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Effect of molecule size on carbon isotope fractionation during biodegradation of chlorinated alkanes by *Xanthobacter autotrophicus* GJ10

Yumiko Abe[a], Jakob Zopfi[b] and Daniel Hunkeler[a]*

[a] *Centre for Hydrogeology;* [b] *Laboratory for Microbiology, University of Neuchâtel, Rue Emile Argand 11, 2009, Neuchâtel, Switzerland*

The effect of the number of carbon and chlorine atoms on carbon isotope fractionation during dechlorination of chlorinated alkanes by *Xanthobacter autotrophicus* GJ10 was studied using pure culture and cell-free extract experiments. The magnitude of carbon isotope fractionation decreased with increasing carbon number. The decrease can be explained by an increasing probability that the heavy isotope is located at a non-reacting position for increasing molecule size. The isotope data were corrected for the number of carbons as well as the number of reactive sites to obtain reacting-site-specific values denoted as apparent kinetic isotope effect (AKIE). Even after the correction, the obtained AKIE values varied (on average 1.0608, 1.0477, 1.0616, and 1.0555 for 1,2-dichloroethane, chloropentane, 1,3-dichloropentane and chlorobutane, respectively). Cell-free extract experiments were carried out to evaluate the effect of transport across the cell membrane on the observed variability in the AKIE values, which revealed that variability still persisted. The study demonstrates that even after differences related to the carbon number and structure of the molecule are taken into account, there still remain differences in AKIE values even for compounds that are degraded by the same pure culture and an identical reaction mechanism.

Keywords: bacterial cultures; carbon-13; chlorinated alkanes; degradation; enzymes; kinetic isotope effect; molecule size

1. Introduction

Stable isotope fractionation analysis is used increasingly to assess *in situ* biodegradation of organic compounds in contaminated subsurface environments [1–4]. The quantitative evaluation of isotope data requires reliable values for isotope fractionation factors. In a number of laboratory studies, isotope fractionation factors for the degradation of different organic compounds such as chlorinated ethenes [1,5–9], methyl tert-butyl ether [10–12] and monoaromatic hydrocarbons [10,13–18] were determined. The concentration and isotope data are usually evaluated using the Rayleigh equation [19]. The resulting isotope fractionation factors are denoted as *bulk* isotope fractionation factors since they are based on the average isotope ratio of the whole molecule.

*Corresponding author. Email: daniel.hunkeler@unine.ch

In enzyme studies, the observed isotope fractionation is the result of a *kinetic isotope effect* during the first irreversible transformation step [20]. The isotope effect associated with this step is usually referred to as *intrinsic isotope effect* [20] and reflects the difference in reaction rate for molecules with light and heavy isotopes, respectively, at the reacting site. In general, the observed bulk isotope effect is smaller than the intrinsic isotope effect for several reasons. First, the heavy isotopes may not be located at the reacting position; thus, molecules with a heavy isotope may react at the same rate as molecules without a heavy isotope [21]. Second, if the catalytic step is fast compared with the preceding binding steps, the intrinsic isotope effect will be partly masked [20]. The relative velocity of reactive to binding steps is often expressed as the *commitment to catalysis* which corresponds to the ratio between the rate of the catalytic step to the rate of substrate–enzyme dissociation step [20]. The larger the commitment to catalysis, the smaller is the observed isotope fractionation. Third, as in the binding steps on the enzymatic level, the rate-limiting transport of a substrate across the cell membrane can lower the observed isotope effect. Such an effect was previously observed for reductive dechlorination of tetrachloroethene (PCE) to trichloroethene (TCE), but not for the transformation of TCE to *cis*-dichloroethene [22,23]. Finally for a given reaction mechanism acting on different substrates, the intrinsic isotope effect may vary due to differences in the structure of the transition state [24], leading to variable degrees of observed isotope fractionation even if all other factors are identical.

