International Biodeterioration & Biodegradation (2020) 55: 105093

https://doi.org/10.1016/j.ibiod.2020.105093

Reductive Debromination by Sponge-Associated Anaerobic Bacteria Coupled to Carbon Isotope Fractionation

Isabel Horna-Gray [a], Nora A. Lopez [a], Ivonne Nijenhuis [b], Youngbeom Ahn [a,c], Hans H. Richnow [b] and Max. M. Häggblom [a]

[a] Department of Biochemistry and Microbiology, School of Environmental and Biological Sciences, Rutgers University, New Brunswick, New Jersey 08901, USA

[b] Department of Isotope Biogeochemistry, Helmholtz-Centre for Environmental Research – UFZ, D-04318 Leipzig, Germany

[c] Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA

Running title: CSIA of reductive debromination

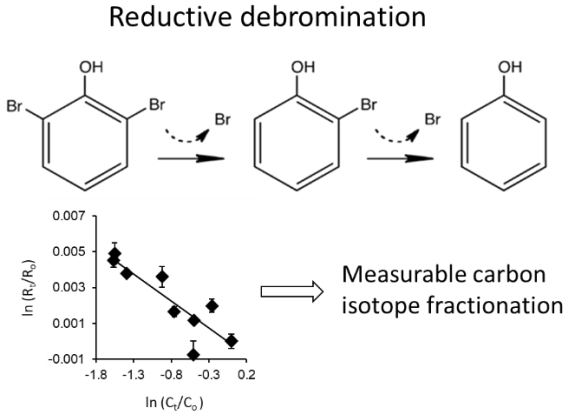
Correspondence to: Max M. Häggblom, haggblom@rutgers.edu

ABSTRACT

Marine sponges contain diverse brominated compounds as secondary metabolites and the sponge habitat appears to enrich for a population of anaerobic dehalogenating bacteria. Hence, there is interest in understanding how these natural and anthropogenic compounds are degraded in the marine environment. Compound specific isotope analysis (CSIA) is a useful tool to monitor and to quantify the degradation and fate of aquatic pollutants. The objective of this study was to evaluate whether reductive dehalogenation of brominated phenols by sponge-associated bacteria, including *Desulfoluna spongiiphila,* can be monitored by CSIA. Debromination of 2,6-dibromophenol to phenol by sponge-associated cultures resulted in measurable stable carbon isotope fractionation. All sponge-associated cultures showed similar isotopic enrichment factors (ε). The ε values for two independent sponge-derived dehalogenating cultures were -3.1 ± 1.5 ‰, and -3.0 ± 0.3 ‰, and that of sponge associated sediment cultures -2.0 ± 0.3 ‰. Thus, we demonstrate that reductive debromination of 2,6-dibromophenol resulted in measurable carbon isotope fractionation and that CSIA can be used to assess reductive debromination and to monitor and estimate *in vivo* dehalogenation in a sponge animal.

Keywords: Anaerobic, bromophenol, microbial reductive dehalogenation, carbon isotope fractionation

Graphical Abstract:



1. Introduction

Organobromine compounds are ubiquitous in marine sedimentary environments (Leri et al. 2010) and organic and inorganic bromine cycling is intertwined with the cycling of organic carbon. Marine organisms produce and transform a myriad of natural organohalides (Gribble 2003; 2010), while the marine environment is also a sink for many organohalide pollutants, including brominated flame retardants, widely used to avoid fire accidents in electronics, indoor house-hold fabrics and even clothing (de Wit 2002; Law et al. 2006). Brominated aromatics, such as tetrabromobisphenol A (TBBPA) and polybrominated diphenyl ethers (PBDEs), are among the most widely used brominated flame retardants (Birnbaum & Staskal 2004). Because of their physicochemical properties, brominated compounds can accumulate in anoxic sediments, have ecotoxicological effects, or may biomagnify in the food chain (de Wit et al. 2010; Fernie & Letcher 2010; Bartrons et al. 2012). For example, several brominated flame retardants are thyroid and estrogen disruptors (Hamers et al. 2006, 2008). The wide use and increasing prevalence of these brominated compounds has prompted the study of their fate and transport in the environment.

Interestingly, the marine environment is also a particularly rich source of biogenic halogenated compounds produced by diverse marine organisms, including sponges (Gribble 2003; 2010). The organohalides present in marine sponges are usually bioactive, with antifouling or/and antibiotic characteristics. Some of these organohalides resemble anthropogenic pollutants, such as halogenated dibenzo-*p*-dioxins (Bowden et al. 2000) and flame retardants (Utkina et al. 2001). This long-term exposure to biogenic organohalides is believed to have supported the evolution of reductive dehalogenating bacteria (Krzmarzick et al. 2012). Marine sponges are some of the most prolific organohalide producers. Sponges are filter feeders, and although microorganisms are a major component of their diet, complex sponge-specific microbial communities thrive within the mesohyl of the sponge (Hentschel et al. 2006, 2012; Taylor et al. 2007a, 2007b; Thacker & Freeman 2012; Webster & Taylor 2012). The combined abundance of organohalides and the high density of their associated microbes, for example in the *Aplysina aerophoba* sponge, prompted Ahn *et al.* (2003) to determine whether sponge-associated microbes were capable of dehalogenation. Subsequently, a novel sponge-associated bacterial species, *Desulfoluna spongiiphila*, capable of organohalide respiration via reductive debromination of tri-, di-, and mono-bromophenols was isolated (Ahn et al. 2009). A novel reductive dehalogenase of *D. spongiiphila* was upregulated during respiration of bromophenols

(Liu et al. 2017, 2020; Peng et al. 2020). Sponge-produced organohalides, thus, appear to select for microorganisms that can utilize these compounds as a source of energy. *Desulfoluna* spp. are a peculiar group of dehalogenating microorganisms active in metazoans and represent a unique contrasting group from the other known dehalogenating bacteria. Reductive dehalogenation of brominated phenolics has also been observed in other habitats, including estuarine sediments (e.g., Monserrate & Häggblom 1997; Boyle et al. 1999; Watson et al. 2000; Fennell et al. 2004; Arbeli et al. 2006), and reductive dehalogenase genes appear to be widely distributed in marine *Desulfobacterota* (Liu & Häggblom 2018). There is still much to understand about the *in situ* activity of dehalogenating microbes in marine and estuarine environments (Zanaroli et al. 2015) and elucidation of the fate and transport of brominated pollutants.

Compound specific isotope analysis (CSIA) is a useful tool to monitor and to quantify the degradation and fate of aquatic pollutants (Meckenstock et al. 1999; Sherwood-Lollar et al. 1999; Hunkeler et al. 2005). Compounds analyzed for effects of biodegradation on isotope composition include aromatic hydrocarbons (Richnow et al. 2003; Braeckevelt et al. 2007), methyl *tert*-butyl ether (Somsamak et al. 2005); organohalides such as tetrachloroethene (Nijenhuis et al. 2005; Fletcher et al. 2011), dichloromethane (Nikolausz et al. 2006) and polychlorinated dibenzo-*p*-dioxins (Ewald et al. 2007; Liu et al 2010). More recently, carbon isotope fractionation for microbial debromination of brominated alkanes and aromatics were published (Bernstein et al. 2013; Sohn *et al*. 2018; Woods et al. 2018). CSIA is based on the different reaction kinetics of bond formed by light vs. heavy isotopes. Chemical bonds containing heavier isotopes need more energy for cleavage than light isotopes and in either abiotic or biological transformations. Therefore, normal isotope effects with a preferential transformation of the ^{12}C substrates are observed. This results in an enrichment of ^{13}C with a change in the C isotope ratio ($^{13}C/^{12}C$) in the remnant substrate over time. Biological transformations commonly lead to a normal isotope effect accompanied with an enrichment of heavier isotopes in the residual fraction of the substrate (for reviews see, Meckenstock et al. 2004; Schmidt et al. 2004; Bombach et al. 2010; Elsner 2010; Renpenning & Nijenhuis 2016). The isotope enrichment factors can potentially be linked to specific biochemical reactions because the extent of fractionation is linked to the degradation pathway of the target compound and type of bonds being affected during initial cleavage (Nijenhuis & Richnow 2016). Accordingly, CSIA represents a powerful tool for assessing biodegradation of organic chemicals in the field.

