results highlight the importance of sustainable supply of electron acceptor for the activity of strain CBDB1, and demonstrate the advantages of the crystalline form feeding approach for the cultivation of *D. mccartyi* strains with highly halogenated compounds.

4.1.2 Growth adaption to brominated compounds with oligocyclic structures

In this study, hexabromobenzene was tested as the first compound because it belongs to the class of brominated flame retardants and it is also the brominated counterpart of hexachlorobenzene. Thus it was not surprising that growth adaption of strain CBDB1 to brominated benzenes was observed. The other four tested brominated compounds had a more complex structure than brominated benzenes. Another aspect of the choice of these electron acceptors was their larger molecular size compared to that of brominated benzenes. The tests were aimed to see reductive debromination but also investigate if there were limitations due to the size of the electron acceptor. Conclusive results were obtained with the presented cultivation approaches.

For bromophenol blue, the debromination activity and cell growth detected in cultures demonstrated that *D. mccartyi* strain CBDB1 could use a complex structured oligocyclic brominated compound for organohalide respiration. Growth yields (3.6×10^{14} and 2.7×10^{14} cells mol⁻¹ of bromide released) observed with the two types of bromophenol blue cultures are in accordance with the growth yields of strain CBDB1 grown with lower brominated benzenes,¹⁴⁰ trichloroethene,¹³² or chlorophenols.¹³⁷ However, the specific dehalogenation rates (1.9×10^{-11} and 3.7×10^{-11} μmol bromide day⁻¹ cell⁻¹) calculated in bromophenol blue cultures were much lower than their parent cultures (hexabromobenzene) and also lower than values previously reported for strain CBDB1 grown with lower brominated benzenes. Although the highest cell density of bromophenol blue cultures only reached around 2×10^7 cells mL⁻¹ by slow feeding, up to three generation transfers were possible by adding bromophenol blue with an initial concentration of 10 μM. This indicated that strain CBDB1 could be adapted to use bromophenol blue as electron acceptor for cell growth at certain cultivation conditions and that strain CBDB1 was able to conserve energy from the debromination of bromophenols.

Reductive debromination of tetrabromobisphenol A by strain CBDB1 was evidenced in slow-feeding cultures. Together with the study in which a *Dehalobacter* strain in mixed culture debrominated tetrabromobisphenol A in the presence of humin,¹⁶² our results further exhibit the dehalogenation ability of organohalide-respiring bacteria. No cell growth was detected for all tested tetrabromobisphenol A cultures indicating tetrabromobisphenol A has a stronger toxic effect on the growth of strain CBDB1 than bromophenol blue which shares a similarity in chemical structure (toxicity will be

discussed in section 4.1.3). The slower decrease in cell density of tetrabromobisphenol A cultures than negative controls indicated that the cells were able to harvest at least some energy from the debromination but it appeared that the toxicity of tetrabromobisphenol A was stronger than the supporting effect. This was also evidenced by the fact that cell density decreased faster when the slow feeding was stopped.

For decabrominated diphenyl ether and hexabromocyclododecane, neither debromination activity nor cell growth was detected in the culture bottles after using all current cultivation methods in our laboratory. A possible explanation is: i) *D. mccartyi* strain CBDB1 cannot either transform decabrominated diphenyl ether and hexabromocyclododecane; ii) decabrominated diphenyl ether and hexabromocyclododecane are highly toxic to strain CBDB1 thus reductive dehalogenation was inhibited; iii) current cultivation parameters are not suitable for decabrominated diphenyl ether and hexabromocyclododecane.

4.1.3 Inhibitory effects on reductive debromination

Although *D. mccartyi* strain CBDB1 can be well cultivated with brominated benzenes as electron acceptor, the accumulation of debromination products could result in inhibitory effects on the cell growth. In culture bottles with different types of septa for the cultivation of strain CBDB1 with hexabromobenzene, clear differences in cell growth were observed (section 3.2.1). A much slower growth rate and a smaller final cell density were detected in the cultures sealed with Teflon-lined rubber septa compared with those that were sealed with butyl rubber septa, indicating that using Teflon-lined rubber septa resulted in an inhibitory effect on the cell growth. Teflon-lined rubber septa were reported to be used for the cultivation of strain CBDB1 with lower brominated benzenes and trichlorobenzenes in previous studies.^{135,140} This septa was applied because the material could not adsorb organic compounds thus the quantification for dehalogenation substrates and products would be accurate. In contrast, butyl rubber septa can continuously adsorb organic compounds during the dehalogenation process. Therefore, it is concluded that large amounts of debromination products especially the volatile ones *e.g.* benzene, monobromobenzene and dibromobenzenes inhibited the growth of strain CBDB1 in the test cultures sealed with Teflon-lined rubber septa. No inhibitory effects were observed in the cultures of strain CBDB1 grown with lower brominated benzenes or trichlorobenzenes which were also sealed with Teflon-lined rubber septa. This could be due to the effect that