The main objective of this study was to evaluate experimentally how bulk isotope fractionation factors are related to the carbon number and molecular structure (number of reactive sites). It is expected that an increase in the carbon number influences the bulk isotope fractionation primarily in two ways: by lowering the probability that the heavy isotope is at the reacting position, and possibly by slowing down the transfer through the cell membrane to the enzyme due to its increased bulkiness. Previous studies investigated the effect of carbon number on isotope fractionation during biodegradation of aromatic compounds by different microorganisms that all make use of the same initial transformation reaction [17]. Even after correction for carbon numbers, the isotope effect expressed in terms of isotope enrichment factors varied by a factor of 9, which may be due to differences in the properties of the enzyme of different organisms. Similarly, in a study on aerobic alkane degradation in alluvial sand with an indigenous microbial population [25], the apparent kinetic isotope effect (AKIE) was smaller when the molecule size was larger. In contrast to these studies that made use of different pure cultures or an indigenous microbial population, we investigated the variation of isotope fractionation of different compounds degraded by the same pure culture. Haloalkane degradation by *Xanthobacter autothrophicus* GJ10, which had been characterised in detail in previous studies [26–31], was chosen as a model system for two reasons. First, transformation of haloalkane by *X. autotrophicus* GJ10 is associated with a substantial carbon isotope effect as shown previously for the degradation of 1,2-dichloroethane (1,2-DCA) [32]. Thus, even if the isotope effect decreases with increasing carbon chain length, it should still be possible to measure the perhaps subtle difference in the isotope effect with a relatively high precision. Second, the haloalkane dehalogenase responsible for the initial transformation step is known to degrade haloalkanes of variable size with one or two reactive positions by hydrolytic dehalogenation using an identical S_N2 mechanism (Figure 1) [27,28].

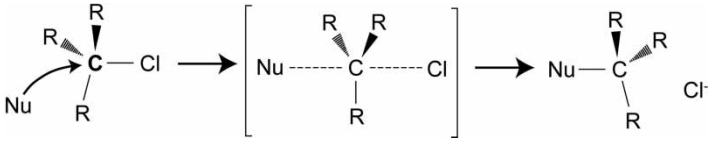

Figure 1. Reaction mechanism of the initial S_N2 nucleophilic substitution of chlorine whereby a carboxylate oxygen of the haloalkane dehalogenase acts as a nucleophile.

The studies involved experiments with the pure culture and corresponding cell-free extracts using chlorinated ethanes with two to four carbon atoms. Bulk isotope fractionation factors were determined using the classical Rayleigh equation. To gain additional insight into the origin of observed variations, the obtained isotope data were corrected for the dilution due to non-reactive positions and multiple reactive sites to obtain the bond-specific AKIE using a procedure recently proposed by Elsner *et al.* [21]. The results of pure culture and cell-free extract studies were compared to evaluate the influence of transport across the cell membrane on the observed variation in isotope effects.

2. Materials and methods

2.1. *Bacterial growth conditions*

X. autotrophicus GJ10 was obtained from D.B. Janssen (Department of Biochemistry, University of Groningen, The Netherlands). The growth medium was prepared as described by Hunkeler and Aravena [32]. Cultures were grown in 250-ml glass bottles, which contained 180 ml of medium and were capped with Mininert-Valves (Vici Precision Sampling, LA, USA). Growth was monitored by measuring the optical density at 570 nm.