In order to develop an approach for understanding the metabolic activities of the sponge endomesohyl microbiota in the cycling of organohalogens in the marine environment we applied CSIA to analyze the bromophenol substrates and dehalogenation products. We tested whether CSIA could be used for assessing the reductive dehalogenation of brominated phenols and for monitoring the activity of anaerobic sponge-associated bacteria, and to eventually estimate the organohalide flux *in vivo*. 2,6-Dibromophenol (2,6-DBP) was used as a model compound to assess whether temporal analysis of carbon isotopic composition during reductive debromination by sponge derived cultures can be used to determine the isotopic enrichment factor (ε) for this process. Here we report on CSIA of brominated phenols during microbial degradation in a marine system.

2. Materials and Methods

2.1. Chemicals

2,6-DBP used as substrate and other phenolic compounds (2-bromophenol, 2-BP; 4-chlorophenol, 4-CP; 4-methylphenol; 2,4-dichlorophenol; phenol) used as standards were obtained from Aldrich Chemical Co. (Milwaukee, Wis., USA) and had a minimum of 99% purity.

2.2. Origin and cultivation of reductively debrominating bacterial cultures

Anaerobic dehalogenating cultures of *Desulfoluna spongiiphila* strain AA1 (AA1), a sponge enrichment culture (PPS) of an unidentified sponge (presumed to be a *Haplosclerida*; collected Bucco Sur, Tumbes, Peru), and a sediment enrichment culture (TS7) prepared from sediment adjacent to a marine sponge (collected near Tuckerton, NJ) were maintained in anaerobic minimum salts media (MSM) (Ahn et al. 2003). The enrichment cultures had been successively transferred 1/10 (v/v) at least 10 times over the course of several years prior to setup of the experiments. The cultures were amended with 200 µM 2,6-DBP as the electron acceptor and a mixture of short chain organic acids (1 mM each of lactate, propionate and butyrate) as electron donors and carbon source (Ahn et al. 2003).

2.3. Experimental set up

The dehalogenating cultures (AA1, PPS and TS7) were established in triplicate in 1 L MSM, with respective killed controls. All cultures were amended with 200-1000 µM 2,6-DBP and a mixture of 1-5 mM (each) lactate, propionate and butyrate under strict anoxic conditions.

Samples (1 mL and 30 mL) were taken over a time course and stored at -20 °C until analyses of dehalogenation product concentrations and carbon isotope composition were performed.

2.4. *In vivo* sponge experiments

Clathria prolifera sponges were collected from a Long Island Sound estuary (41º 02.55' N, 73º 30.37' W) stored in zip-lock bags filled with seawater and transported to the laboratory. Three intact sponges were each placed in 2 L tanks filled with artificial seawater and aerated with an aquarium air pump. To monitor *in vivo* sponge debrominating activity the tanks were amended with 500 µM 2,6-DBP and maintained at room temperature. Triplicate water controls without sponges and 500 µM 2,6-DBP were also established. Samples of water in the tank were analyzed for 2,6-DBP, dehalogenation product concentrations and carbon isotope composition of the phenols. A 1 mL sample was used for HPLC analysis to monitor concentration. Samples for CSIA (between 10 to 100 mL) were stored at -20 °C prior to analysis.

2.5. Bromophenol Analysis

Samples were filtered (0.45 µm pore size filter) prior to analysis. Concentrations of 2,6-DBP, 2-bromophenol and phenol were determined by high performance liquid chromatography using an Agilent 1100 series HPLC (Palo Alto, CA) equipped with a C18 column (Spherisorb, 4.6×250 mm, particle size 5 µm; Phenomenex) with methanol/water/acetic acid (40:58:2; vol:vol:vol) as the eluent at a flow rate of 1 mL/min and a UV absorbance detector set to 280 nm.

2.6. Compound specific isotope analysis

Samples taken from bacterial cultures and *in vivo* sponge experiments were stored at -20 °C and transported to the Helmholtz Centre for Environmental Research in Leipzig, Germany for analysis. A mixture of 2,6-DBP, 2-BP, phenol, 4-chlorophenol and 2,4-dichlorophenol (the last 2 being used as internal standards) dissolved in water was used to validate the method for sample preparation by either solid-phase or solvent extraction. The initial experiments allowed us to determine: 1) the best non-fractionating sample preparation method 2) the appropriate sample concentration for CSIA according to the detection limits of the equipment; and 3) the use of methanol to elute DBP from solid-phase extraction or dichloromethane (DCM) for solvent extractions. After these assays (data not shown) extraction in DCM was found to be the most suitable for CSIA. The samples (3-50 mL) were centrifuged for 20 min at 14000 x g and the supernatant was separated and acidified with 20 µL concentrated HCl. After acidification, 0.5

mL DCM was added and mixed thoroughly by vortex. After a minimum of 10 min settling the DCM fraction was collected with a Pasteur pipette and transferred into GC vials.

The carbon isotope composition of the phenols was measured with a GC-C-IRMS (gas chromatography combustion isotope-ratio-monitoring mass spectrometry) system (FINNIGAN MAT, Bremen, Germany). The system consisted of a GC unit connected to a FINNIGAN MAT combustion unit with a water removal assembly coupled to a FINNIGAN MAT 252 mass spectrometer (Cichocka et al. 2008). Bromophenols in the GC effluent stream were oxidized to CO_2 (at 980 °C) in the combustion interface and were transferred to a mass spectrometer to determine $^{13}CO_2/^{12}CO_2$ ratios (Merritt et al. 1994; Nijenhuis et al. 2016). Bromophenols extracted in DCM were injected into a gas chromatograph (HP 6890, Agilent, Palo Alto, CA, USA) using a split/splitless injector held at 280 °C. The split was adjusted between 1:5 and 1:20 to obtain suitable peak areas for reproducible determination of the $^{13}CO_2/^{12}CO_2$ mass ratio. At least 3 replicate measurements were made for each sample. For chromatographic separation, a ZB-5 capillary column (60 m × 0.32 mm, 0.5 µm film thickness; Phenomenex Inc., Torrance, CA) with the following oven temperature program was used: 2 min at 70 °C; 10 °C/min to 200 °C; 20 °C/min to 300 °C; and hold for 2 min with Helium as the gas carrier.

