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Stable isotope fractionation concepts for characterizing biotransformation of organohalides

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Recent development allows multi-element compound-specific isotope analysis of D, $^{13}$C, $^{35}$Cl and $^{81}$Br for characterizing the degradation pathways of halogenated organic substances in the environment. The apparent kinetic isotope effect (AKIE) obtained in (bio)degradation experiments yields information on the chemical mechanism of the bond cleavage. In biochemical reactions, rate limitation such as uptake into cells, substrate transport in cells and binding to enzymes as well as equilibrium isotope effects (EIE) are modifying the observed isotope fractionation associated with the bond cleavage reactions, thus, complicating mechanistic interpretation of isotope effect. One way for improved analysis of bond cleavage reactions by isotope effects reactions is the combination of isotope effects from two or more elements forming the reactive organic moieties where bond change reaction takes place as indicator. A further option is the combination of enantiomeric with isotope fractionation as complementary indicator for enzymatic bond cleavage reaction governing biodegradation. Finally an interesting concept with large potential for elucidation of the biochemical reaction mechanisms of biological dehalogenation may be quantum mechanical/molecular mechanical (QM/MM) modelling in combination experimentally with multi-element isotope analysis, thus merging quantum chemical theory with experimental observation.

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Introduction and definition of isotope effects

Stable isotope fractionation has been used to characterize the fate of organic contaminants in the environment, particularly halogenated organic chemicals, since more than one decade [1,2]. The ultimate goal of this approach is to interpret stable isotope fractionation patterns to understand degradation of organohalides. The concept for applying ‘compound-specific stable isotope analysis’ (CSIA) for characterizing degradation in the environment has been subject of several previous reviews [3,4] and recently summarized for the in situ degradation of hydrocarbons (Vogt et al., current issue COB). For this work we summarize recent trends in employing isotope fractionation approaches for analyzing mechanisms of biological degradation reactions governing the fate of organohalides in the environment.

The theoretical background and definition of isotope effects in biological reactions have been recently summarized [5 **]. The kinetic isotope effect (KIE) is based on the difference in kinetic rates during chemical bond change reaction of isotopologues in a unidirectional reaction, reflecting the relative stability of bonds formed by the heavy vs. light stable isotopes of an element. A primary kinetic isotope effect can be substantial in reactions where an isotopologue substituted chemical bond is cleaved or formed [6,7 **]. A secondary kinetic isotope effect (SKIE) is observed when an adjacent isotopically substituted bond affects the rate of bond change in the rate-determining step of a reaction but is not directly involved in bond change [8]. SKIE tend to be much smaller than primary kinetic isotope effects. Larger SKIE effects are observed for hydrogen [9] and, more recently, for chlorine [10–12].

The concept for mechanistic interpretations of isotope effects in biological reactions has been established a long time ago making use of transition state theory and considerations for rate limitations in biological reactions [13]. The experimentally determined apparent kinetic isotope fractionation (AKIE) in biological system is considered to contain information of isotope sensitive mode of bond cleavage and all steps prior commitment of catalysis which need to be taken into account when interpreting isotope fractionation pattern (see Scheme 1).

In chemical homogeneous reactions the AKIE characterizes the mode of bond change in transition state providing full expression of the kinetic isotope effect (KIE) in many cases. In heterogeneous chemical reaction, such as reaction of mineral surfaces transport can become rate limiting to a significant extent lowering the AKIE compared to the expected KIE. In contrast, in complex enzymatic reactions the AKIE can be affected by rate limitation prior catalysis leading to masking of isotope effects, which can result in a reduction of the observed
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Scheme 1

\[ S_{\text{out}} \xleftrightarrow{\text{transport over membrane}} S_{\text{in}} \xrightarrow{\text{transport to enzyme}} E+S \xrightarrow{\text{binding to enzyme}} E-S \xrightarrow{\text{irreversible reaction}} E+P \]

Simplified model of steps governing rate limitations in biological reactions such as uptake into the cell and transport to the enzyme, binding to the enzyme and catalysis and product formation (modified after Northrop [19]). \( S_{\text{in}} \) = substrate on the outside of the cell, \( S_{\text{out}} \) = substrate inside the cell, \( E \) = Enzyme, \( P \) = product. BIE = binding isotope effect, KIE = kinetic isotope effect, AKIE = apparent kinetic isotope effect.

isotope fractionation and the AKIE is smaller than the KIE [13].