2.2. *Culture experiments*

Four haloalkanes – 1,2-DCA ($\geq$99.5 % purity, $\delta^{13}C_0 = -28.50 \pm 0.12$ ‰, with uncertainty corresponding to the standard deviation of five replicate measurements), 1-chloropropane (CP) ($\geq$99.5 % purity, $\delta^{13}C_0 = -33.70 \pm 0.15$ ‰), 1,3-dichloropropane (1,3-DCP) ($\geq$98 % purity, $\delta^{13}C_0 = -19.36 \pm 0.18$ ‰) and 1-chlorobutane (CB) ($\geq$99.5 % purity, $\delta^{13}C_0 = -26.80 \pm 0.14$ ‰) – were obtained from Fluka, Switzerland, and used for degradation experiments. Initial isotope ratios were determined after dissolution of the compounds in deionised water as described in *Analytical methods*. Cultures were incubated at 24 °C under continuous shaking, and they were kept upside down to prevent leakage of gas phase through the valve. Before starting the experiments, a 5-ml aliquot of exponentially growing culture was transferred three times to 175 ml of the fresh medium containing one of the substrate haloalkanes at an aqueous phase concentration of 0.5 mM. Each culture was spiked three times with one of the haloalkanes at a concentration of 0.5 mM before each transfer. All experiments were carried out in triplicate for each haloalkane. For concentration and $\delta^{13}C$ analyses of substrates, aqueous samples were taken and preserved with NaN_3 (final concentration of 1 % weight to weight). On average, concentrations and isotope ratios were monitored until the concentration had decreased by more than three orders of magnitude.

2.3. *Preparation of cell-free extracts*

Cells of 1 L of exponentially growing *X. autotrophicus* GJ 10 culture were harvested by centrifugation (10 min at 10,000 $\times$ g), washed once with 10 mM Tris sulphate buffer (pH 7.5), centrifuged again and resuspended in the same buffer as described by Janssen *et al.* [28]. Ultrasonic disruption (Branson Sonifier 450) of the cells was performed in a pulsed manner of 30 s duration (1 pulse per second) in an ice-water bath until the cell lysis was visually confirmed by microscopy. Cell-free extracts were finally obtained after centrifugation at 45,000 $\times$ g for 30 min in the supernatant phase. Protein content of cell-free extracts was determined by the micro-biuret method according to Goa [33].

2.4. *Cell-free extract experiments*

The cell-free extract was added at a final protein concentration of 0.3 mg/ml to 50 mM Tris sulphate buffer containing 0.5 mM of substrate in a 120-ml glass bottle with a Teflon cap. The experiments were performed at 24 °C in bottles without headspace and under constant magnetic stirring. Immediately after the cell-free extracts were introduced into the buffer, samples were taken by a sterile syringe and analysed for the initial concentration and $\delta^{13}C_0$ as described in what follows. During the experiments, samples for concentrations and $\delta^{13}C$ were taken and measured immediately.

2.5. *Analytical methods*

Aqueous concentrations and carbon isotope ratios of the remaining substrate in the liquid phase were measured using a Thermo Finnigan Trace gas chromatograph (GC) coupled to a Thermo Finnigan Delta Plus XP isotope-ratio mass spectrometer (IRMS) *via* a combustion interface set to 940 °C (Thermo Finnigan, Bremen, Germany). The GC was equipped with a DB-5 column (30 m × 0.32 mm) and a Tekmar Velocity Purge and Trap system (Tekmar Dohrmann). Samples from both pure culture and cell-free extract studies were diluted with deionised water to a final volume of 20 ml. Samples were purged for 10 min and the compounds were retained on a VOCARB trap at room temperature. After the 3-min desorption from the trap at 245 °C, the compounds were condensed in a cryogenic unit at −100 °C and injected splitless into the GC-C-IRMS system. Concentrations were quantified on the basis of the mass 44 peak area by comparison with the peak areas of four external standards. The standard uncertainty of the concentration measurement was estimated to be 7 %. Carbon isotope ratios are reported in the δ-notation relative to the VPDB standard. The δ-notation is defined by

$$\delta^{13}C = \left(\frac{R}{R_{std}} - 1\right) \times 1000‰, \tag{1}$$

where R and R_{std} are the carbon isotope ratios of the sample and VPDB standard, respectively.

