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Title

Evaluation of Carbon Isotope Fractionation during Anaerobic Reductive Dehalogenation of Chlorinated and Brominated Benzenes

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Abstract

Compound specific stable isotope analysis (CSIA) has been established as a useful tool to evaluate *in situ* biodegradation. Here, CSIA was used to determine microbial dehalogenation of chloro- and bromobenzenes in microcosms derived from Hackensack River sediments. Gas chromatography-isotope ratio mass spectrometry (GC-IRMS) was used to measure carbon isotope fractionation during reductive dehalogenation of hexachlorobenzene (HCB), pentachlorobenzene (PeCB), 1,2,3,5-tetrachlorobenzene (TeCB), 1,2,3,5-tetrabromobenzene (TeBB), and 1,3,5-tribromobenzene (TriBB). Strong evidence of isotope fractionation coupled to dehalogenation was not observed in the substrate, possibly due to the low solubilities of the highly halogenated benzene substrates and a dilution of the isotope signal. Nonetheless, we could measure a depletion of the $\delta^{13}C$ value in the dichlorobenzene product during dechlorination of HCB, the sequential depletion and enrichment of $\delta^{13}C$ value for trichlorobenzene in TeCB dechlorinating cultures, and the enrichment of $\delta^{13}C$ during debromination of TriBB. This indicates that a measurable isotope fractionation occurred during reductive dehalogenation of highly halogenated chloro- and bromobenzenes in aquatic sediments. Thus, although more quantitative measurements will be needed, the data suggests that CSIA may have application for monitoring *in situ* microbial reductive dehalogenation of highly halogenated benzenes.

Keywords: Compound specific carbon isotope analysis, halogenated benzenes, microbial reductive dehalogenation, anaerobic, aquatic sediments

1. Introduction

Tools for evaluating and demonstrating *in situ* biodegradation are important for the assessment of bioremediation, in particular in the application of natural attenuation as a remediation option of contaminated groundwater plumes. Compound specific stable isotope analysis (CSIA) is a promising option for monitoring and quantification of *in situ* biodegradation of contaminants at polluted sites (Hunkeler et al., 2008). In principle, the rate of biodegradation is reduced by the presence of the heavier isotope since in biological systems lighter isotopes are preferentially reacted. Hence, this results in the accumulation of the heavier isotope in the residual substrate (Hunkeler et al., 1999) and the lighter isotope is enriched in the product of degradation. This is referred to as compound specific stable isotope fractionation. The magnitude of the isotope fractionation during degradation depends on the nature of the bond cleavage and is affected by a number of factors, including the redox condition, temperature and growth kinetics, sorption, evaporation and chemical reactions (for reviews, see Meckenstock et al., 2004; Elsner, 2010).

Over the last decade CSIA has been applied to study degradation of various halogenated compounds. Most studies have focused on carbon stable isotope fractionation during the reductive dehalogenation process. The focus has mainly been on the use of CSIA for assessing dechlorination of chlorinated ethenes (e.g., Hunkeler et al., 1999; Slater et al., 2001; Nijenhuis et al., 2005; Lee et al., 2007; Cichocka et al., 2008; Renpenning et al., 2015), but there are some recent reports on chlorinated benzenes, such as trichlorobenzene (TriCB), dichlorobenzene (DCB), and monochlorobenzene (MCB). Griebler et al. (2004) showed significant carbon isotope fractionation during reductive dehalogenation of 1,2,3-TriCB by *Dehalococcoides* sp. strain CBDB1. The combined results from CSIA and microcosms supported that MCB biodegradation occurred *in situ*. Liang et al. (2014) reported carbon isotope fractionation during anaerobic degradation of DCB isomers. They converted bulk isotopic enrichment factor (ε_{bulk}) values to apparent kinetic isotope effects for carbon ($AKIE_C$) in order to characterize the carbon isotope effect at the reactive positions for the DCB isomers. Braeckevelt et al. (2007) combined carbon stable isotope analysis with a microcosm study to assess *in situ* MCB biodegradation in a wetland system.