A mixture of substituted phenols (2-chlorophenol, 2-methylphenol and 2,4-dichlorophenol) in addition to 2,6-DBP, 2-BP and phenol were tested to establish the ideal internal standards for our samples, the detection limits and the linearity (i.e., isotope ratio obtained is independent of the concentration of the analyte) of the isotope ratio mass spectrometer (IRMS). The GC-IRMS detection limits for 2,6-DBP, 4-CP, 2-BP and phenol were 300, 700, 400 and 900 µM, respectively. The linearity for isotope analysis was satisfactory only in a narrow range between 500 to 1200 µM (Supplementary Information). Not all samples from the various cultures yielded acceptable isotope measurements and these were not included in subsequent data analysis. Samples with low concentrations out of the range where carbon isotope value are independent of the amount where excluded from evaluation.

The isotopic enrichment factor ε was determined with GC-C-IRMS and used to calculate the apparent kinetic isotope effect (AKIE) for comparison of our results with that of other C-Br reaction of other compounds. The carbon isotope composition is reported in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard, V-PDB (Coplen 2011). $^{13}C/^{12}C$ in the sample was measured, R_{sample}, which was then normalized to $R_{standard}$, the $^{13}C/^{12}C$ ratio of the

standard reference material V-PDB. Carbon isotope enrichment factors were calculated according to the Rayleigh equation (Elsner et al. 2005):

$$\varepsilon \times (\ln C_t/C_0) = \ln R_t/R_0 \qquad (1)$$

where C_t and C_0 are the concentrations of 2,6-DBP at time t and time 0, respectively, R_t and R_0 are the $^{13}C/^{12}C$ ratios in at time t and 0, respectively, and $R= \delta +1$.

To characterize the Br-C bond cleavage of the first irreversible reaction of 2,6-DBP, AKIE values were calculated for a particular molecular position. The AKIE for carbon for each debromination reaction was estimated using the following equation (Elsner et al. 2010):

$$AKIE = 1 / (1 + z \times \varepsilon (n/x)) \qquad (2)$$

where n is the number of C atoms of the molecule, x the number of reactive positions, and z is the number of positions in intramolecular competition. For 2,6-DBP, n = 6 for carbon atoms, x = 1 from the stepwise debromination via 2-BP to phenol as represented in Figure 1, and z = 2, the number of positions in intramolecular competition because of the symmetrical positions (2 or 6) available for debromination.

3. Results

3.1. CSIA of debrominating cultures *D. spongiiphila* AA1, sponge enrichment culture PPS and sediment culture TS7

Three sponge-derived anaerobic dehalogenating cultures were compared to determine carbon isotopic fractionation during debromination of 2,6-DBP. All cultures showed sequential debromination of 2,6-DBP to 2BP and phenol in less than 14 days (Fig.1a and 1b). In cultures of *D. spongiiphila* AA1 the $\delta^{13}C$ of 2,6-DBP became enriched from an initial value of -30 to -25.6 ‰ at 79% debromination. Similarly, the $\delta^{13}C$ of 2,6-DBP in the Peruvian sponge enrichment culture (PPS) and the Tuckerton NJ sediment culture (TS7) was enriched from -31.1 to -28.2 ‰ at 81% debromination and from -30.2 to -25.4 ‰ at 79% debromination, respectively (Fig. 1c). The relationships between the change in 2,6-DBP concentration and isotope composition during dehalogenation by the three cultures is shown in Fig. 2.

The isotope fractionation of the debromination reaction could be quantified by the Rayleigh equation. Isotopic enrichment (ε) factors as well as the AKIE for each debrominating culture are summarized in Table 1. Similar ε (-3.1 ± 1.5 ‰ and -3.0 ± 0.5 ‰) were observed for the two sponge derived-cultures: AA1 (*Aplysina aerophoba* sponge) and PPS (unidentified *Haplosclerida* sponge), respectively. The culture of sponge associated sediment (TS7) exhibited

slightly different ε (-2.0 ± 0.3 ‰). The overall similar ε values observed for the dehalogenating cultures suggest that transformation of 2,6-DBP is mediated by similar mechanisms, comprising similar steps.

3.2. Carbon isotope fractionation during debromination of 2,6-DBP by sponges *in vivo*

Three intact *C. prolifera* sponges were incubated in aerated artificial seawater tanks, each amended with 500 µM 2,6-DBP and monitored for debrominating activity over two weeks. Artificial seawater tanks without a sponge were used as a control. Complete loss of 2,6-DBP accompanied with production of 2-BP and phenol with the *C. prolifera* sponges was observed within 100 to 180 hours, while the 2,6-DBP concentration remained stable in the controls. A set of 2,6-DBP samples from one of the sponge cultures and controls were analyzed for isotope composition (the data analyses failed or were inconclusive for the samples of the two other sponge sets). Over the course of debromination concomitant carbon isotope enrichment in the residual 2,6-DBP was observed from an initial $\delta^{13}C$ value of -31.1 to -29.8 ‰ with a standard deviation of 0.3 ‰ (Fig. 3). This corresponds to an isotopic enrichment factor (ε) of -5.28 ± 9.52‰, which is comparable to what was observed in the anaerobic cultures (Figs. 1 and 2). The dehalogenation products 2-BP and phenol were detected in the open tank cultures with *C. prolifera* sponges, although their enrichment factors (ε) were not calculated due to insignificant shifts, lower than the analytical uncertainty for these two compounds. There was minimal loss of 2,6,-DBP in the no-sponge water column controls, and neither dehalogenation products, nor substantial change in the $\delta^{13}C$ of 2,6-DBP were detected .

4. Discussion

Three sponge-derived anaerobic dehalogenating cultures sets showed sequential debromination of 2,6-DBP to 2BP and phenol with measurable stable carbon isotope fractionation (Figs 1 and 2). All sponge-associated cultures showed similar isotopic enrichment factors (ε) (Table 1). The values observed for the dehalogenating cultures from *Aplysina* and *Haploscleridae* sponges indicates that transformation of 2,6-DBP is mediated by similar mechanisms, comprising similar steps. CSIA provides an approach to differentiate transformation pathways (Elsner et al. 2005), such as in the dechlorination of tetracholoethane (Nijenhuis et al 2005) and degradation of 1,2-dichloroethane (Hirschorn et al. 2004), tribromoneopentyl alcohol (Kozell et al. 2015) and ethylene dibromide (Kuntze et al. 2016).

In order to compare the results obtained for 2,6-DBP with those for different organohalides it is useful to focus on the AKIE, to examine the isotope effect of the C-Br bond cleavage at the reactive site. AKIE values can reflect the similarities of dehalogenating pathways of similar microbes, by eliminating the dilution of the isotope effect of higher number of non-reactive carbon atoms and intramolecular competition (Fletcher et al. 2011; Abe et al. 2009). From the microbiological point of view, subtle differences in AKIE could reflect microbial membrane transport effects, enzyme location in the cell (membrane or cytoplasm), enzymatic activity, and as recently demonstrated, growth stages and type of membranes (Gram positive vs. Gram negative) of the microorganisms involved (Nijenhuis et al. 2005; Imfeld et al. 2008; Cichocka et al. 2007; Renpenning et al. 2015). The AKIE values (Table 1) observed for the *Aplysina* and *Haplosclerida* sponge-derived cultures (1.036) suggest that reductive debromination of 2,6-DBP by these cultures is identical. The TS7 culture, although from sponge associated sediment, shows a different AKIE (1.024). The dehalogenating population of the sediment culture (TS7) has not been identified, but the overall microbial community composition of TS7 differed from the sponge associated cultures (unpublished data), suggesting that the differences in AKIE values may be due to differences in the dehalogenating species.

