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Stable carbon isotope fractionation during aerobic and anaerobic transformation of trichlorobenzene

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Abstract

Fractionation of stable carbon isotopes upon degradation of trichlorobenzenes was studied under aerobic and anaerobic conditions. Mineralization of 1,2,4-trichlorobenzene by the aerobic strain *Pseudomonas* sp. P51 which uses a dioxygenase for the initial enzymatic reaction was not accompanied by a significant isotope fractionation. In contrast, reductive dehalogenation by the anaerobic strain *Dehalococcoides* sp. strain CBDB1 revealed average isotope enrichment factors (ε) between −3.1 and −3.7 for 1,2,3- and 1,2,4-trichlorobenzene, respectively. The significant isotope fractionation during reductive dehalogenation would allow tracing the in situ biodegradation of halogenated benzenes in contaminated anoxic aquifers, whereas the lack of isotope fractionation during aerobic transformation limits the use of this approach in oxic environments.

Keywords: Chlorobenzene; Stable isotope fractionation; Reductive dechlorination

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1. Introduction

Chlorinated benzenes are used as solvents, pesticides, degreasers, as additives to oils and plastics, and as an intermediate for the synthesis of dyes, pharmaceutical products and plastic materials [1]. Today chlorobenzenes are widely distributed in the environment due to point pollution sources as well as diffusive contamination.

Several aerobic microorganisms have been described to metabolize chlorinated benzenes with one to four chlorine substituents using these substrates as the sole source of carbon and energy [2]. Chlorobenzenes with more than two chlorine substituents show increasing persistence against aerobic biodegradation [2]. Highly chlorinated benzenes such as hexachlorobenzene, pentachlorobenzene, 1,2,3,5-tetrachlorobenzene and 1,3,5-trichlorobenzene (1,3,5-TCB) are still considered to be non-biodegradable under oxic conditions [3]. Transformation of di- and trichlorobenzenes by a chlorobenzene dioxygenase leads to *cis*-1,2-dihydroxycyclohexadiene which successively re-aromatizes to the corresponding chlorinated catechol [4]. Similarly, degradation of 1,2,3,4- and 1,2,4,5-tetrachlorobenzene via the dioxygenase pathway was shown with *Burkholderia* sp. [5,6]. Substantial oxidation of chlorobenzenes under nitrate-, sulfate-, manganese- or iron-reducing conditions has not been demonstrated yet [3].

Under strictly anoxic conditions, some microbial consortia are able to reductively dehalogenate chlorobenzenes to less chlorinated benzene congeners (see [2] for a review). No efficient reductive dehalogenation of monochlorobenzene has been found yet. The different reductive dehalogenation pathways during anaerobic microbial transformation of chlorobenzenes have

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recently been summarized by Adrian and Görisch [3]. Only in some bacteria the reductive dehalogenation of chlorinated compounds is coupled to energy conservation known as dehalorespiration [7]. Dehalorespiration with chlorinated benzenes has been demonstrated with a mixed culture and with the pure culture *Dehalococcoides* sp. strain CBDB1 [8,9]. *Dehalococcoides* sp. strain CBDB1 is able to dechlorinate 1,2,3,5-tetrachlorobenzene to 1,3,5-TCB, as well as 1,2,3,4- and 1,2,4,5-tetrachlorobenzene via 1,2,4-trichlorobenzene (1,2,4-TCB) to a mixture of 1,3- and 1,4-dichlorobenzene. 1,2,3-TCB is dehalogenated to 1,3-dichlorobenzene (1,3-DCB). Recently, also the reductive dechlorination of hexachlorobenzene and pentachlorobenzene to tri- and dichlorobenzenes by *Dehalococcoides* sp. strain CBDB1 and *Dehalococcoides ethenogenes* strain 195 by has been reported [10,11]. Dehalogenation of chlorinated benzenes by *Dehalococcoides* sp. strain CBDB1 is catalyzed by a membrane associated dehalogenase. Crude cell extracts showed enzymatic activity towards 1,2,3-TCB, 1,2,4-TCB, all three tetrachlorobenzene congeners, penta- and hexachlorobenzene when methyl viologen was added as an artificial electron donor. Product patterns obtained with crude extracts and methyl viologen as an artificial electron donor were identical to patterns obtained in cultures growing with hydrogen as electron donor [12]. Similar to most other reductive dehalogenases described earlier [7], the reductive dehalogenase of *Dehalococcoides* sp. strain CBDB1 seems to contain a corrinoid co-factor. The distantly related strain DF-1 that has been shown to dehalogenate polychlorinated biphenyls in a mixed culture was also shown to dehalogenate hexachlorobenzene, pentachlorobenzene and 1,2,3,5-tetrachlorobenzene [13,14]. To date, no other bacteria have been described that dehalogenate chlorobenzenes at a significant respiratory rate.