To date, there have been no CSIA studies reported for highly halogenated benzenes. For compounds such as halogenated benzenes the magnitude of measurable fractionation is substantially diluted by the number of carbon atoms in the compound. In addition, the limited solubility, bioavailability of highly chlorinated and brominated benzenes may mask any isotope effects (Thullner et al., 2013). We set out to determine whether measurable C isotope fractionation could be detected during reductive dehalogenation of highly chlorinated and brominated benzenes. We previously demonstrated that indigenous bacteria in sediments of the Hackensack River, New Jersey were capable of dehalogenating the persistent halogenated aromatic compounds, hexachlorobenzene (HCB) and hexabromobenzene (HBB) (Sohn and Häggblom, 2016). Dechlorination of HCB and other chlorinated benzenes has also been observed in other aquatic sediments and soils (Holliger et al., 1992; Masunaga et al., 1996; Susarla et al., 1996; Middeldorp et al., 1997; Chen et al., 2000; Pavlostathis and Prytula, 2000; Brahushi et al., 2004; Fung et al., 2009; Liang et al., 2013). HCB dechlorination pathways via pentachlorobenzene (PeCB), tetrachlorobenzenes (TeCBs), TriCBs and finally DCBs have been observed (Fathepure et al., 1988; Ramanand et al. 1993; Beurskens et al., 1994; Chang et al., 1997; Chen et al., 2000; Pavlostathis and Prytula, 2000; Wu et al., 2002; Jayachandran et al., 2003; Brahushi et al., 2004; Fennell et al., 2004; Tas et al., 2011; Duan and Adrian, 2013; Sohn and Häggblom, 2016). Reductive dehalogenation of tribromobenzenes (TriBBs) has also been observed (Wagner et al., 2012). Hence, there is an interest in developing CSIA for monitoring dechlorination of highly chlorinated and brominated benzenes. In this study, carbon isotope fractionation during reductive dehalogenation of highly halogenated benzenes was determined with anaerobic cultures originating from Hackensack River sediment. CSIA was conducted for dehalogenation of HCB and its intermediates, PeCB and 1,2,3,5-TeCB, and brominated benzenes including, 1,2,3,5-tetrabromobenzene (TeBB) and 1,3,5-TriBB.

2. Materials and Methods

2.1. Establishment of dehalogenating cultures

The dehalogenating cultures used in this study were established with sediment from the Hackensack River, NJ, from sites designated H1, H2 and H3 (for site description see Sohn and Häggblom 2016). The microcosms were set up as described below to examine the potential of indigenous microorganisms to dehalogenate chlorinated and brominated benzenes. Two sets of

carbon isotope fractionation experiments were established. The first set was prepared to examine carbon isotope fractionation for HCB and HBB. The second experiment investigated HCB, and three main intermediates of HCB dechlorination, namely PeCB, 1,2,3,5-TeCB and 1,3,5- TriCB. In addition, two bromobenzenes, 1,2,3,5-TeBB and 1,3,5-TriBB were examined. Halogenated benzenes were purchased from Sigma-Aldrich (St. Louis, MO).

For the first set, sediment slurries were made with sediment in anoxic media, amended with a mixture of electron donors and then transferred to each bottle for a final culture volume of 40 mL as described (Sohn and Häggblom, 2016). Each serum bottle contained 10 % sediment from H1 in media with 500 μM of electron donors (a 1:1 molar mixture of propionic acid and butyric acid) and 10 μM of the halogenated electron acceptors. Two halogenated benzenes, HCB and HBB, were used as electron acceptors. A set of triplicate monitoring cultures amended with HCB or HBB were also prepared. Thirty replicate cultures were prepared for each halogenated electron acceptor. Three of these cultures were monitored over the time course to determine the time points for sampling for CSIA, when separate whole cultures were sacrificed for analysis. At each time point selected for CSIA, three replicate cultures were randomly chosen and sacrificed. Autoclaved killed controls were also prepared in triplicate for two time points (6 bottles) for each electron acceptor. A 0.5 mL culture sample was taken for GC-MS analysis to determine the extent of dechlorination and the remainder of the culture was frozen at -20 °C for later CSIA.

The second set of experiments was prepared to examine HCB and three main intermediates of HCB dechlorination (PeCB; 1,2,3,5-TeCB; or 1,3,5-TriCB). In addition, since no HBB debromination was observed in the first set of analysis, cultures amended with less brominated benzenes were set up to monitor debromination. During HCB dechlorination, 1,3,5-TriCB was detected as one intermediate and 1,2,3,5-TeCB was predicted as the TeCB intermediate. Based on this information on the HCB dechlorination pathway, the corresponding bromobenzenes, 1,2,3,5-TeBB and 1,3,5-TriBB, were chosen for CSIA experiments. Twenty cultures were prepared with H2 sediment for each of TeBB and TriBB for ten time points (duplicated). At each time point, two culture bottles were sacrificed. Killed controls containing both TeBB and TriBB were also prepared for two time points by autoclaving for 30 min at 121 °C on three successive days. A 0.5 mL sample was taken for GC-MS analysis and the remainder of the culture was frozen at -20 °C for later CSIA.

For CSIA of HCB and intermediates of HCB dechlorination, a set of 80 cultures was prepared with a sediment mixture of sites H2 and H3 (0.77:1; w:w). Four halogenated benzenes (HCB; PeCB; 1,2,3,5-TeCB; 1,3,5-TriCB) detected/predicted as intermediates in HCB dechlorination were tested. Twenty cultures were prepared for each substrate to yield duplicate cultures for 10 time points. At each time point, two cultures were sacrificed, a 0.5 mL sample was taken for GC-MS analysis and the remainder of the culture was frozen at -20 °C for later CSIA. Duplicate killed controls containing HCB, PeCB, 1,2,3,5-TeCB, and 1,3,5-TriCB were prepared for two time points as described above.

