STABLE ISOTOPE FRACTIONATION OF HEXACHLORCYCLOHEXANES DURING REDUCTIVE DECHLORINATION

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Introduction

Heavy use of organochlorine insecticides, especially in the past, has led to the dispersal of these pollutants throughout the global environment. Among them, one compound of major concern is 1,2,3,4,5,6-hexachlorocyclohexane (HCH). Organochlorine insecticides are of major concern for human health and the environment due to their hazardous nature and persistence and often constitute ground water contamination at productions sites and industrial waste deposits (1).

Mixed chlorobenzenes (CBs) and benzene are anaerobic transformation products of γ-HCH produced by marine microorganisms, cyanobacteria, sulfate-reducing bacteria and other unidentified anaerobes (2). These compounds have also been observed in soil slurries under different redox conditions (2). Monochlorobenzene (MCB) and benzene also appeared to be dominant metabolites produced during biotransformation of HCHs under anoxic conditions (2). Tetrachlorocyclohexane (TCCH) was determined as a transient methabolite (2). Under aerobic conditions the formation of gamma-pentachlorocyclohexene (γ-PCCH) indicatates a β-elimination suggesting a dehydrohalogenation reaction (12,13). Under anoxic conditions anaerobic dechlorination lead to the formation of tetrachlorocyclohexane (TCCH) which is may react via the putative unstable intermediate 1,3 dichlorocyclohexadiene (1,3-DCDN) to form either spontaneously MCB or enzymatically benzene. The first step of the anaerobic dehalogenation reaction may be a dehydrohalogenation reaction similar to aerobic degradation, however, to our knowledge was not shown proved in experiments with pure anaerobic cultures. There is controversy in the literature over the persistence and transformation pathways of HCHs in soil, water and air, probably due to contrasting data resulting from the complex interactions of environmental factors affecting rates of both abiotic and biotic removal (2). Reductive dechlorination seems to be the dominant transformation pathway of HCHs under anoxic conditions as indicated by laboratory studies with anaerobic strains (2,11). Dehalogenated xenobiotic compounds become less toxic and more readily degradable under oxic conditions. However, anaerobic environmental biodegradation of HCHs leads frequently to the accumulation of its metabolites (especially MCB), forming large plumes in aquifers (3). Chlorinated benzenes (especially MCB) are less toxic but more soluble in water, and less hydrophobic compared to HCHs, often leading to contamination plume extension in contaminated aquifers (9). At HCH contaminated field site, however, beside reductive dechlorination, the dehydrohalogenation may be a likely transformation pathway under anoxic conditions. Under oxic conditions biologically and photochemical induced dehydrohalogenation mechanisms may be dominant (12,13). For example in the case of dehydrohalogenation of γ-HCH the transformation product gamma-pentachlorocyclohexene (γ-PCCH) will be obtained. Under anoxic conditions HCH transformation leads to halogenated benzenes in contrast to oxic conditions where halogenated phenols may be abundant.

Stable isotope fractionation has been increasingly considered for characterizing *in situ* biodegradation processes (4) and reductive dehalogenation of chlorinated ethenes has been shown to be accompanied by fractionation of both chlorine (5) and carbon isotopes (6, 7). Compound specific stable isotope fractionation analysis (CSIA) is taking advantage of the preferential transformation of lighter isotopomers during a degradation reaction, thus leading to an enrichment of heavier isotopes in the residual phase in the course of biodegradation (for an overview of the method see (4),(9)), CSIA may be a promising tool for assessing the anaerobic dehalogenation of HCH in field studies, however, to our knowledge, no studies investigated this aspect.

In this study, we investigated the isotope fractionation of γ-HCH as a model compound for technical HCH to elucidate the potential of CSIA for characterizing of the *in situ* dehalogenation under anoxic condition.