Microbiologically mediated transformation of polychlorinated benzenes is suggested to take place also in polluted environments as was shown for sewage sludge [15,16], sediments [17–19] and contaminated aquifers [20].

The microbial transformation of pollutants by autochthonous microorganisms is an important process for the natural attenuation of contaminated sites. Thus, the use of natural attenuation processes as a remediation strategy requires the analysis of the in situ degradation of individual contaminants. A promising indicator to monitor in situ degradation of individual contaminants in aquifers is stable isotope fractionation during biodegradation.

This approach has been successfully applied to demonstrate in situ bioremediation of chlorinated ethenes and fuel related compounds at various contaminated sites [21–26]. Beside the qualitative indication of biodegradation, some reports demonstrated that for individual compounds a quantification of in situ microbial degradation by determining ratios of stable isotopes is feasible [27–30]. A prerequisite for quantification of biodegradation in the field is the availability of isotope enrichment factors which are commonly obtained from laboratory degradation experiments with well-defined bacterial enrichment cultures or pure microbial strains. To date, isotope enrichment factors are available for the aerobic and anaerobic transformation of a number of chlorinated and non-chlorinated hydrocarbons [21–24,27,31], but not for chlorinated benzenes.

The objective of this work was to investigate carbon isotope fractionation during aerobic and anaerobic transformation of chlorinated benzenes using 1,2,3- and 1,2,4-TCB as model substrates. 1,2,3- and 1,2,4-TCB were chosen because they are amenable to microbial transformation both under aerobic and anaerobic conditions. Degradation experiments were carried out with the aerobic strain *Pseudomonas* sp. P51 and the anaerobic strain *Dehalococcoides* sp. strain CBDB1. Results are discussed in the context of using isotope signatures of halogenated benzenes to assess in situ biodegradation in contaminated aquifers.

2. Materials and methods

2.1. Aerobic trichlorobenzene transformation

Experiments for aerobic degradation of 1,2,4-TCB were carried out with strain *Pseudomonas* sp. P51 obtained from J.R. van der Meer (EAWAG, Zürich, Switzerland). Cells were grown aerobically in 100 mL serum bottles with 60 mL of Z3 minimal medium adjusted to pH 7.2–7.4 and 1,2,4-TCB as sole carbon and energy source [32]. Prior to the experiments, 1,2,4-TCB was added to 500 mL growth medium in excess (20 μL) and the bottle was closed with a butyl rubber stopper (Maag Technik, Switzerland), and incubated for one week on a shaker at 30 °C in the dark. Then, 60 mL medium aliquots with TCB dissolved to a concentration of about 35 μM were transferred into 100-mL serum bottles and closed with viton stoppers (Maag Technik, Switzerland). Several parallel batches were inoculated (10% v/v) with a pre-cultured batch ($OD_{360} = 0.045$) with sterile syringes through the stoppers and incubated on a shaker at 30 °C in the dark. Samples of 3–10 mL were collected periodically with a sterile syringe and extracted with at least 0.5 mL *n*-hexane. The compound-specific stable carbon isotope composition, the concentration of 1,2,4-TCB and Cl^- as well as the optical density (OD) were determined. Amendment of NaOH to a final concentration of 100 mM after subsampling was used to stop biological activity.

2.2. Anaerobic trichlorobenzene transformation

Anaerobic dehalogenation of trichlorobenzenes was examined using strain *Dehalococcoides* sp. strain

CBDB1. Cells were grown under anaerobic conditions in 60-mL serum bottles with 30 mL of a synthetic bicarbonate buffered and Ti(III) citrate-reduced mineral medium [33]. The medium further contained 1,2,3- or 1,2,4-TCB in concentrations of about 30 μM, hydrogen as electron donor (7.5 mM nominal concentration) and acetate (5 mM) as the carbon source. The gas phase was N_2/CO_2 (80:20 v/v). For inoculation, 0.5 mL of a *Dehalococcoides* sp. strain CBDB1 culture pre-grown on 1,2,3-TCB to a cell number of about 10^7 cells mL^{-1} was injected into the serum flasks containing 30 mL medium with a sterile glass syringe pre-flushed with nitrogen to remove traces of oxygen. The about 60-fold dilution of the inoculum by the medium ensures that only minor amounts of compounds (>1 μM) were transferred from the pre-culture. The cultures were incubated statically at 29 °C in the dark. Samples were taken with N_2-flushed glass syringes.