the accumulated concentrations of dehalogenation products were not high enough to cause inhibitory effects. While in the cultures of strain CBDB1 fed with crystalline hexabromobenzene (see section 4.1.1), a high bromide concentration was detected meaning a large amount of debromination products was also formed during debromination which would result in an inhibitory effect on the cell growth if they could not be removed (adsorbed by butyl rubber septa). This inhibitory effect was also evidenced by the cultivation of strain CBDB1 with crystalline hexachlorobenzene in which both butyl rubber septa were used and the headspace of the cultures was exchanged periodically.¹³⁶ However, so far by our results we could not clarify which debromination products at which concentration threshold cause the inhibitory effect or if the toxicity is a concerted effect by all products and substrates together.

Obvious inhibitory effects were observed in cultures of strain CBDB1 with the two brominated phenolic compounds as electron acceptor. No debromination activity but a decrease in cell density was detected in the cultures with an initial 10 μM of tetrabromobisphenol A, suggesting a clear inhibition due to the molecule structure since at this concentration chlorinated and brominated benzenes were not inhibitory to strain CBDB1. Reductive debromination of tetrabromobisphenol A was only seen in the slow feeding cultures, and still no cell growth but a constant decrease in cell density of strain CBDB1 was detected in such cultures. The results revealed that the inhibitory effects existed in spite of the debromination of tetrabromobisphenol A and were much stronger than the supporting energy harvested for cell growth, indicating that the phenol group was the reason causing toxicity.

Relatively low inhibitory effects were observed for cultures of strain CBDB1 with bromophenol blue as electron acceptor. As described in section 4.1.2, with the same concentration level for electron acceptor, similar growth yields of strain CBDB1 but lower specific dehalogenation rates were detected in bromophenol blue cultures compared to brominated benzenes cultures. Test cultures started with either varying concentrations of bromophenol blue or different cell densities confirming that the inhibitory effect was related to the initial concentration of bromophenol blue per cell of strain CBDB1. Higher initial concentration of bromophenol blue per cell in cultures resulted in a longer lag time before debromination was initiated. Possible explanations could be that: i) the number of viable cells catalyzing debromination was lower due to the toxicity; ii) stronger toxicity influenced the debromination efficiency of each cell.

Although no further transfer cultures were carried out for both slow feeding cultures due to the limitation in obtaining high cell density, previous reports of strain CBDB1 incubated with chlorophenols¹³⁷ and bromophenols¹⁰⁹ demonstrated that such inhibitory effect was due to the toxicity from phenol group to which strain CBDB1 was not adapting with generation transfers. Phenols have been shown to be toxic to bacteria by changing the lipid-to-lipid or lipid-to-protein ratios in cell membranes, thereby altering the membrane permeability and activity of membrane-associated proteins.^{182,183} Substituted phenols can also destroy the electrochemical proton gradient by transporting protons back across the membrane as uncouplers or inhibit the electron flow by binding directly to specific components of the electron transfer chain in energy transducing membranes.¹⁸⁴⁻¹⁸⁶ Since *D. mccartyi* strain CBDB1 only gain energy via organohalide respiration for cell growth, it is assumed that the inhibitory effect onto cell growth and delay in debromination process were because of the disruption by brominated phenolic compounds in proton gradient across the cell membrane. Tetrabromobisphenol A and bromophenol blue have similar structures but different pK_a values (7.0 and 4.0, respectively. <http://pubchem.ncbi.nlm.nih.gov/>) showing that tetrabromobisphenol A is more hydrophobic at pH 7.0. During the cultivation, tetrabromobisphenol A might more easily cross the cell membrane and dissipate the proton gradient, therefore resulting in a stronger toxic effect than bromophenol blue. The balance between the debromination reaction which builds the proton gradient and the concentration of hydrophobic phenols which dissipate the proton gradient then determines if debromination continues and cell growth occurs. On the other hand, bromophenol blue is less hydrophobic and can be completely deprotonated in the medium then resulting in less effects on proton gradient because the debrominated product is even less hydrophobic than the substrate. Thereby, the growth adaption of strain CBDB1 to bromophenol blue was possible and the toxic effect was represented as a delay in the activating debromination process.

