1 *Methods in Enzymology:*

2 Measurement of Enzyme Isotope Effects

3 **Characterization of substrate, co-substrate, and**
4 **product isotope effects associated with enzymatic**
5 **oxygenations of organic compounds based on**
6 **compound-specific isotope analysis**

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Abstract

Enzymatic oxygenations are among the most important biodegradation and detoxification reactions of organic pollutants. In the environment, however, such natural attenuation processes are extremely difficult to monitor. Changes of stable isotope ratios of aromatic pollutants at natural isotopic abundances serve as proxies for isotope effects associated with oxygenation reactions. Such isotope fractionations offer new avenues for revealing the pathway and extent of pollutant transformation and provide new insights into the mechanisms of catalysis by Rieske non-heme ferrous iron oxygenases. Based on compound-specific C, H, N, and O isotope analysis, we present a comprehensive methodology with which isotope effects can be derived from the isotope fractionation measured in substrates, the co-substrate O₂, and organic oxygenation products. We use dioxygenation of nitrobenzene and 2-nitrotoluene by nitrobenzene dioxygenase as illustrative examples to introduce different mathematical procedures for deriving apparent substrate and product isotope effects. We present two experimental approaches to control reactant and product turnover for isotope fractionation analysis in experimental systems containing purified enzymes, *E. coli* clones, and pure strains of environmental microorganisms. Finally, we present instrumental procedures and sample treatment instructions for analysis of C, H, and N isotope analysis in organic compounds and O isotope analysis in aqueous O₂ by gas and liquid chromatography coupled to isotope ratio mass spectrometry.

Keywords Compound-specific isotope analysis, apparent kinetic isotope effects, nitrobenzene dioxygenase, non-heme Rieske iron oxygenase, oxygen activation, pollutant biodegradation, isotope ratio mass spectrometry, enzyme assays.

1 Introduction

Enzymatic oxygenations are among the most important biodegradation and detoxification reactions of organic pollutants (Gibson & Parales, 2000; Kohler, 2011; Neilson & Allard, 2008). The oxidation of organic pollutants through the insertion of one or two oxygen atoms from molecular O₂ makes these compounds more polar and more bioavailable. The oxygenated organic products may be channeled into common metabolic pathways, which ultimately enable the mineralization of the pollutants (Boyd & Bugg, 2006; Bugg & Winfield, 1998; Schwarzenbach, Gschwend, & Imboden, 2016). Such processes enable natural attenuation of pollutants but are inherently difficult to monitor in contaminated soil and water. In the environment, oxidative biodegradation typically occurs through multiple different reaction pathways and takes place over timescales of years to decades. Moreover, the concentration dynamics of organic pollutants are often determined by non-reactive processes such as dilution and phase transfers, making it almost impossible to assess biodegradation quantitatively (Schwarzenbach et al., 2006).

Compound-specific isotope analysis (CSIA) of organic pollutants has become an alternative avenue to reveal the type of degradation reaction and to quantify the extent of transformation by measuring changes of pollutant isotope ratios at *natural isotopic abundances* (Aelion, Höhener, Hunkeler, & Aravena, 2010; Elsner, 2010; Elsner et al., 2012; Hofstetter & Berg, 2011; Hofstetter, Schwarzenbach, & Bernasconi, 2008). CSIA relies on the phenomenon of kinetic isotope effects, KIE (eq. 1), that arises from small differences in activation free energies of reactant molecules with different isotopic substitution (Kohen & Limbach, 2006; Wolfsberg, Hook, Paneth, & Rebelo, 2010).

$$\text{KIE} = \frac{{}^l k}{{}^h k} \quad (1)$$

where ${}^l k$ and ${}^h k$ denote reaction rate constants of light and heavy isotopologues, respectively. As a consequence, bonding differences between the ground state and transition state, especially at sites undergoing bond cleavage reactions, give rise to changes of stable isotope ratios, the so-called “isotope fractionation”, in the reacting pollutant. Isotope fractionation associated with pollutant biodegradation serves as proxy for isotope effects of reactive processes, from which both the pathway and extent of pollutant degradation can be derived. As will be shown in Section 2 of this chapter, the link between pollutant isotope fractionation and the mechanisms

of biodegradation through oxygenation reactions can be made in a number of different ways. The link can be established most rigorously in laboratory experiments when isotope fractionation of several elements in the pollutant, such as C, H, and N, are considered simultaneously together with the O isotope fractionation of aqueous O₂.

However, a widespread application of CSIA to assess enzymatic oxygenation reactions of organic pollutants is currently compromised. At present, we lack comprehensive knowledge of the mechanisms of mono- and dioxygenations of organic pollutants and the magnitude of isotope effects that are associated with the initial steps of the reactions. Moreover, the kinetic complexity of enzymatic O₂ activation and its consequences for the expression of isotope fractionation in the organic substrate is poorly understood (Wijker, Pati, Zeyer, & Hofstetter, 2015). The isotope fractionation observed during enzyme-catalyzed oxygenations of organic pollutants can vary substantially even for identical reactions because of multiple rate-limiting steps. We recently observed highly variable kinetic isotope effects of dioxygenations of different nitroarenes by nitrobenzene and nitrotoluene dioxygenases (Pati, Kohler, Bolotin, Parales, & Hofstetter, 2014; Pati, Kohler, et al., 2016). In these works, we hypothesized that different steps of the pathway for activation of molecular O₂ contribute in a substrate-specific manner to the catalytic cycle of Rieske non-heme ferrous iron oxygenases. Based on these observations, we developed methodologies for assessing isotope effects of oxygenation reactions (1) in aromatic pollutants, (2) their oxygenated organic products, as well as (3) in aqueous O₂ based on measuring isotope fractionation.

The approach presented here allows a comprehensive characterization of isotope effects in substrates, co-substrates, and products. Our examples focus on oxygenations, but similar procedures may be used to study isotope effects on other enzymatic processes based on isotope fractionation observed at natural isotopic abundances and measured by gas and liquid chromatography in combination with isotope ratio mass spectrometry. First, we summarize the different mathematical procedures with which isotope effects can be derived from data on the fractionation of stable isotopes of organic substrates and their reaction products. Thereafter, we describe experiments in laboratory model systems at different levels of biological complexity covering pure cultures of pollutant-degrading microorganisms, *E. coli* clones expressing enzyme system of interest, crude cell extracts thereof, as well as enzyme assays of purified nitroarene

dioxygenases. In the fourth section, we illustrate customized analytical methods for quantification of C, H, N, and O isotope ratios in different analytes before concluding with a summary.

2 Deriving apparent kinetic isotope effects from measurements of stable isotope ratios

2.1 Instrumental approaches and observable quantities

Isotope ratios of individual compounds present in a solution or gas mixture are typically measured with continuous flow isotope ratio mass spectrometry by coupling gas or liquid chromatography with an isotope ratio mass spectrometer (GC/IRMS and LC/IRMS, [Elsner et al. \(2012\)](#); [Jochmann and Schmidt \(2012\)](#); [Schmidt and Jochmann \(2012\)](#); [Sessions \(2006\)](#)). Such measurements at natural isotopic abundances involve, in most cases, the conversion of analytes to small molecules such as CO₂, N₂, and H₂ with a limited number of stable isotopologues. Measured isotope ratios therefore reflect the averages of all atoms of the studied element in a molecule. Recent instrumental developments extend the scope to molecules with multiple heavy isotopic substitutions (“clumped isotopes”) and different isotopomers, but those techniques are not discussed here ([Bernstein et al., 2011](#); [Eiler, 2013](#); [Magyar, Orphan, & Eiler, 2016](#); [Piasecki et al., 2016](#)).

Due to these instrumental boundary conditions, one observes changes of isotope ratios as isotope fractionation of all atoms of one element in a molecule. Isotope effects can only be inferred indirectly by making explicit assumptions with regard to the mechanisms of reaction and the number and position of reactive atoms in a molecule. The mathematical procedures for obtaining such “observed” or “apparent” isotope effects have been established for a while ([Melander & Saunders, 1980](#); [Singleton & Thomas, 1995](#)) and are now applied extensively for CSIA based on a formalism introduced by [Elsner \(2010\)](#); [Elsner, Zwank, Hunkeler, and Schwarzenbach \(2005\)](#). The evaluation of isotope fractionation with CSIA is now used for two purposes. (i) In laboratory model systems, quantification of isotope fractionation of (model) pollutants enables one to assess the magnitude and variability of apparent kinetic isotope effects of (bio)degradation pathways. In combination with theoretical analyses, for example through computational investigation of reaction sequences, such data are key to assess the extent, to which intrinsic isotope

Figure 1 Oxygenation of nitrobenzene (**1**) by nitrobenzene dioxygenase (NBDO) leading to a *cis*-dihydrodiol intermediate (**2**) and two products, that is catechol (**3**) and nitrite (NO_2^-).

effects of bond cleavage reactions are observable as isotope fractionation because isotope effects may be masked by kinetic complexity in the chemical or enzymatic reaction (i.e. the isotopically sensitive step is not rate limiting, Cook and Cleland (2007); Northrop (1981)) or through phase transfer processes (Aeppli et al., 2009; Eckert, Qiu, Elsner, & Cirpka, 2013). (ii) Derivation of apparent kinetic isotope effects from isotope fractionation measured in (contaminated) environments and its comparison with data from laboratory investigations are one of the most promising means to identify if, how, and to what extent pollutants are transformed in complex systems.

In the following sections, we illustrate the mathematical procedures for evaluating isotope fractionation data and deriving apparent kinetic isotope effects for enzymatic oxygenations based on two examples, in which the pollutants nitrobenzene and 2-nitrotoluene function as substrates for nitrobenzene dioxygenase. The two data sets from Pati et al. (2014) show how different evaluation procedures for substrate and product isotope fractionation data can be applied to obtain insights into the mechanisms of pollutant oxygenation and catalytic cycle of a Rieske non-heme ferrous iron oxygenase.

2.2 Substrate isotope fractionation

Measurements of substrate isotope fractionation is the most widespread application of CSIA. Here, we consider the enzymatic dioxygenation of nitrobenzene, compound **1** in Figure 1, by nitrobenzene dioxygenase (NBDO). This reaction leads to a *cis*-dihydrodiol intermediate (**2**), which undergoes spontaneous elimination of nitrite (NO_2^-) to catechol (**3**). In laboratory experiments, we determined the dynamics of substrate and product concentrations by conventional methods, that is gas chromatography / mass spectrometry (GC/MS) for **1**, high-performance liquid chromatography (HPLC) for **3**, and a colorimetric assay for nitrite (Pati et al., 2014).

Figure 2 (a) Time-course of nitrobenzene concentration and change in C isotope signature during the dioxygenation by NBDO. (b) Non-linear correlation between change in C isotope signature and carbon isotope ratios and fraction of remaining nitrobenzene concentration (c/c_0). Note that the two y-axes, $\delta^{13}\text{C}$, and R/R_0 , in panel (b) are equivalent.

Isotope ratios of **1** and **3** are measured by GC/IRMS and LC/IRMS, respectively. A typical time-course of concentrations and $^{13}\text{C}/^{12}\text{C}$ ratios of nitrobenzene during its dioxygenation by NBDO is illustrated in Figure 2a.