Materials and Methods:

Biodegradation experiments with γ hexachlorocyclohexane. The carbon isotope fractionation factors upon anaerobic dehalogenation of gamma hexachlorocyclohexane (γ-HCH) by the sulfate-reducing bacteria

Desulfococcus multivorans DSM 2059 and *Desulfovibrio gigas* DSM 1382 was determined. Both strains are known for co-metabolic HCH dehalogenation. Biodegradation experiments with γ-HCH in concentrations of 22 to 25 µM were carried out using benzoate (final concentrations: 3-5 mM) (for *D. multivorans*) and lactate (final concentration: 10 mM) (for *D. gigas*) as electron donors, respectively, and different amounts of sulfate (final concentrations ranging from 5 to 30 mM).

Biodegradation experiments for determining isotope fractionation factors were performed in 1 L bottles crimped gas-tight by Teflon-coated butyl septa. 900 mL medium were inoculated with 50 mL freshly outgrown pre-culture. The cultures were incubated first at 30°C until the stationary phase was reached due to electron donor limitation, in order to get high cell numbers for the dechlorination experiment. Additional quantities of electron donors (sodium lactate for *D. gigas* and sodium benzoate for *D. multivorans*) and γ-HCH were further added. Because of its low solubility γ-HCH was difficult to solve in water and the culture bottles were permanently shaken on a horizontal shaker during the entire dechlorination experiment: for 3-4 hours at 150-200 rpm before the first samples were taken, and afterwards at 125 rpm.

For sampling, aliquots of the culture medium were regularly taken by syringes for stable isotope analysis (14 mL). Syringes were always flushed with nitrogen before use to avoid oxygen contaminations inside the culture bottles. Removed volume was always balanced by sterile nitrogen to avoid negative pressure inside the bottles. Samples for isotope analysis were conserved with 0.5 mL concentrated hydrochloric acid. The extraction of γ-HCH was done in 16 mL vials (Supelco) with 1 mL DCM containing 100 µM hexachlorobenzene (HCB) as internal standard for γ-HCH and 100 µM toluene as internal standard for metabolites. The vials were shaken for 12-36 hours at 110 rpm and 12 ºC. The organic phase was separated with Pasteur pipettes and transferred in 2 mL vials containing anhydrous Na_2SO_4 for drying. The Na_2SO_4 was dried several hours prior usage in an air oven at 150-250ºC. Finally, 100 µL of this dry organic phase where transferred in vials for injection in the GC-MS and GC-IRMS system.

Biodegradation experiments with β hexachlorocyclohexane. Biodegradation experiments of β-HCH with *D. gigas* for determining isotope fractionation factors were performed in 2 L bottles crimped gas-tight by Teflon-coated butyl septa. 1800 mL medium were inoculated with 100 mL freshly outgrown pre-culture of *D. gigas*. The experiments were performed in the presence of different amounts of sulfate: 30 mM both for the active bottle and also for autoclaved control and 5 mM, and the same amount of lactate (10 mM). β-HCH was added from a stock solution of acetone (50 mM), in a final concentration of about 1.5 µM that did not exceed its very low solubility in water (about 0.4-0.5 mg/L respectively 1.3-1,7 µM). The cultivation procedure for *D. gigas* was described previously (11).

For sampling, aliquots of the culture medium were regularly taken by syringes for stable isotope analysis (100 mL). Samples for isotope analysis were conserved with 1 mL concentrated hydrochloric acid. The extraction of β- HCH was done in 100 mL bottles crimped with 3 mL DCM containing 100 µM hexachlorobenzene (HCB) as internal standard for β-HCH and 100 µM toluene as internal standard for metabolites. The details of the analytical procedure were performed as was described previously (11).

Results and Discussion:

Biodegradation of γ hexachlorocyclohexane by *D. gigas* and *D. multivorans*. γ-HCH was transformed by both bacterial strains in incubation times of up to four weeks (Figure 1). The main metabolites formed during biodegradation were benzene, monochlorbenzene, and as transient metabolite γ TCCH was tentatively characterized by GC-MS. Thus suggesting that γ-HCH was indeed reductively dechlorinated under sulfate-reducing conditions, as previously observed (2) . After an initial increase, the relative concentration of γ-TCCH decreased after 2 to 3 days of incubation, while benzene and monochlorbenzene accumulated. The absolute concentration (µM) was calculated only for benzene and monochlorbenzene because a standard of γ-TCCH was not available for precise calibration. For both strains, the sum of the final concentrations of benzene and MCB was close to the theoretical spiked γ-HCH concentration (Figure 1A and B). We were not able to detect γ-PCCH to support the hypothesis that dehydrodehalogenation was involved in the anaerobic dehalogenation reaction and the biochemical mechanisms of the initial step of anaerobic transformation reaction remains to be elucidated.