2.3. Whole cell enzymatic assay

Cultures with about 10^7 of cells mL^{-1} were used to determine the dehalogenation with methyl viologen as an electron donor and TCB as an electron acceptor. In these experiments, 200 μL of the culture suspension was used without prior lysis as described previously [12]. Chlorobenzene concentrations and isotopic compositions were analyzed after an incubation time of 5–120 min.

2.4. Analytical methods

Chlorinated benzenes were extracted from samples with *n*-hexane (SupraSolv, Merck, Darmstadt, Germany). The ratio of solvent to medium varied according to the expected concentration between 1:1 and 0.01:1 (v:v). Concentrations of chlorinated benzenes were determined either by GC–FID or GC–MS analysis (HP 6890; MS: HP 5973, Paolo Alto, USA). Samples were injected in splitless mode and a capillary column (DB-5 MS, 30 m × 0.25 mm, 0.25 μm film, J&W Scientific, USA) was used for separation of target compounds. The flow rate of the carrier gas (Helium) was 1 mL min^{-1}. The oven temperature was held at 40 °C for 3 min, then increased at a rate of 10 °C min^{-1} to 130 °C and held for 1 min. The MS was operated in the selected ion mode (SIM). 1,4-Dichlorobenzene and 2,4-dichlorotoluene served as internal standards in the extraction solvent for quantification of trichlorobenzenes in aerobic and anaerobic degradation experiments, respectively.

Stable carbon isotope ratios were determined from the *n*-hexane extracts by gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS). The GC-C-IRMS system consisted of a GC unit (6890 Series, Agilent Technology, USA) connected to a Finnigan MAT GC III combustion device with a modified water removal assembly (Nafion® membrane, 50 cm long, $T = 0$ °C) coupled to a Finnigan MAT 252 mass spectrometer (ThermoFinnigan, Germany). The organic substances of the GC effluent stream were oxidized to CO_2 on a CuO/Ni/Pt catalyst in the combustion furnace that was held at 960 °C. CO_2 was transferred online to the mass spectrometer to determine carbon isotope ratios. Aliquots (1–4 μL) of *n*-hexane extracts were injected in split mode using a split/splitless injector held at 250 °C and targeted compounds were separated on a capillary column (DB5 60 m × 0.32 mm, 0.5 μm film, J&W Scientific, USA). The temperature was held at 60 °C for 2 min and was then increased with a rate of 4 °C min^{-1} to 270 °C, where it was held for 5 min. Helium was used as carrier gas at a flow rate of 1.5 mL min^{-1}. All samples were measured in at least five replicates.

Chloride concentrations were determined by ion-chromatography (Dionex 120, Dionex, Sunnyvale, USA). The optical density of cultures was determined photometrically (Varian Cary 50, Varian Inc., Palo Alto, USA) at a wavelength of 360 nm. Cell numbers of CBDB1 were estimated by direct cell counting [11].

2.5. Calculations

The carbon isotope composition (R) is reported in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna).

The isotope fractionation accompanied with the microbial degradation of chlorinated benzenes was calculated applying the Rayleigh equation [34],

$$\ln\left(\frac{R_t}{R_0}\right) = (\alpha_c - 1) \times \ln\left(\frac{C_t}{C_0}\right). \tag{1}$$

The kinetic carbon isotope fractionation factor α_c is a constant for the reaction at given experimental conditions. R_t and R_0 are the isotope compositions, and C_t and C_0 are the concentrations of the compound at times t and zero, respectively. The isotope fractionation factor α_c relates the changes in the isotope composition to changes in concentration of the residual fraction during the transformation. Many authors prefer to express the isotope fractionation as the enrichment of the heavier isotope with the enrichment factor ε which is related to α_c by the following equation [35]:

$$\varepsilon = (\alpha_c - 1) \times 1000. \tag{2}$$

The intrinsic isotope fractionation factor $\alpha_{\text{intrinsic}}$ was calculated according to Eq. (3). The intrinsic isotope fractionation factor normalizes the isotope fractionation of the isotopomer to the atom at which the biochemical reaction takes place. α is the fractionation factor obtained using Eq. (1). The equation was deduced from the Rayleigh equation (1) to consider the number of carbon atoms (n) which do not undergo isotope fractionation and remain isotopically stable during the reaction. This may allow a comparison of isotope fractionation of organic compounds with different numbers of carbon atoms,

$$\alpha_{\text{intrinsic}} = 1 \Big/ \left(\left(\frac{n}{\alpha_c} \right) - (n-1) \right). \tag{3}$$