4.2 Dehalogenation Patterns of *D. mccartyi* strain CBDB1

4.2.1 Complete removal of bromine in reductive debromination

Removal of halogens from the electron acceptor is a direct result to evaluate the extent of reductive dehalogenation. In previous studies, the reductive dehalogenation of chlorinated benzenes catalyzed by *D. mccartyi* strain CBDB1 was reported to stop at

monochlorobenzene, 1,3-, 1,4-dichlorobenzene and 1,3,5-trichlorobenzene.^{135,136} Such incomplete removal of chlorine was also observed in the cultures of strain CBDB1 incubated with dioxins,¹³⁹ chlorophenols,¹³⁷ or polychlorinated biphenyls¹³⁸ and reported for other organohalide respiring bacteria.^{116,126} Our results demonstrate the complete debromination of 1,3,5-tribromobenzene to benzene via 1,3-dibromobenzene showing a different dehalogenation pattern with the chlorinated counterpart 1,3,5-trichlorobenzene which was viewed as the most stable chlorinated benzene against dehalogenation catalyzed by strain CBDB1. Although not all brominated benzenes were commercially available for testing in this study, results of the resting cell activity test demonstrated that strain CBDB1 could transform 1,2,4,5-tetrabromobenzene to 1,2,4-tribromobenzene and 1,2,3,5-tetrabromobenzene to 1,2,4-tribromobenzene and 1,3,5-tribromobenzene. Wagner. et al¹⁴⁰ previously reported the complete debromination of lower brominated benzenes by strain CBDB1 and pointed out that doubly flanked bromine substituents were preferentially removed, followed by singly flanked then isolated bromine substituents. Together with this, it is concluded that all congeners of brominated benzenes can be completely debrominated to benzene by strain CBDB1 with a removal of bromine stepwise. The complete removal of bromine was also presented in the reductive debromination of two oligocyclic brominated phenols by strain CBDB1 showing that such complete debromination was not influenced by the molecular size of electron acceptor or the toxicity caused by the phenol group. To our knowledge, the complete removal of bromine happened in so far all reported reductive debromination reactions catalyzed by strain CBDB1 while in contrast no complete reductive dechlorination by strain CBDB1 was reported. This indicates that such different patterns in the extent of reductive dehalogenation by strain CBDB1 were mainly affected by the types of halogens. Our findings are accordance with electron density modeling study on reductive dehalogenation by strain CBDB1.¹⁰⁹ According to the study, the dehalogenation reaction preferentially took place in the position having the most positive (i.e. the least negative) charge on the halogen atom (Q_X) and a more negative partial charge on the carbon atom (Q_C) by partial charge models. Based on this, the study pointed out higher electronegativity for chlorine compared to bromine and suggested that an enhanced removal order in the sequence $-Br > -Cl > -F$ was followed. This could explain why further and stronger extent of bromine vs chlorine removal was observed in the current study and also implies that brominated compounds might be more available as electron acceptor for strain CBDB1 than chlorinated or fluorinated compounds.