2.2.1 Isotope ratios and isotope signatures

We express the isotope ratios of an element E as the ratio of heavy (^hE) and light (^lE) element concentrations, R_E , and as isotope signature, $\delta^h\text{E}$. Equations 2 and 3 show the relationship between R_E and $\delta^h\text{E}$. $\delta^h\text{E}$ is derived from the ratio of $^h\text{E}/^l\text{E}$ for the analyte and internationally accepted reference material (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014; Coplen, 2011). Eq. 4 illustrates this relationship for C isotope signatures, $\delta^{13}\text{C}$, of an analyte and the international standard (Vienna Pee Dee Belemnite, VPDB).

$$R_E = \frac{^h\text{E}}{^l\text{E}} \quad (2)$$

$$\delta^h\text{E} = \frac{(^h\text{E}/^l\text{E})_{\text{analyte}}}{(^h\text{E}/^l\text{E})_{\text{reference}}} - 1 \quad (3)$$

$$\delta^{13}\text{C} = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{analyte}}}{(^{13}\text{C}/^{12}\text{C})_{\text{VPDB}}} - 1 \quad (4)$$

Referencing of isotope ratios to standard materials not only matters for the comparison of measurements across studies and quality assurance of stable isotope laboratories but also for the quantification of isotope fractionation (Coleman & Meier-Augenstein, 2014). Different initiatives make organic reference materials available for CSIA (Schimmelmann et al., 2009, 2016) and simple spreadsheet templates facilitate correct referencing (Dunn, Hai, Malinovsky, & Goenaga Infante, 2015). The use of isotope signatures simplifies handling of data for isotope fractionation at natural isotopic abundances. For example, nitrobenzene used in this study exhibits an initial $\delta^{13}\text{C}$ -value of -0.0284 or -28.4‰ . However, nitrobenzene has a $\delta^{13}\text{C}$ -value of -0.0196 or -19.6‰ after 94% conversion by NBDO. This difference of $^{13}\text{C}/^{12}\text{C}$ ratio is small and more difficult to keep track with the R_{C} notation ($R_{\text{C},0} = 0.011047$ vs. $R_{\text{C},94\%} = 0.011147$). Note, however, that isotope signatures and isotope ratios are interconvertible and despite general recommendations by Coplen (2011), terminologies may vary slightly among different scientific disciplines.

2.2.2 Quantifying isotope fractionation

We derive kinetic isotope effects from the fractionation of stable isotopes in the substrate and the reaction progress, which is quantified as fraction of remaining substrate, c/c_0 . The calculations are shown here for C isotope fractionation of nitrobenzene during its transformation to catechol but procedures apply equally to H, N, and O isotope fractionation data. Changes of $\delta^{13}\text{C}$ -values are related to c/c_0 of nitrobenzene through the C isotope enrichment factor, ϵ_{C} , as shown in eq. 5 for the experimental data shown in Figure 2b. We calculate the ϵ_{C} -value associated with the dioxygenation of nitrobenzene by NBDO by two procedures; (a) the observational data ($\delta^{13}\text{C}$ vs. c/c_0) is modeled directly by the function given in eq. 5 and best fit parameters are obtained through non-linear regression analysis. (b) The same data ($\delta^{13}\text{C}$ vs. c/c_0) is transformed according to eq. 6 and parameters are derived by linear regression analysis. Note that normalization of isotope fractionation as $R_{\text{C}}/R_{\text{C},0}$ is used extensively in CSIA even though more accurate estimates of parameter uncertainties are obtained through linear regression of the non-normalized form (eq. 7, Scott, Lu, Cavanaugh, and Liu (2004), application examples in Hofstetter, Neumann, et al. (2008); Tobler, Hofstetter, and Schwarzenbach (2007)). Applying linear and non-linear regressions using data shown in Figure 2 results in identical ϵ_{C} -values

205 within uncertainty, that is $-3.28 \pm 0.15\%$ and $-3.25 \pm 0.13\%$ using eqs. 5 and 6, respectively.
 206 Note that all uncertainties shown in this article represent 95% confidence intervals.

$$\frac{R_C}{R_{C,0}} = \frac{\delta^{13}\text{C} + 1}{\delta^{13}\text{C}_0 + 1} = \left(\frac{c}{c_0}\right)^{\epsilon_C} \quad (5)$$

$$\ln\left(\frac{R_C}{R_{C,0}}\right) = \ln\left(\frac{\delta^{13}\text{C} + 1}{\delta^{13}\text{C}_0 + 1}\right) = \epsilon_C \cdot \ln\left(\frac{c}{c_0}\right) \quad (6)$$

$$\ln(\delta^{13}\text{C} + 1) = \epsilon_C \cdot \ln(c) + \ln\frac{\delta^{13}\text{C}_0 + 1}{c_0^{\epsilon_C}} \quad (7)$$

207 2.2.3 Apparent kinetic isotope effects

208 Apparent kinetic isotope effects, AKIEs, are derived from isotope enrichment factors with an
 209 explicit assumption made regarding the reaction mechanism. In most cases, those assumptions
 210 are made to account for the presence of atoms that do not participate in the reaction (isotopic
 211 dilution). If a substrate exhibits multiple reactive positions, heavy isotopologues will react with
 212 both rates for light and heavy isotopes (eq. 1) because the heavy isotope is located only in one
 213 of several equivalent reactive sites (intramolecular isotopic competition). The resulting isotope
 214 fractionation is then smaller than predicted from the intrinsic KIE necessitating correction for
 215 the intramolecular isotopic competition effect (Elsner, 2010). The procedure leading to AKIEs
 216 shown in eq. 8 apportions the observed isotope fractionation to reactions, in which bonds
 217 are broken and formed and results in estimates for primary KIEs. Because secondary isotope
 218 effects due to isotopic substitution at atoms that are not at the reactive site of a molecule
 219 are neglected, AKIE-values may slightly exceed those determined by alternative means (e.g.,
 220 through experiments with site-specifically labelled compounds or computed KIEs).

For the dioxygenation of nitrobenzene leading to the *cis*-dihydrodiol (**2** in Figure 1), we assume that changes of C hybridization and formation of C–O bonds reflect the rate-limiting reaction step. The ^{13}C -AKIE is calculated based on the ϵ_C -value according to eq. 8.

$$^{13}\text{C-AKIE} \approx \frac{1}{1 + n/x \cdot z \cdot \epsilon_C} \quad (8)$$

221 where ϵ_C is the isotope enrichment factor derived with eqs. 5 to 7, n is the total number of C

atoms of nitrobenzene, x is the number of reactive sites, and z denotes the number of reactive sites that are subject to intramolecular isotopic competition. For dioxygenation of nitrobenzene n is 6 while x and z depend on whether one or both C–O bonds are considered to form in the rate-limiting reaction step. If we assume that the two C–O bonds are formed simultaneously in a synchronous dioxygenation (DelMonte et al., 1997; Houk & Strassner, 1999), the number of reactive sites, x , is 2 (C-1 and C-2 or C-1 and C-6 in Figure 1), z is 1, and the ^{13}C -AKIE equals 1.011 ± 0.001 . If, however, the two C–O bonds form in subsequent step as asynchronous dioxygenation, of which only one is rate-limiting, then $x = z = 2$ and the ^{13}C -AKIE amounts to 1.023 ± 0.002 (Pati et al., 2014). The larger ^{13}C -AKIE has indeed been confirmed by hybrid quantum mechanical/molecular mechanical (QM/MM) calculations (Pati, Kohler, et al., 2016). ^{15}N -AKIE-values calculated according to eq. 8 with $n = x = z = 1$ reflects a secondary isotope effect regardless of the mechanistic assumption because the N atom is not involved directly in the reaction. The ^{15}N -AKIE-value (1.001 ± 0.001) is not significantly different from unity. A primary ^2H KIE arises if one assumes an asynchronous dioxygenation initiated at C-2. In this case, the primary ^2H -AKIEs of 1.029 ± 0.007 was obtained using the parameters $n = 5$, $x = 2$, $z = 2$.

Note that eq. 8 is a good approximation of the relationship between AKIE and ϵ , which does not hold for large H isotope fractionation due to large ^2H -KIEs (Dorer, Höhener, Hedwig, Richnow, & Vogt, 2014; Elsner, 2010). Wijker, Adamczyk, Bolotin, Paneth, and Hofstetter (2013) proposed a kinetic model, in which a set of ordinary differential equation account explicitly for all isotopomers of singly substituted heavy isotopologues of the substrate and thereby accounts for the phenomena of isotopic dilution and intramolecular isotopic competition.

2.3 Product isotope fractionation

Apparent kinetic isotope effects of enzymatic oxygenations may not only be derived from analysis of the *substrate* but are also reflected in the isotope fractionation of the oxygenated organic reaction *product(s)*. This analysis by CSIA is often based on the implicit assumptions that the isotope fractionation of the catalytic step exhibiting bonding changes is not obscured by the following steps of the catalytic cycle (e.g., product release from the enzyme) before isotopic analysis of the dissolved reaction *product* can be carried out. Here, we illustrate the information

Figure 3 (a) $\delta^{13}\text{C}$ of nitrobenzene (substrate) and catechol (final product) vs. fraction of remaining substrate, c/c_0 , during dioxygenation by NBDO. (b) $\delta^{13}\text{C}$ of 2-nitrotoluene, 3-methylcatechol, and 2-nitrobenzyl alcohol vs. fraction of remaining substrate, c/c_0 , during simultaneous dioxygenation and CH_3 -group oxidation by NBDO. Reprinted with permission from [Pati et al. \(2014\)](#), Copyright 2014, American Chemical Society.

that can be obtained from product isotope fractionation for two simple cases, in which oxygenation of a nitroaromatic substrate leads to one or two oxygenated organic products. These two cases are encountered, for example, when NBDO transforms nitrobenzene and 2-nitrotoluene to catechol and a mixtures of 3-methylcatechol and 2-nitrobenzyl alcohol, respectively (Figures 1 and 4). We focus our discussions on the derivation of C isotope enrichment factors, ϵ_C , from measured isotope signatures, $\delta^{13}\text{C}$. All ϵ_C -values can be converted to AKIEs according to eq. 8 bearing in mind the same assumptions made for the analysis of substrate isotope fractionation.

2.3.1 Simple substrate to product relationships

The C isotope fractionation of catechol formed through dioxygenation of nitrobenzene is shown in Figure 3a. All C atoms of nitrobenzene end up in catechol and the total number of heavy and light C atoms is conserved during any state of the reaction. Therefore, the initial C isotope ratio of the substrate should match the one measured in the product after complete substrate conversion. This requirement is almost met for the dioxygenation of nitrobenzene to catechol with an initial $\delta^{13}\text{C}$ -value of nitrobenzene of $-28.4 \pm 0.4\text{‰}$ as compared to a final $\delta^{13}\text{C}$ -value of $-29.6 \pm 0.2\text{‰}$ measured for catechol. The difference, however, is small and in the range of

the total instrumental uncertainty of C isotope ratio measurements [Sherwood Lollar, Hirschorn, Chartrand, and Lacrampe-Couloume \(2007\)](#).