2.6. *Calculations*

Isotope fractionation during the biodegradation of haloalkanes was quantified using the classical Rayleigh equation to yield bulk isotope enrichment factors [19,34] as follows:

$$\ln\left(\frac{R}{R_0}\right)_{bulk} = \ln\frac{(1000 + \delta^{13}C)}{(1000 + \delta^{13}C_0)} = \frac{\varepsilon_{bulk}}{1000} \times \ln f, \tag{2}$$

where R is the measured isotope ratio ($^{13}C/^{12}C$), R_0 the initial isotope ratio, $\delta^{13}C$ the measured isotope ratio, $\delta^{13}C_0$ the initial isotope ratio, ε_{bulk} the bulk isotope enrichment factor for the whole molecule and $f = C/C_0$ the fraction of substrate remaining. The isotope enrichment factors were quantified by least square linear regression according to Equation (2) without forcing the regression through the origin [35]. The bulk isotope enrichment factor is related to the bulk isotope fractionation factor (ε_{bulk}) by

$$\varepsilon_{bulk} = (\alpha_{bulk} - 1) \times 1000. \tag{3}$$

The AKIE was calculated using the following two equations [21]:

$$\ln\left(\frac{R}{R_0}\right)_{\mathrm{rp}} = \ln\frac{[1000+\delta^{13}\mathrm{C}_0+(n/x)(\Delta\delta^{13}\mathrm{C})]}{(1000+\delta^{13}\mathrm{C}_0)} = \frac{\varepsilon_{\mathrm{rp}}}{1000}\times\ln f, \tag{4}$$

$$\mathrm{AKIE} = \left(\frac{{}^{\mathrm{L}}k}{{}^{\mathrm{H}}k}\right)_{\mathrm{app}} = \frac{1}{z\times\varepsilon_{\mathrm{rp}}/1000+1}, \tag{5}$$

where n is the number of carbons per molecule, x the number of reacting sites per molecule, $\Delta\delta^{13}$C the difference between the actual δ^{13}C and $\delta^{13}\mathrm{C}_0$, $\varepsilon_{\mathrm{rp}}$ the isotopic enrichment factor for a specific reacting site, α_{rp} the corresponding isotope fractionation factor, z the number of identically reacting site undergoing intramolecular competition, ${}^{\mathrm{L}}k$ and ${}^{\mathrm{H}}k$ are the reaction rate constants for light and heavy isotopes in the reacting bond, respectively. Equation (4) corrects for the presence of heavy isotopes at the non-reacting position, while Equation (5) accounts for multiple reacting sites as present in chlorinated alkanes with two chlorine atoms.

3. Results and discussion

3.1. *Pure culture studies*

In the pure culture studies, *X. autotrophicus* GJ10 degraded 1,2-DCA, 1,3-DCP, CP and CB as expected on the basis of previous studies [28]. The δ^{13}C value of 1,2-DCA shifted from −28‰ to 250‰. The other haloalkanes demonstrated somewhat smaller but significant increases in δ^{13}C (final δ^{13}C values for 1,3-DCP 170‰, CP 120‰ and CB 125‰). Using the Rayleigh equation (Equation (2)), the bulk isotope fractionation factors were calculated. Figure 2 illustrates representative experimental results for one replicate experiment of each compound. The reported variance among triplicate experiments was calculated on the basis of the variance of regression from each experiment according to the method proposed by Scott *et al.* [35]. The inverse value of α_{bulk} is reported (Table 1) in order to facilitate the comparison with AKIE values (see what follows). In general, the magnitude of isotope fractionation decreased with increasing number of carbon atoms as expected. For 1,2-DCA, $1/\alpha_{\mathrm{bulk}}$ (1.030) corresponded well to the value observed

