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C and N Isotope Fractionation during Biodegradation of the Pesticide Metabolite 2,6-Dichlorobenzamide (BAM): Potential for Environmental Assessments

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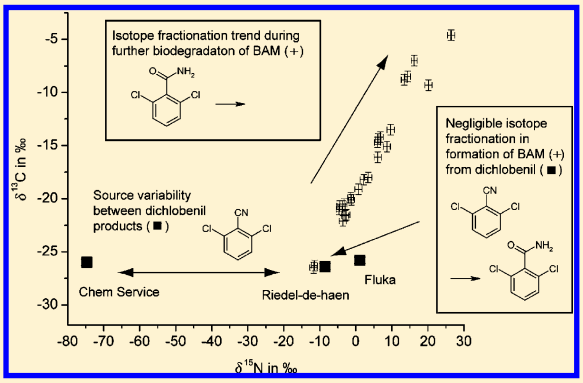
Supporting Information

ABSTRACT: 2,6-Dichlorobenzamide (BAM) is a metabolite of the herbicide 2,6-dichlorobenzonitrile (dichlobenil), and a prominent groundwater contaminant. Observable compound-specific isotope fractionation during BAM *formation*—through transformation of dichlobenil by *Rhodococcus erythropolis* DSM 9685—was small. In contrast, isotope fractionation during BAM *degradation*—with *Aminobacter* sp. MSH1 and ASI1, the only known bacterial strains capable of mineralizing BAM—was large, with pronounced carbon (ε_C = −7.5‰ to −7.8‰) and nitrogen (ε_N = −10.7‰ to −13.5‰) isotopic enrichment factors. BAM isotope values in natural samples are therefore expected to be dominated by the effects of its degradation rather than formation. Dual isotope slopes Λ (=$\Delta\delta^{15}N/\Delta\delta^{13}C \approx \varepsilon_N/\varepsilon_C$) showed only small differences for MSH1 (1.75 ± 0.03) and ASI1 (1.45 ± 0.03) suggesting similar transformation mechanisms of BAM hydrolysis. Observations are in agreement with either a tetrahedral intermediate promoted by OH^- or H_3O^+ catalysis, or a concerted reaction mechanism. Therefore, owing to consistent carbon isotopic fractionation, isotope shifts of BAM can be linked to BAM biodegradation, and may even be used to quantify degradation of this persistent metabolite. In contrast, nitrogen isotope values may be rather indicative of different sources. Our results delineate a new approach to assessing the fate of BAM in the environment.



INTRODUCTION

The herbicide 2,6-dichlorobenzonitrile (dichlobenil) was used in many countries on nonagricultural areas such as court yards, plant nurseries and fruit orchards. Although dichlobenil-containing herbicide formulations were banned in EU in 2008 (EU, 2008), the concentration of its main metabolite 2,6-dichlorobenzamide (BAM) in groundwater is still increasing.[1−3] Dichlobenil concentrations in groundwater are low, or even negligible [1−3] due to its immobilization through sorption in soil (K_{OC} of between 500 and 896 L kg^{-1}).[4] Once this compound gets degraded to BAM, however, the metabolite is more mobile (K_{OC} of 33 to 35 L kg^{-1})[4] and can leach into groundwater. Degradation of dichlobenil to BAM is known to be catalyzed by numerous microorganisms through nitrile hydratases (Scheme 1) and is well described in recent work.[5] Further transformation of BAM, in contrast, has been observed only recently in soil previously exposed to dichlobenil.[6] So far, only two BAM-mineralizing bacteria (*Aminobacter* sp. strains ASI1 and MSH1) have been isolated and characterized.[6,7] Although *Aminobacter* spp. are ubiquitous in the environment,[8] in aquatic systems, BAM degradation seems to be limited.[9] Consequently, the environmental fate of dichlobenil and BAM is of great concern.

Scheme 1. Hydrolysis of Dichlobenil to 2,6-Dichlorobenzamide (BAM) Catalysed by Nitrile Hydratase and Further Degradation of BAM to 2,6-Dichlorobenzoic Acid Catalysed by Amidase

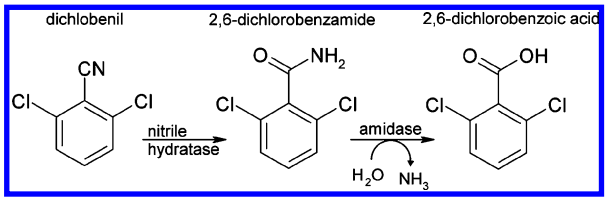


Figure 1 gives an overview of environmental scenarios of BAM after its formation from dichlobenil as given by its physical and chemical properties and dependent on the presence of microorganisms capable of degrading the compound. The concentration of BAM in groundwater is determined through (i) its degradation potential in soil and subsequent leaching to