Biological kinetic isotope fractionation has to be considered as a sum of processes which includes the discrimination of stable isotopes during a unidirectional reaction comprising bond cleavage at the irreversible step, and masking effects resulting from the rate limiting steps related to uptake into the cell, transport within the cell, binding to the enzyme (Scheme 1). In this simplified model of biological reactions the last step is the isotope sensitive bond change after which the reaction becomes irreversible (commitment to catalysis) and all rate limitations of previous reaction steps modify (mostly lower) the KIE of the bond change. However, biological reactions may not always be completely unidirectional in a strict sense [13] and backward reactions are possible to some extent favoring equilibrium isotope effects (EIEs). The EIE describes the equilibrium constants for isotopologues present in two phases as well as the reversible binding of a ligand to a receptor [5**]. EIEs are connected with any physical process or chemical reaction that reached equilibrium. EIEs could be relevant in reactions in which a ligand binds to the binding pocket of an enzyme and has then been termed binding isotope effects (BIE) [5**].

Today, the progress in quantum mechanical/molecular mechanical (QM/MM) modelling allows to model kinetic isotope effects of enzymatic catalysis at various levels of theory and this calculated isotope fractionation can be used for a quantitative interpretation of isotope effects during degradation of organohalides in biological systems. The comparison of experimental examined isotope effects with quantum chemical modelling of carbon, chlorine and hydrogen isotope effects thus offers a perspective for mechanistic interpretation of dehalogenation reaction in future studies [14,15].

Next steps for improvement of interpretation of isotope fractionation patterns for analyzing reaction mechanisms of dehalogenation reactions may be a combination of carbon, hydrogen and chlorine isotope fractionation and their evaluation by molecular modelling. QM/MM modelling may improve the interpretation of AKIEs of complex biological bond cleavage reactions as expected for the degradation of halogenated organic contaminants when using considerations from theoretical calculation for interpretation of experimental results.

Compound-specific stable isotope analysis for the evaluation of microbial reactions

Analytical techniques for determination of \(^{2}H\), \(^{13}C\), \(^{37}Cl\), \(^{81}Br\) of organohalides

The progress in CSIA during the last decade allowed the evaluation of the origin and fate, particularly the in situ transformation pathways, of halogenated environmental contaminants [16–18]. Recent analytical developments for applying CSIA to Cl, Br and H isotopes of organohalides [19,20,21*,22–24] was subject of a recent review (Nijenhuis et al., unpublished data). Whereas CSIA of carbon is routine for halogenated organics, methods for hydrogen, chlorine or bromine stable isotope analyses are recent and not routine yet. Determination of D/H isotope composition was recently made possible by the combination of pyrolysis with chromium reduction, preventing the formation of by-products containing hydrogen such as HCl, HBr, HCN, H₂S from heteroatom (N, Cl, Br, S) containing organics [21,25]. Main bottleneck is the relatively high amount of analytes required. Several approaches to analyze chlorine stable isotope compositions were recently presented: one approach uses the direct analysis of the compound [19] requiring standards for each of the substances of interest with known isotope composition. Another, more universal, concept was presented where chlorinated compounds were pyrolyzed at high temperatures in an excess of hydrogen in the carrier gas to obtain HCl as analyte gas [20,26]. Though promising, due to instabilities and memory effects, this method cannot be applied routinely yet. The CSIA of bromine isotopes required an inductively coupled plasma (ICP)-multi collector-mass spectrometer (MC-MS) linked to a GC [23,24]. This technique is currently only sparsely applied, thus far, mainly due to its cost.

Understanding of isotope fractionation for evaluation of in situ biodegradation

The carbon isotope fractionation for a large variety of common halogenated groundwater contaminants was investigated in laboratory studies as a reference for the interpretation of isotope fractionation in field studies (for an overview see [27*]). By evaluation of the carbon isotope fractionation, the reductive and oxidative degradation of, for example, dichloroethene (DCE) could be distinguished even in complex model systems like constructed wetlands [28]. Providing knowledge of a groundwater flow path, hydrological conditions and a fractionation factor representing the mode of biodegradation of the microbial community, the extent of biodegradation may, furthermore, be estimated within an aquifer system [27*,29]. The use and
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Furthermore, the transfer and resulting uptake needs to be taken into account. The variation in the extent of isotope fractionation could be to some extent related to the type of microbial cell envelope, with higher masking related to the more hydrophobic envelope of Gram-negative cells [31,35]. In addition, strains with a similar enzyme inventory dehalogenating PCE and TCE exhibit similar fractionation suggesting that dehalogenation is governed by a similar mechanism [31,33]. This indicates that for characterization of mechanisms, experiments with pure strains and isolated enzymes are needed to understand the mechanisms and variabilities of isotope fractionation. Mixed cultures or enrichments might contain several species degrading a contaminant and thus the AKIE of these represents a mixture of different pathways [37], probably very dependent of experimental conditions and not very suitable for mechanistic interpretation.