The related intrinsic enrichment factor is calculated according to Eq. (4),

$$\varepsilon_{\text{intrinsic}} = (\alpha_{\text{intrinsic}} - 1) \times 1000. \tag{4}$$

3. Results

In aerobic batch experiments, *Pseudomonas* sp. strain P51 degraded initial 1,2,4-TCB concentrations of 25–38 μM to less than 1 μM within 40 h (Fig. 1). Decrease of TCB was in all cases accompanied by an increase of the Cl^- concentration. At the end of the experiments the recorded chloride accounted for 95–116% of the chloride initially bound in TCB indicating complete mineralization. An at least threefold increase in optical density within the incubation time indicated bacterial biomass production. During the course of the aerobic batch experiments, the stable carbon isotope composition of 1,2,4-TCB did not change significantly although more than 99% of the substrate was degraded (Fig. 1). The evaluation of concentration and isotopic compositions according to the Rayleigh model (Eq. (2)) revealed no significant isotope fractionation considering the analytical error of the isotope measurement (Fig. 2).

Dehalococcoides sp. strain CBDB1 anaerobically dechlorinated 27 μM 1,2,3-TCB to below 1.0 μM within 30 days of incubation. Dechlorination of 1,2,3-TCB was accompanied by an enrichment of ^{13}C in the residual TCB fraction (Fig. 3). The initial isotope composition was −29.42 ± 0.4‰ and at day 25 when 1,2,3-TCB was dechlorinated to a relative amount of 26.6%, the residual TCB was enriched in ^{13}C to −24.55 ± 0.2‰. During the transformation, 1,2,3-TCB was nearly dehalogenated stoichiometrically to 1,3-DCB indicated by a recovery of about 80% of the initial TCB as 1,3-DCB. No further dehalogenation products could be detected. The produced 1,3-DCB was depleted in ^{13}C compared to the parent 1,2,3-TCB.

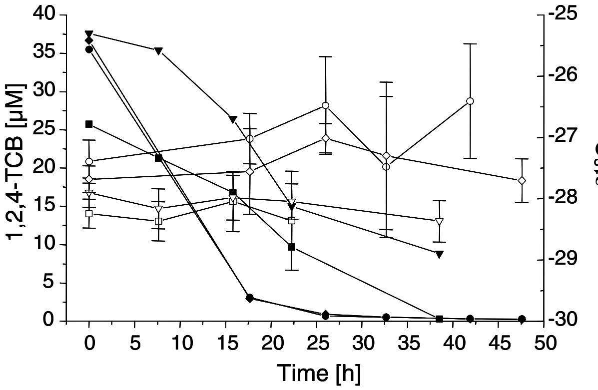

Fig. 1. Aerobic degradation of 1,2,4-TCB by *Pseudomonas* sp. strain P51 in four parallel experiments. Closed symbols show concentrations, open symbols depict the isotope compositions (±SD 1σ; $r \geqslant 5$) of the respective cultures.

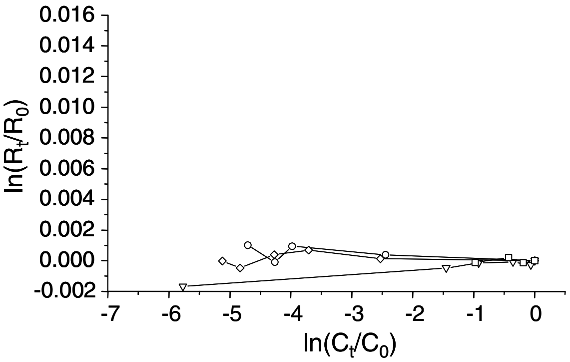

Fig. 2. Double logarithmic plot of isotopic composition versus concentration of 1,2,4-TCB according to the Rayleigh equation. Each curve shows an individual degradation experiment under aerobic conditions.