The AKIE values reported for the sponge-associated cultures in this study are in agreement within the limits for the cleavage of a C−Br bond, with a theoretical AKIE= 1.042 estimated by using the semiclassical Streitwieser limit model (Kozell et al. 2015). Our data suggest fewer rate-limitations prior to the bond cleavage reaction for sponge associated cultures than for the sediment enrichment. AKIE values as high as 1.062 ± 0.005 have been reported for aliphatic brominated compounds in a nucleophilic substitution (S_N2) reaction of ethylenebromide under alkaline conditions (Kuntze et al. 2016). Similar values, 1.038 ± 0.004, were observed in the abiotic reductive dehalogenation of tribromoneopentyl alcohol by zero-valent iron nanoparticles (Kozell et al. 2015) and in the dehalogenation of ethylene dibromide by norpseudo B12 and cyano B12 enzymes by dibromoelimination (Kuntze et a. 2016). The lowest AKIE value for ethylene dibromide dehalogenation (1.0056) was reported for anaerobic microcosms (Henderson et al. 2008). Aerobic degradation of methylbromide following an S_N2 mechanism yielded AKIE values of 1.004 to 1.042 (Elsner et al. 2005; Miller et al. 2001), while values for aerobic biodegradation by pure cultures of facultative methylotrophs and soil microcosms ranged from 1.057 to 1.072 (Elsner et al. 2005). The differences in AKIE for 2,6-DBP might be explained by the intrinsic variation in the catalysis of aromatic vs. aliphatic molecules, in which halogen bonds of aliphatic molecules tend to be easier to break than aromatic ones.

Some dechlorinating organisms, such as *Dehalococcoides mccartyi* CBDB1, are capable of dehalogenating aromatic organobromides, including 1,2,4-tribromobenzene, and hepta- or lower brominated diphenyl ethers (Wagner et al. 2012; Xu et al. 2014). Recently, Sohn *et al*. (2018) measured isotope fractionation during dehalogenation of penta-, tetra- and tri-bromobenzenes by cultures comprising a *Dehalococcoides* spp. The ε value for tribromobenzene debromination was -5.5 ‰. Although measurable isotope fractionation occurred during reductive dehalogenation of highly halogenated bromobenzenes in aquatic sediments, it was minimal probably due the high insolubility of the higher brominated compounds. Bromine isotope analysis of brominated organic compounds is also being developed, and kinetic bromine isotope effects have been observed for debromination of bromophenol (Bernstein et al. 2013) and transformation of brominated ethenes (Kunze et al. 2016; Woods et al. 2018), suggesting the application of dual carbon-bromine analysis in environmental monitoring.

The *C. prolifera in vivo* experiment (Fig. 3) demonstrated that dehalogenation of 2,6-DBP occurred in the sponge with isotopic enrichment of the substrate in contrast to the control. We hypothesize that 2,6-DBP fractionation was due to activity of sponge-associated dehalogenating bacteria (although we cannot exclude activity also by the sponge animal), since the enrichment of 2,6-DBP isotope composition ($\Delta\delta^{13}C$) of aerated water control was negligible ($\Delta\delta^{13}C$ =0.2) compared to the sponge system ($\Delta\delta\ ^{13}C$= 4.5). The sponge is compartmentalized and we expect that the microbes capable of dehalogenation are active in anoxic sections of the animal. With changes in the redox state of the animal we would also expect fluctuation in dehalogenation activity.

Desulfoluna spp. are a peculiar group of dehalogenating microorganisms active in metazoans and represent a unique contrasting group from the other known dehalogenating bacteria. Much has been clarified regarding organochloride dehalogenation, but there is still much to understand about the fate of natural and anthropogenic organobromides in the marine environment. CSIA can be used to gain understanding of the mechanisms of dehalogenation by sponge-associated bacteria. These studies used 2,6-DBP as a model, but it is feasible to assess dehalogenation of other brominated aromatics with CSIA, such as TBBPA and PBDE, compounds of urgent interest because of their wide occurrence and extensive production. Future dual-element investigation of carbon and bromine may help to exclude rate limiting

effects for more precise characterization of reaction mechanisms of biodegradation of organohalides (Nijenhuis & Richnow 2016).

In conclusion, the reductive debromination of 2,6-DBP resulted in measurable carbon isotope fractionation both in sponge-derived microbial cultures and in sponges *in vivo.* The results of these experiments show that CSIA can be used to analyze microbial reductive debromination of bromophenols and for detecting the activity of sponge-associated bacteria and to estimate debromination in a sponge *in vivo*.

Author Contribution Statement

IH-G, IN and MMH conceived and designed the research. IH-G, NAL and YA conducted the experiments. HHR contributed analytical tools. IH-G, IN and MH analyzed the data. IH-G, IN and MMH wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments

We thank Dr. Matthias Gehre, Dr. Stefanie S. Weber, Ursula Gunther and the crew of the isotope lab of the Department of Isotope Biogeochemistry, Helmholtz-Centre for Environmental Research – UFZ, Leipzig, Germany for their assistance with CSIA. The work was supported in part by the USDA National Institute of Food and Agriculture Hatch project accession numbers 205270 and 1012785 through the New Jersey Agricultural Experiment Station (Hatch projects NJ00138 and NJ01160) and by the European Union under the 7th Framework Programme (project acronym CSI: ENVIRONMENT, contract number PITN-GA-2010-264329). The views expressed herein do not necessarily reflect those of the US Food and Drug Administration or the US Department of Health and Human Services.

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

References

Abe Y, Zopfi J, Hunkeler D (2009) Effect of molecule size on carbon isotope fractionation during biodegradation of chlorinated alkanes by *Xanthobacter autotrophicus* GJ10. Isotopes Environ Health Stud 45:18-26.

Ahn YB, Rhee, SK, Fennell DE, Kerkhof LJ, Hentschel U, Häggblom MM (2003) Reductive dehalogenation of brominated phenolic compounds by microorganisms associated with the marine sponge *Aplysina aerophoba*. Appl Environ Microbiol 69:4159-4166.

Ahn YB, Kerkhof LJ, Häggblom MM (2009) *Desulfoluna spongiiphila* sp. nov., a dehalogenating bacterium in the *Desulfobacteraceae* from the marine sponge *Aplysina aerophoba*. Int J System Evol Microbiol 59:2133-2139.

Arbeli Z, Ronen Z, Diaz-Baez MC (2006) Reductive dehalogenation of tetrabromobisphenol-A by sediment from a contaminated ephemeral streambed and an enrichment culture. Chemosphere 64:1472-1478.

Bernstein A, Ronen Z, Levin E, Halicz L, Gelman F (2013) Kinetic bromine isotope effect: example from the microbial debromination of brominated phenols. Anal Bioanal Chem 405:2923-2929.

Bartrons M, Grimalt JO, de Mendoza G, Catalan J (2012) Pollutant dehalogenation capability may depend on the trophic evolutionary history of the organism: PBDEs in freshwater food webs. Plos One 7:e41829.

Birnbaum LS, Staskal DF (2004) Brominated flame retardants: Cause for concern? Environ Health Perspect 112:9-17.

Boyle AW, Phelps CD, Young LY (1999) Isolation from estuarine sediments of a *Desulfovibrio* strain which can grow on lactate coupled to the reductive dehalogenation of 2,4,6-tribromophenol. Appl Environ Microbiol 65:1133-1140.

Bowden BF, Towerzey L, Junk PC (2000) A new brominated diphenyl ether from the marine sponge *Dysidea herbacea*, Aust J Chem 53:299-301.