4.2.3 Influence of the chemical property of halogenated compounds on reductive dehalogenation

In this study, a total of eight brominated organic compounds with different chemical properties and chemical structures were tested as electron acceptor (six in cultivation and two in resting cell enzymatic assay) for *D. mccartyi* strain CBDB1. Only six of the tested compounds (hexabromobenzene, 1,3,5-tribromobenzene, 1,2,3,5- and 1,2,4,5-tetrabromobenzene, tetrabromobisphenol A and bromophenol blue) were debrominated by strain CBDB1 while no sign of cell growth and debromination activity was observed for the other two compounds (decabromodiphenyl ether and hexabromocyclododecane). So far, all research on the reductive dehalogenation catalyzed by strain CBDB1 showed that the strain only removed halogen substituents on carbons with a π bond. Observed debromination processes in our study were in accordance with such a phenomenon, and it was also evidenced by the fact that no debromination activity was detected in cultures of strain CBDB1 incubated with hexabromocyclododecane which does not have π bonds on its structure. However, also no debromination activity was observed in cultures of strain CBDB1 incubated with decabromodiphenyl ether. This is in contrast to previous reports on dehalogenation of chlorinated dioxins¹³⁹ and polychlorinated biphenyls¹³⁸ (which have similarity in structure with decabromodiphenyl ether) by strain CBDB1 and the preferential halogen removal theory in electron density models.¹⁰⁹ In fact, decabromodiphenyl ether has a much bigger molecular weight and higher hydrophobicity (computed octanol / water partition coefficient $\text{LogK}_{\text{ow}} = 10.4$) than 1,2,3,4-tetrachlorodibenzo-p-dioxin ($\text{LogK}_{\text{ow}} = 7.2$) and decachlorobiphenyl ($\text{LogK}_{\text{ow}} = 8.3$) (<http://pubchem.ncbi.nlm.nih.gov/>). Reasons for the results that strain CBDB1 was not able to debrominate decabromodiphenyl ether could be: i) the molecular size of decabromodiphenyl ether is too big for the reductive dehalogenases of strain CBDB1 thereby it could not pass through the substrate channel of the reductive dehalogenase protein to reach the active center of the enzyme; ii) the strong hydrophobicity of decabromodiphenyl ether results in an inhibitory effect on strain CBDB1, or the strong hydrophobicity results in a very low concentration of decabromodiphenyl ether so that the reductive dehalogenases cannot work efficiently by taking up the compound.

4.3 Evaluation on resting cell enzymatic activity assay

Resting cell enzymatic activity assays can help to evaluate the transformation of halogenated compounds with the expressed set of dehalogenases especially from well-grown cultures. Based on this, resting cells from the cultures of strain CBDB1 incubated with hexabromobenzene or 1,3,5-tribromobenzene were used for a photometric activity assay to test the specific activity on four brominated benzenes and two chlorinated benzenes. Both cultures showed the highest specific activities on 1,2,4-tribromobenzene at a higher specific activity than found with any other halogenated benzenes so far tested by strain CBDB1. No activity was found with monobromobenzene with both cultures. This could be due to that either the debromination reactions for monobromobenzene was too slow in the activity assays or that the expressed reductive dehalogenases did not catalyze the required reactions under *in vitro* conditions. This is accordance with previous activity studies on cultures of strain CBDB1 using either lower brominated benzenes or trichlorobenzenes as the electron acceptor.¹⁴⁰ Similar results have been also observed for the purified trichloroethene reductive dehalogenase of *D. mccartyi* strain 195 which had a much higher specific activity on 1,2-dibromoethane than the specific activity on the original electron acceptor for cultivation.¹⁸⁷ From this point, the activity assay can be a useful tool for selecting better candidates of electron acceptor for organohalide respiring bacteria. Higher specific activities detected on 1,2,4-tribromobenzene and 1,2-dibromobenzene than on 1,3,5-tribromobenzene for both tested cultures of strain CBDB1 indicated that the chemical property of an electron acceptor showed a strong impact on the activity test. The impact was represented as that the halogenated benzene which was preferentially dehalogenated (see section 3.3.2 and 4.2.1) by strain CBDB1 would show a higher specific activity. The results also implied that the reductive dehalogenases in *D. mccartyi* strain CBDB1 might not tightly determine the reaction specificity on halogenated benzenes (will be discussed section 4.4). Additionally, more reductive dehalogenases were detected to be expressed in the cultures grown with hexabromobenzene than in cultures grown with 1,3,5-tribromobenzene (see section 3.4.1). These additionally expressed reductive dehalogenases could have contributed activities on halogenated benzenes in activity assays. This might explain that a similar specific activity on 1,2,3-trichlorobenzene and a relative low specific activity on 1,3-dibromobenzene compared to 1,2-dibromobenzene were detected for the cultures grown with hexabromobenzene but not for the cultures grown with 1,3,5-tribromobenzene.