The ϵ_C value for the transformation of nitrobenzene to catechol is determined by eq. 9. ϵ_C can then be derived by non-linear regression analysis. In eq. 9, $\delta^{13}C_P$ is the product isotope signature and $\delta^{13}C_{S,0}$ is the initial isotope signature of the substrate. The ϵ_C determined in this way for the data set shown in Figure 3b was $-4.5 \pm 0.1\%$ ([Pati et al., 2014](#)), which is slightly larger (i.e., more negative) than the value determined by substrate isotope fractionation. A comparison of approaches follows in section 2.4.

$$\frac{\delta^{13}C_P + 1}{\delta^{13}C_{S,0} + 1} = \frac{1 - (c/c_0)^{(\epsilon_C + 1)}}{1 - (c/c_0)} \quad (9)$$

$$\lim_{c/c_0 \rightarrow 1} \epsilon_C \approx \delta^{13}C_{P,0} - \delta^{13}C_{S,0} \quad (10)$$

Eq. 9 can be simplified to eq. 10 for low substrate conversion when c/c_0 is close to unity. In this case, ϵ_C equals the difference between the isotope signature of product at early stages of the reaction, $\delta^{13}C_{P,0}$, and the initial substrate isotope signature, $\delta^{13}C_{S,0}$. In practice, this approach is applied to $\delta^{13}C_P$ measured for substrate turnovers below 10% when $\delta^{13}C_P$ is still close to $\delta^{13}C_{P,0}$ ([Melander & Saunders, 1980](#)). In the example of catechol formation from nitrobenzene, we determined a ϵ_C -value of $-3.76 \pm 0.24\%$.

2.3.2 Multiple reaction products

Here, we describe a procedure based on a modified form of eq. 9 that can be used for the analysis of isotope fractionation of multiple oxygenated organic products (eq. 11). Note that this procedure does not enable one to distinguish reaction mechanisms in which two (or more products) form directly from the substrate molecule from cases, in which the reaction products result from a common intermediate. An in-depth discussion of these cases can be found in [Elsner, Chartrand, VanStone, Lacrampe Couloume, and Sherwood Lollar \(2008\)](#); [Pati, Kohler, et al. \(2016\)](#).

Figure 4 shows the NBDO-catalyzed oxygenation of 2-nitrotoluene (**4**) to 3-methylcatechol (**6**) and 2-nitrobenzyl alcohol (**7**). The two products are formed in almost equal amounts (see

Figure 4 Dioxygenation (top) and CH₃-group oxidation (bottom) of 2-nitrotoluene (**4**) catalyzed simultaneously by NBDO with the products 3-methylcatechol (**6**) and NO₂[−], which form spontaneously from a *cis*-dihydrodiol intermediate (**5**), and 2-nitrobenzyl alcohol (**7**), respectively.

data in Pati et al. (2014)). The ϵ_C -value determined for substrate isotope fractionation of 2-nitrotoluene according to eq. 5 was $-1.3 \pm 0.1\%$ (Pati et al., 2014), which is much smaller than the one for nitrobenzene. The difference is caused by two different KIEs that are responsible for the substrate isotope fractionation of 2-nitrotoluene; one KIE for the dioxygenation of aromatic C atoms and one for the oxidation of the CH₃-group. The ¹³C-AKIE for each of the two independent reactions cannot be derived from analysis of the ϵ_C -value of the substrate. Instead, isotope enrichment factors for dioxygenation and CH₃-group oxidation must necessarily be obtained from the isotope fractionation measured in the two products (Figure 3b).

We calculate a *concentration-weighted average* of the isotope signatures of 3-methylcatechol and 2-nitrobenzyl alcohol according to eq. 11, which is a modified form of eq. 9 (Elsner et al., 2008). Figure 3b shows that the “average” product isotope signature calculated with eq. 11 follows the same trend as the isotope signatures of the two products, but with a constant offset. In eq. 11, ϵ_C denotes the isotope enrichment factor of the “average” product isotope signature and D_{3-MC} is the offset between the $\delta^{13}\text{C}$ -value of 3-methylcatechol and the “average” product isotope signature.

$$\frac{\delta^{13}\text{C}_{3-MC} + 1}{\delta^{13}\text{C}_{2-NT,0} + 1} = (1 + D_{3-MC}) \frac{1 - (c/c_0)^{(\epsilon_C+1)}}{1 - (c/c_0)} \quad (11)$$

where $\delta^{13}\text{C}_{3-MC}$ is the C isotope signature of 3-methylcatechol, $\delta^{13}\text{C}_{2-NT,0}$ is the initial isotope signature of 2-nitrotoluene, c/c_0 is the fraction of remaining substrate.

Assuming that the dioxygenation and CH₃-group oxidation of 2-nitrotoluene are two inde-

pendent reactions that do not share a common intermediate, the reaction-specific isotope enrichment factor for the dioxygenation reaction of 2-nitrotoluene to 3-methylcatechol ($\epsilon_{\text{C}}^{2\text{NT}\rightarrow 3\text{-MC}}$) is derived from $D_{3\text{-MC}}$ according to eq. 12.

$$\epsilon_{\text{C}}^{2\text{NT}\rightarrow 3\text{-MC}} = D_{3\text{-MC}} + \epsilon_{\text{C}} \quad (12)$$

The identical procedure can be applied to the data for C isotope signatures of 2-benzyl alcohol instead of 3-methylcatechol. The offset of fitted $\delta^{13}\text{C}$ -values relative to the average product signatures would then correspond to $D_{2\text{-NBA}}$ and enable one to calculate $\epsilon_{\text{C}}^{2\text{NT}\rightarrow 2\text{-NBA}}$ with $\delta^{13}\text{C}$ -values of 2-nitrobenzyl alcohol with the same outcome. Using the data shown in Figure 3b, one obtains $\epsilon_{\text{C}}^{2\text{NT}\rightarrow 3\text{-MC}}$ and $\epsilon_{\text{C}}^{2\text{NT}\rightarrow 2\text{-NBA}}$ -values of $-2.0 \pm 0.4\text{‰}$ and $-0.2 \pm 0.4\text{‰}$, respectively (Pati et al., 2014).

It follows from the reasoning in section 2.3.1 that the ϵ_{C} -values for each reaction pathway can also be obtained using the approximation at low substrate turnover (eq. 10) introduced above. This procedure leads to $\epsilon_{\text{C}}^{2\text{NT}\rightarrow 3\text{-MC}}$ and a $\epsilon_{\text{C}}^{2\text{NT}\rightarrow 2\text{-NBA}}$ values of $-3.2 \pm 1.0\text{‰}$ and $-0.2 \pm 0.9\text{‰}$, respectively (Pati et al., 2014). Eqs. 10 and 11 lead to identical numbers but uncertainties are larger when the low substrate turnover approximation of eq. 10 is used.

2.4 Method comparison for deriving isotope effects

The different approaches for calculation of ϵ and AKIE-values are best benchmarked against a set of ordinary differential equations that describe the system of interest (13). In its most simple form, two differential equations for species with light and heavy isotopic substitution, respectively, are used for deriving eq. 5 and calculating ϵ and AKIE-values as shown above (Hunkeler & Elsner, 2010; Melander & Saunders, 1980). More comprehensive numerical models include several chemical species and isotopic elements as well as multiple isotopologues and isotopomers thereof (e.g., Höhener and Atteia (2014); Jin, Haderlein, and Rolle (2013); Maggi and Riley (2010); Wijker, Adamczyk, et al. (2013)). Those models do not require corrections for isotopic dilution and intramolecular isotopic competition, but applying eqs. 5 and 8 for interpretation of isotope fractionation seems more popular in large parts of the stable isotope community.

A combined evaluation of substrate and product isotope fractionation for the dioxygenation and CH₃-group oxidation of different substrates by NBDO was carried out with eqs. 13 and 14.

$$\frac{dc_{\kappa}}{dt} = \sum_j \nu \cdot k_j \cdot c_{\kappa} \quad (13)$$

$$\epsilon_j = \frac{h k_j}{l k_j} - 1 \quad (14)$$

where c_{κ} is the concentration of species κ . Here, we considered one heavy and one light isotope for each substrate and product. k_j is the rate constant for reaction j , and ν is the stoichiometric coefficient of reaction j . The ratio of reaction rate constants for heavy and light species, which relates to the enrichment factor of reaction j (eq. 14), was estimated by fitting the model to measured concentrations and isotope signatures of all available species. We used Aquasim (Reichert, 1994) to implement the model and estimate enrichment factors but many other software packages (e.g. R (R Core Team, 2014), Matlab, etc.) are equally suited.

Table 1 shows a compilation of all ϵ_C -values derived for nitrobenzene dioxygenation as well as 2-nitrotoluene dioxygenation and CH₃-group oxidation by NBDO (Figures 1-4, Pati et al. (2014)). In the case of nitrobenzene dioxygenation to catechol, 4 differential equations according to eq. 13 were considered for light and heavy C isotopologues of nitrobenzene as well as light and heavy C isotopologues of catechol. The resulting ϵ_C -value derived according to eq. 14 was $-4.1 \pm 0.2\%$ (Pati et al., 2014), which is in between the ϵ_C -values derived with eqs. 6 and 9, respectively (see Table 1). For the simultaneous dioxygenation and CH₃-group oxidation of 2-nitrotoluene to 3-methylcatechol and 2-nitrobenzyl alcohol, the number of differential equations according to eq. 13 was 6 and the model resulted in reaction-specific C isotope enrichment factors of $\epsilon_C^{2\text{-NT} \rightarrow 3\text{-MC}} = -2.5 \pm 0.2\%$ and $\epsilon_C^{2\text{-NT} \rightarrow 2\text{-NBA}} = -0.4 \pm 0.2\%$ (see Table 1 and Pati et al. (2014)).

All approaches for deriving enrichment factors, and thus AKIE-values, gave consistent results for both nitrobenzene and 2-nitrotoluene dioxygenation by NBDO and the estimated parameters match within their 95% confidence intervals (see Table 1). Besides providing a general and powerful internal check on AKIE accuracy, there are, however, two advantages for combining substrate and product isotope fractionation for parameter estimation. First, uncer-

tainties associated with reaction-specific enrichment factors were smaller. Second, in the case of 2-nitrotoluene, the concentration-weighted average of the two reaction-specific enrichment factors (i.e. of $\epsilon_C^{2\text{-NT}\rightarrow 3\text{-MC}}$ and $\epsilon_C^{2\text{-NT}\rightarrow 2\text{-NBA}}$) was in best agreement with the substrate isotope enrichment factor ($\epsilon_C^{2\text{-NT}}$).

Table 1 Compilation of C isotope enrichment factors derived with different evaluation methods and isotope fractionation data for NBDO-catalyzed reactions of nitrobenzene (NB) and 2-nitrotoluene (2-NT) to catechol (CAT), 3-methylcatechol (3-MC), and 2-nitrobenzyl alcohol (2-NBA), respectively. ^a Original data can be found in [Pati et al. \(2014\)](#).