2.2. Analytical methods

For the first experiment conducted, a 0.5 mL sample was taken every few days from the three monitoring cultures and analyzed by GC-MS as described below in order to determine time points for destructive sampling of cultures for CSIA. (The second experimental set did not have monitoring cultures, and sample time points were estimated from previous data.) At each time point, 0.5 mL sample was taken to determine the extent of dehalogenation by GC-MS, and the whole remaining culture was sacrificed for CSIA. For analysis of chlorinated benzenes, the culture was extracted three times with 5 mL hexane. For analysis of brominated benzenes, 7.5 mL of a mixture of toluene and acetone (1:1; v:v) was used.

The extraction procedures for CSIA were started with addition of solvent to the culture. Then, the bottle was sealed with Teflon coated rubber stopper and crimped with an aluminum seal. Sealed bottles were laid on shaker at 180 rpm overnight. The supernatant was then removed to a clean glass test tube. This extraction procedure was repeated twice, but for 2 hours each. All three extracts (~15 mL total) were combined and briefly sonicated in a water bath for clearer separation of solvent and any residual aqueous phase. The solvent phase was transferred to a clean glass extraction bottle, concentrated by evaporation of the solvent to less than 0.5 mL, and then sealed, packed on ice in a Styrofoam box, and shipped to Germany for CSIA.

The concentration of brominated benzenes and chlorinated benzenes was determined with 0.5 mL samples taken from cultures. Chloro- and bromobenzenes were extracted into hexane and toluene, respectively by shaking at 180 rpm for two hours. To extract chlorobenzenes, 500 µL of

hexane was used. For extraction of bromobenzenes, 750 μL of a mixture of toluene and acetone (1:1(v:v)) was used. The supernatant was transferred to a 2 mL vial. When the solvent and aqueous/sediment phase were not clearly separated, brief sonication in a water bath was applied. Samples were analyzed with an Agilent 6890 gas chromatograph equipped with a HP-5MS capillary column (0.250 mm × 60 m, 0.25 μm film thickness, J&W Scientific, Folsom, CA) and an Agilent 5973 Network Mass selective detector. The GC column temperature was first held at 50 °C for 2 min, increased to 300 °C at a rate of 12 °C min^{-1}, and then held for 5 min. For chlorinated benzenes, 1,2,3,5-TeBB, and 1,3,5-TriBB, intermediates were identified by comparison of their retention times to authentic standards. Dibromobenzene (DBB) and monobromobenzene (MBB) were determined by their mass spectra. Standard curves for each compound except DBB and BB were generated with standards of 10, 20, 30, 40, 50 μM. For DBB and MBB, response curves were generated with the calculated average value of standard sets of TeBB and TriBB.

To determine whether the extraction and concentration process affected isotope composition, two sets of controls were prepared for CSIA. To test for potential extraction effects, triplicate serum bottles were prepared with mixture of 10 μM HCB, PeCB, 1,2,3,4-TeCB, 1,2,4-TriCB, and 1,3-dichlorobenzene (DCB) and 500 μM electron donor mix in 40 mL anaerobic media. Extraction was conducted as described above. Extracts were concentrated by evaporation in fume hood to a final volume of less than 0.5 mL. To test for any effects resulting from solvent evaporation, the mixture of HCB, PeCB, TeCB, TriCB, and DCB was added in 15 mL hexane. The volume of mixture added was same with previously described extraction test. Hexane containing the mixture was evaporated in a fume hood till its final volume reached less than 0.5 mL. Evaporation and extraction tests were performed in triplicate and analyzed by CSIA as described below. No changes in C isotope composition were observed due to the extraction, sample concentration or Florisil clean-up procedures (data not shown).

2.3. Stable Carbon Isotope Analysis

Samples concentrated to a volume of less than 0.5 mL were shipped to Germany and stable carbon isotope analyses conducted at the Stable Isotope Laboratory of the Helmholtz-Centre for Environmental Research (UFZ), Leipzig-Halle, Germany. Before analyses, sample cleanup through a Florisil column (~1 g of Florisil in a Pasteur pipette eluted with 5 mL of pentane-

