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Compound specific isotope analysis of the pesticides bentazone, MCPA, dichlobenil and its main metabolite BAM: Method validation and degradation studies

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Summary

Pesticides are chemical substances widely used for pest control in agricultural, horticultural, forestry and road industries. They are repeatedly detected in groundwater systems and considered as groundwater pollutants. Their fate is therefore of interest for environmental authorities. For fate assessment in groundwater, detecting and observing concentration changes of the pesticides and their metabolites is often not conclusive. Processes like dilution and sorption may lead to a concentration decrease but not to a transformation of the contaminant into non toxic substances. This thesis brings forward compound specific isotope analysis (CSIA) as a new tool to asses, verify, identify and even quantify transformation reactions of organic contaminants in soil and groundwater systems. My work aimed to develop and validate CSIA of the pesticides bentazone, MCPA, dichlobenil, its metabolite 2,6-dichlorobenzamid (BAM) and atrazine and its metabolite desethylatrazine (DEA) to determine the carbon as well as nitrogen isotope composition. Goal of this study was to provide an additional approach for an enhanced risk assessment of the frequently detected environmental pollutants.

Chapter 2 of this work deals with negatively charged analytes that must be derivatized prior to GC-IRMS, with a stringent control of isotope fractionation. Current methods require offline sample preparation. This study tests for the first time trimethylsulfonium hydroxide (TMSH) as online derivatization agent prior to compound specific isotope analysis, addressing the herbicides bentazone and MCPA. Fully automated derivatization was achieved in a temperature programmable GC injector, where reactants were injected into a packed liner. After the removal of the solvent via a split flow a subsequent flash heating triggered the derivatization followed by a transfer of the derivates onto the chromatographic column. Stoichiometric addition of TMSH resulted in complete conversion giving accurate and reproducible nitrogen isotope values. In contrast, reproducible carbon isotope values of bentazone and MCPA required TMSH in ≥ 250-fold excess. Contrary to expectations, $\delta^{13}C$ values become more negative at smaller TMSH excess. This indicates that elevated methyl group concentration in the pore space of the injection liner facilitated close-to-equilibrium rather than kinetic isotope fractionation. $\delta^{13}C$ results under these conditions compared favourably with liquid chromatography (LC)-IRMS. Both methods showed low standard deviations with 0.3 ‰ for GC-IRMS and 0.1 ‰ for LC-IRMS and a comparable offset of 1 ‰ compared to elemental analyzer-

IRMS. This demonstrates that both methods represent expedient ways for online isotope analysis of anionic compounds.

In Chapter 3 multiple oxidation reactor tubes of two different kinds were tested for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope analysis of selected target compounds that are particularly difficult to target with GC-IRMS: atrazine, DEA, dichlobenil, BAM and caffeine. Besides the conventional reactor with CuO-NiO-Pt wires, a newly developed reactor with NiO tube/CuO-NiO wires (Thermo Fisher Scientific, Bremen, Germany) was tested. Advantages of the new reactor design were the absence of an additional reduction reactor, the possibility for routine reoxidation in nitrogen isotope analysis, and reliable atrazine and DEA measurements over several hundred injections. However, both reactor types showed notable variations in the accuracy of nitrogen and, to a less extent, also of carbon isotope values. Trueness of isotope values was studied according to reactor design, reactor conditions and reactor lifetime for compounds with a different content of hetero atoms. Improved accuracy was achieved by bracketing samples with external compound-specific standards and subsequent offset correction. My study therefore shows that instrument data should never be taken as “face value”, but must consistently be validated with compound-specific standards of the respective analyte.

In Chapter 4 of my thesis, the newly established method was used to measure for the first time carbon and nitrogen isotope fractionation during biodegradation of dichlobenil and BAM. Carbon and nitrogen isotope fractionation during BAM degradation using the two bacterial strains *Aminobacter* sp. MSH1 and ASI 1 was remarkably pronounced. My work therefore demonstrated that changes in isotope ratios of BAM can be used for qualitative detection of BAM degradation. Moreover, the remarkable consistent carbon isotope fractionation observed in my study highlights the potential to quantitatively estimate the BAM degradation in the environment. Since the parent compound dichlobenil is present as recalcitrant pool in the soil, isotope fractionation associated to its degradation has to be considered, when assessing the fate of BAM in the environment using CSIA. A first dichlobenil degradation experiment using *Rhodococcus erythropolis* DSM 9685 showed only small nitrogen and a hardly significant carbon isotope effect. The originating BAM would in fact show largely the same $\delta^{13}C$ value as the dichlobenil from which it is formed until BAM degradation is initiated. The enrichment of ^{13}C in BAM could then directly be linked to its transformation. However, due to the multiplicity of present dichlobenil degraders further investigations are necessary to perform a reliable fate assessment of dichlobenil and BAM in the environment using CSIA.