Isotope fractionation of	ϵ_C^{NB}	$\epsilon_C^{\text{NB}\rightarrow\text{CAT}}$	$\epsilon_C^{2\text{-NT}}$	$\epsilon_C^{2\text{-NT}\rightarrow 3\text{-MC}}$	$\epsilon_C^{2\text{-NT}\rightarrow 2\text{-NBA}}$
substrate (eq. 6)	-3.7 ± 0.2		-1.3 ± 0.1		
product (eq. 9)		-4.5 ± 0.1			
product (eq. 10)		-3.6 ± 0.6		-3.2 ± 1.0	-0.2 ± 0.9
product (eqs. 11-12)				-2.0 ± 0.4	-0.2 ± 0.4
substrate and product (eqs. 13-14)		-4.1 ± 0.2		-2.5 ± 0.2	-0.4 ± 0.2

^a All values given in ‰ with uncertainties as 95%-confidence intervals

2.5 Multidimensional isotope fractionation analysis

If intrinsic KIEs of enzymatic bond cleavage reactions are masked by non-isotopic steps of catalytic cycles and independent information, such as from computational theory, is unavailable, ambiguities of AKIE interpretations can be circumvented with CSIA of two (or more) isotopic elements. This so-called multi-element isotope fractionation analysis is based on the linear correlation of the observed isotope fractionation of different elements of a compound. Slopes of such correlations will be independent of masking because all elements in the breaking bond will be affected by masking in the same way. The formalism is shown for C and H isotope fractionation in eq. 15 and illustrates how the correlation slope, $\Lambda_{\text{H/C}}$, is related to the intrinsic KIEs ([Elsner, 2010](#)).

$$\Lambda_{\text{H/C}} = \frac{\Delta\delta^2\text{H}}{\Delta\delta^{13}\text{C}} \approx \frac{\epsilon_{\text{H}}}{\epsilon_{\text{C}}} \approx \frac{(n/x)_{\text{H}}}{(n/x)_{\text{C}}} \cdot \frac{{}^2\text{H-KIE} - 1}{{}^{13}\text{C-KIE} - 1} \cdot \frac{1 + {}^{13}\text{C-KIE} \cdot (z_{\text{C}} - 1)}{1 + {}^2\text{H-KIE} \cdot (z_{\text{H}} - 1)} \quad (15)$$

An example for multi-element isotope fractionation analysis is shown in Figure 5 for the $\delta^2\text{H}$ vs $\delta^{13}\text{C}$ -values of 2-, 3-, and 4-nitrotoluene during their transformation by NBDO ([Pati, Kohler, et al., 2016](#)). Both ϵ -values and AKIEs differ significantly for these three substrates despite a

Figure 5 $\delta^2\text{H}$ vs $\delta^{13}\text{C}$ values of 2-, 3-, and 4-nitrotoluene during dioxygenation by NBDO. Similar correlation slopes of C and H isotope fractionation (eq. 15) are independent evidence for a common reaction mechanism. Reprinted with permission from Pati, Kohler, et al. (2016), Copyright 2016, American Chemical Society.

common reaction mechanism. For example, ϵ_{H} -values for 3- and 4-nitrotoluene dioxygenation by NBDO were $-2.6 \pm 1.6\text{‰}$ and $-5.5 \pm 2.3\text{‰}$, respectively. Nevertheless, slopes of the linear regressions of $\delta^2\text{H}$ vs $\delta^{13}\text{C}$, $\Lambda_{\text{H/C}}$, are identical within uncertainties ($1.5 \pm 2.2\text{‰}$ for 3-nitrotoluene and $2.1 \pm 0.6\text{‰}$ for 4-nitrotoluene). Identical $\Lambda_{\text{H/C}}$ -values confirm that the observable C and H isotope fractionation originate, for both substrates, from the dioxygenation of aromatic carbon atoms (Figure 1). In fact, our most recent work suggests that masking occurs through rate-limiting O_2 activation and that contributions of masking are substrate dependent (Pati, Kohler, et al., 2016). Note that eq. 15 is a good approximation but not valid for reactions associated with large primary ^2H -KIE (Dorer et al., 2014; Wijker, Adamczyk, et al., 2013).

3 Experimental approaches for determining isotope fractionation during oxygenation reactions

3.1 Experiment design and sampling strategies

The purpose of the experiments described below is to obtain samples for concentration and isotope ratio measurements of substrate, products, and co-substrate (O_2). In contrast to experiments for assessment of concentration dynamics, such as those used for determining kinetic

parameters of enzymatic reactions, samples for stable isotope analysis at natural abundance requires: (i) large amounts of analytes, and (ii) an adequate extent of substrate conversion and product formation concomitant with strong isotopic enrichment (or depletion) to determine isotope fractionation parameters reliably. For substrate isotope fractionation, large extents of conversion of $> 90\%$ are preferred whereas substrate conversion $< 10\%$ is suited for quantifying product isotope fractionation.

To obtain sufficient amounts of analytes in a sample, one can either use high initial substrate concentrations provided that no substrate inhibition occurs or large sample volumes for subsequent automated analyte enrichment by solid-phase microextraction (SPME, see section 4). We identified two separate approaches, with which samples with $> 90\%$ substrate conversion can be generated. (a) In “continuous transformation assays”, the reaction progress is controlled by stopping a reaction, for example through acidification or analyte extraction. Those experiments can be conducted in one large volume reactor (> 100 mL), from which adequate sample volumes are withdrawn at different time points. Alternatively, experiments are run in multiple reactors of smaller volumes (< 30 mL), which are sacrificed at specific time points. (b) In the “limited turnover assays”, the extent of transformation is controlled by limiting the amount of available cofactors (e.g., NADH). Both approaches were used to study the oxygenation of nitroaromatic compounds and three example procedures are illustrated below for isotopic analyses of substrates, products, and co-substrate (O_2).

3.2 Enzyme assays for isotope analysis of substrates

Isotope effects on the oxygenation of (nitro)aromatic substrates, such as nitrobenzene, 2,6-dinitrotoluene, and naphthalene, by nitrobenzene dioxygenase (NBDO) can be obtained in assays with the purified, three-component enzyme system, as illustrated here. Those experiments follow the “limited turnover” approach illustrated in Figure 6 (left-hand side, “1. Substrate isotope analysis”) but alternative approaches with “continuous transformation assays” (Figure 7) are also possible when working with whole cell cultures and cell extracts (Pati et al., 2014; Pati, Kohler, et al., 2016). Using purified enzymes instead of whole cells of *E. coli* clones can be required to avoid interferences, for example, from substrate loss through sorption to cell material when working at high substrate concentrations (up to 1 mM) and high cell densities.

The activity of NBDO depends on the type of buffer used, concentration of dissolved Fe^{2+} , and initial substrate concentration. Consequently, the most practical approach to ensure adequate substrate conversion are experiments at high enzyme concentrations ($0.3\ \mu\text{M}$ oxygenase) and addition of dissolved Fe^{2+} . Because substrate dioxygenation is too fast to be quenched at selected time points under these conditions, substrate turnover was limited by adding different sub-stoichiometric amounts of NADH to different reactors. Purification procedures of the three enzyme components of NBDO (reductase, ferredoxin, and oxygenase) are reported elsewhere [Parales et al. \(2005\)](#).

Procedure

1. Mix the three purified enzyme components of NBDO in 50 mM MES buffer at pH 6.8 containing $100\ \mu\text{M}$ $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ to final concentrations of $0.3\ \mu\text{M}$ reductase, $3.6\ \mu\text{M}$ ferredoxin, and $0.3\ \mu\text{M}$ oxygenase.
2. Dissolve the nitroaromatic substrate (e.g., nitrobenzene) in MES buffer and add an appropriate volume to the enzyme mixture to give an initial substrate concentration of $200\ \mu\text{M}$.
3. Fill nine 10-mL serum vials with 5 mL buffer solution containing enzymes and substrate and cap them with Viton rubber stoppers. The stoppers limit the loss of volatile substrate, however, the rubber material should be tested to avoid sorptive losses of the substrate into the stoppers.
4. Amend all but one vial, which is used as a control as is, with different amounts of the co-factor NADH ranging from $100 - 980\ \mu\text{M}$.
5. Incubate all reactions at 30°C while shaking at 100 rpm for 30 min. Thereafter, all NADH should be oxidized and the transformation of the substrate terminated.
6. Store vials at 4°C until chemical and isotopic analyses. The concentrations of substrate and reaction products should be determined immediately by high-performance liquid chromatography (HPLC). Analysis of C, H, and N isotopic ratios of the substrate by GC/IRMS is described in section 4.

Limited Turnover Assays

Figure 6 Schematic view of experimental procedures for “limited turnover assays” where the extent of substrate turnover and product formation is controlled through the sub-stoichiometric addition of NADH. Procedures for *substrate* isotope analysis, for example of nitrobenzene, and for the *co-substrate*, that is aqueous O₂, are shown on the left- and right-hand side, respectively.

3.3 Whole cell assays for C isotope analysis of organic reaction products

Procedures for the quantification of isotope fractionation in the organic products of enzymatic oxygenation reactions take into account that these polar compounds are often only amenable to isotopic analysis by LC/IRMS. Isotopic analysis by LC/IRMS was restricted to C isotope analysis until recently (Godin & McCullagh, 2011; Zhang, Kujawinski, Jochmann, & Schmidt, 2011) but may be extended to N in the near future (Federherr et al., 2016). For these purposes, suitable experimental systems include enzyme assays and whole cells assays of *E. coli* clones because in both systems the products are not transformed further. Products of substituted nitroaromatic compound oxygenation, that is substituted catechols, phenols, and benzyl alcohols, however, are not necessarily stable in the different experimental systems. In particular catechols, are easily oxidized abiotically. We found that oxygenation products were stable in whole cell assays with *E. coli* clones expressing NBDO (Pati et al., 2014). Note that addition of acid or base to stop reactions always resulted in artifacts and product concentration were substoichiometric. The procedure described below follows the “continuous transformation assay” with few reactors (Figure 7) and can also be applied to determine the substrate isotope fractionation in assays with whole cells of *E. coli* clones as well as with pure strains of environmental microorganisms and their crude cell extracts.

There are two major differences between assays for product and substrate analysis shown in Figure 7. First, in assays for nitroaromatic compound analysis, reactor and sample volumes are usually 4 – 10 times larger than for analysis of the hydroxylated products. The difference is due to the requirements for instrumental analysis (see GC/IRMS vs LC/IRMS in section 4). Second, samples for analysis of nitroaromatic compounds can be acidified and thus stored at 4°C for days to a few weeks whereas samples for analysis of the hydroxylated products cannot be preserved and need be analyzed immediately.

Procedure

1. Grow and induce cultures of *E. coli* clones according to standard methods (overnight growth in nutrient rich medium) and dilute cell to appropriate densities (3 – 9 g/L) with phosphate buffer (40 mM, pH 7.0).
2. Fill two 25-mL serum flasks with 20 mL cell suspension and cap them with Viton rubber

Continuous Transformation Assays

Figure 7 Schematic view of experimental procedures for “continuous transformation assays”. The extent of substrate turnover and product formation is controlled by withdrawing samples from large reactors at pre-defined time points. Procedures for *substrate* and *product* isotope analysis are shown on the left- and right-hand side, respectively.

470 stoppers. An additional serum flask, that only contains phosphate buffer, should be run
471 simultaneously as a control.

- 472 3. Initiate reactions by adding small volumes of a nitroaromatic substrate in a methanolic
473 solution resulting in initial substrate concentrations of 0.5 – 1.0 mM.
- 474 4. After vigorous shaking, withdraw the first sample of 1.5 mL immediately with a glass
475 syringe. Subsequently, incubate the reactors at 30°C while shaking at 200 rpm.
- 476 5. Take additional 6 – 8 samples after time intervals that enable sufficient isotope fraction-
477 ation of the target analyte. Withdraw samples with gas tight glass syringes through the
478 stopper after injecting an equivalent volume of air into the reactors. In most experiments,
479 90% substrate conversion was achieved after 8 – 10 h. However, in reactors with larger
480 volumes reactions were often slower due to O₂ limitation.
- 481 6. Transfer samples into 1.5-mL plastic tubes and centrifuge them at 13.000 rpm for 5 min.
482 The supernatant should be transferred into HPLC vials and stored at 4°C until concen-
483 tration (by HPLC) and isotope analysis (see LC/IRMS in section 4).