4.4 Expression of reductive dehalogenases

D. mccartyi strains contain multiple *rdhA* genes but so far only a few putative reductive dehalogenases encoded by these genes were characterized in regard to their substrate spectrum.¹⁰⁴ Since reductive debromination catalyzed by *D. mccartyi* strain CBDB1 showed both similarities and differences with reductive dechlorination, it is necessary to find out the relations between dehalogenation patterns and the diverse reductive dehalogenases and especially, if dechlorination and debromination reactions are catalyzed by the same enzymes. Here, shotgun proteomics were used for cultures grown with brominated benzenes or incubated with brominated phenolic compounds to investigate whether the same reductive dehalogenases were expressed and have a preliminary understanding on their potential functions. In the sixth transfer cultures of strain CBDB1 grown with hexabromobenzene, six RdhA proteins were found to be simultaneously expressed which were also detected in cultures of strain CBDB1 grown with 1,2,4-tribromobenzene except for CbdbA187. Cultures of strain CBDB1 grown with 1,3,5-tribromobenzene were initially inoculated from hexabromobenzene cultures. Thus it was not surprising that the first generation of 1,3,5-tribromobenzene cultures shared a same subset of five RdhA proteins with hexabromobenzene cultures. Only four of these RdhA proteins were still expressed in the second transfer of 1,3,5-tribromobenzene cultures indicating more reductive dehalogenase encoded genes were induced by higher brominated benzenes. According to obtained emPAI values, the most abundant reductive dehalogenase expressed in both brominated benzene cultures was CbrA (CbdbA84) which was previously demonstrated as the enzyme responsible for the dehalogenation of trichlorobenzenes.¹⁵¹ The second highest expressed RdhA protein was CbdbA80 which was also highly expressed in cultures of strain CBDB1 cultivated with trichlorobenzenes, 2,3-dichlorophenol and lower brominated benzenes.^{140,151,188} All other RdhA proteins expressed in brominated benzene cultures were found at much lower abundances. Therefore, it was concluded that the dominant reductive dehalogenases of strain CBDB1 catalyzing the dehalogenation of brominated benzenes and chlorinated benzenes are the same. This indicates that these reductive dehalogenases are not strictly substrate specific. Our result is supported by the functional heterologous expression of the tetrachloroethene reductive dehalogenase (PceA) which dechlorinated 2,3-, 2,4- and 3,5-dichlorophenols,¹⁸⁹ and also by the heterologous expression of vinyl chloride reductive dehalogenase (VcrA) which dechlorinated 1,2-dichloroethane to ethane.¹⁹⁰

The induction of CbdbA1092 and CbdbA1503 was shown in cultures of strain CBDB1 incubated with bromophenol blue or tetrabromobisphenol A but was never observed for brominated and chlorinated benzenes indicating the two RdhA proteins might be specifically involved in the dehalogenation of oligocyclic phenolic compounds. Again, CbrA and CbdbA80 were the two most abundant RdhA proteins in all analyzed cultures based on emPAI values suggesting they are the essential reductive dehalogenases for dehalogenation catalyzed by strain CBDB1. The abundances of CbdbA1092 and CbdbA1503 were as high as CbdbA80 (especially in the third transfer of bromophenol blue cultures) showing the substrate structure might influence the RdhA protein expression. Similarly, two specific but different RdhA enzymes (CbdbA1588 and CbdbA88) were reported to be highly expressed in cultures of strain CBDB1 incubated with the monocyclic phenolic compound 2,3-dichlorophenol.¹⁸⁸ All together this suggested that not only the phenol group but also the compound molecule size results in different expression of RdhA proteins. Structures of the PceA reductive dehalogenase¹⁹¹ in *Sulfurospirillum multivorans* and a chlorophenol RdhA enzyme¹⁹² from *Nitrateductor pacificus* suggested that the corrinoid-containing active site of the enzyme was deep inside the core of the RdhA protein, and that the compound needs to go through a “letter box”-like selective substrate channel to reach the active center. Such a selective channel could contribute to the specificity a RdhA protein displays towards chemical structure or molecule size of the substrate. However, such a highly restricted substrate binding pocket was only described in an enzyme purified from non-obligate organohalide respiring bacteria. In fact, single dominant RdhA proteins which catalyzed distinct dehalogenation reactions have been reported to be highly transcribed in the cultures of three different *Dehalococcoides* strains grown with polychlorinated biphenyls.¹⁹³ Cooper et al¹⁰⁹ demonstrated that the electron densities in halogenated compounds highly influenced the dehalogenation process and proposed that reductive dehalogenases in *Dehalococcoides* strains do not have tight substrate specificity. Together with our results, this suggests that reductive dehalogenases in *Dehalococcoides* strains have broader substrate specificity than non-obligate organohalide respiring bacteria. Remarkably, a clear downregulation of CbrA was observed during the transfers of strain CBDB1 with bromophenol blue as electron acceptor. CbrA was expressed as the second abundant RdhA protein in the first generation cultures but not detectable in the third transfer cultures. Meanwhile, the expression of CbdbA80 was constantly at high levels in all generation cultures suggesting that its regulation is impaired or that it is more broadly regulated. Such finding is in contrast to the results from