Furthermore, substrate properties (hydrophobicity) and related bioavailability were observed to significantly affect measured isotope fractionation [30,35,38]. Mass transfer of a substrate from a second phase, for example, from a non-aqueous liquid phase, from dissolving solids or desorption of substrate from organic matter, reduced the observed isotope effect significantly [38] showing limitations for mechanistic studies in complex environments. Mass transfer related to bioavailability is also important factor governing isotope effects in the field [30].

Multi-element isotope analysis for characterizing chemical mechanisms of organohalide biotransformation

Over recent years, focus of CSIA has been on the microbial reductive dehalogenation of mainly chlorinated ethenes. The reductive dehalogenation reaction is a bond cleavage of the carbon–halogen bond with subsequent protonation of the carbon compared to oxidation of the carbon which forms CO₂ and chlorine. These processes exhibit specific isotope fractionation patterns for hydrogen, carbon and chlorine at the position of reaction allowing elucidating aerobic transformation pathways for chlorinated ethenes [11,12]. Initial reaction of 1,2-dichloroethane during oxidation via C–H bond cleavage resulted in relatively lower carbon isotope fractionation compared to C–Cl bond cleavage via an S_N2 mechanism while chlorine isotope fractionation was similar for both pathways [11,12]. Furthermore, expected, that is, significant carbon and low chlorine, isotope effects during the aerobic biodegradation of dichloroethene and vinyl chloride were observed in agreement with the initial reaction via epoxidation [39]. However, in case of the reductive dechlorination of PCE, two typical C/Cl isotope fractionation patterns were observed [40,41,42] suggesting that at least two types of reductive dehalogenases are at work and isotope fractionation pattern has the potential for the identification specific reactivity of dehalogenases.

Dual element (C/Cl) isotope fractionation patterns were analyzed for characterizing organohalide respiration mechanism of PCE [40,41] and TCE [10,41,43]. The dual element analysis of PCE, comparing dehalogenation by the enzyme with purified corrinoids, suggests a preceding rate-limiting step (e.g., enzyme–substrate association) prior catalysis governing isotope fractionation. Apparently, due to the higher intrinsic turn-over rate of the enzyme relative to the rate of transport of the substrate to the reactive site, the PCE isotope fractionation pattern does not reflect the chemical reaction. In this case the observed isotope effects may be mainly the result of preceding rate-limiting steps such as enzyme–substrate association, accompanied with a binding isotope effect (BIE). In the case of TCE, enzymatic bond cleavage reaction mainly governs the AKIE and the rate of substrate transport apparently exceeded the intrinsic reaction rate of the enzyme so that AKIE ≈ KIE [10,41,43].

These examples show that isotope fractionation patterns have to be used with caution and that it is possibly not suitable to deduce reaction mechanisms in many cases. By combining observed isotope effects of several elements of a specific reaction, and compare experimental results with QM/MM modelling, stable isotope patterns may be applied to test and validate reaction mechanisms and microbial reactions. Isotope analysis of not only the elements (i.e., C and Cl) involved in the bond cleavage but also the addition of elements, that is, protonation during dehalogenation as was done for TCE [44], may provide further insights into the reaction. As the methods for H isotope analysis of halogenated substances now become routinely available, the hydrogen isotope fractionation analysis might provide further mechanistic insights on dehalogenation reactions.

Enantiomeric and isotope fractionation for analyzing mode of bond cleavage and binding to enzymes

Enantiomers are stereoisomers that are mirror images of each other, are non-superimposable and exhibit almost identical physical and chemical properties. The chemical reactions with the same reaction mechanism are expected to result in similar degradation kinetic and isotope effects, however, the biological reactivity can lead to a preferential...
degradation of individual enantiomers and individual AKIE. The combination of carbon isotope fractionation studies with enantiomeric fractionation, that is, the preferential conversion of one stereoisomer over the other, may be, on one hand, a diagnostic concept for distinguishing biodegradation from chemical degradation [48], and yields on the other hand information of uptake, binding and catalysis upon biodegradation. Recent studies have focused on aerobic biotransformation of α-hexachlorocyclohexane (α-HCH) by Sphingomonas spp. Chiral GC columns enable the separation of α-HCH enantiomers for CSIA [45]. The aerobic degradation of α-HCH by Sphingomonas spp. was employed as a model to study enantiomeric and carbon isotope fractionation [46]. The variable enantiomeric and isotope fractionation factors show potential for tracking the reaction in the environment. However, the potential of isotope fractionation patterns for studying biochemical mechanisms and binding to enzymes has not been fully exploited yet. The proteins for dehalogenation are coded by linA1 and linA2 genes present in various compositions in different bacterial strains and regulation of enantiomeric degradation remains unknown, thus far. Thus, the isotope fractionation and enantiomer specificity of isolated enzymes catalyzing the degradation of α-HCH remains a topic of future studies.