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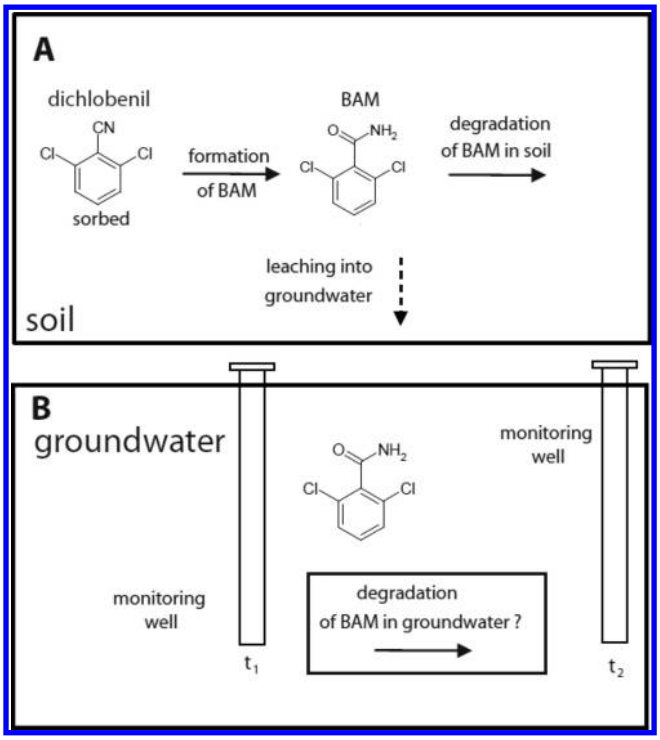

Figure 1. Environmental scenarios of dichlobenil and BAM. Dichlobenil is sorbed to soil, where it can be transformed to BAM. BAM is more mobile and persistent than dichlobenil so that it leaches into groundwater and is found there in the absence of dichlobenil. While evidence for BAM degradation has been obtained in soil, it is unknown whether degradation of BAM occurs in groundwater.

groundwater and (ii) through possible further degradation in groundwater.

Although the potential for BAM degradation in soil (Figure 1 A) has been demonstrated,[6,7] the extent of such natural attenuation is yet uncertain. Even less is known about the potential for BAM degradation in groundwater (Figure 1B). Conventional approaches which rely on identifying and quantifying BAM and its metabolite(s) are not conclusive: Dilution can decrease concentrations, but does not cause pollutant elimination. In addition, further metabolites such as 2,6-dichlorobenzoic acid from BAM (Scheme 1) may be rapidly degraded so that they are not accessible to analysis. It is, therefore, difficult to obtain mass balances and, hence, to assess the fate of BAM in the environment. Compound specific isotope analysis (CSIA) by gas chromatograph isotope ratio mass spectrometry (GC-IRMS) is a new, independent approach which determines the isotope composition of a compound (e.g., $^{13}C/^{12}C$ and $^{15}N/^{14}N$) at natural abundance. Due to the kinetic isotope effect (KIE) during biochemical reactions, molecules with light isotopes typically react slightly faster than molecules containing a heavy isotope in the reactive position.[10−12] As a consequence, the heavier isotopes usually become enriched in the remaining substrate. Since the method relies on isotope analysis of the reactant, enrichment of ^{13}C and ^{15}N in BAM can even give evidence of its degradation even if further metabolites (e.g., 2,6-dichlorobenzoic acid from BAM) cannot be detected.[13]

Besides *detection* of degradation the isotopic enrichment offers potential to *quantify* degradation of organic contaminants in the environment. Such quantification is based on isotope enrichment factor ε which can be determined in laboratory experiments according the Rayleigh equation (see Materials and Methods) and can then be used to quantify biodegradation B in samples of contaminated sites from measured isotope ratios according to the following:

$$B = 1 - f = \left[\frac{R}{R_0}\right]^{1/\varepsilon} \quad (1)$$

where R_0 and R are the isotope ratio at the source and at a specific location in the field and f is the fraction of the remaining contaminant at the given time. Such quantification based on changes in stable isotope ratios has been successfully conducted in numerous cases of point source pollution.[14−16]

The situation is more complex in cases of metabolites such as BAM, because these compounds may be simultaneously formed and degraded. To interpret their isotope ratios, it is, therefore, important to consider not only isotope fractionation occurring during their degradation reaction (i.e., mineralization of BAM), but also due to their formation (i.e., formation of BAM from dichlobenil). Evolution of isotope ratios during simultaneous formation and degradation of intermediates has been thoroughly considered, particularly in the case of chlorinated ethenes and including the dependence on underlying rate constants.[17−22] In the case of BAM in groundwater, this consideration can be simplified, as sketched in Figure 1.