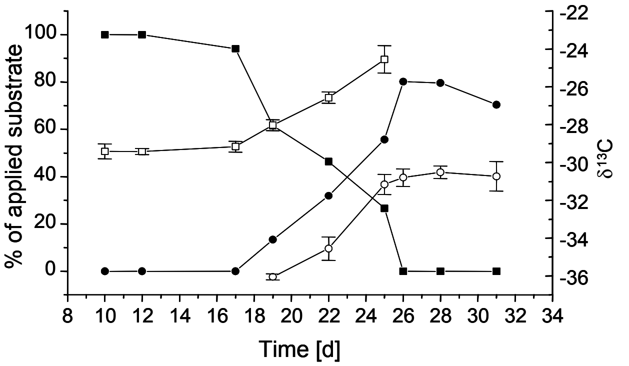

Fig. 3. Concentration (■) and isotope composition (□; ±SD 1σ; $r \geqslant 5$) of 1,2,3-TCB in an anaerobic growth experiment with *Dehalococcoides* sp. strain CBDB1. Concentration (●) and isotopic composition (○; ±SD 1σ; $r \geqslant 5$) of 1,3-DCB indicate the product formation.

The increase of the relative amounts of 1,3-DCB from 13% to 80% of the initial TCB concentration between day 19 and 26 was associated with an enrichment in ^{13}C from −36.01 ± 0.2‰ to −30.78 ± 0.5‰ which remained stable until the end of the experiment.

The initial isotope composition of the parent 1,2,3-TCB (−29.42‰) and the final isotope composition of 1,3-DCB (−30.78‰) at the end of the experiment differed by 1.36‰ illustrating that the isotope balance was not closed. This indicates an incomplete transformation of 1,2,3-TCB to 1,3-DCB reaction during the experiment. The dechlorination of 1,2,3-TCB was associated with a carbon isotope enrichment (ε) factor of

−3.1 ± 0.4‰ to −3.8 ± 0.2‰ in two parallel experiments revealing an average fractionation factor of $\varepsilon = -3.4 \pm 0.5$ (Fig. 4).

Three additional dehalogenation experiments were performed with 1,2,4-TCB using initial concentrations between 20 and 30 μM. 1,4-DCB was the major product of the dehalogenation of 1,2,4-TCB and showed similar isotope values as the 1,3-DCB formed during the dehalogenation of 1,2,3-TCB (data not shown). To obtain an isotope enrichment factor, a concentration change from about 25 μM to below 1 μM was monitored for the isotope composition of 1,2,4-TCB. The isotope enrichment factors determined in the individual experiments were −2.8 ± 0.3, −3.1 ± 0.4 and −3.7 ± 0.3, and revealed an average enrichment factor of −3.2 ± 0.5 (Fig. 4). Concerning the statistical uncertainty (1σ) within the regression curves according to the Rayleigh equation, no significant difference was found between the ^{13}C enrichment during the reductive dehalogenation of 1,2,3- and 1,2,4-TCB.

Incubation of whole cells of *Dehalococcoides* sp. strain CBDB1 with 76 μM 1,2,3-TCB and methyl viologen as an artificial electron donor resulted in a complete transformation of the 1,2,3-TCB to 1,3-DCB within 4.0 h (Fig. 5). The initial stable carbon isotope composition of 1,2,3-TCB was −30.00 ± 0.24‰ which was enriched in ^{13}C to −24.93 ± 1.55‰ in the course of the experiment when 80% of 1,2,3-trichlorobenzene was transformed. At a more advanced stage of transformation, i.e., at TCB concentration less than 15 μM, a reliable determination of the isotopic composition was not possible. The isotope composition of the product 1,3-DCB could be determined when about 26% of the TCB was degraded and showed an isotope composition of −33.83 ± 1.15‰ indicating clear depletion in ^{13}C compared to the parent compound. At the end of the experiment when TCB was completely dechlorinated, the carbon isotope composition of 1,3-DCB was enriched in ^{13}C to 29.82 ± 0.46‰. The final isotope composition of 1,3-CB was almost identical with the initial isotope composition of 1,2,3-TCB exhibiting a closed isotope balance and a complete transformation of 1,2,3-TCB to 1,3-DCB. The ^{13}C enrichment factor determined for the dehalogenation of 1,2,3-TCB with methyl viologen as an electron donor according to the Rayleigh model was $\varepsilon = -3.4 \pm 0.3$ (Fig. 4).