D. mccartyi strain DCMB5 where Dcmb_81 (the ortholog of CbdbA80) was not expressed.¹⁹⁴ The up and down regulation of the expression of RdhA proteins further proved that multiple RdhA proteins are involved and apart from CbdbA80 are strictly controlled in a complex network for reductive dehalogenation.

4.5 Potential of applying CSIA to detect reductive debromination

In order to investigate carbon isotope effects of different debromination activities, 1,2-, 1,3-, 1,4-dibromobenzene and 1,3,5-, 1,2,4-tribromobenzene were chosen as model compounds. CSIA was applied in enzymatic activity assay by using resting cells of strain CBDB1 from well-grown hexabromobenzene cultures and the artificial electron donor methyl viologen, as well as applied during the growth of strain CBDB1 incubated with the respective brominated benzenes. For enzymatic activity assays, almost identical carbon isotope enrichment factors were determined during reductive debromination of 1,2- and 1,3-dibromobenzene suggesting that the same reaction mechanism affected the debromination of the two dibromobenzenes even though strain CBDB1 showed distinct specific activities on the two compounds (see section 3.5). In addition, during the growth of strain CBDB1 with 1,3-dibromobenzene as terminal electron acceptor, an identical carbon isotope enrichment factor was calculated in comparison to the enzymatic activity assay with an artificial electron donor indicating that carbon isotope fractionation was mainly affected by the biochemical reaction rather than by the physiological status of the cells. This can also be explained by that there were no masking effects during the reductive debromination so that the enzyme is relatively freely accessible at the outside of the membrane according to the study of Renpenning et al.¹⁹⁵ Our results are in accordance with the study by Griebler et al.¹⁷⁴ on carbon isotope fractionation during dehalogenation of 1,2,3- and 1,2,4-trichlorobenzene by strain CBDB1 revealing identical carbon isotope enrichment factors of $\epsilon_C = -3.1$ to -3.7‰ with the two different congeners, although different kinetics between the two isomers were observed. A significantly lower carbon isotope enrichment factor was determined for 1,3,5- and 1,2,4-tribromobenzene in the current work compared to the two dibromobenzenes exhibiting a weaker carbon isotope effect during reductive debromination of tribromobenzene congeners by strain CBDB1. Here, the chemical properties such as hydrophobicity resulting in rate-limiting steps for the electron acceptor might be responsible for the influence on the variability in isotope fractionation. Such rate-limiting steps prior to carbon-halogen cleavage were also reported

in previous researches.¹⁹⁵⁻¹⁹⁷ During the dibromoelimination of dibromoethane in an anaerobic microcosm a carbon isotope enrichment factor of $\epsilon_C = -5.6 \pm 1.0\text{‰}$ was calculated.¹⁷⁵ Calculated carbon isotope enrichment factors during abiotic transformation of brominated organic compounds ranged from -2.4 to -31‰.¹⁹⁸⁻²⁰⁰ The estimated carbon isotope enrichment factors for the reductive debromination of dibromobenzenes by strain CBDB1 are in the same range as for the dibromoelimination of dibromoethane although two different reaction mechanisms must be involved because during the dibromoelimination reaction both carbons are contributing whereas in the reductive debromination of tribromobenzene to dibromobenzene and dibromobenzene to monobromobenzene, only one carbon atom is involved. Accordingly, the actual isotope effect on the reacting carbon is diluted by the non-reacting carbons and an $\epsilon_{C(\text{reactive position})}$ of around -6‰ for tribromobenzene and around -33‰ ($\epsilon_{C(\text{reactive position})} = n \times \epsilon_C$)²⁰¹ represents the actual carbon isotope effect during the reductive debromination.