484 3.4 Enzyme assays for O isotope analysis of aqueous O₂

485 Oxygen isotope effects for the activation of O₂ by NBDO can be obtained from the analysis
486 of O isotope fractionation in aqueous O₂. [Pati, Bolotin, et al. \(2016\)](#) developed a procedure
487 for “limited turnover assays” with purified enzyme component similar to the one described in
488 the first example for substrate isotope fractionation (Figure 6, right-hand side). The major
489 differences between enzyme assay for co-substrate vs. substrate isotope analysis are that (i)
490 vials need to be filled with solution without headspace, (ii) one 10-mL reactor is sacrificed for
491 every measurement of O isotope composition (no replicate measurements possible), and (iii)
492 O₂ concentrations are monitored continuously during the reaction with an optical micro-sensor.
493 Details on the sampling procedure and reaction progress monitoring will be shown below in
494 section 4.3 and Figure 9.

Procedure

1. Completely fill 5 – 10 10-mL glass vials with MES buffer (50 mM, pH 6.8) containing 0.15 μ M reductase, 1.8 μ M ferredoxin, 0.15 μ M oxygenase, 100 μ M $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, and 300 – 500 μ M of a nitroaromatic substrate. Enzymes were purified according to [Pati et al. \(2014\)](#).
2. Close the vials with butyl rubber stoppers and aluminum crimp caps and ensure that no air bubbles are entrapped within the reactors.
3. Insert a fiber-optic oxygen microsensor (PreSens Precision Sensing GmbH, Germany; [Pati, Bolotin, et al. \(2016\)](#)) with a stainless needle through the stopper for monitoring O_2 concentrations.
4. Initiate the reaction by adding different amounts of NADH (50 – 250 μ M) with a syringe through the stopper to all but one reactor (control). The reaction solution should be stirred slightly during the reaction with a magnetic stir bar inside the vial.
5. Monitor O_2 concentrations until a stable value is reached. The decrease in O_2 concentration was usually proportional to the amount of NADH added.
6. Remove the microsensor from the vessel and prepare the reactor for analysis. Due to potential leaking of ambient O_2 into the vials, measurements of O isotope composition by GC/IRMS should be performed on the same day the experiment is conducted (see section 4.2 for details).

4 Instrumentation for stable isotope analysis by isotope ratio mass spectrometry

4.1 Instrumental strategies

Compound-specific isotope analysis relies on specialized isotope ratio mass spectrometers with sector field mass analyzers for the measurement of stable isotope ratios at natural isotopic abundance. Details on instrumentation and functioning of isotope ratio mass spectrometers can be found in many compilations such as [Amrani, Sessions, and Adkins \(2010\)](#); [Bernstein](#)

et al. (2011); Eiler (2013); Elsner et al. (2012); Gelman and Halicz (2010); Jochmann and Schmidt (2012); Said Ahmad et al. (2017); Sessions (2006); Zakon, Halicz, and Gelman (2014); Zakon, Halicz, Lev, and Gelman (2016). Here we highlight only the most important features of particular relevance for studying isotope effects of enzyme-catalyzed reactions.

Due to the instrumental requirement to focus isotopic ratio measurements on a few small molecules and the fact that analytes are present as mixture of compounds, analytes need to be converted, in most cases, into sample gases through the use of chemical reaction interfaces under continuous flow conditions. GC/IRMS devices are most versatile and enable one to measure C, H, N, and O isotope ratios of (semi-)volatile organic compound because analytes can be converted to CO₂, H₂, N₂, and CO through oxidation, reduction, and pyrolysis. A simplified scheme for this instrumental setup is shown in Figure 8a. Most applications are based on commercially available standard instrumentations, but customized reactors may be required for the chemical conversion of highly oxidized compounds (Gehre et al., 2015; Nijenhuis, Renpenning, Kümmel, Richnow, & Gehre, 2016; Renpenning, Hitzfeld, et al., 2015; Renpenning, Kümmel, Hitzfeld, Schimmelmann, & Gehre, 2015) or complexing agents (Spahr et al., 2013). On the other hand, LC/IRMS devices apply wet chemical oxidation interfaces of analytes in aqueous solutions (Federherr et al., 2016; Godin & McCullagh, 2011; Krummen et al., 2004; Zhang et al., 2011). This approach is largely restricted to measuring C, and very recently, N isotope ratios.

The instrumental setup is selected primarily based on the physical-chemical properties of the analyte molecules. GC/IRMS is suitable for (semi-)volatile organic compounds extracted from water or injected in organic solvents as well as for gases. Conversely, LC/IRMS enables one to analyze polar and ionic organic compounds exclusively from aqueous solutions. Because enzyme assays are generally conducted in aqueous solution, direct injection of aqueous sample to an LC/IRMS system is most straightforward. For GC/IRMS measurements, analytes need to be extracted into organic solvents, or adsorbed to solid phases, for example by solid phase (micro)extraction, and by purge and trap concentrators (Zwank, Berg, Schmidt, & Haderlein, 2003). Many of these steps can be automated. Details for sample preparation, instrumental settings, and data evaluation are illustrated below in three practical examples related to the three assays described in section 3.

Figure 8 Simplified schematic view of instrumentation for compound-specific isotope analysis by gas and liquid chromatography / isotope ratio mass spectrometry (adapted from [Elsner et al. \(2012\)](#)). (a) Instrumental setup for measurement of C, H, N, O isotope ratios in organic compounds after chemical conversion to small molecule gases by oxidation, reduction, and pyrolysis. Examples are shown here for determination of $\delta^{13}\text{C}$ -values. “M” would correspond to H_2 , N_2 , and CO for measurements of $\delta^2\text{H}$, $\delta^{15}\text{N}$, $\delta^{18}\text{O}$, respectively. (b) Instrumental setup for isotope ratio measurements without chemical conversion interface as exemplified for $\delta^{18}\text{O}$ of O_2 . Note that gas purification devices used to remove, for example, H_2O and S-containing gases, are not shown here for simplicity.

Finally, the isotope ratios of a series of gases and organic vapors is accessible by continuous-flow isotope ratio mass spectrometry without the use of chemical conversion interfaces. This approach is chosen here for measurement of $^{18}\text{O}/^{16}\text{O}$ ratios of O_2 as shown in Figure 8b because O_2 isotopologues can be ionized and measured directly in a isotope ratio mass spectrometer ([Pati, Bolotin, et al., 2016](#)). The same strategy is pursued, for example, for analysis of halogen isotopes in organic compounds where isotopologue ratios are determined in fragments of the target analyte ([Bernstein et al., 2011](#); [Elsner & Hunkeler, 2008](#); [Zakon et al., 2016](#)).

4.2 Substrate isotope analysis by GC/IRMS

C, H, and N isotope ratios of nitroaromatic compounds can be measured by GC/IRMS after solid phase microextraction (SPME) from different matrices and similar analytical procedures apply for aminoaromatic compounds and alkylated and halogenated benzenes and phenols ([Berg, Bolotin, & Hofstetter, 2007](#); [Ratti, Canonica, McNeill, Bolotin, & Hofstetter, 2015](#); [Ratti,](#)

Canonica, McNeill, Erickson, et al., 2015; Skarpeli-Liati et al., 2011; Wijker, Bolotin, Nishino, Spain, & Hofstetter, 2013; Wijker, Kurt, et al., 2013; Wijker, Zeyer, & Hofstetter, 2017). The range of analyte concentrations ($3 - 200\mu\text{M}$) in samples from enzyme assays makes SPME the ideal method for introducing analytes into the gas chromatograph. SPME fibers with different sorbate materials are commercially available for a range of organic compounds. Analytes are desorbed from the solid phase during a bake-out in the heated injector of the GC resulting in the transfer of analytes onto an analytical column. A GC compatible autosampler device can perform the whole extraction and desorption procedure automatically. The analyte(s) of interest are separated on an appropriate GC column from any other compounds that were extracted onto the SPME fiber so that for each compound a separate CO_2 , N_2 , or H_2 peak can be measured. The isotopic composition of the nitroaromatic substrates is derived from peak area ratios of heavy and light isotopologues of the corresponding analyte gas peaks. It is best practice to dilute samples for GC/IRMS analysis to a range of concentrations in which isotope ratios can be measured without mass bias from non-linear detector responses (Jochmann, Blessing, Haderlein, & Schmidt, 2006; Sherwood Lollar et al., 2007).

4.2.1 Sample preparation procedure

1. Dilute samples from enzyme assays with different degree of substrate conversion with buffer to a specific substrate concentration that is ideal for GC/IRMS analysis. Such ranges depend on the element studied, e.g., $2\mu\text{M}$ for C and $10\mu\text{M}$ for H isotope analysis of nitrobenzene. Note that different instrumental runs are required for the various elements.
2. Adjust sample pH to 7.0 with NaOH or HCl to prolong the lifetime of the SPME fibers and to obtain analytes as neutral species.
3. Filter samples with precipitates or particulate matter to prevent clogging and distortion of the SPME fibers. Additional test should be performed to ensure that filtration does not change the isotopic composition of the analyte.
4. For each sample taken from an assay, prepare three identical analysis samples by mixing 1.3 mL diluted sample solution with 0.303 mg NaCl (4.0 M final concentration) to increase adsorption efficiency of nitroaromatic compounds onto the SPME fiber.

5. In addition to assay samples, prepare control samples containing the analyte(s) of interest in buffered solutions or water at the same analyte and NaCl concentrations. Analyzing control samples can reveal whether isotope fractionation occurred during sample preparation.
6. Additional samples containing in-house isotope standards, compounds with known isotopic composition that have been calibrated against certified standard material, need to be measured to ensure accuracy (Werner & Brand, 2001).

4.2.2 Instrumental parameters

The automated SPME extraction method is performed by a GC autosampler. Analysis vials are 1.5-mL glass vials with magnetic screw or crimp caps. The first sample is transported from the sample rack into an agitator that heats and agitates the vials during analyte extraction. For SPME of nitroaromatic compounds the fiber is immersed into the sample solution for 45 min at an agitator temperature of 40°C (Berg et al., 2007; Wijker, Bolotin, et al., 2013). Thereafter, nitroaromatic compounds are desorbed from the SPME fiber in the injector of the gas chromatograph for 5 min at 270°C. Nitroaromatic compounds are separated by means of gas chromatography using standard columns and temperature programs. Complete base-line separation of the analyte(s) is a prerequisite for GC/IRMS analysis. For C isotope analysis, the interface between GC and IRMS consists of a combustion oven operated at 1000°C. The setup for N isotope analysis requires an additional reduction step for conversion of NO_x species to N₂ at 650°C (Berg et al., 2007; A. Hartenbach, Hofstetter, Berg, Bolotin, & Schwarzenbach, 2006; A. E. Hartenbach et al., 2008). H isotope analysis is carried out with a pyrolysis reactor at 1200°C (Wijker, Adamczyk, et al., 2013). The ion masses that are recorded by the Faraday cups in the mass analyzer are m/z 2 and 3 for H isotope analysis, m/z 28, 29, and 30 for N isotope analysis, as well as m/z 44, 45, and 46 for C isotope analysis.