diethyl ether; 90:10 (v:v)) was conducted to remove organic matter that would interfere with CSIA. Samples were concentrated to a volume of 50 to 100 µL prior to analysis. The GC-IRMS system consisted of a gas chromatograph (7890 series, Agilent Technology) coupled via a Conflow IV Interface combustion oven (ThermoFinnigan GC-combustion IV, Bremen, Germany) to a Finnigan MAT 252 isotope ratio mass spectrometer (ThermoFinnigan, Bremen). The organic substances in the GC effluent were oxidized to CO_2 on a CuO/Ni/Pt catalyst held at 960 °C. A Zebron ZB5 column (60 m × 0.32 m; 0.25 µm film thickness) was used for separation with helium at a flow rate of 1.5 mL min^{-1} used as the carrier gas. The GC temperature program was held at 60 °C for 5 min, then increased to 320 °C at a rate of 10 min^{-1}, and then held for 5 min isothermally. Samples were injected (2 µL) in split mode with a split ratio 1:1 into a hot injector held at 320 °C. Each sample was analyzed at least in triplicate. The carbon isotope composition of halogenated benzene levels below 1 µM in the microcosms samples could not be determined. CSIA data for 1,3,5-TriCB cultures was not obtained.

The carbon isotope composition of the laboratory isotope reference HCB used for method validation was determined directly by elemental-analyser-IRMS as described previously (Coplen et al., 2006). HCB used for the experiments had a $\delta^{13}C$ value of -26.096 ± 0.062 ‰ vs. V-PDB.

The carbon isotopic compositions (R) are reported as δ notation in parts per thousand (denoted as ‰) enrichments or depletions relative to a standard (V-PDB; Vienna Pee Dee Belemnite standard) of known composition. δ values of carbon were calculated as follows:

$$\delta(^{13}C)\ ‰ = (R_{sample}/R_{standard} - 1) \times 1000$$

where R_{sample} and $R_{standard}$ represent the ratios of the heavy isotope to light isotope in sample and the international standard, respectively (Coplen, 2011).

The extent of isotope fractionation during degradation can be expressed as an isotopic enrichment factor (ε) calculated based on the Rayleigh equation for a closed system (Rayleigh, 1896; Mariotti et al., 1981):

$$\ln (R_t/R_0) = \varepsilon \cdot \ln(C_t/C_0)$$

where R is the isotope ratio, C is the concentration, and the index (0 and t) describes the incubation time of experiment (t). The isotope ratios (R_t/R_0) are determined from the equation $R_t/R_0 = (\delta_t + 1)/(\delta_0 + 1)$. When ln R_t/R_0 versus ln C_t/C_0 is plotted, the isotopic enrichment factor (ε) can be determined from the slope of the curve. Linear regression was used to estimate the slope.

3. Results

3.1. Reductive dehalogenation of chlorobenzenes

HCB was sequentially dechlorinated via PeCB, 1,2,3,5-TeCB and 1,3,5-TriCB (and a minor amount of another unidentified TriCB isomer) to 1,3-DCB over a period of 33 days. At select time points (Fig. 1), cultures were sacrificed for CSIA. Due to differences in the dechlorination rate of the monitoring cultures and CSIA sample cultures (data not shown) we only had two early time points and thus data for isotope composition at residual substrate concentrations of HCB between 100 and 50 mole % were not obtained. From the CSIA, no clear C isotopic enrichment in HCB (at ~ -26 ‰) could be observed over the course of dechlorination. However, PeCB detected at approximately 50 % dechlorination of HCB had a $\delta^{13}C$ value of -29 to -26 ‰ (standard deviation 0.41 to 1.18 ‰). Similarly, DCB detected at 70 to 90 % dechlorination of HCB also had a depleted $\delta^{13}C$ value, at around -28 to -30 ‰ with approximately 12 % of residual HCB (Fig. 1b). For the 1,3,5-TriCB dechlorination intermediate, there was no measurable change in isotope composition over the course of reductive dechlorination.

In a follow-up experiment with the suite of HCB, PeCB, 1,2,3,5-TeCB and 1,3,5-TriCB as substrates, dechlorination of PeCB and TeCB occurred over 30 days, while HCB dechlorination took longer (Fig. 2a, b). After 30 days, the mole fraction of HCB was still at 50 % of all CBs. No significant change in the $\delta^{13}C$ value of HCB was observed over the course of dechlorination. The PeCB intermediate was slightly depleted in $\delta^{13}C$, as observed in the first experiment, however, changes in the isotope composition for the other dechlorination products were inconclusive. Based on the isotope separation comparing the substrate and the instantaneous products (Fig. 1b and Fig 2b) we can estimate an ε for HCB dechlorination of approximately -3 ‰. The isotope balance (not shown) was not closed and the average isotope composition of substrate and

analyzed products was enriched by approximately 4.5 ‰ over the time-course of the experiment, indicating that not all products were detected in the isotopic analysis.

During PeCB dechlorination (Fig. 2c, d) 1,2,3,5-TeCB, 1,3,5-TriCB and 1,3-DCB were the main intermediates. Additional minor TeCB and TriCB intermediates were below the detection limit for CSIA. The $\delta^{13}C$ value for the 1,2,3,5-TeCB intermediate was slightly depleted from -26 to -27 ‰ (standard deviation 0.03 to 0.30 ‰) at around 70 % of dechlorination of PeCB, and the value was more enriched at -24 ‰ (standard deviation 0.30 ‰) when PeCB dechlorination was almost completed. However, no significant changes in the isotope composition of the substrate, other intermediates and products, PeCB, 1,3,5-TriCB, and 1,3-DCB, were observed over the course of PeCB dechlorination.