The BAM formed from dichlobenil leaches into the saturated zone and is trapped in the freshly formed groundwater with an isotope ratio that can be considered like an initial BAM signature R_0 of eq 1. In the absence of further BAM input, subsequent changes in this signature (R in eq 1) can be used to quantify BAM degradation according to eq 1. To assess R_0, however, isotope fractionation caused by the degradation of dichlobenil must also be considered. Is R_0 to be equal to the original dichlobenil source, does it contain less ^{13}C or even more? Two cases can be distinguished. (i) Transformation of dichlobenil is associated with significant carbon isotope fractionation such that dichlobenil becomes enriched in ^{13}C when it is degraded and the product BAM is initially depleted in ^{13}C. At later stages of dichlobenil degradation, the freshly formed BAM contains more ^{13}C reflecting the trend of the remaining parent compound from which it is formed. In such a case, the R_0 of BAM would be difficult to predict, and would depend on the stage of dichlobenil degradation at which it was formed. (ii) Alternatively, if degradation of dichlobenil is associated with no or negligible isotope fractionation, R_0 of freshly formed BAM would always correspond to the original source signature of the dichlobenil from which it is formed.

The first objective of this study was, therefore, to investigate carbon and nitrogen isotope fractionation associated with BAM formation studying dichlobenil degradation with the bacterial strain *Rhodococcus erythropolis* DSM 9685.[23] The second aim was to study for the first time carbon and nitrogen isotope fractionation during BAM degradation using the only bacterial strains (*Aminobacter* sp. strains MSH1 and ASI1) known to mineralize BAM to date.[6,7] Isotope enrichment factors and dual isotope plots of the degradation reactions were determined in order to obtain insight into the underlying reaction mechanisms.

MATERIALS AND METHODS

Chemicals. Dichlobenil (2,6-dichlorobenzonitrile, CAS No.: 1194−65−6) was from Fluka and BAM (2,6-dichlorobenzamide, CAS No.: 2008−58−4) from Riedel-de Haën (>98%)

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supplied by Sigma Aldrich (Taufkirchen, Germany). Methanol (99.9%, Merck, Germany) was used as solvent for the BAM standards and the enrichment of BAM extracts. Dichloromethane (>99.8%, Fluka, Germany) was used to extract BAM from water. Acetonitrile (HPLC gradient grade, Roth, Germany) was used as eluent for HPLC-UV measurements.

Degradation Studies. *Precultivation.* The degradation study of dichlobenil was performed using the pure culture *Rhodococcus erythropolis* DSM 9685. Degradation of BAM was studied using two different bacterial strains, *Aminobacter* sp. ASI1 and MSH1. The strains MSH1 and DSM 9685 were pregrown in R2B medium (Difco, Detroit, Michigan, U.S.) and the strain ASI1 was pregrown in mineral salt medium (MS)[24] supplied with a carbon source (200 mg L^{-1} butanedioic acid) and 1 mg L^{-1} BAM. All three strains were incubated at 20 °C under orbital shaking (125 rpm) in the dark. The precultures were harvested by centrifugation at 4000 rpm (Heraeus Megafuge 1.0R) in the late exponential growth phase and washed three times in MS. For the degradation experiments, the cell pellets were dissolved in MS to obtain an optical density (OD600 nm) of 0.9 for MSH1, 0.8 for ASI1 and 0.6 for DSM 9685 determined in a Varian Cary 50 Bio UV−vis Spectrophotometer.

Transformation Experiments. Triplicates of each degradation experiment were conducted in 1000 mL sterilized Erlenmeyer flasks containing 500 mL MS supplemented with either 9 mg L^{-1} dichlobenil or 12 mg L^{-1} BAM. For dichlobenil degradation the flasks were closed with a Viton seal. MS (475 mL) were inoculated with 25 mL of the washed cell suspension of ASI1 and DSM 9685, respectively. BAM degradation using MSH1 was performed with 497.5 mL MS inoculated with 2.5 mL of a washed cell suspension. The dichlobenil degradation experiment was spiked with a stock solution of 9000 mg L^{-1} dichlobenil dissolved in dimethyl sulfoxide to yield an initial concentration of 9 mg L^{-1}. For degradation studies of BAM sterilized Milli-Q water with a concentration of 1200 mg L^{-1} was spiked to yield an initial concentration of 12 mg L^{-1}. BAM provided the only carbon and nitrogen source for MSH1 and ASI1.[6,7] The degradation experiments were performed under orbital shaking (125 rpm) and were incubated at room temperature in the dark. The concentrations of BAM and dichlobenil in nonsterile experiments were compared to sterile controls without cells.

Sampling. For quantification purposes 0.5 mL aliquots were centrifuged for 2 min at 14 000 rpm (Centrifuge 5417R, Eppendorf, Hamburg, Germany). 200 μL of the supernatant were used for quantification of BAM and dichlobenil. For isotope analysis of dichlobenil direct immersion solid phase microextraction (SPME) was employed in 10 to 20 mL aliquots using a PDMS/DVB fiber from Supelco analytical supplied by Sigma Aldrich (Taufkirchen, Germany). Until SPME extraction aliquots were stored frozen. For isotope analysis BAM was extracted from aliquots of water (10 to 250 mL) according to the following procedure. Aliquots were centrifuged for 15 min with 4000 rpm (Heraeus Megafuge 1.0R) and the supernatants were frozen at −18 °C in 20 to 80 mL vials and subsequently lyophilized. The remains (salts and BAM) were reconstituted in 1 mL water and extracted with 10 mL dichloromethane. Dichloromethane was dried at room temperature under a gentle stream of nitrogen and the extract was redissolved in 0.4 to 1 mL methanol to reach concentrations of BAM between 300 to 500 mg L^{-1}.