The rate of γ-HCH biodegradation by *D. gigas* was strongly affected by the concentrations of the electron acceptor sulfate in the growth medium, since *D. gigas* degraded γ-HCH completely only under sulfate limitation (5 mM sulfate) (Figure 1A). In contrast to the results for *D. gigas*, the rate of γ-HCH degradation by *D. multivorans* was not influenced by limiting amounts of sulfate.

In the biodegradation experiments of β-HCH with *D. gigas* the concentration of β-HCH remained stable and no metabolites were detected even in the highest sulfate limitation medium (5 mM sulfate) and even after 53 days of incubation and the isotope composition remains stable during the experiment. This result seems to confirm the hypothesis of high stability of β-HCH because it has all equatorially oriented chlorine atoms ('eeeeee'), resulting in a more structurally stable compound (2). This may be the reason that the *D. gigas* is not able to attack HCH isomers with equatorially oriented chlorine atoms.

Carbon isotope fractionation in the course of γ-HCH degradation. In all experiments with living cells, the carbon isotope composition γ-HCH changed in the course of biodegradation. In Figure 1A and 1B, data of a single experiment for both *D. gigas* and *D. multivorans* are shown. For *D. gigas* under sulfate-limitation (5 mM sulfate), the carbon isotope composition of γ-HCH increased from -27.6 ± 0.1 ‰ to -16.4 ± 0.8 ‰, whereas the γ-HCH concentrations decreased from 19.4 μM to 0.9 μM (Figure 1A). MCB was detected after six days in a concentration of 9.2 μM and a carbon isotopic composition of -29.6 ± 0.7 ‰, which is slightly lighter than the isotopic signature of the parent compound γ-HCH. The concentration of MCB increased to 24.4 μM and correlated with carbon isotope enrichment at end of the experiment.

For *D. multivorans,* the carbon isotope signatures of γ-HCH were enriched to a similar extent during biodegradation as observed for *D. gigas*: in the experiment initially spiked with 20 mM sulfate, the carbon isotope composition of γ-HCH increased from -27.2 ± 0.3 ‰ to -16.1 ± 0.5 ‰, whereas the γ-HCH concentrations decreased from 20.5 μM to 0.8 μM. (Figure 1B). Isotopically depleted MCB up to -36.5 ± 3.8 ‰ was detected after two days and was always significantly depleted compared to the parent compound in the course of the experiment. The MCB concentrations increased to around 12 μM, and the corresponding carbon isotope signatures got enriched to -25.7 ± 0.4 ‰, which is roughly the initial isotope signature of the parent compound γ-HCH.

Quantitative assessment of isotope fractionation. The carbon stable isotope composition of γ-HCH during biodegradation was determined by compound specific stable isotope analysis (CSIA). The isotope fractionation process was quantified by the isotope fractionation factor (αC) using the Rayleigh model, obtaining fractionation factors of 1.0040 ± 0.0002 (εC=4.0) for *D. gigas* and 1.0034 ± 0.0002 (εC=3.4) for *D. multivorans* (Figure 2).

Environmental implications. Our results (11) clearly demonstrate that carbon isotope fractionation occurs during the anaerobic dechlorination of γ-HCH. Thus, monitoring the carbon isotope signatures of γ-HCH is a promising tool for the qualitative and quantitative assessment of γ-HCH biogradation in anoxic contaminated areas and might be also applicable to other HCH isomers. Assuming that a HCH contamination in the field originates from a single source, variations in the carbon isotope composition between zones and depths may serve as indication for *in situ* anaerobic dechlorination of HCH isomers. Our current studies are conducted to the assessment of the fractionation factors for other HCHs and especially on enantioselective transformation of α HCH. To evaluate the environmental fate of alpha HCH is necessary to combine enantiomeric specific fractionation of α HCH with isotope fractionation processes that appear during its anaerobic dechlorination including dehydrochlorination (12,13).