4.2.3 Sample sequence and data evaluation

To compensate for signal drifts and offsets, control samples containing unreacted analyte should be dispersed throughout the sequence of measurements. Ideally, the analyte of interest is available as a calibrated in-house standard. If the isotopic composition of the analyte has not been

calibrated externally, control samples should be amended with an additional compound of known isotopic composition. A typical sequence includes 3 replicate vials with control samples followed by 3 replicate vials of 2-3 assay samples (total of 6-9 injections). Thereafter, another 3 replicate vials with control samples are measured followed by 3 replicate vials of 2-3 assay samples, and, finally, another 3 replicate vials with control samples. With a run time of approximately 50 min per sample, this sequence takes 18 – 23 hours. An experiment with 9 assay samples requires 6 full days of GC/IRMS instrument time to determine C, H, and N isotope fractionation. As a quality control within and between measurement sequences, the control samples allow to detect signal drift over time as well as off-sets, e.g., due to the SPME extraction procedure. Data can be reconciled using spreadsheet templates such as described by [Dunn et al. \(2015\)](#).

4.3 Oxygen isotope analysis of aqueous O₂ by GC/IRMS

For determining O isotope ratios in aqueous O₂ a similar GC/IRMS setup as for substrate isotope analysis is used. The major differences are (i) mode of injection, (ii) operation of the conversion interface, and (iii) data evaluation strategy. O₂-containing gases can be analyzed with the isotope ratio mass spectrometer without chemical conversion. Major challenge for using a GC/IRMS are potential contamination of samples before and during analysis with atmospheric O₂. Because atmospheric O₂ has a constant isotopic composition ([Barkan & Luz, 2005](#)), small amounts of contamination from ambient O₂ can be corrected for with a blank sample subtraction. With this approach, accurate $\delta^{18}\text{O}$ -values can be determined for aqueous O₂ concentrations ranging from 20 – 250 μM (0.6 – 8 mg/L) ([Pati, Bolotin, et al., 2016](#)).

4.3.1 Sample preparation procedure

1. To quench the reaction, create a headspace in every 10-mL assay vial by manually replacing 3 mL reaction solution with N₂ gas while holding the vials upside down (see [Figure 9](#)). The 3 mL excess solution can be used to quantify substrate consumption and product formation to establish reaction stoichiometries.
2. Once all vials contain a headspace, place them upside down on an orbital shaker for 30 min at 200 rpm to facilitate the transfer of O₂ into the gas phase.
3. Afterwards, place samples onto the autosampler and automatically inject aliquots of the

Figure 9 Procedure for extraction of aqueous O₂ from “limited turnover assays” containing NBDO in aqueous solution. See section 3.4 and sample preparation procedure 4.3.1 for details. An O₂ microsensor (optode) is used for continuous monitoring of O₂ consumption during the experiment. $\delta^{18}\text{O}$ is measured in O₂ of the N₂ headspace.

headspace from the same vials, in which the enzyme assays were run. Because of a danger of small contaminations of the vials with ambient air, only one injection should be made per vial.

4. Prepare control samples containing air-equilibrated water and blank samples containing O₂-free water in the same way as assay samples (make headspace manually and shake for phase transfer, see Figure 9). O₂-free water can be produced by purging water with N₂ gas for 3 h and subsequently filling 10-mL vials headspace free in an anaerobic glove box under a N₂ atmosphere (O₂ < 0.1 ppm). Blanks should undergo the same sample preparation scheme as assay and control samples (including headspace creation).

4.3.2 Instrumental parameters

After equilibration of gas and water phases in the vial, the O₂ concentration in the headspace should range between 50 – 600 μM (2 – 20 mg/L) or 0.12 – 1.5 vol-%. The autosampler injects 250 μL of the headspace with a large-volume headspace syringe into the split/splitless injector of the gas chromatograph. Before piercing the septum, the syringe should be flushed for 1 min

Figure 10 $\delta^{18}\text{O}$ of aqueous O_2 after partial O_2 conversion during the dioxygenation of nitrobenzene by NBDO using “limited turnover assays” using the procedure shown in Figure 9.

with N_2 gas and automated procedures can be programmed with an autosampler. Setting the injector temperature up to 200°C will ensure a better reproducibility and precision of measured isotope ratios compared to having the injector operated at ambient temperature (Pati, Bolotin, et al., 2016). Separation of O_2 from other gases, in particular N_2 is achieved by means of gas chromatography with a molecular sieve PLOT column ($5\text{\AA}$, $30\text{m} \times 0.32\mu\text{m}$ I.D.) at a constant oven temperature of 30°C . The combustion reactors in the interface between gas chromatograph and mass spectrometers are turned off (ambient temperature) and O_2 isotopologues can be analyzed directly with recording ion masses m/z of 32, 33, and 34. $^{18}\text{O}/^{16}\text{O}$ isotope ratios of O_2 are derived from peak area ratios at m/z 32 and 34. $^{17}\text{O}/^{16}\text{O}$ ratios can, in principle, be derived from peak area ratios at m/z 32 and 33, however, special multipliers for the middle Faraday cup are required to do so accurately.

4.3.3 Sample sequence and data evaluation

The measurement sequence for O isotope ratio analysis is similar to the one described for substrate isotope analysis above with the exception that only one measurement per assay sample is performed. As an example, the fractionation of O isotopes during dioxygenation of nitrobenzene by NBDO is shown in Figure 10. Measurements lasted 6 min per sample. Triplicates of control samples containing air-equilibrated water should be injected at the beginning and the end of the sequence and, depending on the sequence length, also in regular intervals within the

sequence. Three blank samples (O₂-free water) should be injected at the end of the sequence to ensures that the maximum amount of potential contamination with ambient air is captured for the blank correction (eq. 16). Control samples allow for corrections of signal drifts within a sequence as well as between sequences. One O₂ reference gas was used for calibrating isotope signatures of O₂ and the use of a second isotope standard with a different O isotope signature could improve accuracy of measurements. The procedure for correcting for ambient O₂ contamination is described in detail in Pati, Bolotin, et al. (2016) and is performed according to eq. 16.

$$\delta^{18}\text{O}_{\text{corr}} = \frac{\delta^{18}\text{O}_{\text{meas}} \cdot A_{\text{meas}} - \delta^{18}\text{O}_{\text{blank}} \cdot A_{\text{blank}}}{A_{\text{meas}} - A_{\text{blank}}} \quad (16)$$

where $\delta^{18}\text{O}_{\text{meas}}$ and A_{meas} are the measured isotope signature of O₂ and m/z 32 peak area in an assay sample, while $\delta^{18}\text{O}_{\text{blank}}$ and A_{blank} are the averages of the same parameter in the three blank samples. $\delta^{18}\text{O}_{\text{corr}}$ is the blank-corrected isotope signature for the given assay sample, which is used for further data evaluation.

4.4 Product isotope analysis by LC/IRMS

Measuring isotope ratios of oxygenation products by LC/IRMS circumvents the need for derivatization. Injection volumes are larger than with a GC/IRMS. With a 100 μL syringe, the lowest concentration for reliable isotope analysis for catechols and nitrobenzyl alcohols was approx. 30 μM and thus 10 times higher than for C isotope analysis of nitroaromatic compounds by GC/IRMS.

Sample preparation procedure

1. Filter assay samples containing particles or precipitates prior to analysis by LC/IRMS to protect the column.
2. Adjust the pH of assay samples to ≤ 7.0 to ensure that the hydroxylated analytes are protonated (Pati et al., 2014).
3. Fill 500 μL of each assay sample into analysis vials so that multiple injections can be

made from the same vial.

4. Prepare control samples by dissolving analytes in an appropriate buffer solution.

As with GC/IRMS, measurements should be performed with constant amounts of analytes in all samples (including controls). With LC/IRMS, however, constant amounts of injected analytes can be achieved by adjusting injection volumes instead of diluting samples for analysis by GC/IRMS. If multiple analytes are present in the same sample, the compounds need to be present at comparable concentrations or repeated measurements with different injection volumes should be performed. Measurement sequences and strategies for isotopic calibration and referencing are analogous to those for substrate isotope analysis by GC/IRMS.

4.4.1 Instrumental parameters

Organic analytes are separated on an appropriate column by LC and converted to compound-specific CO₂-peaks in the wet oxidation interface before entering the isotope ratio mass spectrometer. The principle of the chemical conversion interface requires that organic solvent cannot be used as eluents for chromatographic separation and eluents consist of aqueous solution or inorganic buffers. Ion exchange chromatography and high-temperature reversed-phase chromatography are valuable alternatives (Godin & McCullagh, 2011; Zhang et al., 2011). In fact, high-temperature reversed-phase chromatography led to equal or better separation of substituted catechols and nitrobenzyl alcohols from each other and the corresponding nitroaromatic substrates than conventional HPLC with organic solvents.

Temperature gradients from 30 to 160°C can be applied to change the retention of analytes on the analytical column (Zhang et al., 2011). Here, we used a 10 mM phosphate buffer solution at pH 2.5 to minimize the amount of CO₂ dissolved in our solutions. The effluent of the LC column (0.5 mL/min) is channelled into a wet chemical oxidation interface. The reactor in the oxidation interface is heated to 100°C and supplied continuously with phosphoric acid and Na₂S₂O₈. Analytes need to be base-line separated from all interfering compounds, so that compound-specific CO₂ peaks are formed in the interface. The CO₂ is subsequently stripped from the eluent when flowing through gas-permeable membranes and introduced with a He stream into the isotope ratio mass spectrometer. The IRMS settings are identical to those of C isotope analysis by GC/IRMS, CO₂ background levels, however, are considerably higher with LC/IRMS. It is

therefore very important that CO₂ background levels do not change during elution of the target analyte peak. This issues can be circumvented by evaluating different temperature programs accordingly and testing reference compounds with known isotopic composition as mixtures in the LC/IRMS and through direct injection of the pure compounds into the oxidation interface. Note that the organic matter contents of the sample, for example, enzymes, cell components etc. can compromise the analysis if that material was converted into CO₂. It may be advantageous to use columns that retain the analytes sufficiently long so that for the first 1 – 2 min after injection the eluent with high organic backgrounds can bypass the wet oxidation interface.

5 Summary and conclusion

Enzymatic oxygenations are among the most important biodegradation reactions in the environment, and they also contribute to initial transformation of numerous organic pollutants through co-metabolic oxygenations. Despite its relevance, knowledge of enzymatic mechanisms of O₂ activation and oxygenation of organic pollutants are scarce (e.g., [Wijker et al. \(2015\)](#)). It is therefore very challenging to assess whether such processes will happen unless the stable isotope fractionation of soil and water pollutants is understood in greater detail. The theoretical, experimental, and instrumental procedures illustrated here enable a researcher to investigate isotope enrichment factors and kinetic isotope effects that come along with biodegradation of many persistent organic pollutants. Because oxygenases are widely involved in oxidative biodegradation and -transformation and exhibit a wide spectrum of possible substrates, the presented methods can be applied beyond the compounds and enzymes discussed here.