Quantification with HPLC. Concentrations of dichlobenil and BAM were determined in a Shimadzu LC-10A series HPLC system and quantified with CLASS VP V6.10 software (Shimadzu). The system was equipped with a Luna 5u C18(2) column, 100 × 2.00 mm, 5 μm (Phenomenex, Aschaffenburg, Germany) and a UV detector. The eluents were a phosphate buffer and acetonitrile as described in Berg et al.[25] The initial gradient contained 15% acetonitrile (1 min) and was increased to 70% acetonitrile (2−6 min), after which the level was maintained for 2 min. The gradient was returned to 15% acetonitrile (8−13 min) with a post-time run (13−16 min). The injection volume was 20 μL, the flow rate was 0.7 mL min^{-1} and the oven temperature was set to 45 °C. Detection and quantification were accomplished measuring the absorbance at 220 nm including a comparison with pure standards.

Isotope Analysis. The GC-C-IRMS system consisted of a TRACE GC Ultra gas chromatograph (GC) (Thermo Fisher Scientific, Milan, Italy) coupled to a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS) (Thermo Fisher Scientific, Bremen, Germany) via a Finnigan GC Combustion III interface (Thermo Fisher Scientific, Bremen, Germany). Emission energy was 1.5 mA for C isotope analyses and 2.0 mA for N isotope analyses. He of grade 5.0 was used as the carrier gas with a flow rate of 1.4 mL min^{-1}. Liquid samples were injected via a GC Pal autosampler (CTC, Zwingen, Switzerland) and SPME of dichlobenil was performed manually with an extraction time of 15 min. The split/splitless injector was held at 250 °C and operated for BAM and Dichlobenil analysis for 2 and 10 min, respectively, in splitless mode, after which it was switched to split mode (split ratio 1:10). Standard measurements verified that this approach caused no isotope fractionation.[26] The analytical column was a DB-5 (30 m × 0.25 mm; 1 μm film; J&W Scientific, Folsom; CA). The GC program was as follows: 120 °C (hold 1 min), ramp 8 °C min^{-1} to 200 °C (hold 1 min) and ramp 15 °C min^{-1} to a final temperature of 280 °C (hold for 2 min). A commercial ceramic tube filled with CuO/NiO/Pt-wires (Thermo Fisher Scientific, Bremen, Germany) operated at 940 °C and a recently introduced Ni tube/NiO-CuO reactor with a silcosteel capillary (Thermo Fisher Scientific, Bremen, Germany) operated at 1030 °C were used for carbon and nitrogen isotope analysis, respectively. A CuO/NiO/Pt reactor was used without further treatment for nitrogen isotope measurements (up to 80 measurements) and prior to carbon isotope analysis the reactor was oxidized for 6 h at 940 °C as recommended by the manufacturer. The Ni tube/NiO−CuO reactor was initially oxidized for 2 h at 600 °C, and 12 h at 1030 °C. Compared to the CuO/NiO/Pt reactor, the new product bears the potential to a longer lifetime, a better performance and it allows an easy switching between nitrogen and isotope measurements.

During carbon isotope analysis by GC-IRMS, $\delta^{13}C$ values were automatically calculated relative to a laboratory CO_2 standard gas that was introduced at the beginning and the end of each run. The laboratory standard was calibrated to V-PDB by reference CO_2 standards (RM 8562, RM 8563, RM 8564) and were reported without any further correction. $\delta^{13}C$ values of standards agreed with those determined by elemental analyzer-isotope ratio mass spectrometry (EA-IRMS, see below). Nitrogen isotope analysis via GC-IRMS of BAM and dichlobenil involved standard bracketing and a drift correction using in-house standards of BAM and diclobenil. This approach was recently validated in our laboratory.[26] The carbon and nitrogen isotopic composition of dichlobenil and BAM in-