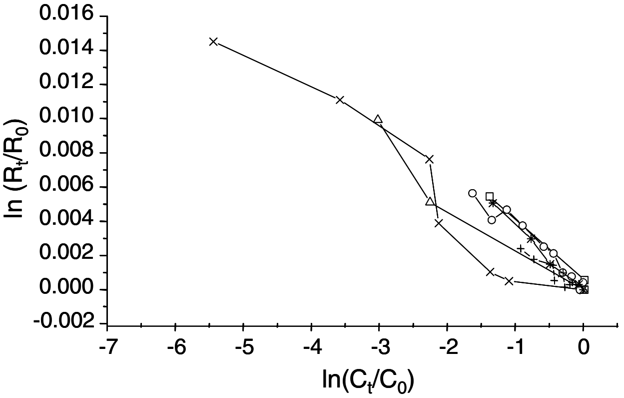

Fig. 4. Double logarithmic plot according to the Rayleigh equation of the isotopic composition versus residual concentration of TCB. Each curve represents a single anaerobic transformation experiment. (○), incubated with methyl viologen as an electron donor and 1,2,3-TCB as the electron acceptor. Cultures growing with 1,2,4-trichlorobenzene are represented by (+; ×; □), and cultures growing and with 1,2,3-TCB by (∗; △).

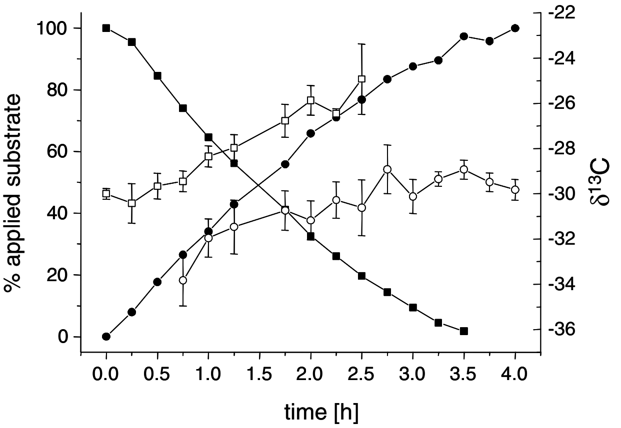

Fig. 5. Concentration (■) and isotope composition (□) of 1,2,3-TCB after incubation with whole cells of *Dehalococcoides* sp. strain CBDB1 and methyl viologen as an artificial electron donor. The concentration (●) and isotope composition (○; ±SD 1σ; $r \geqslant 5$) of 1,3-DCB formation demonstrates a stoichiometric transformation.

4. Discussion

Several bacteria have been described that degrade mono-, di-, tri- and tetra-chlorinated benzenes under oxic conditions using a chlorobenzene dioxygenase for the initial attack [4]. However, no carbon isotope fractionation data were so far available concerning the aerobic degradation of chlorinated aromatic compounds. Our laboratory experiments with the aerobic strain *Pseudomonas* sp. P51 and 1,2,4-TCB as the model compound illustrated that the chloro-dioxygenase reaction is not accompanied by a significant carbon isotope fractionation. Similar results were previously obtained with other ring dioxygenases transforming non-chlorinated aromatic compounds. Aerobic toluene degradation via the ring-dioxygenase pathway by *P. putida* strain F1 as well as aerobic naphthalene degradation by *P. putida* strain NCIB 9816 revealed no significant $^{13}C/^{12}C$ isotope fractionation [31]. The family of aromatic-ring-hydroxylating dioxygenases is characterized by Rieske [2Fe–2S] centers providing electrons and ferrous iron at the catalytic sites, which catalyze the transfer of two activated oxygen atoms to the substrate [36].

Dioxygen is activated by accepting an electron from the iron and may form a superoxide radical like species or a peroxide [36]. The rate-limiting step in the dioxygenase reaction is probably an interaction of the reactive oxygen species with the π-electron system of the aromatic substrate leading to formation of a *cis*-dihydrodiol. In this process no σ-bond is formed or cleaved. Reactions involving exclusively π-electron systems do not lead to large primary isotope effects as shown for the toluene and naphthalene dioxygenase reaction [31]. Subsequent to the formation of the *cis*-dihydrodiol, the molecule is re-aromatized in a dehydrogenase reaction cleaving two hydrogen atoms from the ring system, however, this is not the rate-limiting step of the dioxygenase reaction. The very small primary isotope effects during the aerobic degradation of 1,2,4-TCB by *P. putida* strain P51 suggests that the aerobic degradation of chlorinated benzenes by the dioxygenase pathway cannot be traced by isotope studies in the field.