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References

- Aelion, C. M., Höhener, P., Hunkeler, D., & Aravena, R. (2010). *Environmental Isotopes in Biodegradation and Bioremediation*. Taylor & Francis Group, Boca Raton, FL.
- Aeppli, C., Berg, M., Cirpka, O. A., Holliger, C., Schwarzenbach, R. P., & Hofstetter, T. B. (2009). Influence of mass-transfer limitations on carbon isotope fractionation during microbial dechlorination of trichloroethene. *Environ. Sci. Technol.*, *43*(23), 8813–8820. doi: 10.1021/es901481b
- Amrani, A., Sessions, A. L., & Adkins, J. F. (2010). Compound-specific $\delta^{34}\text{S}$ analysis of volatile organics by coupled GC/multicollector-ICPMS. *Anal. Chem.*, *81*(21), 9027–9034. doi: 10.1021/ac9016538
- Barkan, E., & Luz, B. (2005). High precision measurements of $^{17}\text{O}/^{16}\text{O}$ and $^{18}\text{O}/^{16}\text{O}$ ratios in H_2O . *Rapid Commun. Mass Spectrom.*, *19*(24), 3737–3742. doi: 10.1002/rcm.2250
- Berg, M., Bolotin, J., & Hofstetter, T. B. (2007). Compound-specific nitrogen and carbon isotope analysis of nitroaromatic compounds in aqueous samples using solid-phase microextraction coupled to GC/IRMS. *Anal. Chem.*, *79*(6), 2386–2393. doi: 10.1021/ac0622577
- Bernstein, A., Shouakar-Stash, O., Ebert, K. A., Laskov, C., Hunkeler, D., Jeannotat, S., ... Elsner, M. (2011). Compound-specific chlorine isotope analysis: A comparison of gas chromatography/isotope ratio mass spectrometry and gas chromatography/quadrupole mass spectrometry methods in an interlaboratory study. *Anal. Chem.*, *83*(20), 7624–7634. doi: 10.1021/ac200516c
- Boyd, D. R., & Bugg, T. D. H. (2006). Arene *cis*-dihydrodiol formation: from biology to application. *Org. Biomol. Chem.*, *4*(2), 181–192. doi: 10.1002/chin.200618269
- Brand, W. A., Coplen, T. B., Vogl, J., Rosner, M., & Prohaska, T. (2014). Assessment of international reference materials for isotope-ratio analysis (IUPAC Technical Report). *Pure Appl. Chem.*, *86*(3), 425–467. doi: 10.1515/pac-2013-1023
- Bugg, T. D. H., & Winfield, C. J. (1998). Enzymatic cleavage of aromatic rings: mechanistic aspects of the catechol dioxygenases and later enzymes of bacterial oxidative cleavage pathways. *Nat. Prod. Rep.*, *15*(5), 513–530. doi: 10.1039/a815513y
- Coleman, M., & Meier-Augenstein, W. (2014). Ignoring IUPAC guidelines for measurement and reporting of stable isotope abundance values affects us all. *Rapid Commun. Mass*

Spectrom., 28(17), 1953–1955. doi: 10.1002/rcm.6971

Cook, P. F., & Cleland, W. W. (2007). *Enzyme kinetics and mechanism*. Garland Science: New York.

Coplen, T. B. (2011). Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Commun. Mass Spectrom.*, 25(17), 2538–2560. doi: 10.1002/rcm.5129

DelMonte, A. J., Haller, J., Houk, K. N., Sharpless, K. B., Singleton, D. A., Strassner, T., & Thomas, A. A. (1997). Experimental and theoretical kinetic isotope effects for asymmetric dihydroxylation. evidence supporting a rate-limiting "(3 + 2)" cycloaddition. *J. Am. Chem. Soc.*, 119(41), 9907–9908.

Dorer, C., Höhener, P., Hedwig, N., Richnow, H.-H., & Vogt, C. (2014). Rayleigh-based concept to tackle strong hydrogen fractionation in dual isotope analysis - the example of ethylbenzene degradation by aromatoleum aromaticum. *Environ. Sci. Technol.*, 48(10), 5788–5797. doi: 10.1021/es404837g

Dunn, P. J. H., Hai, L., Malinovsky, D., & Goenaga Infante, H. (2015). Simple spreadsheet templates for the determination of the measurement uncertainty of stable isotope ratio delta values. *Rapid Commun. Mass Spectrom.*, 29(22), 2184–2186. doi: 10.1002/rcm.7376

Eckert, D., Qiu, S., Elsner, M., & Cirpka, O. A. (2013). Model complexity needed for quantitative analysis of high resolution isotope and concentration data from a toluene-pulse experiment. *Environ. Sci. Technol.*, 47(13), 6900–6907.

Eiler, J. M. (2013). The Isotopic Anatomies of Molecules and Minerals. *Annu. Rev. Earth Planet. Sci.*, 41(1), 411–441. doi: 10.1146/annurev-earth-042711-105348

Elsner, M. (2010). Stable isotope fractionation to investigate natural transformation mechanisms of organic contaminants: principles, prospects and limitations. *J. Environ. Monit.*, 12(11), 2005–2031. doi: 10.1039/c0em00277a

Elsner, M., Chartrand, M., VanStone, N., Lacrampe Couloume, G., & Sherwood Lollar, B. (2008). Identifying abiotic chlorinated ethene degradation: characteristic isotope patterns in reaction products with nanoscale zero-valent iron. *Environ. Sci. Technol.*, 42(16), 5963–5970. doi: 10.1021/es8001986

Elsner, M., & Hunkeler, D. (2008). Evaluating chlorine isotope effects from isotope ratios

and mass spectra of polychlorinated molecules. *Anal. Chem.*, 80(12), 4731–4740. doi: 10.1021/ac702543y

Elsner, M., Jochmann, M., Hofstetter, T., Hunkeler, D., Bernstein, A., Schmidt, T., & Schimmelmann, A. (2012). Current challenges in compound-specific stable isotope analysis of environmental organic contaminants. *Anal. Bioanal. Chem.*, 403, 2471–2491. doi: 10.1007/s00216-011-5683-y

Elsner, M., Zwank, L., Hunkeler, D., & Schwarzenbach, R. P. (2005, September). A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. *Environ. Sci. Technol.*, 39(18), 6896–916. doi: 10.1021/es0504587

Federherr, E., Willach, S., Roos, N., Lange, L., Molt, K., & Schmidt, T. (2016). A novel high-temperature combustion interface for compound-specific stable isotope analysis of carbon and nitrogen via high-performance liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.*, 30(7), 2537–2544. doi: 10.1002/rcm.7524

Gehre, M., Renpenning, J., Gilevska, T., Qi, H., Coplen, T. B., Meijer, H. A. J., ... Schimmelmann, A. (2015). On-line hydrogen-isotope measurements of organic samples using elemental chromium: An extension for high temperature elemental-analyzer techniques. *Anal. Chem.*, 87(10), 5198–5205. doi: 10.1021/acs.analchem.5b00085

Gelman, F., & Halicz, L. (2010). High precision determination of bromine isotope ratio by GC-MC-ICPMS. *Int. J. Mass Spectrom.*, 289(2-3), 167–169. doi: 10.1016/j.ijms.2009.10.004

Gibson, D. T., & Parales, R. E. (2000). Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr. Opin. Biotech.*, 11(3), 236–243. doi: 10.1016/s0958-1669(00)00090-2

Godin, J. P., & McCullagh, J. S. O. (2011). Review: Current applications and challenges for liquid chromatography coupled to isotope ratio mass spectrometry (LC/IRMS). *Rapid Commun. Mass Spectrom.*, 25(20), 3019–3028. doi: 10.1002/rcm.5167

Hartenbach, A., Hofstetter, T. B., Berg, M., Bolotin, J., & Schwarzenbach, R. P. (2006). Using nitrogen isotope fractionation to assess abiotic reduction of nitroaromatic compounds. *Environ. Sci. Technol.*, 40(24), 7710–7716. doi: 10.1021/es061074z

Hartenbach, A. E., Hofstetter, T. B., Aeschbacher, M., Sander, M., Kim, D., Strathmann, T. J., ... Schwarzenbach, R. P. (2008). Variability of nitrogen isotope fractionation during the

reduction of nitroaromatic compounds with dissolved reductants. *Environ. Sci. Technol.*,
42(22), 8352–8359. doi: 10.1021/es801063u

Hofstetter, T. B., & Berg, M. (2011). Assessing transformation processes of organic contam-
inants by compound-specific stable isotope analysis. *TrAC-Trends Anal. Chem.*, *30*(4),
618–627. doi: 10.1016/j.trac.2010.10.012

Hofstetter, T. B., Neumann, A., Arnold, W. A., Hartenbach, A. E., Bolotin, J., Cramer, C. J., &
Schwarzenbach, R. P. (2008). Substituent effects on nitrogen isotope fractionation during
abiotic reduction of nitroaromatic compounds. *Environ. Sci. Technol.*, *42*(6), 1997–2003.
doi: 10.1021/es702471k

Hofstetter, T. B., Schwarzenbach, R. P., & Bernasconi, S. M. (2008). Assessing transformation
processes of organic compounds using stable isotope fractionation. *Environ. Sci. Technol.*,
42(21), 7737–7743. doi: 10.1021/es801384j

Höhener, P., & Atteia, O. (2014). Rayleigh equation for evolution of stable isotope ratios in
contaminant decay chains. *Geochim. Cosmochim. Acta*, *126*(0), 70–77. doi: 10.1016/
j.gca.2013.10.036

Houk, K. N., & Strassner, T. (1999). Establishing the (3 + 2) mechanism for the permanganate
oxidation of alkenes by theory and kinetic isotope effects. *J. Org. Chem.*, *64*(3), 800–802.

Hunkeler, D., & Elsner, M. (2010). Principles and mechanisms of isotope fractionation. In
Environmental Isotopes in Biodegradation and Bioremediation, C. M. Aelion, P. Höhener,
D. Hunkeler, & R. Aravena (Eds.), (pp. 43–77). Taylor & Francis Group, Boca Raton,
FL.

Jin, B., Haderlein, S. B., & Rolle, M. (2013). Integrated carbon and chlorine isotope modeling:
Applications to chlorinated aliphatic hydrocarbons dechlorination. *Environ. Sci. Technol.*,
47(3), 1443–1451. doi: 10.1021/es304053h

Jochmann, M. A., Blessing, M., Haderlein, S. B., & Schmidt, T. C. (2006). A new approach
to determine method detection limits for compound-specific isotope analysis of volatile
organic compounds. *Rapid Commun. Mass Spectrom.*, *20*(24), 3639–3648. doi: 10.1002/
rcm.2784

Jochmann, M. A., & Schmidt, T. C. (2012). *Compound-specific stable isotope analysis*. RSC
Publishing.