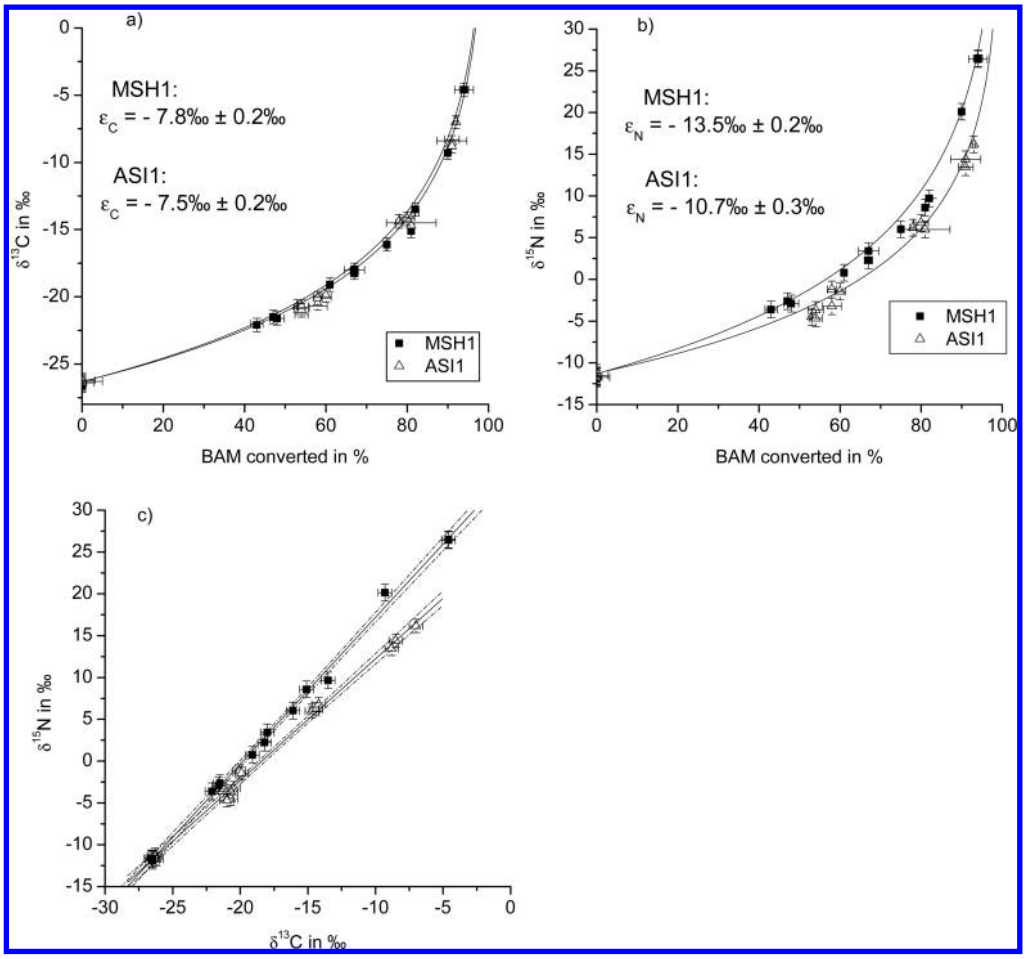

Figure 2. Changes in isotope ratios of carbon (a) and nitrogen (b) during biotic degradation of BAM using two pure bacterial strains, MSH1 (filled rectangle) and ASI1 (open triangle). *Y* error bars represent the total uncertainty of carbon (±0.5 ‰) and nitrogen (±1 ‰) analysis. *X* error bars represent the uncertainty of concentration measurements. Panel (c) represents the two-dimensional isotope plot of carbon and nitrogen of BAM isotope ratios for *Aminobacter* sp. ASI1 (open triangle) and MSH1 (filled rectangle). The slope of regression is determined using bulk data of all strains and is given as Δ. *Y* error bars indicate the total uncertainty of carbon isotope (±0.5 ‰) and nitrogen isotopes (±1 ‰). Dotted lines represent 95% confidence interval of the linear regression.

house standards was determined by EA-IRMS consisting of a EuroEA (EuroVector, Milano, Italy) coupled to a Finnigan TM MAT253 IRMS (Thermo Fisher Scientific, Bremen, Germany) by a FinniganTM ConFlow III interface (Thermo Fisher Scientific, Bremen, Germany). A calibration was performed against organic reference materials (USGS 40, USGS 41, IAEA 600) provided by the International Atomic Energy Agency (IAEA, Vienna).

δ^{13}C- and δ^{15}N-values are reported in the delta notation relative to Vienna PeeDee Belemnite (V-PDB) and air, respectively:

$$\delta^{13}C = \frac{(^{13}C/^{12}C_{Sample} - {}^{13}C/^{12}C_{Standard})}{^{13}C/^{12}C_{Standard}} \quad (2)$$

$$\delta^{15}N = \frac{(^{15}N/^{14}N_{Sample} - {}^{15}N/^{14}N_{Standard})}{^{15}N/^{14}N_{Standard}} \quad (3)$$

Carbon and nitrogen enrichment factors were determined as the slope of the linear regression according to the Rayleigh equation:

$$\ln \frac{R}{R_0} = \left(\frac{1 + \delta^h E}{1 + \delta^h E_0}\right) = \varepsilon \cdot \ln f \quad (4)$$