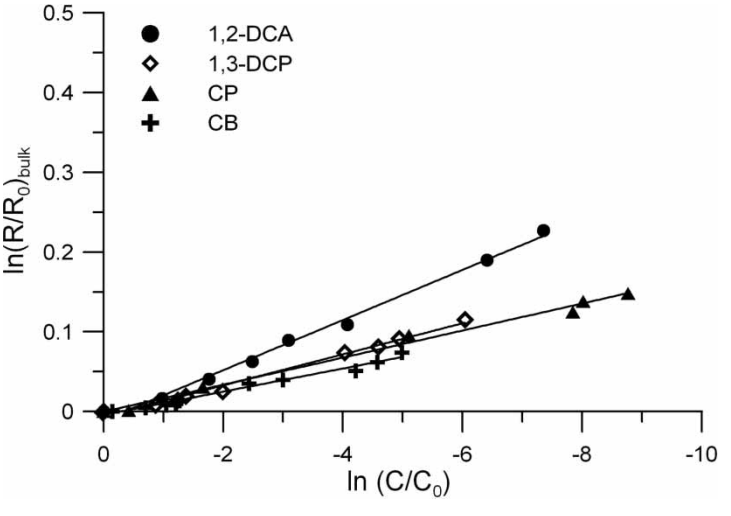

Figure 2. Rayleigh plots for one replicate of the pure culture experiments with 1,2-DCA, 1,3-DCP, CP and CB.

Table 1. Bulk isotope effect for pure culture studies with *X. autotrophicus* GJ 10. The isotope effect is expressed as $1/\alpha_{bulk}$ to facilitate comparison with AKIE values (n, number of replicates).

	n	Number of C	Number of Cl	$1/\alpha_{bulk}$	Variance
1,2-DCA	3	2	2	1.0295	0.0003
CP	3	3	1	1.0172	0.0004
1,3-DCP	3	3	2	1.0203	0.0001
CB	3	4	1	1.0149	0.0002

in a previous study (1.032, [32]). For compounds with three carbon atoms, double-chlorinated 1,3-DCP showed slightly larger $1/\alpha_{bulk}$ than the single-chlorinated CP.

In order to compare the magnitude of isotope effects independent of molecule structure and size, AKIE values for all experiments were calculated using Equations (4) and (5) (Figure 3 and Table 2). The comparison between Figures 2 and 3 suggests that after the corrections for molecular structure and size, the differences in the slopes (or the isotope enrichment factors) became less obvious. The calculated AKIE values based on pure culture studies were in rather narrow range between 1.0477 and 1.0616, corresponding to the typical KIE of S_N2 reactions (1.03–1.09, [36]). In previous studies with different aromatic compounds transformed by the same reaction mechanism but different organisms, a larger variability of AKIE values between 1.0035 and 1.0273 was observed [17]. The higher variability might be due to differences in the enzymes between different organisms, which may be associated with differences in the commitment to

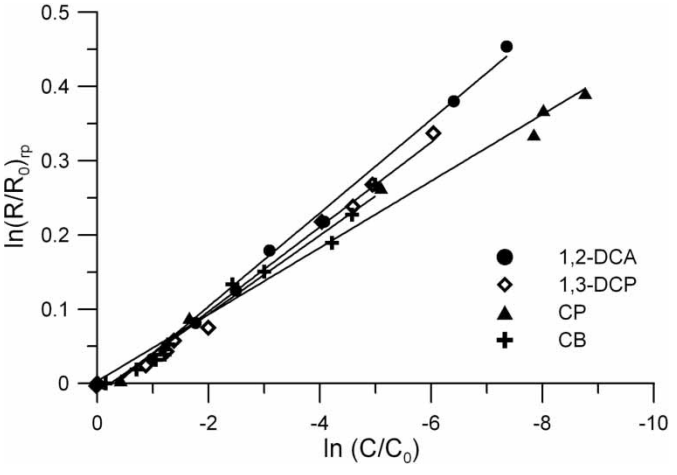

Figure 3. Rayleigh plots for reacting positions according to Equation (4) for one replicate of the pure culture experiments with 1,2-DCA, 1,3-DCP, CP and CB.

Table 2. AKIE values from pure culture, AKIE cell-free extract experiments and p-value for pairwise t-test between pure culture and cell-free extract AKIE values.