- Kohen, A., & Limbach, H.-H. (2006). *Isotope Effects in Chemistry and Biology*. New York: CRC Press / Taylor & Francis.
- Kohler, H.-P. E. (2011). Biochemistry of environmental contaminant transformation: Nonylphenolic compounds and hexachlorocyclohexanes - Two case studies. In *Biophysico-Chemical Processes of Anthropogenic Organic Compounds in Environmental Systems*, B. Xing, N. Senesi, & P. M. Huang (Eds.), (p. 465-481). John Wiley & Sons, Inc.
- Krummen, M., Hilker, A. W., Juchelka, D., Duhr, A., Schlüter, H.-J., & Pesch, R. (2004). A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.*, 18(19), 2260–2266. doi: 10.1002/rcm.1620
- Maggi, F., & Riley, W. J. (2010). Mathematical treatment of isotopologue and isotopomer speciation and fractionation in biochemical kinetics. *Geochim. Cosmochim. Acta*, 74(6), 1823–1835. doi: 10.1016/j.gca.2009.12.021
- Magyar, P. M., Orphan, V. J., & Eiler, J. M. (2016). Measurement of rare isotopologues of nitrous oxide by high-resolution multi-collector mass spectrometry. *Rapid Commun. Mass Spectrom.*, 30(17), 1923–1940. doi: 10.1002/rcm.7671
- Melander, L. C. S., & Saunders, W. H. (1980). *Reaction rates of isotopic molecules*. John Wiley & Sons: New York.
- Neilson, A. H., & Allard, A.-S. (2008). *Environmental Degradation and Transformation of Organic Chemicals*. CRC Press.
- Nijenhuis, I., Renpenning, J., Kümmel, S., Richnow, H.-H., & 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. *Trends in Environmental Analytical Chemistry*, 11, 1–8. doi: 10.1016/j.teac.2016.04.001
- Northrop, D. B. (1981). The expression of isotope effects on enzyme-catalyzed reactions. *Annu. Rev. Biochem.*, 50, 103-131. doi: 10.1146/annurev.bi.50.070181.000535
- Parales, R. E., Huang, R., Yu, C.-L., Parales, J. V., Lee, F. K. N., Lessner, D. J., ... Gibson, D. T. (2005). Purification, characterization, and crystallization of the components of the nitrobenzene and 2-nitrotoluene dioxygenase enzyme systems. *Appl. Environ. Microbiol.*, 71(7), 3806–3814. doi: 10.1128/aem.71.7.3806-3814.2005

- Pati, S. G., Bolotin, J., Brennwald, M. S., Kohler, H.-P. E., Werner, R. A., & Hofstetter, T. B. (2016). Measurement of oxygen isotope ratios ($^{18}\text{O}/^{16}\text{O}$) of aqueous O_2 in small samples by gas chromatography/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.*, 30(6), 684–690. doi: 10.1002/rcm.7481
- Pati, S. G., Kohler, H.-P. E., Bolotin, J., Parales, R. E., & Hofstetter, T. B. (2014). Isotope effects of enzymatic dioxygenation of nitrobenzene and 2-nitrotoluene by nitrobenzene dioxygenase. *Environ. Sci. Technol.*, 48(18), 10750–10759. doi: <http://dx.doi.org/10.1021/es5028844>
- Pati, S. G., Kohler, H.-P. E., Pabis, A., Paneth, P., Parales, R. E., & Hofstetter, T. B. (2016). Substrate and enzyme specificity of the kinetic isotope effects associated with the dioxygenation of nitroaromatic contaminants. *Environ. Sci. Technol.*, 6708–6716. doi: <http://dx.doi.org/10.1021/acs.est.5b05084>
- Piasecki, A., Sessions, A. L., Lawson, M., Ferreira, A. A., Neto, E. V. S., & Eiler, J. M. (2016). Analysis of the site-specific carbon isotope composition of propane by gas source isotope ratio mass spectrometer. *Geochim. Cosmochim. Acta*, 188, 58–72. doi: 10.1016/j.gca.2016.04.048
- R Core Team. (2014). R: A language and environment for statistical computing [Computer software manual]. Retrieved from <http://www.R-project.org/>
- Ratti, M., Canonica, S., McNeill, K., Bolotin, J., & Hofstetter, T. B. (2015). Isotope fractionation associated with the indirect photolysis of substituted anilines in aqueous solution. *Environ. Sci. Technol.*, 49(21), 12766–12773. doi: <http://dx.doi.org/10.1021/acs.est.5b03119>
- Ratti, M., Canonica, S., McNeill, K., Erickson, P. R., Bolotin, J., & Hofstetter, T. B. (2015). Isotope fractionation associated with the direct photolysis of 4-chloroaniline. *Environ. Sci. Technol.*, 49(7), 4263–4273. doi: <http://dx.doi.org/10.1021/es505784a>
- Reichert, P. (1994). Aquasim - a tool for simulation and data-analysis of aquatic systems. *Water Sci. Technol.*, 30, 21–30.
- Renpenning, J., Hitzfeld, K. L., Gilevska, T., Nijenhuis, I., Gehre, M., & Richnow, H.-H. (2015, February). Development and validation of an universal interface for compound-specific stable isotope analysis of chlorine ($^{37}\text{Cl}/^{35}\text{Cl}$) by GC-high-temperature conversion (HTC)-

MS/IRMS. *Anal. Chem.*, 87(5), 2832–2839. doi: 10.1021/ac504232u

Renpenning, J., Kümmel, S., Hitzfeld, K. L., Schimmelmänn, A., & Gehre, M. (2015). Compound-specific hydrogen isotope analysis of heteroatom-bearing compounds via gas chromatography-chromium-based high-temperature conversion (Cr/HTC)-isotope ratio mass spectrometry. *Anal. Chem.*, 87(18), 9443–9450. doi: 10.1021/acs.analchem.5b02475

Said Ahmad, W., Wong, K., Mcnall, M., Shawar, L., Jacksier, T., Turich, C., . . . Amrani, A. (2017). Compound-Specific Sulfur Isotope Analysis of Petroleum Gases. *Anal. Chem.*, 89(5), 3199–3207. doi: 10.1021/acs.analchem.6b05131

Schimmelmänn, A., Albertino, A., Sauer, P. E., Qi, H., Molinie, R., & Mesnard, F. (2009). Nicotine, acetanilide and urea multi-level ^2H -, ^{13}C -and ^{15}N -abundance reference materials for continuous-flow isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.*, 23(22), 3513–3521. doi: 10.1002/rcm.4277

Schimmelmänn, A., Qi, H., Coplen, T. B., Brand, W. A., Fong, J., Meier-Augenstein, W., . . . Werner, R. A. (2016). Organic reference materials for hydrogen, carbon, and nitrogen stable isotope-ratio measurements: Caffeines, n-alkanes, fatty acid methyl esters, glycines, l-valines, polyethylenes, and oils. *Anal. Chem.*, 88(8), 4294–4302. doi: 10.1021/acs.analchem.5b04392

Schmidt, T. C., & Jochmann, M. A. (2012). Origin and Fate of Organic Compounds in Water: Characterization by Compound-Specific Stable Isotope Analysis. *Annu. Rev. Anal. Chem.*, 5(1), 133–155. doi: 10.1146/annurev-anchem-062011-143143

Schwarzenbach, R. P., Escher, B. I., Fenner, K., Hofstetter, T. B., Johnson, C. A., von Gunten, U., & Wehrli, B. (2006, August). The challenge of micropollutants in aquatic systems. *Science*, 313(5790), 1072–7. doi: 10.1126/science.1127291

Schwarzenbach, R. P., Gschwend, P. M., & Imboden, D. M. (2016). *Environmental organic chemistry* (2nd ed.). John Wiley & Sons Inc.: Hoboken, New Jersey.

Scott, K. M., Lu, X., Cavanaugh, C. M., & Liu, J. S. (2004). Optimal methods for estimating kinetic isotope effects from different forms of the Rayleigh distillation equation. *Geochim. Cosmochim. Acta*, 68(3), 433–442. doi: 10.1016/s0016-7037(03)00459-9

Sessions, A. L. (2006). Isotope-ratio detection for gas chromatography. *J. Sep. Sci.*, 29(12), 1946–61. doi: 10.1002/jssc.200600002

- Sherwood Lollar, B., Hirschorn, S. K., Chartrand, M. M. G., & Lacrampe-Couloume, G. (2007).
An approach for assessing total instrumental uncertainty in compound-specific carbon
isotope analysis: Implications for environmental remediation studies. *Anal. Chem.*, *79*(9),
3469–3475. doi: 10.1021/ac062299v
- Singleton, D. A., & Thomas, A. A. (1995). High-precision simultaneous determination of
multiple small kinetic isotope effects at natural-abundance. *J. Am. Chem. Soc.*, *117*(36),
9357–9358.
- Skarpeli-Liati, M., Turgeon, A., Garr, A. N., Arnold, W. A., Cramer, C. J., & Hofstetter,
T. B. (2011). pH-Dependent equilibrium isotope fractionation associated with compound-
specific nitrogen and carbon isotope analysis by SPME-GC/IRMS. *Anal. Chem.*, *83*(5),
1641–1648. doi: 10.1021/ac102667y
- Spahr, S., Huntscha, S., Bolotin, J., Maier, M. P., Elsner, M., Hollender, J., & Hofstetter, T. B.
(2013). Compound-specific isotope analysis of benzotriazole and its derivatives. *Anal.
Bioanal. Chem.*, *405*(9), 2843–2856. doi: 10.1007/s00216-012-6526-1
- Tobler, N. B., Hofstetter, T. B., & Schwarzenbach, R. P. (2007). Assessing iron-mediated
oxidation of toluene and reduction of nitroaromatic contaminants in anoxic environments
using compound-specific isotope analysis. *Environ. Sci. Technol.*, *41*(22), 7773–7780. doi:
10.1021/es071129c
- Werner, R. A., & Brand, W. A. (2001). Referencing strategies and techniques in stable isotope
ratio analysis. *Rapid Commun. Mass Spectrom.*, *15*(7), 501–519. doi: 10.1002/rcm.258
- Wijker, R. S., Adamczyk, P., Bolotin, J., Paneth, P., & Hofstetter, T. B. (2013). Isotopic anal-
ysis of oxidative pollutant degradation pathways exhibiting large H isotope fractionation.
Environ. Sci. Technol., *47*(23), 13459–13468. doi: 10.1021/es403597v
- Wijker, R. S., Bolotin, J., Nishino, S. F., Spain, J. C., & Hofstetter, T. B. (2013). Using
compound-specific isotope analysis to assess biodegradation of nitroaromatic explosives
in the subsurface. *Environ. Sci. Technol.*, *47*(13), 6872–6883. doi: 10.1021/es3051845
- Wijker, R. S., Kurt, Z., Spain, J. C., Bolotin, J., Zeyer, J., & Hofstetter, T. B. (2013). Isotope
fractionation associated with the biodegradation of 2-and 4-nitrophenols via monooxy-
genation pathways. *Environ. Sci. Technol.*, *47*(24), 14185–14193. doi: 10.1021/es403876u
- Wijker, R. S., Pati, S. G., Zeyer, J., & Hofstetter, T. B. (2015). Enzyme kinetics of different

types of flavin-dependent monooxygenases determine the observable contaminant stable isotope fractionation. *Environ. Sci. Technol. Lett.*, 2(11), 329–334. doi: 10.1021/acs.estlett.5b00254

Wijker, R. S., Zeyer, J., & Hofstetter, T. B. (2017). Isotope fractionation associated with the simultaneous biodegradation of multiple nitrophenol isomers by *Pseudomonas putida* B2. *Environ. Sci.-Process Impacts*, 74(19), 775–784. doi: 10.1039/C6EM00668J

Wolfsberg, M., Hook, W. A. V., Paneth, P., & Rebelo, L. P. N. (2010). *Isotope effects in chemical, geological, and bio sciences*. Springer: Heidelberg.

Zakon, Y., Halicz, L., & Gelman, F. (2014). Isotope Analysis of Sulfur, Bromine, and Chlorine in Individual Anionic Species by Ion Chromatography/Multicollector-ICPMS. *Anal. Chem.*, 86(13), 6495–6500. doi: 10.1021/ac5010025

Zakon, Y., Halicz, L., Lev, O., & Gelman, F. (2016). Compound-specific bromine isotope ratio analysis using gas chromatography/quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.*, 30(17), 1951–1956. doi: 10.1002/rcm.7672

Zhang, L., Kujawinski, D. M., Jochmann, M. A., & Schmidt, T. C. (2011). High-temperature reversed-phase liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.*, 25(20), 2971–2980. doi: 10.1002/rcm.5069

Zwank, L., Berg, M., Schmidt, T. C., & Haderlein, S. B. (2003). Compound-specific carbon isotope analysis of volatile organic compounds in the low-microgram per liter range. *Anal. Chem.*, 75(20), 5575–5583. doi: 10.1021/ac034230i