Bombach P, Richnow HH, Kastner M, Fischer A (2010) Current approaches for the assessment of *in situ* biodegradation. Appl Microbiol Biotechnol 86:839-852.

Braeckevelt M, Rokadia H, Imfeld G, Stelzer N, Paschke H, Kuschk P. Kaestner M, Richnow HH, Weber S (2007) Assessment of in situ biodegradation of monochlorobenzene in contaminated groundwater treated in a constructed wetland, Environ Pollut 148:428-437.

Cichocka D, Siegert M, Imfeld G, Andert J, Beck K, Diekert G, Richnow HH, Nijenhuis I (2007) Factors controlling the carbon isotope fractionation of tetra- and trichloroethene during

reductive dechlorination by *Sulfurospirillum ssp* and *Desulfitobacterium sp* strain PCE-S, FEMS Microbiol Ecol 62:98-107.

Cichocka D, Imfeld G, Richnow HH, Nijenhuis I (2008) Variability in microbial carbon isotope fractionation of tetra- and trichloroethene upon reductive dechlorination. Chemosphere 71:639-648.

Coplen TB (2011) Guidelines and recommended terms for expression of stable - isotope-ratio and gas-ratio measurement results. Rapid Commun Mass Spectrom 25:2538–2560.

de Wit CA (2002) An overview of brominated flame retardants in the environment, *Chemosphere* 46:583-624.

de Wit CA, Herzke D, Vorkamp K (2010) Brominated flame retardants in the Arctic environment - trends and new candidates. Sci Total Environ 408:2885-2918.

Elsner M, Zwank L, Hunkeler D, Schwarzenbach RP (2005) A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. Environ Sci Technol 39:6896-6916.

Elsner M (2010) Stable isotope fractionation to investigate natural transformation mechanisms of organic contaminants: principles, prospects and limitations, J Environ Monit 12:2005-2031.

Ewald EM, Wagner A, Nijenhuis I, Richnow HH, Lechner U (2007) Microbial dehalogenation of trichlorinated dibenzo-*p*-dioxins by a *Dehalococcoides*-containing mixed culture is coupled to carbon isotope fractionation. Environ Sci Technol 41:7744-7751.

Fennell DE, Rhee SK, Ahn YB, Häggblom MM, Kerkhof LJ (2004) Detection and characterization of a dehalogenating microorganism by terminal restriction fragment length polymorphism fingerprinting of 16S rRNA in a sulfidogenic, 2-bromophenol-utilizing enrichment. Appl Environ Microbiol 70:1169-1175.

Fernie KJ, Letcher RJ (2010) Historical contaminants, flame retardants, and halogenated phenolic compounds in peregrine falcon (*Falco peregrinus*) nestlings in the Canadian Great Lakes Basin. Environ Sci Technol 44:3520-3526.

Fletcher KE, Nijenhuis I, Richnow HH, Löffler FE (2011) Stable carbon isotope enrichment factors for cis-1,2-dichloroethene and vinyl chloride reductive dechlorination by *Dehalococcoides*. Environ Sci Technol 45:2951-2957.

Gribble GW (2003) The diversity of naturally produced organohalogens, Chemosphere 52:289-297.

Gribble GW (2010) Naturally Occurring Organohalogen Compounds - A Comprehensive Update, 1 ed. Springer-Verlag Wien, doi:10.1007/978-3-211-99323-1.

Hamers T, Kamstra JH, Sonneveld E. Murk AJ, Kester MHA, Andersson PL, Legler J, Brouwer A (2006) In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol Sci 92:157-173.

Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Visser TJ, Van Velzen MJM, Brouwer A, Bergman A (2008) Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2,2 ',4,4 '-tetrabromodiphenyl ether (BDE-47). Mol Nutr Food Res 52:284-298.

Henderson JK, Freedman DL, Falta RW, Kuder T, Wilson JT (2008) Anaerobic biodegradation of ethylene dibromide and 1,2-dichloroethane in the presence of fuel hydrocarbons. Environ Sci Technol 42:864−870.

Hentschel U, Usher KM, Taylor MW (2006) Marine sponges as microbial fermenters, FEMS Microbiol Ecol 55:167-77.

Hentschel U, Piel J, Degnan SM, Taylor MW (2012) Genomic insights into the marine sponge microbiome. Nat Rev Microbiol 10:641-675.

Hirschorn SK, Dinglasan SK, Elsner MJ, Mancini SA, Lacrampe-Couloume G, Edwards EA, Sherwood-Lollar B (2004) Pathway dependent isotopic fractionation during aerobic biodegradation of 1,2-dichloroethane. Environ Sci Technol 38:4775-4781.

Hunkeler D, Aravena R, Berry-Spark K, Cox E (2005) Assessment of degradation pathways in an aquifer with mixed chlorinated hydrocarbon contamination using stable isotope analysis. Environ Sci Technol 39:5975-5981.

Imfeld G, Nijenhuis I, Nikolausz M, Zeiger S, Paschke H, Drangmeister J, Grossmann J, Richnow HH, Weber S (2008) Assessment of in situ degradation of chlorinated ethenes and bacterial community structure in a complex contaminated groundwater system. Water Res. 42:871-882.

Kozell AY, Yecheskel N, Balaban I, Dror L, Halicz L, Gelman F (2015) Application of dual carbon-bromine isotope analysis for investigating abiotic transformations of tribromoneopentyl alcohol (TBNPA). Environ Sci Technol 49:4433-4440.

Krzmarzick MJ, Crary BB, Harding JJ, Oyerinde OO, Leri AC, Myneni, SCB, Novak PJ (2012) Natural niche for organohalide-respiring Chloroflexi. Appl Environ Microbiol 78:393-401.

Kuntze K, Kozell AY, Richnow HH, Halicz L, Nijenhuis I, Gelman F (2016) Dual carbon–bromine stable isotope analysis allows distinguishing transformation pathways of ethylene dibromide. Environ Sci Technol 50:9855-9863.

Law RJ, Allchin CR, de Boer J, Covaci A, Herzke D, Lepom P, Morris S, Tronczynski J, de Wit CA (2006) Levels and trends of brominated flame retardants in the European environment.

Chemosphere 64:187-208.

Leri AC, Hakala JA, Marcus MA, Lanzirotti A, Reddy CM, Myneni SCB (2010) Natural organobromine in marine sediments: New evidence of biogeochemical Br cycling. Global Biochem. Cycles 24: GB4017.

Liu F, Cichocka D, Nijenhuis I, Richnow HH, Fennell DE (2010) Carbon isotope fractionation during dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin by a *Dehalococcoides*-containing culture. Chemosphere 80:1113-1119.

Liu J, Lopez N, Ahn Y, Goldberg T, Bromberg Y, Kerkhof LJ, Häggblom MM (2017) Novel reductive dehalogenases from the marine sponge associated bacterium *Desulfoluna spongiiphila*. Environ Microbiol Rep 9:537-549.

Liu J, Häggblom MM (2018) Genome guided identification of organohalide-respiring Deltaproteobacteria from the marine environment. mBio 9:e02471-18.

Liu J, Adrian L, Häggblom MM (2020) Transcriptomic and proteomic response of the organohalide respiring bacterium *Desulfoluna spongiiphila* to growth with 2,6-dibromophenol as electron acceptor. Appl Environ Microbiol 86:e02146-19.

Meckenstock RU, Morasch B, Warthmann R, Schink B, Annweiler E, Michaelis W, Richnow HH (1999) $^{13}C/^{12}C$ isotope fractionation of aromatic hydrocarbons during microbial degradation. Environ Microbiol 1:409–414.