Over the course of dechlorination of 1,2,3,5-TeCB (Fig. 2e, f), changes in the $\delta^{13}C$ value for the TeCB substrate and 1,3,5-TriCB intermediate were detected. A second minor TriCB intermediate was below the detection limit for CSIA. The $\delta^{13}C$ value for 1,2,3,5-TeCB was enriched from -29±0.16 to -27±0.74 ‰ at approximately 10 % dechlorination of TeCB. The $\delta^{13}C$ value for 1,3,5-TriCB was depleted to -33 to -34 ‰ when TeCB decreased to residual concentrations of ~90 to 70 %. Then, the $\delta^{13}C$ value was enriched to -28 ‰ at 4 % residual TeCB in the culture. Based on the isotope separation comparing the substrate and the instantaneous products (Fig. 2f) we can estimate an ε for 1,2,3,5-TeCB dechlorination of approximately -3 to 5 ‰. In this experiment, the isotope balance was also not closed (relative enrichment over the time-course approximately 15 ‰), indicating that some products were below the detection limit for isotope analysis.

3.2. Reductive dehalogenation of bromobenzenes

Previous experiments with HBB (Sohn and Häggblom, 2016) and the first CSIA culture set (data not shown) had shown only limited debromination activity, possibly due to the very low solubility of HBB or to steric hindrance that blocked reductive dehalogenation. We therefore tested 1,2,3,5-TeBB and 1,3,5-TriBB as electron acceptors in microcosms originating from the Hackensack River sediment (H2). 1,2,3,5-TeBB and 1,3,5-TriBB were selected based on the dechlorination pattern of HCB shown in previous experiments. Debromination was observed for

both compounds, with TriBBs, DBBs, and MBB detected as dehalogenation intermediates and end-products (see below).

1,2,3,5-TeBB and 1,3,5-TriBB were debrominated via DBB to MBB. At 31 days of incubation, approximately 50 % of the substrates remained (the last sampling point for CSIA; Fig. 3a, c). From the CSIA of TeBB, no clear C isotopic enrichment in TeBB (at an average of approx. -26 ‰) could be observed over the course of reductive debromination (Fig. 3b). However, a sequential depletion and enrichment of the $\delta^{13}C$ value was detected for the TriBB intermediate. The $\delta^{13}C$ value of TriBB decreased from -25 to -27 ‰ during the decrease of the residual TeBB concentration from 80 to 70 mol %, indicating a slight ^{13}C depletion, whereas it increased again to -25 ‰ during the further dehalogenation.

Over the course of TriBB debromination (Fig. 3c), the value of $\delta^{13}C$ for TriBB in cultures became more enriched from an initial value of -27 ‰ and increased to -23 ‰ when approximately 68 % of TriBB remained in the culture (Fig. 3d). For the DBB intermediate detected, there was no substantial change of isotope fractionation over the course of debromination. MBB was below the detection limit for CSIA. The isotope enrichment factor (ε) was calculated to describe the debromination of TriBB (Fig. 4), yielding an estimated ε value for TriBB debromination of -5.5 ‰ ($r^2 = 0.48$).

4. Discussion

In this study, carbon stable isotope signatures were obtained for the reductive dehalogenation of highly chlorinated benzenes (HCB, PeCB and 1,2,3,5-TeCB) and brominated benzenes (1,2,3,5-TeBB and 1,3,5-TriBB). Compound specific stable isotope analysis has been shown to be an effective tool to evaluate *in situ* biodegradation in many studies (for review, see Meckenstock et al., 2004; Nijenhuis et al. 2016). Stable carbon isotope fractionation during aerobic and anaerobic transformation of MCB, DCBs, and TriCBs has been reported previously (Griebler et al., 2004; Braeckevelt et al., 2007; Liang et al., 2014). In addition, multi-dimensional CSIA, by combining the analysis of two or more different stable isotopes within a molecule, can be used to identify specific pathways of *in situ* biodegradation (Vogt and Richnow, 2014; Nijenhuis et al. 2016). Previously, there are only a few studies measuring isotope fractionation of brominated

compounds (e.g., Kuder et al. 2012; Wilson et al. 2008; Kuntze et al., 2016). This is thus the first attempt of measuring carbon isotope fractionation during anaerobic dehalogenation of highly chlorinated and brominated benzenes. Theoretically, the substrate of the reaction is expected to show $\delta^{13}C$ enrichment; the product of the reaction to show $\delta^{13}C$ value depletion; and the intermediates to show a sequential depletion and enrichment of the $\delta^{13}C$ value. Not all of our CSIA data are in agreement with the theory. There are some possible reasons to explain this. A high intrinsic rate of reaction, a high hydrophobicity and low solubility of the substrate can cause masking of the isotope fractionation (Renpenning et al., 2015). In addition, for halogenated benzenes the magnitude of measurable fractionation is substantially diluted by the number of carbon atoms in the compound.