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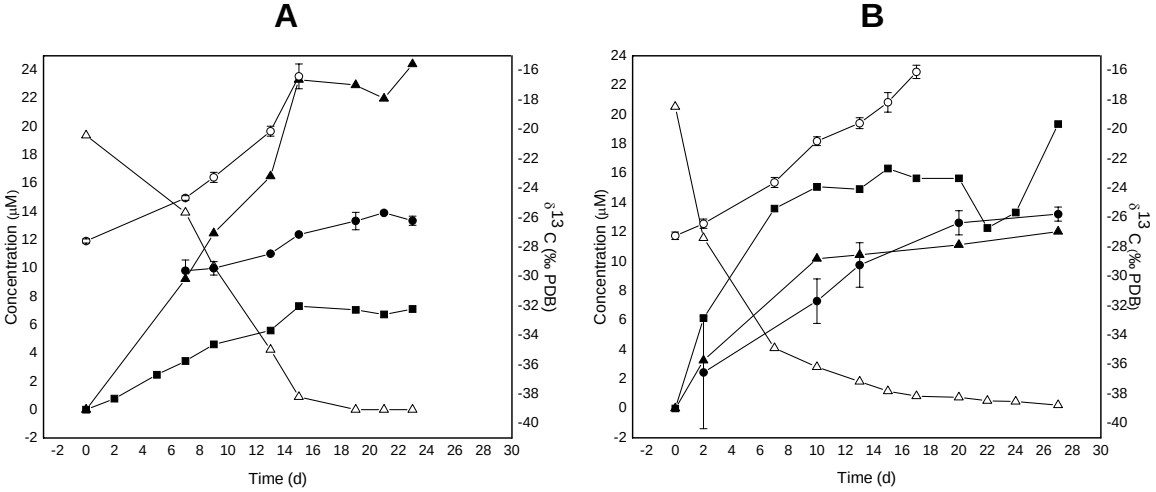

Figure 1. Change in concentration (Δ) and carbon isotope composition (○) of γ-HCH, in concentration (▲) and carbon isotope composition (●) of MCB, and in concentration (■) of benzene during anaerobic dechlorination by *D. gigas* (initially spiked with 5 mM sulfate) (A) and *D. multivorans* (initially spiked with 20 mM sulfate) (B). (submitted to *Environmental Science & Technology*)

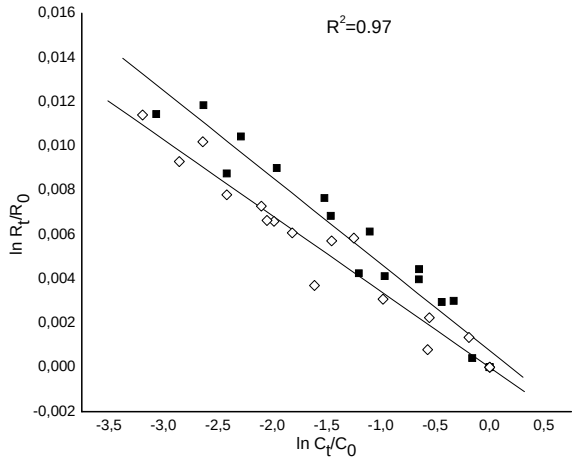

Figure 2. Quantitative assessment of isotope fractionation of γ-HCH in the biodegradation experiments with *D. gigas* (■) and *D. multivorans* (◊) by the use of the Rayleigh model. Data of all biodegradation experiments were taken for calculating the isotope fractionation factors: 1.0040 ± 0.0002 for *D. gigas* and 1.0034 ± 0.0002 for *D. multivorans.*