Meckenstock RU, Morasch B, Griebler C, Richnow HH (2004) Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. J Contam Hydrol *75*:215-255

Merritt DA, Brand WA, Hayes JM (1994) Isotope-ratio-monitoring gas-chromatography mass-spectrometry methods for isotopic calibration. Org Geochem 21:573-583.

Miller LG, Kalin RM, McCauley SE, Hamilton JTG, Harper DB, Millet DB, Oremland RS, Goldstein AH (2001) Large carbon isotope fractionation associated with oxidation of methyl halides by methylotrophic bacteria. Proc Natl Acad Sci USA. 98:5833−5837.

Monserrate E, Häggblom MM (1997) Dehalogenation and biodegradation of brominated phenols and benzoic acids under iron-reducing, sulfidogenic, and methanogenic conditions. Appl Environ Microbiol 63:3911-3915.

Nijenhuis I, Andert J, Beck K, Kastner M, Diekert G, Richnow HH (2005) Stable isotope fractionation of tetrachloroethene during reductive dechlorination by *Sulfurospirillum multivorans* and *Desulfitobacterium sp* strain PCE-S and abiotic reactions with cyanocobalamin. Appl Environ Microbiol 71:3413-3419

Nijenhuis I, Richnow HH (2016) Stable isotope fractionation concepts for characterizing

biotransformation of organohalides. Curr Opin Biotechnol 41:108-113

Nijenhuis I, Renpenning J, Kümmel S, Richnow HH, Gehre M (2016) Recent advances in multi-element compound-specific stable isotope analysis of organohalides: Achievements, challenges and prospects for assessing environmental sources and transformation. Tr Env Anal Chem 11:1-8.

Nikolausz M, Nijenhuis I, Ziller K, Richnow HH, Kästner M (2006) Stable carbon isotope fractionation during degradation of dichloromethane by methylotrophic bacteria. Environ Microbiol 8:156-164.

Renpenning J, Rapp I, Nijenhuis I (2015) Substrate hydrophobicity and cell composition influence the extent of rate limitation and masking of isotope fractionation during microbial reductive dehalogenation of chlorinated ethenes. Environ Sci Technol 49:4293-4301.

Renpenning J, Nijenhuis I (2016) Evaluation of the microbial reductive dehalogenation reaction using compound-specific stable isotope analysis (CSIA). p 429- 453, In: Adrian L, Löffler F. (eds) Organohalide-Respiring Bacteria. Springer, Berlin, Heidelberg.

Richnow HH, Annweiler E, Michaelis W, Meckenstock RU (2003) Microbial *in situ* degradation of aromatic hydrocarbons in a contaminated aquifer monitored by carbon isotope fractionation. J Contam Hydrol 65:101-120.

Peng P, Goris T, Lu Y, Nijsse B, Burrichter A, Schleheck D, Koehorst J, Liu J, Detmer Sipkema D, Sinninghe Damste JS, Stams AJM, Häggblom MM, Smidt H, Atashgahi S (2020) Organohalide-respiring Desulfoluna species isolated from marine environments. ISME Journal 14:815–827.

Schmidt TC, Zwank L, Elsner M, Berg M, Meckenstock RU, Haderlein SB (2004) Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges, Anal Bioanal Chem 378:283-300.

Sherwood-Lollar B, Slater GF, Ahad J, Sleep B, Spivack J, Brennan M, MacKenzie P (1999) Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: Implications for intrinsic bioremediation. Org Geochem 30:813-820.

Sohn SY, Kuntze K, Nijenhuis I, Häggblom MM (2018) Evaluation of carbon isotope fractionation during dehalogenation of chlorinated and brominated benzenes. Chemosphere 193:785-792.

Somsamak P, Richnow HH, Häggblom MM (2005) Carbon isotopic fractionation during anaerobic biotransformation of methyl tert-butyl ether and tert-amyl methyl ether. Environ Sci Technol 39:103-109.

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: Evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 71:295-347.

Taylor MW, Hill RT, Piel J, Thacker RW, Hentschel U (2007) Soaking it up: the complex lives of marine sponges and their microbial associates. ISME Journal 1:187-190.

Thacker RW, Freeman CJ (2012) Sponge-microbe symbioses: recent advances and new directions. Adv Mar Biol 62:57–111.

Utkina NK, Denisenko VA, Scholokova OV, Virovaya MV, Gerasimenko AV, Popov DY, Krasokhin VB, Popov AM (2001) Spongiadioxins A and B, two new polybrominated dibenzo-*p*-dioxins from an Australian marine sponge *Dysidea dendyi*. J Nat Prod 64:151-153.

Wagner A, Cooper M, Ferdi S, Seifert J, Adrian L (2012) Growth of *Dehalococcoides mccartyi* strain CBDB1 by reductive dehalogenation of brominated benzenes to benzene. Environ Sci Technol 46:8960-8968.

Watson J, Matsui GY, Leaphart A, Wiegel J, Rainey FA, Lovell CR (2000) Reductively debrominating strains of *Propionigenium maris* from burrows of bromophenol-producing marine infauna. Int J Syst Evol Microbiol 50:1035-1042.

Webster NS, Taylor MW (2012) Marine sponges and their microbial symbionts: love and other relationships, Environ Microbiol 14:335-346.

Woods A, Kuntze K, Gelman F, Halicz L, Nijenhuis I (2018) Variable dual carbon-bromine stable isotope fractionation during enzyme-catalyzed reductive dehalogenation of brominated ethenes. Chemosphere 190:211-217.

Xu G, Wang J, Lu M (2014) Complete debromination of decabromodiphenyl ether using the integration of *Dehalococcoides* sp. strain CBDB1 and zero-valent iron. Chemosphere 117:455-461.

Zanaroli G, Negroni A, Häggblom, MM, Fava F (2015) Microbial dehalogenation of organohalides in marine and estuarine environments. Curr Opin Biotechnol 33:287–295.