R and R_0 are the compound specific isotope ratios of heavy versus light isotopes of an element E in the contaminant at a given time and at the beginning of the reaction. δ^hE and $\delta^h E_0$ are the corresponding isotopic signatures of E (either C or N in the per mill notation and f is the fraction of the remaining contaminant (C_t/C_0). The enrichment factor ε is caused by the kinetic isotope effects in the reactive bond and gives an average (bulk) isotope enrichment over all positions of an element in the compound. Normal isotope effects (i.e., light isotopes react faster) are expressed as negative ε-values whereas inverse isotope effects (i.e., preference for heavy isotopes) are expressed as positive ε-values. Dilution of observable isotope fractionation due to nonreactive atoms of the same element can be taken into account when considering ε as composed of the kinetic isotope effects KIE from all different molecular positions i[13]

$$\varepsilon \approx \frac{1}{n} \cdot \sum \left(\frac{1}{KIE_i} - 1\right) \quad (5)$$

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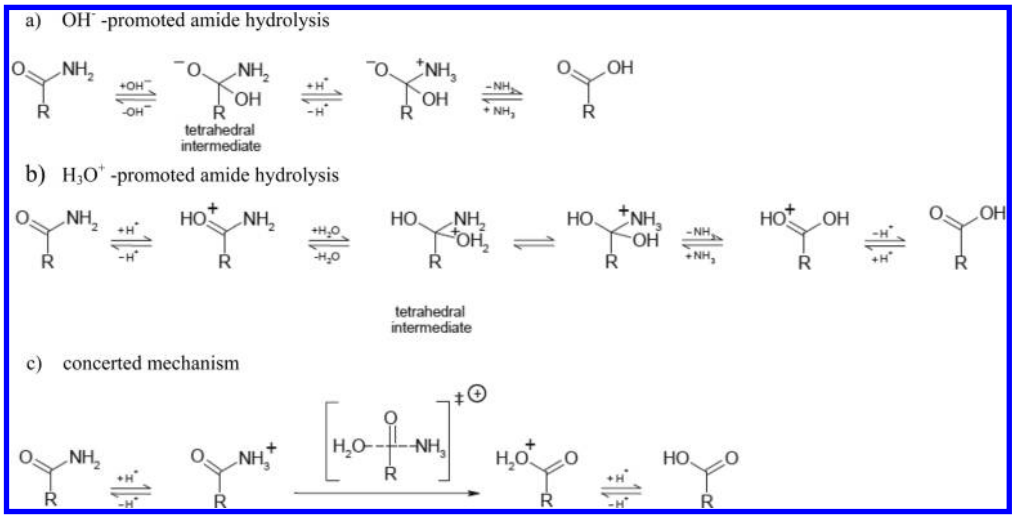
Table 1. Carbon and Nitrogen Isotope Enrichment Factors of BAM Degradation with the Bacteria *Aminobacter* sp. MSH1 and ASI1

	enzyme	ε_{carbon} [‰]	$\varepsilon_{nitrogen}$ [‰]	$\Delta = \delta^{15}N/\ \delta^{13}C = \varepsilon_{nitrogen}/\varepsilon_{carbon}$	$AKIE_{carbon}$[a]	$AKIE_{nitrogen}$[a]
Aminobacter sp. MSH1	amidase	−7.8 ± 0.2	−13.5 ± 0.2	1.75 ± 0.03	1.06	1.01
Aminobacter sp. ASI1	amidase	−7.5 ± 0.2	−10.7 ± 0.3	1.45 ± 0.03	1.06	1.01

[a]Calculated from ε_{carbon} and $\varepsilon_{nitrogen}$ of this study according to eq 6.

Scheme 2. Schematic Illustration of Possible BAM Hydrolysis to 2,6-Dichlorobenzoic Acid with a Nucleophilic Attack on the Carbonyl Carbon



In this equation, positions which do not show isotope effects ($KIE_i = 1$) contribute with zero. If secondary isotope effects are assumed to be absent, then an apparent position-specific kinetic isotope effect (AKIE) in the reactive bond is therefore calculated according to the following:

$$\varepsilon \approx \frac{1}{n}\cdot\left(\frac{1}{AKIE} - 1\right)$$

$$AKIE \approx \frac{1}{(1 + n\cdot\varepsilon)} \quad (6)$$

[27] where n is the number of atoms of the element present in the molecule.

Independent of the effect of dilution, the term "apparent" accounts for the fact that observable isotope fractionation may be smaller than expected when the intrinsic kinetic isotope effect KIE of the (bio)chemical bond conversion is suppressed ("masked"). The reason is that observable isotope fractionation contains contributions from all processes leading up to and including the first irreversible step in a (bio)chemical process. Masking can therefore occur if the bond-changing step is relatively fast compared to other rate-determining steps, which do not cause isotope fractionation themselves ("commitment to catalysis").