At contaminated field sites, the estimation of contaminant degradation is problematic due to the difficulty in quantification of substrate and degradation products. CSIA may help to overcome these difficulties, however, the knowledge of isotope enrichment factors characteristic for the specific process is necessary. Our work provides for the first time carbon isotope enrichment factors connected to the anaerobic transformation of brominated benzenes. The combination with application approaches could be useful to gain more insights on the dehalogenation processes. However, an isotope dilution was also observed influencing the evaluation of carbon isotope fractionation on 1,2,4-tribromobenzene which was mainly due to the initial high concentration supplied to obtain measureable data on GC-IRMS. So far such problem was not well taken care since most electron acceptor (*e.g.* chloroethene and chloromethane) studied before had relatively low hydrophobicity while reductive dehalogenation was normally taken place in the water phase. Therefore, balancing the detection limit for GC-IRMS and the water solubility of electron acceptor is essential for applying carbon stable isotope analysis onto novel halogenated compounds in the future.

5 Conclusions

This research investigated the reductive debromination of several brominated flame retardants by *Dehalococcoides mccartyi* strain CBDB1. Strain CBDB1 has been shown to debrominate all brominated benzenes and two oligocyclic brominated phenols. These results extend the range of electron acceptors for strain CBDB1 and also show the potential of applying strain CBDB1 in the remediation of sites contaminated with brominated flame retardants. Cell growth was coupled to the release of bromide ions during the incubation time indicating strain CBDB1 was well adapted to use either hexabromobenzene or 1,3,5-tribromobenzene as electron acceptor. The crystalline form feeding of halogenated benzenes was demonstrated to be a useful approach to obtain high cell density cultures of strain CBDB1. By this approach, cultures of strain CBDB1 incubated with either hexabromobenzene or 1,3,5-tribromobenzene obtained high cell densities after three months of cultivation without exchanging the headspace of culture bottles suggesting that the two brominated benzenes were better suited as electron acceptor than chlorinated benzenes for the cultivation of strain CBDB1. Two oligocyclic brominated phenols were shown to be toxic to strain CBDB1. The extent of toxicity was associated with the ratio of the electron acceptor concentration to the cell density in the cultures and presented as either a complete inhibition or as a delay on the debromination process and cell growth. Under the same cultivation conditions, tetrabromobisphenol A with a higher hydrophobicity than bromophenol blue had a stronger toxicity to strain CBDB1 suggesting the toxicity was affecting cell membrane integrity or proton permeability, because a proton gradient is essential for energy conservation via organohalide respiration. The results also highlight that the chemical properties of the electron acceptor could influence the debromination process.

The observed debromination processes revealed a complete removal of bromine substituents and showed a further dehalogenation extent than the reductive dechlorination catalyzed by *D. mccartyi* strain CBDB1. This complete debromination indicated brominated compounds were more extensively dehalogenated than chlorinated compounds, and confirmed the advantage of using brominated benzenes as electron acceptor for the cultivation of strain CBDB1. Reductive debromination catalyzed by strain CBDB1 had the same characteristics as dechlorination in dehalogenation pathway in which doubly flanked halogen substituents were preferentially removed. Shotgun proteomics revealed that the same dominant reductive dehalogenases were expressed in debromination and

dechlorination catalyzed by strain CBDB1 suggesting these reductive dehalogenases have a versatile electron acceptor range. Additionally, specific reductive dehalogenases expressed in cultures of strain CBDB1 incubated with hexabromobenzene or two oligocyclic phenols indicated that the molecular size and chemical property of electron acceptor could induce specific reductive dehalogenases.

Resting cell enzymatic activity assays and compound specific isotope analysis were shown to be promising tools for a quick investigation on reductive debromination. GC-format based activity assays demonstrated the debromination of two tetrabromobenzenes by strain CBDB1 and proved that the approach was useful in identifying the dehalogenation pathway for halogenated compounds which had limitations to be used as electron acceptor for the cultivation. Photometric activity assays revealed that strain CBDB1 had distinct specific activities on brominated and chlorinated benzenes although the dominant reductive dehalogenases expressed in the cultures were shown to be same. This indicated that the chemical properties of an electron acceptor had a strong influence on the debromination rate. Identical carbon isotope enrichment factors were determined during the reductive debromination of 1,2-dibromobenzene and 1,3-dibromobenzene indicating the reaction mechanism for the debromination of the two dibromobenzenes is similar. Additionally, carbon isotope fractionation was shown to be mainly affected by the biochemical reaction rather than by the physiological status of the cells suggesting the combination of resting cell activity assay and compound specific isotope analysis can be applied for a fast investigation on the debromination process catalyzed by strain CBDB1.

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