CSIA of the HCB dechlorinating cultures showed depletion in the $\delta^{13}C$ value of the DCB, dechlorination product, even though there was a negligible isotope fractionation for HCB (Fig. 1). From the isotope separation comparing the substrate and the instantaneous products (Figs. 1 and 2), we estimated ε values of approximately -3 to 5 ‰ for dechlorination of HCB and 1,2,3,5-TeCB. There are some studies representing $\delta^{13}C$ depletion in the product. For example, Hunkeler et al. (1999) conducted CSIA for microcosm and field study of groundwater, and they observed the $\delta^{13}C$ depletion of dechlorination products in tetrachloroethene (PCE) dechlorination. Ewald et al. (2007) analyzed carbon isotope composition of trichlorinated dibenzo-*p*-dioxins during reductive dehalogenation by an anaerobic mixed culture. The dehalogenation product, monochlorinated dibenzo-*p*-dioxin (MCDD), showed a significant depletion in ^{13}C and may thus be monitored for evidence of transformation.

In our CSIA data, two clear changes of isotope composition were observed. One is from TriCB in TeCB dechlorinating cultures, and the other is from TriBB in TriBB debrominating cultures. First, the $\delta^{13}C$ value for the 1,3,5-TriCB intermediate from 1,2,3,5-TeCB dechlorination showed a sequential depletion and enrichment as TeCB dechlorination proceeded (Fig. 2f). This might be explained by the role of TriCB in TeCB dechlorinating cultures. As an intermediate of 1,2,3,5-TeCB dechlorination, 1,3,5-TriCB is both a product of TeCB dechlorination and the substrate of subsequent TriCB dechlorination. In the initial stage of TeCB dechlorination, $\delta^{13}C$ depletion for TriCB occurred, reflecting the accumulation of ^{12}C in the product, 1,3,5-TriCB, as a result of TeCB dechlorination. In the later stage, 1,3,5-TriCB is the substrate for dechlorination and the

$\delta^{13}C$ value of 1,3,5-TriCB was enriched. Liang et al. (2013) reported the same tendency in the $\delta^{13}C$ for benzene during degradation of MCB; the $\delta^{13}C$ values for benzene decreased until 45 % of the MCB was degraded. As both MCB and benzene were further degraded, the $\delta^{13}C$ values of benzene became enriched. Second, the $\delta^{13}C$ value for TriBB in TriBB debrominating cultures became more enriched as debromination advanced (Fig. 3d). This suggests that CSIA can be used to monitor the dehalogenation of brominated substances. Kaschl et al. (2005) reported the enrichment in $\delta^{13}C$ for MCB during anaerobic MCB degradation. Liang et al. (2014) showed that the $\delta^{13}C$ value for DCB became enriched during anaerobic DCB degradation. Thus, CSIA of the highly halogenated substrates may not be possible, but products/intermediates can be monitored for evidence of transformation.

The isotope enrichment factor (ε) was calculated to describe the debromination of 1,3,5-TriBB (Fig. 4), yielding an ε value for TriBB debromination of -5.5 ‰ (r^2 = 0.48). The low r^2 value suggested that the Rayleigh model is not suitable for carbon isotope fractionation during reductive dehalogenation of TriBB. Since there was no reference of ε values for brominated benzenes, ε values are compared instead to those reported for chlorinated benzenes. Griebler et al. (2004) reported carbon isotope enrichment factor (ε) for TriCB dechlorination. The values are between -3.1 and -3.7 ‰ for 1,2,3-TriCB and 1,2,4-TriCB during anaerobic dechlorination by *Dehalococcoides* sp. strain CBDB1. Liang et al. (2011) reported ε values of -5.0 ± 0.2 ‰ and -3.0 ± 0.4 for MCB and 1,2,4-TriCB, respectively, during anaerobic degradation. In their follow-up report, ε values of -0.8 ± 0.1 ‰; -5.4 ± 0.4 ‰; and -6.3 ± 0.2 ‰ for anaerobic transformation of 1,2-DCB, 1,3-DCB, and 1,4-DCB, respectively, were obtained (Liang et al., 2014). Our estimated ε value for TriBB dehalogenation was more in agreement with the ε value for DCB or MCB than the ε value for TriCB. Dual element analysis (C and Br) might improve the assessment (e.g., Kuntze et al., 2016).