Under anoxic conditions, with the exception of monochlorobenzene, the major biochemical pathway to degrade chlorinated benzenes is probably a reductive dehalogenation. The dechlorination of 1,2,3- and 1,2,4-TCB by *Dehalococcoides* sp. strain CBDB1 leads to the formation of ^{13}C depleted 1,3-DCB and 1,4-DCB illustrating the isotope fractionation during the dehalogenation reaction. The chlorine substitution pattern of chlorinated benzene had some effect on the dehalogenation reaction in vitro and in vivo. The dehalogenation of 1,2,3-TCB with a doubly flanked chlorine substituent is faster than the dehalogenation of 1,2,4-TCB with a singly flanked chlorine substituent. Experiments with cell extracts of *Dehalococcoides* sp. strain CBDB1 revealed a significantly lower specific enzymatic activity for the transformation of the latter compound [12]. In order to investigate isotope effects of different dehalogenation activities 1,2,3- and 1,2,4-TCB were chosen as model compounds. Although 1,2,3-TCB was dechlorinated faster, no significant difference in the ^{13}C/^{12}C fractionation of these two compounds were detected. Thus, the different kinetics of the reaction due to the stereochemistry of different isomers did not show a significant effect on ^{13}C/^{12}C fractionation. In addition, almost identical enrichment factors were obtained in growth experiments and with whole cells of *Dehalococcoides* sp. strain CBDB1 using methyl viologen as an artificial electron donor to accelerate the dehalogenation reaction. This may indicate that isotope fractionation was predominantly affected by the biochemical reaction mechanism rather than by other factors such as physiological status of the cells, growth rate or uptake of the substrate and that the enzymatic mechanism is the rate-limiting step causing the isotope fractionation.

The enzymatic mechanism of the reductive dehalogenation reaction catalyzed by the dehalogenases of *Dehalococcoides* sp. has not yet been elucidated. Up to 17 putative dehalogenase gene homologues have been detected in the genome sequence of *Dehalococcoides ethenogenes* strain 195 which is able to dehalogenate a broad spectrum of substrates [10]. However, there are indications that a corrinoid is an important co-factor of dehalogenases in organisms able to dehalorespire [7,12]. The catalytic dehalogenation by cobalamin observed for halogenated ethenes may proceed by alkylating a super reduced corrinoid containing a Co(I) species at the reactive site with the chloroethene [37]. The alkylation would result in a chloroethyl–cob(II)alamin complex as an intermediate leading to the formation of σ–carbon bonds. The σ–carbon bound chlorine may be then cleaved by α,β-elimination and leave the enzyme complex as chloride ion, whereby the reaction becomes thermodynamically irreversible. This cleavage may produce the primary carbon isotope effect during dehalogenation by cobalamin containing dehalogenases. This reaction scheme may explain the carbon isotope and chlorine isotope fractionation [27,38] typically associated with the dehalogenation of perchloroethene and trichloroethene. The alkyl residue may be subsequently protonolytically eliminated depriving the cobalt of the σ-bond electron and cob(III)-alamin is subsequently formed [37]. A moderate solvent kinetic effect of deuterated water in dehalogenation reaction of aryl substituted chlorine has shown that the protonation reaction of the substrate is only to some extent a reaction step which controls the overall kinetics of the reaction [39]. However, the solvent kinetic isotope effect suggests that in the transition state, protonation and solvatization of the chloride is closely associated. In total, two electrons are transferred from cob(I)alamin complex to reduce the substrate.

We speculate that a similar mechanism may be at work for the dehalogenation of halogenated benzenes, whereby a cob(II)alamin chlorobenzene complex may be formed as an intermediate leading to the formation of carbon bonds with a σ-type characteristic. This type of reaction would induce a pronounced primary isotope effect. However, a more detailed investigation is necessary to support this suggestion.