RESULTS AND DISCUSSION

Formation of BAM from Dichlobenil. A degradation experiment with dichlobenil was conducted using *Rhodococcus erythropolis* DSM 9685 with nitrile hydratase enzyme activity[23] to obtain insight into the magnitude of isotope fractionation related to dichlobenil degradation. The transformation was followed over 312 days up to 71% of dichlobenil degradation (Figure S2 of the Supporting Information, SI). Only very small carbon isotope fractionation was observed, with an overall enrichment of ^{13}C by 0.7 ‰ at 61% degradation. This value is hardly significant within the analytical uncertainty of the method. Likewise, very small nitrogen isotope fractionation was observed, with a depletion of ^{15}N by 2.3 ‰ after 71% dichlobenil degradation. This is consistent with other studies reporting an inverse isotope effect if an additional bond is formed to the element of concern in the transition state.[28,29] The resultant picture is that BAM shows largely the same $\delta^{13}C$ value as the dichlobenil from which it is formed. Only during subsequent BAM degradation would an enrichment of ^{13}C, or ^{15}N in BAM take place.

Degradation of BAM. The strains *Aminobacter* sp. MSH1 and ASI1 effectively degraded BAM, consistent with previous studies [6,7] (for details see Figure S1 of the SI). As shown in Figure 2, degradation of BAM by both bacterial strains induced an enrichment of ^{13}C (Figure 2a) and ^{15}N (Figure 2b) in the remaining BAM. No isotope fractionation was observed in the sterile control, confirming that no isotope fractionation was associated with lyophilization of BAM and subsequent extraction with dichloromethane. Isotope fractionation was quantified in Rayleigh plots and given in terms of the corresponding enrichment factors (see Figure S3 of the SI, Table 1). Nitrogen isotope fractionation of BAM was slightly stronger with *Aminobacter* sp. MSH1 than with ASI1, (ε = −13.5 ± 0.2 versus ε = −10.7 ± 0.3). Carbon isotope fractionation, in contrast, was similar for both strains (ε = −7.8 ± 0.2 and −7.5 ± 0.2, see Table 1).

To elucidate degradation pathways and even reaction mechanisms, dual element isotope plots are often useful. Values of $\delta^{13}C$ and $\delta^{15}N$ (i.e., values of the y-axes in Figure 2a,b, respectively) are plotted against each other such as shown in Figure 2c. The slope $\Delta\delta_{nitrogen}/\Delta\delta_{carbon} \approx \varepsilon_{nitrogen}/\varepsilon_{carbon}$ is a measure of the relative extent of isotope fractionation of the two elements.[30−32] It reflects underlying carbon and nitrogen

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kinetic isotope effects [13,28,33,34] and is, therefore, a sensitive mechanistic indicator.[27,28] It is particularly useful if intrinsic isotope effects are masked by nonfractionating steps (e.g., diffusive mass transfer, etc.) so that KIE of both elements are suppressed in their respective AKIE values. In contrast, if isotope effects in the (bio)chemical bond conversion are constant, and if the other rate-determining steps do not show isotope fractionation, dual isotope slopes are not affected by masking, because ε values of both elements are diminished to the same extent.[13] Figure 2c shows that the slopes are similar, yet distinguishable for ASI1 and MSH1 (Table 1). This raises the question whether there are differences in the initial transformation mechanism despite the same net reaction (amide hydrolysis), and how they arise.

The microbes are phylogenetically closely related, but show differences in physiology.[7] A possible reason for a distinguishable dual isotope plot could therefore be substrate-specific uptake mechanisms for BAM binding to transporter molecules. To test this hypothesis, AKIE values of nitrogen and carbon were calculated according to eq 6 using the enrichment factors of both reactions (Table 1). If secondary isotope effects are neglected, then AKIEs obtained for carbon are 1.06, of comparable magnitude as the theoretical maximum KIE values for C−N bond cleavage given by the Streitwieser limit of 1.06.[27] Even when allowing for secondary isotope effects of 1.001 or 1.002 at all other positions according to eq 5, our experimental data still results in an estimated large primary carbon isotope effect of 1.05 or 1.04 in the reacting bond. This pronounced isotope effect suggests that the reaction was not masked and therefore isotope fractionation was largely representative of chemical reactions rather than of binding to proteins.

Mechanistic hypotheses for chemical reaction steps involved in amide hydrolysis are either formation of a tetrahedral intermediate, through OH^- or H_3O^+ catalysis (Scheme 2a,b) or a concerted mechanism (Figure 2c). A nucleophilic attack on the carbonyl carbon atom next to an amide bond [35−37] is well established to show a rather large primary carbon isotope effect of up to 1.05[38] and a small nitrogen isotope effect in the range of ^{15}k = 1.01 − 1.03,[39] consistent with the results of our study.