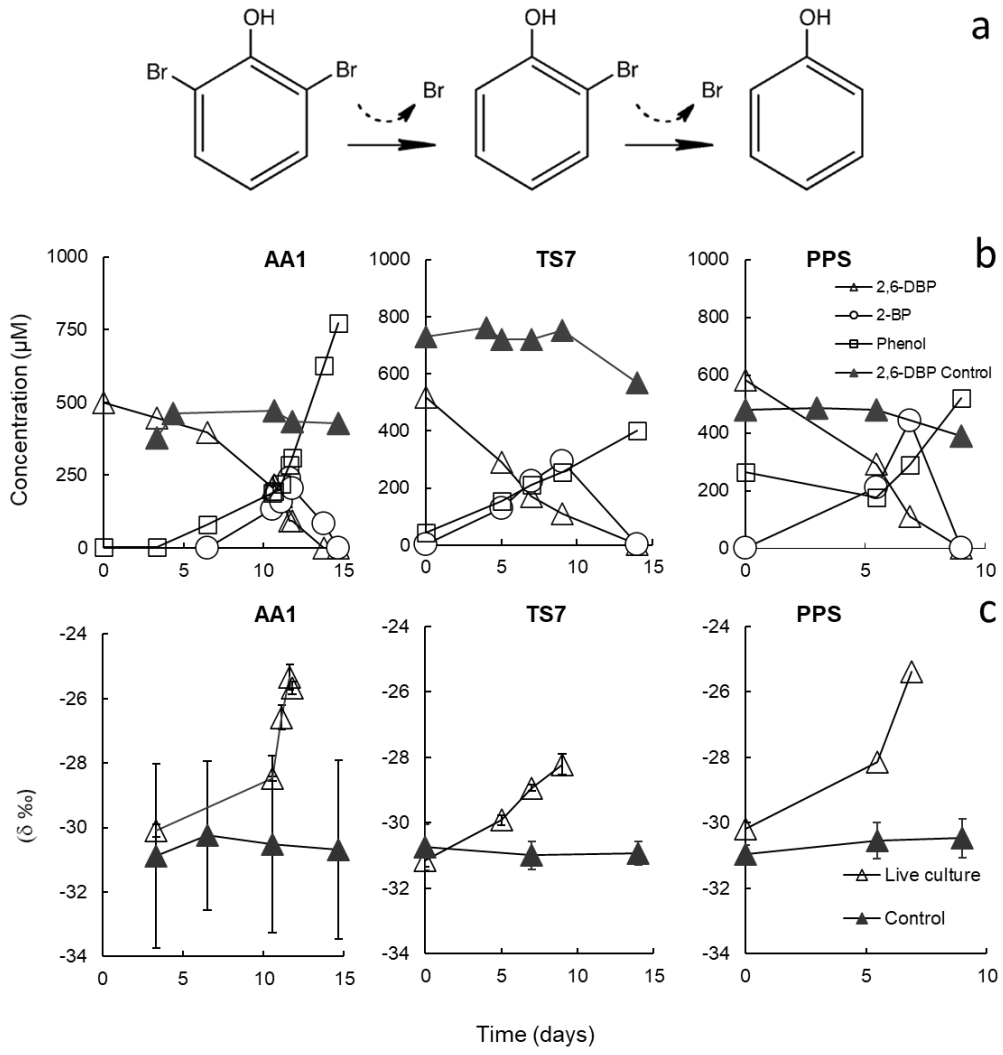


FIGURE 1. Sequential dehalogenation (a) of 2,6-DBP via 2-BP with accumulation of phenol as the final product (b) and $\delta^{13}C$ values (c) obtained for residual 2,6-DBP over time. Data shown (left to right) for dehalogenation by culture of *D. spongiiphila* AA1, sponge enrichment culture PPS, and sediment culture TS7.

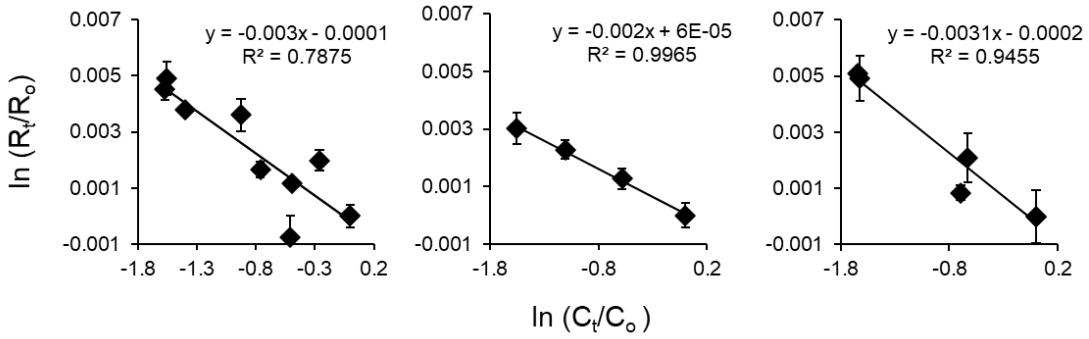


FIGURE 2. Relationship between isotope composition and concentration of 2,6-DBP for *D. spongiiphila* AA1, sponge enrichment culture PPS, and sediment culture TS7. The correlation of change in stable isotope composition to change in concentration was used to calculate the enrichment factor (ε).

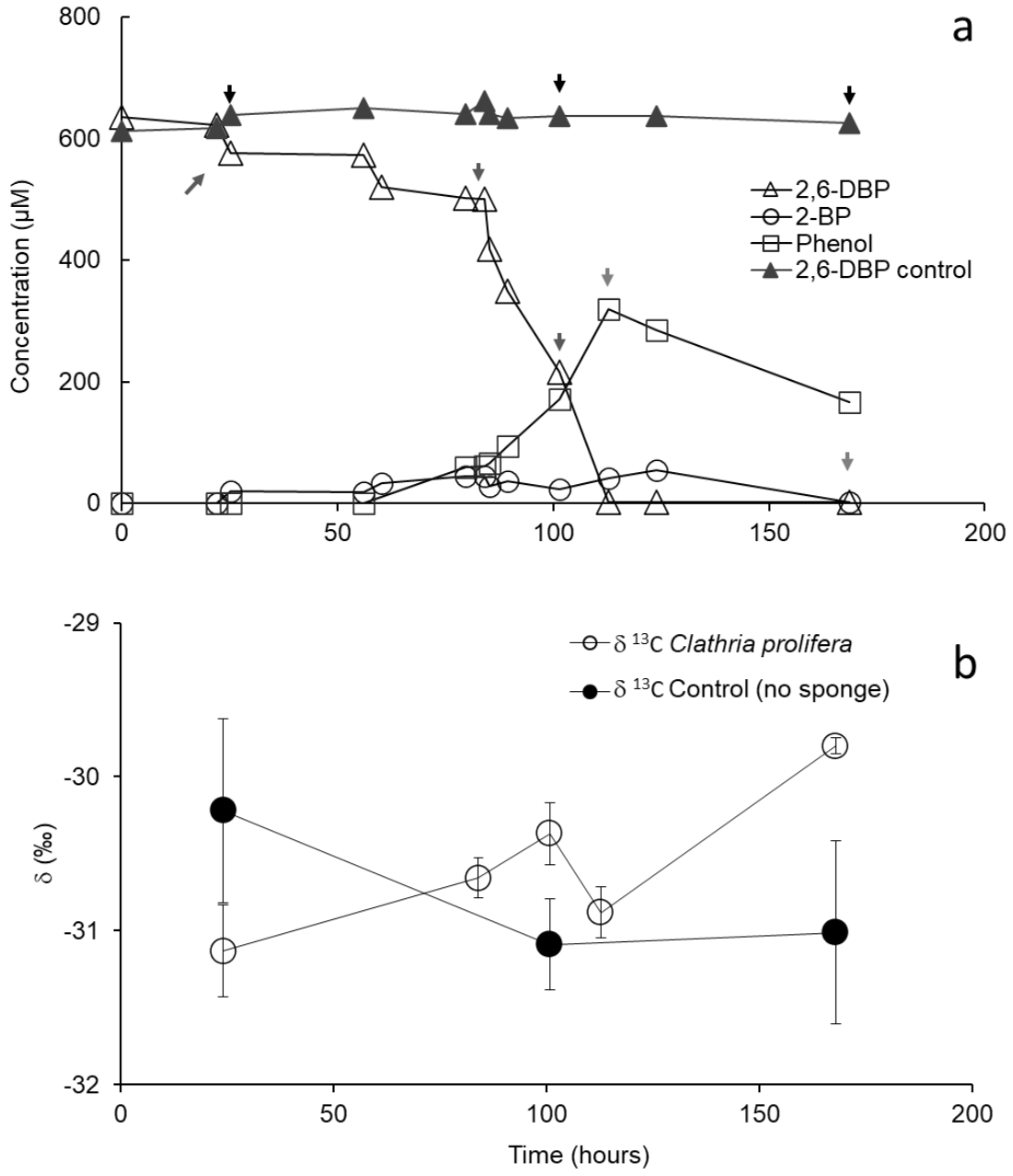


FIGURE 3. Concentration of 2,6-DBP and its debromination products and $\delta^{13}C$ composition of 2,6-DBP during incubation with the sponge *Clathria prolifera* compared to a no-sponge control. Data shown for one sponge animal.