To compare isotope fractionation data of the reductive dehalogenation of different chlorinated compounds with similar enzymatic reaction mechanisms but different numbers of carbon atoms, intrinsic enrichment ($\varepsilon_{\text{intrinsic}}$) factors were calculated (Eq. (4)). The intrinsic enrichment factor presents the isotope fractionation effect at the atom of the molecule where the reaction takes place and may allow another view on the mechanistic aspects of the dehalogenation reaction. Intrinsic enrichment factors for anaerobic TCB transformation as determined in our study ranged between $\varepsilon_{\text{intrinsic}} = -19.5$ and -22.1, and thus are in the same range as previously determined for tetra- and tri-chlorinated ethenes (Table 1). Reductive dehalogenation of perchloroethene and

Table 1
Carbon isotope enrichment factors (ε) and the intrinsic enrichment factors ($\varepsilon_{\mathrm{intrinsic}}$) of selected halogenated compounds

Compound	Microorganism	Condition	ε	$\varepsilon_{\mathrm{intrinsic}}$	Reference
Chlorinated benzenes					
1,2,4-Trichlorobenzene	*Pseudomonas* sp. P51	Oxic	–	–	This study
	Dehalococcoides sp. CBDB1	Anoxic	−3.2	−19.5	This study
1,2,3-Trichlorobenzene	*Dehalococcoides* sp. CBDB1	Anoxic	−3.5	−22.1	This study
	Dehalococcoides sp. CBDB1 with methyl viologen	Anoxic	−3.4	−20.8	This study
Chlorinated ethenes					
Perchloroethene	Methanogenic consortium	Anoxic	−5.5	−10.9	[26]
Trichloroethene	Methanogenic consortium	Anoxic	−13.8	−27.2	[26]
	Mixed facultative anaerobic culture	Anoxic	−7.1	−14.1	[40]
	Methanogenic consortium	Anoxic	−6.6	−13.1	[41]
cis-Dichloroethene	Methanogenic consortium	Anoxic	−20.4	−39.9	[26]
	Microcosm	Anoxic	−19.9	−39.0	[24]
	Methanogenic consortium	Anoxic	−16.1	−31.7	[41]
			−14.1	−27.8	[41]
trans-1,2-Dichloroethene	Microcosm	Anoxic	−30.3	−58.8	[24]
Vinylchloride	Methanogenic consortium	Anoxic	−22.4	−43.8	[26]
	Microcosm	Anoxic	−31.1	−60.3	[24]
			−21.5	−42.1	[41]

trichloroethene in mixed methanogenic consortia revealed intrinsic enrichment factors between $\varepsilon_{\mathrm{intrinsic}} = -10.9$ and −27.2 [26,27,40,41]. Dechlorination of perchloroethene by mixed cultures containing *D. ethenogenes* strain 195 resulted in an intrinsic enrichment factor of $\varepsilon_{\mathrm{intrinsic}} = -12.8$ falling in this range (Nijenhuis, Personal communication). Intrinsic ^{13}C-enrichment factors for the anaerobic dechlorination of *cis*- and *trans*-dichloroethene as well as vinylchloride generally show higher values ranging from $\varepsilon_{\mathrm{intrinsic}} = -13.1$ up to −60.3 [24,26,41] (Table 1). The pronounced primary isotope effect may point to a common mechanism inducing isotope fractionation although there is a significant variability in the isotope fractionation. This may provide evidence that the biochemical reaction, possibly at the corrinoid, may lead to isotope fractionation during dehalogenation. In addition, physiological factors certainly modify the extent of isotope fractionation. However, the overall isotope fractionation during reductive dehalogenation in living cells may be superimposed by the biochemical mechanism in many cases.

Because *Dehalococcoides* sp. strain CBDB1 also catalyzes the dehalogenation of hexa-, penta- and all three tetrachlorobenzenes [11,12], we speculate that carbon isotope fractionation in the order of magnitude as observed in this study may also be obtained during the dehalogenation of other chlorinated benzene congeners. A mixed culture containing strain DF1 and a number of uncultured microorganisms able to dehalogenate double flanked PCB isomers did not show isotope fractionation during dehalogenation of PCBs [14,42]. The enrichment contained relatives of *Dehalococcoides* sp. *Dehalococcoides* spp. employ cob(I)alamin containing enzymes for dehalogenation [43] which lead to isotope fractionation. Thus, more research in particular on the mechanism is needed to understand the isotope fractionation of specific organic halogens.

The pronounced ^{13}C/^{12}C fractionation patterns observed during the reductive dehalogenation of TCB offer a promising approach for the qualitative and quantitative evaluation of in situ bioremediation of chlorinated benzenes. Carbon isotope fractionation factors obtained in this study may further be used to quantify in situ biodegradation of TCB in anoxic aquifers as described for chlorinated ethenes [27]. In contrast, the lack of significant isotope fractionation in the dioxygenase pathway would not allow tracing of in situ degradation under oxic conditions. However, a carbon isotope enrichment of chlorinated benzenes in contaminated aquifers may provide indications that reductive dechlorination is at work.

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