For dehalogenation intermediates, it is difficult to observe isotope enrichment or depletion since the intermediates have both a role as a substrate and a product. Thus, Stelzer et al. (2009) proposed a concept of isotope balance to interpret CSIA during chlorobenzene biodegradation. Sequential dehalogenation of chlorinated benzenes complicates the evaluation with the Rayleigh model because the isotope signature of single chlorobenzene species is affected by both simultaneous depletion and enrichment of ^{13}C. The isotope balance can be calculated by the

equation $\delta^{13}C_{CB}[‰] = \Sigma(C_i \times \delta^{13}C_i) / C_{CB}$, where $\delta^{13}C_{CB}$ is the isotope signature of the total CBs, C_i is the molar concentration of each compound, $\delta^{13}C_i$ is the respective carbon isotope signature of each compound, and C_{CB} is the total molar concentration of all chlorinated benzenes. With isotope balance, *in situ* biodegradation was determined by the enrichment of the cumulative isotope composition of all chlorobenzenes. Stelzer et al. (2009) used this concept to interpret CSIA of MCB and confirm biodegradation of MCB in both laboratory and field. Our calculations of isotope balances suggest the losses of relatively depleted products (e.g., during the extraction) or the production of further dehalogenation products (e.g., MCB and benzene) which were not detected in our analytical procedures. In addition, the different isomers produced during dechlorination may have different isotope signatures, for example where one isomer might be the end product of dechlorination, whereas another might be subject to further dechlorination. This might be a further explanation for the gap in the calculated isotope balance, particularly, when the isomers in small concentrations escaped detection by CSIA.

In this study, CSIA data were obtained for dehalogenation of halogenated benzenes, including HCB, PeCB, 1,2,3,5-TeCB, 1,2,3,5-TeBB, and 1,3,5-TriBB with anaerobic cultures originating from Hackensack River sediment. Although strong isotope fractionation was not observed, we could measure a depletion of the $\delta^{13}C$ value in the DCB product during dechlorination of HCB, the sequential depletion and enrichment of $\delta^{13}C$ value for trichlorobenzene in TeCB dechlorinating cultures, and the enrichment of $\delta^{13}C$ in TriBB during debromination of TriBB. This indicates that a measurable isotope fractionation occurred during reductive dehalogenation of highly halogenated chloro- and bromobenzenes. The low signal is likely due to the low solubility of the halogenated benzene substrates, which can mask the isotope fractionation, combined with dilution of the fractionation by the number of carbon atoms in the compound. Although more quantitative measurements will be needed, this experimental data suggests that CSIA may have application for monitoring *in situ* microbial reductive dehalogenation of highly halogenated benzenes.

5. Conclusions

- CSIA data represents carbon isotope fractionation coupled to reductive dehalogenation of highly halogenated benzenes including HCB, PeCB, TeCB, TeBB, and TriBB

- The evidence of carbon isotope fractionation during reductive dehalogenation was detected even though there were little changes of $\delta^{13}C$ values
- The limited solubilities of highly halogenated substrates may mask intrinsic isotope effects, causing low observed isotope effects.

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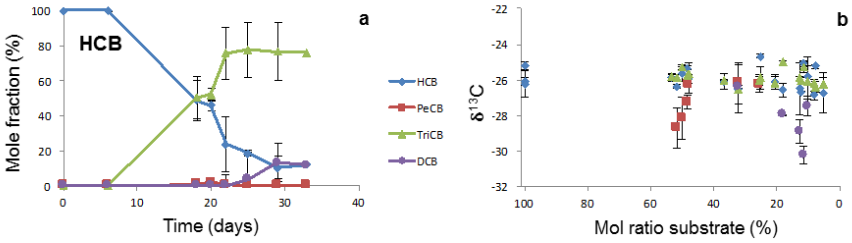

Figure 1. Dechlorination of HCB. a) mole fraction of remaining HCB and generated dechlorination intermediates (Each time point represents the mean of triplicate cultures; TeCB, TriCB and DCB are the sum of all detected isomers). b) CSIA of HCB. X-axis represents residual HCB substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value of HCB and dechlorination products (Each data point represents data from a single culture bottle with mean and SD of triplicate measurements).
Abbreviations: HCB, hexachlorobenzene; PeCB, pentachlorobenzene; TeCB, tetrachlorobenzene; TriCB, trichlorobenzene; and DCB, dichlorobenzene.