QM/MM modelling has potential for deeper investigation of multi-element isotope effects in order to theoretically predict carbon, chlorine and hydrogen KIEs during aerobic degradation of HCH isomers [47]. For example, a small model of the HCH dehydrochlorinase (LinA) for the active site on the receptor–ligand complexes allows detailed analysis of chlorine and hydrogen KIEs patterns. QM/MM calculations suggest that all isomers seem to undergo a concerted E2 mechanism but the contribution of proton transfer and carbon–chlorine leaving group bond stretch in the transition states differs for the different isomers giving rise to totally different magnitudes of predicted isotope effects. The slightly lower carbon isotope fractionation obtained experimentally in comparison to quantum chemical calculations indicates that masking effects play a role and may be related to rate limitation induced by enzyme–substrate binding [48]. The difference in isotope fractionation of the α-HCH enantiomers may characterize the masking effect on carbon isotope fractionation by binding to the enzyme and suggests that binding of enantiomers to the enzyme played a role in the overall process of isotope fractionation [48].

The comparison of D, 13C and 32Cl isotopes of QC/QM calculation with experimental results will offer exiting future perspectives for CSIA. For example the LinA enzyme (HCH dehydrochlorinase) in Sphingobium japonicum strain UT26 catalyzes dehydrochlorination of both enantiomers by the same catalytic mechanism [49]. The comparison of isotope fractionation between α-HCH enantiomers may allow studying rate limitations associated with enzyme–substrate binding. First QM/MM investigation of LinB dehalogenase from Sphingomonas japonicum strain UT26 propose a S_{2}_2 displacement mechanism for the dechlorination reaction of β-HCH and predict a concerted mechanism for the reaction [47,50]. Similarly, QM/MM calculations of dehydrochlorination reactions of γ/β isomers in LinA allows prediction of two distinct reaction pathways (concerted and stepwise) for γ-HCH and β-HCH, respectively [51]. Both examples imply that comparisons of modelling and isotope fractionation experimental studies are the next step for elucidating dehalogenation reactions.

**Future objectives for diagnosis of degradation processes**

The extent of AKIE observed in experimental studies has been used for prediction of degradation mechanisms [9,52] suggesting that the KIE can be used to predict bond cleavage mechanisms. In chemical reactions the order of magnitude of isotope effects may reflect the kinetic rate limitation of bond cleavage directly as a result of the stability bonds formed by heavy and light isotopologues. In this case, the transition state of the bond cleavage governs the rate limitation (commitment to catalysis) on the reaction coordinate. However, even in chemical reactions masking might occur by commitment to catalysis in bimolecular reactions if diffusion of the reactant becomes rate limiting. In contrast, in biochemical reactions with complex transitions states, isotope fractionation is masked by rate limitation due to substrate binding to enzymes and the AKIE is not solely reflecting bond cleavage alone. In addition, BIE can be theoretically substantial and EIE in reactions with a complex reaction coordinate need to be taken into account [53]. Thus, the mechanistic interpretation of AKIE must be done with caution and cannot be done employing the order of the AKIE alone. The AKIE is affected by rate limitations of enzyme binding but also processes such as uptake of substrate into the cell and transport within the cell which makes interpretation of AKIE difficult for analyzing rate limitations prior to bond cleavage. The correlation of fractionation factors of two or more elements for canceling out rate limitations prior commitment to catalysis as an indicator of bond cleavage seems to work in many cases [52], however, is only correct if rate limitations previous to catalysis are not isotope sensitive. Rate limitations caused by transport (e.g., diffusion) and binding are not per se without any isotope effects or couple the isotope effects of two elements in an identical way, which allows canceling out masking for an improved description of bond change reactions. However, if information on the biochemical reaction mechanism is available, AKIE of multi element isotope studies could be valuable to test the hypothesis of reactions and to analyses rate limitations. For future studies multi-element isotope analysis from experimental studies can be calculated using QM/MM modelling at higher levels of theory for a precise...
characterization of reaction mechanism of biodegradation of organohalides.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

1. of special interest
2. of outstanding interest


Recent review on isotope effects in biological reaction, state of the art definition of isotope effects and an overview on molecular modelling approaches.


Overview on principles of isotope fractionation.


New concept for routine analysis of hydrogen isotope fractionation of halogenated compounds.


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Application of isotope fractionation for the assessment of natural attenuation in applied studies.


Concept for the analysis of rate limitations governs by uptake and transport during biodegradation.