Also the small variation in dual isotope plots observed in Figure 2c is consistent with the mechanistic scenarios of Scheme 2. If a (bio)chemical transformation does not consist of a one-step reaction, but involves two or more consecutive bond conversions such as in Scheme 2a,b, then kinetic isotope effects are likely different in the different steps, and they typically change in a different way for each element. For example, in Scheme 2, substantial carbon isotope effects are expected for both the initial attack of a nucleophile (OH^- or H_2O) and extrusion of NH_3, because both steps involve the central carbon atom. In contrast, nitrogen isotope fractionation is primarily expected in the second step (i.e., the extrusion of NH_3). Observable isotope fractionation does not just reflect one step, but is composed of the contributions from all steps weighed with their reciprocal rate constants.[13] Consequently, small differences in the kinetics of two or more isotopically sensitive transformation steps can already lead to a significant variability of dual isotope slopes such as observed in Figure 2c,[34,40] despite the fact that the same transformation mechanism prevails.

Environmental Significance. The results of our study highlight a remarkably pronounced and constant carbon isotope fractionation when the herbicide metabolite BAM is biodegraded by bacterial strains. Besides these degradation-induced changes, however, stable isotope ratios of BAM in groundwater can also be influenced by (1) the isotope effect associated with BAM formation and (2) the source isotope ratio of the pesticide from which it is formed.[41,42] These factors must also be considered for environmental assessments.

Our results indicate that isotope fractionation associated with formation of BAM is small. This facilitates assessments, because the R_0 of freshly formed BAM corresponds to the original source signature of the dichlobenil from which it is formed (scenario (ii) of the Introduction). The question remains what influence has an additional variability introduced by dichlobenil signatures from different manufacturers. Figure 3 shows carbon

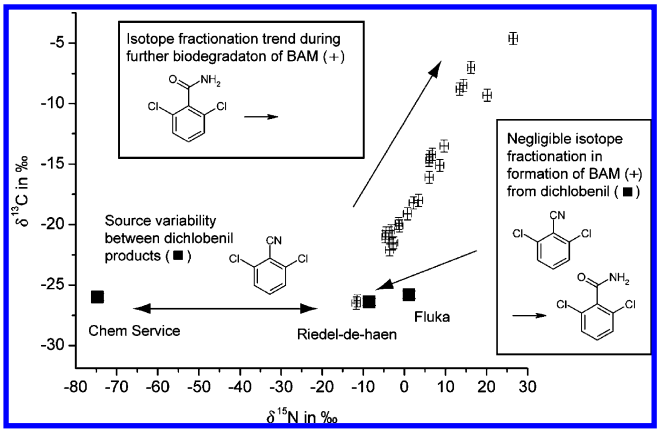

Figure 3. Carbon and nitrogen isotope values of different commercial dichlobenil products (filled squares), as well as of the BAM isotope values observed during BAM degradation (crosses). Note that we found BAM formation from dichlobenil to be associated with negligible carbon isotope fractionation and very small nitrogen isotope fractionation, corresponding to the difference in the data points between the dichlobenil from Riedel-de-haen and the adjacent BAM. This illustrates a realistic scenario of different dichlobenil source values and of resulting BAM which is subsequently degraded.

and nitrogen isotope ratios for three different commercial 2,6-dichlorobenzonitrile standards, which we characterized by EA-IRMS in our laboratory. In addition, also dual isotope slopes of BAM from Figure 2c are plotted. Large differences in nitrogen isotope values contrast with small variations in carbon isotope ratios. The carbon isotope ratios are characteristic of petroleum-derived feedstock originating from C_3 plants. The greater variability in nitrogen isotope values, in contrast, may be explained by the fact that (a) the nitrogen atom can be introduced through different synthesis routes and (b) it is the only atom of the element in the molecule so that position-specific isotope effects are fully reflected in the compound average. Therefore, our results delineate the following general pattern of expected isotope ratio variations in BAM:

(i) Carbon isotope source signatures are expected to vary little because petroleum (C_3-plant) -derived feedstock is typically used in industrial synthesis. Nitrogen isotope ratios, in contrast, have the potential to delineate different sources of BAM.

(ii) Isotope ratios of freshly formed BAM are expected to reflect the composition of the dichlobenil source from which it is formed.

(iii) Pronounced carbon and nitrogen isotope fractionation is expected for further BAM degradation. Because of the

small variation in carbon isotopic source signatures, changes in carbon isotope ratios of BAM can therefore be used (a) as a qualitative indicator of its natural biotransformation and (b) to even quantitatively estimate the extent of BAM degradation from measured isotope values $\delta^{13}C$ of field samples according to eq 1. Further, if isotope values are measured also for nitrogen, and are plotted in dual element isotope plots (Figure 3), (c) the additional information from nitrogen isotope measurements can make it possible to delineate also different sources.

These results delineate a new approach to assessing the fate of BAM in the environment.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1: Degradation kinetics of BAM using *Aminobacter* sp. MSH1 and ASI1. This material is available free of charge via the Internet at http://pubs.acs.org. Figure S2: (a) Degradation kinetics of dichlobenil using the pure bacterial strain *Rhodococcus erythropolis* DSM 9685. (b) Carbon and nitrogen isotopic changes of dichlobenil during transformation to BAM. Figure S3: Logarithmic plots according to the Rayleigh equation of carbon (a) and nitrogen (b) isotope ratios during BAM degradation

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