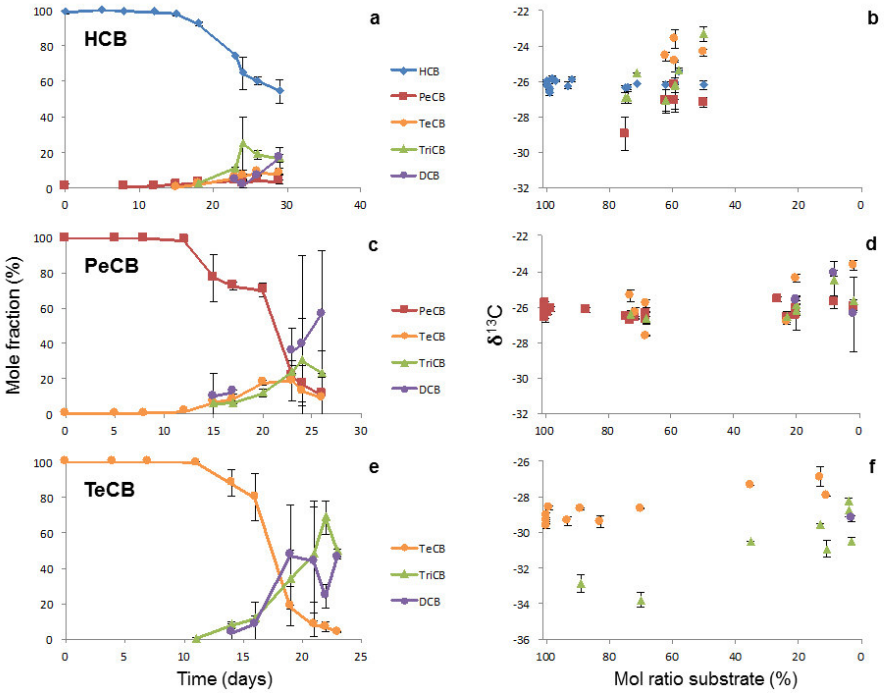

Figure 2. Dehalogenation of HCB, PeCB and 1,2,3,5-TeCB. a, c & e) mole fraction of chlorinated benzenes (Each time point represents data from duplicate cultures; TeCB, TriCB and DCB are the sum of all detected isomers). b, d, & f) CSIA data. X-axis represents residual substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value (Each data point represents data from a single culture bottle with mean and SD of triplicate measurements). Abbreviations: HCB, hexachlorobenzene; PeCB, pentachlorobenzene; TeCB, tetrachlorobenzene; TriCB, trichlorobenzene; and DCB, dichlorobenzene.

Figure 3

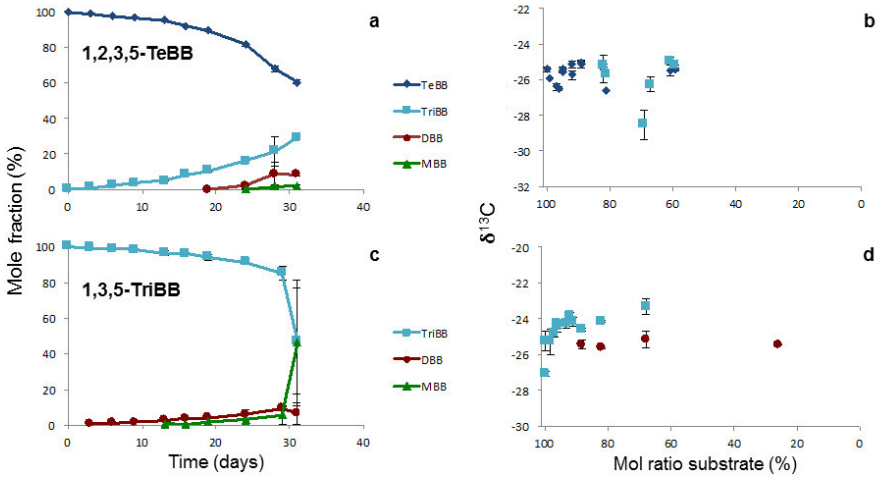

Figure 3. Dehalogenation of 1,2,3,5-TeBB and 1,3,5-TriBB. a & c) mole fraction of brominated benzenes (Each time point represents data from duplicate cultures). b & d), CSIA data. X-axis represents residual substrate concentration in culture and Y-axis represents the measured $\delta^{13}C$ value (Each data point represents data from a single culture bottle with mean and SD of triplicate measurements).
Abbreviations: TeBB, tetrabromobenzene; TriBB, tribromobenzene; DBB, dibromobenzene; and MBB, monobromobenzene.

Figure 4

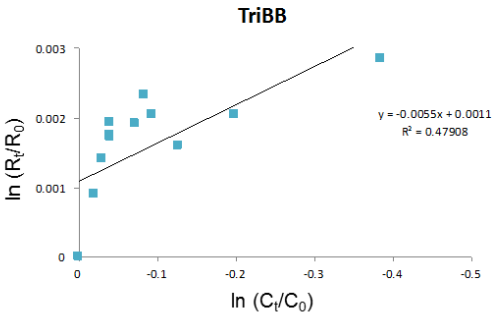

Figure 4. A double logarithmic plot for TriBB in 1,3,5-TriBB debrominating cultures according to Rayleigh equation of the isotopic composition versus the residual concentration of substrate.