TABLE 1. The isotopic enrichment factor (ε) with 95% confidence intervals and the apparent kinetic isotope effect ($\varepsilon_{reactive}$) for debromination of 2,6-DBP by sponge-associated cultures AA1, PPs and TS7

Culture	E	AKIE ‰ ($\varepsilon_{reactive}$)
AA1	-3.1 ± 1.5, R^2=0.93	1.036 ± 0.010 R^2=0.78
PPS	-3.0 ± 0.5, R^2= 0.99	1.036 ± 0.015 R^2=0.92
TS7	-2.0 ± 0.3 R^2=0.934	1.024 ± 0.0017 R^2=0.99

Supplementary Information

Reductive Debromination by Sponge-Associated Anaerobic Bacteria is Coupled to Carbon Isotope Fractionation

Isabel Horna-Gray [a], Nora A. Lopez [a], Ivonne Nijenhuis [b], Youngbeom Ahn [a,c], Hans H. Richnow [b] and Max. M. Häggblom [a]

Method optimization for isotope composition analysis

A mixture of 2,6-DBP, 2-BP, phenol, 4-chlorophenol and 2,4-dichlorophenol (the last 2 being used as internal standards) dissolved in water was used to validate the method for sample preparation by either solid phase or solvent extraction. The initial experiments allowed us to determine: 1) the best non-fractionating sample preparation method 2) the appropriate sample concentration for CSIA according to the detection limits of the equipment; and 3) the use dichloromethane (DCM) for solvent extractions.

The carbon isotope composition of the phenolic compounds was measured with a GC-C-IRMS (gas chromatography combustion isotope-ratio-monitoring mass spectrometry) system (FINNIGAN MAT, Bremen, Germany). The system consisted of a GC unit connected to a FINNIGAN MAT combustion unit with a water removal assembly coupled to a FINNIGAN MAT 252 mass spectrometer as described in Materials and Methods. Bromophenols extracted in DCM were injected into a gas chromatograph (HP 6890, Agilent, Palo Alto, CA) using a split/splitless injector held at 280 °C. The split was adjusted to obtain suitable peak areas for reproducible determination of the $^{13}CO_2/^{12}CO_2$ mass ratio. Bromophenols in the GC effluent stream were oxidized to CO_2 (at 980 °C) in the combustion interface and were transferred to a mass spectrometer to determine $^{13}CO_2/^{12}CO_2$ ratios. At least 3 replicate measurements were made for each sample. For chromatographic separation, a ZB-5 capillary column (60 m × 0.32 mm, 0.5 µm film thickness; Phenomenex Inc., Torrance, CA) with the following oven temperature program was used: 2 min at 70 °C; 10 °C/min to 200 °C; 20 °C/min to 300 °C; and hold for 2 min with Helium as the gas carrier.

A mixture of substituted phenols (2-chlorophenol, 2-methylphenol and 2,4-dichlorophenol) in addition to 2,6-DBP, 2-BP and phenol were tested to establish the ideal internal standards for our samples, the detection limits and the linearity (i.e., isotope ratio obtained is independent of the concentration of the analyte) of the IRMS. The GC-IRMS detection limits for 2,6-DBP, 4-CP, 2-BP and phenol were 300, 700, 400 and 900 µM, respectively. In isotope analysis stability rather than sensitivity is sought. Therefore, the minimum concentration of the compound was used were the accuracy and reproducibility of the stable isotope composition ($\delta^{13}C$) measurements for 2,6-DBP, 2-BP and phenol were within acceptable limits (0.5 ‰ of standard deviation). These conditions were met for all of the phenolic compounds in the range of 500 to 1200 µM, except for 2,6 DBP, for which the upper limit was 500 µM.

The detection limit for bromophenols in CSIA was between 300 – 900 µM. The initial amount of bromophenols inoculated with the cultures was 200 µM 2,6-DBP. Furthermore, the isotope fractionation is ideally studied between 90% to 10% of degradation, requiring that samples had to be concentrated from 20 µM to at least 300 µM without changes to the original isotope

composition. The detection limit for phenol (the product of dehalogenation) was 900 µM, requiring a further concentration.

Extraction into DCM was found to be the most suitable sample concentration method for CSIA. The samples (3-50 mL) were centrifuged for 20 minutes at 14000 x g and the supernatant was separated and acidified with 20 µL concentrated HCl. After acidification, 0.5 mL of DCM was added and mixed thoroughly by vortex. After a minimum of 10 minutes settling the DCM fraction was collected with a Pasteur pipette and transferred into GC vials.

The concentration interval was determined in which the isotope value was not affected by the amount of target compound injected, defined as the linear range in this manuscript. To determine the linear range of GC-C-IRMS analysis of the halophenols the amplitude and isotope composition ($\delta\ ^{13}C/^{12}C$) values were measured at least in triplicate for each compound at concentrations from 500 to 2500 µM (Fig. S1).

The linearity range of 4 chlorophenol and 2 bromophenol was between 2000 and 8000 mV amplitude while carbon isotope values of phenol and 2,6 bromophenol were stable at >4000 mV amplitude.

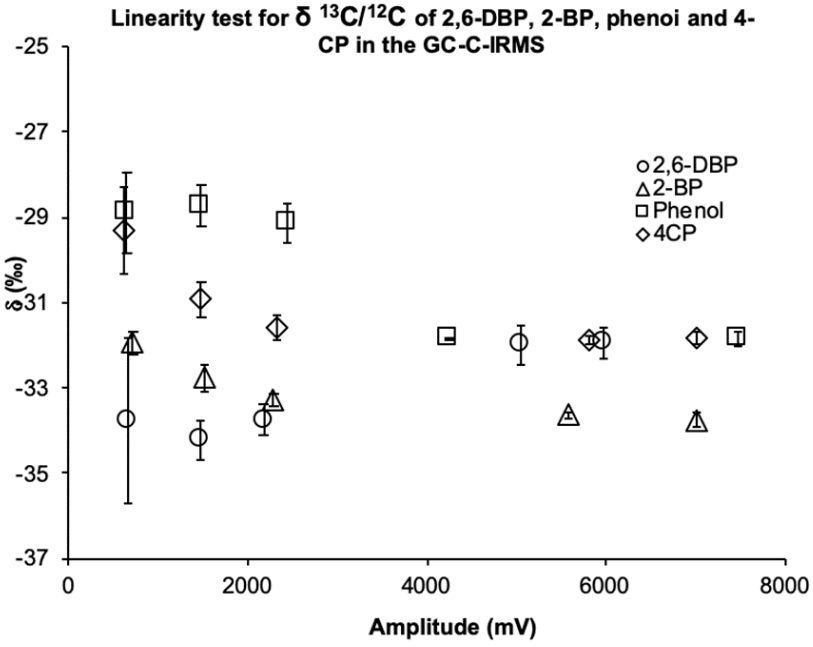


Figure S1: $\delta\ ^{13}C/^{12}C$ values versus amplitude obtained from a mixture of standards of halophenols at different concentrations showing the linearity of the compounds in GC-C-IRMS.