	Pure culture experiments			Cell-free extract experiments			
	n	Average	Variance	n	Average	Variance	p-value
1,2-DCA	3	1.0608	0.0003	3	1.0597	0.0003	0.85
CP	3	1.0477	0.0011	2	1.0454	0.0014	0.80
1,3-DCP	3	1.0616	0.0002	3	1.0621	0.0002	0.94
CB	3	1.0555	0.0008	3	1.0527	0.0007	0.42

catalysis. Furthermore, in previous studies on aerobic alkane degradation, the AKIE decreased with increasing molecule size [25, 37].

Although the AKIE values observed in this study were in a quite narrow range, there is a statistically significant difference between the AKIE for different compounds (single-factor analysis of variance at 95 % confidence level). Haloalkanes with two chlorine atoms had larger AKIE values than those with one chlorine atom.

The observed variations in the magnitude of AKIE values for pure cultures could have several reasons: slow transfer of the substrate across the cell membrane could partly mask the isotope effect, commitment to catalysis could be variable for different substrates or the location of the transition state along the reaction coordinate could vary for the different substrates, leading to different intrinsic KIEs. By disrupting the cell membrane to expose the cytoplasmic enzyme, haloalkane dehalogenase, the substrates do not need to permeate through the membrane before transformation takes place. Therefore, the cell-free extract experiments could reveal the effect of substrate transport on the isotope effect. A previous study on PCE transformation to TCE had demonstrated an increase in observed isotope fractionation for cell-free extracts compared with whole cell experiments [22].

3.2. *Cell-free extract studies*

In the cell-free extract studies, transformation of all substrates was observed as in the pure culture studies. Triplicate experiments yielded AKIE values with a precision comparable with the pure culture experiments (Table 2). Comparing pure culture and cell-free extract experiments for each compound using paired t-tests revealed that there was no statistically significant difference for each pair at the 95 % confidence level. This observation suggests that substrate transport across the cell membrane is fast compared with substrate transformation in the cell for all the substrates. Such a conclusion was previously drawn for 1,2-DCA by comparing the kinetics of the haloalkane dehalogenase with the velocity of compound degradation in continuous cultures [27,28]. Hence the observed difference in AKIE was caused by another factor such as differences in commitment to catalysis and/or differences in the intrinsic KIE for different substrates.

An alternative explanation for the variability of the AKIE values could be an uneven distribution of ^{13}C in the starting substrate owing to isotope fractionation during the production of chlorinated hydrocarbons. To evaluate this possibility, theoretical calculations were made in an attempt to reproduce the observed variability of the AKIE by solely varying the intramolecular distribution of heavy isotopes while assuming that for all compounds the actual kinetic isotope effect was equal. The actual kinetic isotope effect was assumed to correspond to that of 1,2-DCA because the molecule is symmetrical and contains only reacting positions and hence is not affected by an uneven distribution of heavy isotopes. For the other compounds, the initial $\delta^{13}C$ of reacting (chlorinated) and non-reacting (non-chlorinated) positions was varied while keeping the overall $\delta^{13}C$ constant at the measured value until the calculated AKIE corresponded to the measured AKIE. The obtained $\delta^{13}C$ values for reacting and non-reacting carbons were $-252\,‰/76\,‰$, $-109\,‰/2.5\,‰$ and $81\,‰/-219\,‰$ for CP, CB and 1,3-DCP, respectively. These differences are much larger than what can be expected for different positions in an organic molecule. Furthermore, even if chlorination leads to isotope fractionation, it would be expected that the non-chlorinated carbon retains an isotope ratio typical for biomass, which is not the case for the calculated values. Hence, while an uneven distribution may contribute to the observed difference in AKIE, it cannot explain it alone.

In summary, the study demonstrates that even for compounds that are degraded by the same initial transformation step and the same pure culture, some variability in AKIE remains after corrections for carbon numbers and reacting positions are made. Comparison with other studies

indicates that if different cultures are involved, the variability further increases. Hence, it is necessary to evaluate the degree of isotope fractionation for each substrate and each culture separately, and the variability of isotope fractionation factors has to be taken into account when quantifying biodegradation at the field scale.

Acknowledgements

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