In addition, the result of this study gave additional insight on the reaction chemistry of enzymatic BAM transformation catalyzed by the amidase enzyme. The isotope fractionation of both degraders gave similar dual isotope plots which likely reflect similar kinetics and transition states corresponding either to a tetrahedral intermediate promoted by OH^- or H_3O^+ amidase hydrolysis or reflecting a concerted reaction mechanism.

My thesis has therefore taken an important step towards assessing pesticides degradation in groundwater:

(i) The analytics was developed and validated for the pesticides bentazone, MCPA, dichlobenil, its metabolite BAM and atrazine and its metabolite DEA to provide an additional line of evidence for assessing the fate of the pollutants in the environment.

(ii) The first carbon and nitrogen enrichment factors during biodegradation of the pesticide metabolite BAM were determined using the only bacterial strains (*Aminobacter* sp. strains MSH1 and ASI1) known to degrade BAM.

Zusammenfassung

Pestizide sind Pflanzenschutz- und Schädlingsbekämpfungsmittel, die weltweit Anwendung finden und als gesundheitsgefährdend eingestuft sind. Pestizide bzw. ihre Metabolite werden gehäuft in Aquiferen detektiert und stellen somit eine Gefahr für die Umwelt und letztendlich für den Menschen dar. Die Frage nach dem Schicksal der Pestizide und deren teilweise persistenten Metaboliten rückt daher immer mehr in den Fokus von Umweltbehörden. Um Aussagen über das Umweltverhalten treffen zu können, ist es nicht immer ausreichend die Konzentrationen der Substanzen und deren Metabolite zu bestimmen. Prozesse wie Verdünnung und Sorption äußern sich ebenfalls in einem messbaren Konzentrationsrückgang, führen aber zu keiner Transformation in nicht toxische Substanzen. Diese Arbeit bringt die substanzspezifische Isotopenanalyse (CSIA) von Pestiziden als einen weiteren Ansatz voran, um das Umweltverhalten dieser Substanzen in Boden und Grundwasser gezielter beurteilen zu können, indem Abbaureaktionen nachgewiesen, identifiziert und sogar quantifiziert werden. Im speziellen, zielt diese Arbeit darauf ab, CSIA für die Pestizide Bentazon, MCPA, Dichlobenil, dessen Metabolit 2,6-Dichlorbenzamid (BAM) und Atrazine und dessen Metabolit Desethylatrazin (DEA) zu entwickeln, zu validieren und zu optimieren, um die isotopische Zusammensetzung sowohl für Kohlenstoff als auch für Stickstoff bestimmen zu können.

Kapitel 2 dieser Arbeit präsentiert die substanzspezifische Isotopenanalyse negativ geladener Verbindungen. Vor der Bestimmung mit einem Gaschromatografie-Isotopenmassenspektrometer (GC-IRMS) müssen die anionischen Substanzen derivatisiert werden, was eine strikte Kontrolle der Isotopenfraktionierung voraussetzt. Das Methylierungsreagenz Trimethylsulfoniumhydroxid (TMSH) wurde hier zum ersten Mal im Zusammenhang mit CSIA für eine online-Derivatisierung getestet. Bisherige Derivatisierungsmethoden erforderten teilweise eine aufwendige offline-Probenvorbereitung. Die Pestizide Bentazon und MCPA wurden als Zielsubstanzen verwendet und während einer vollautomatisierten Derivatisierung in einem temperaturprogrammierbaren GC-Injektor methyliert. Die Injektion der Reaktanten erfolgte in einen gepackten Liner. Anschließend wurde durch einen Split-Fluss das Lösungsmittel abgeblasen. Das darauf folgende schnelle Hochheizen des Injektors löst die Derivatisierung aus und führt letztendlich zu einem Transfer des Derivates auf die GC-Säule. Eine stöchiometrische Zugabe von TMSH resultierte in einer vollständigen Umsetzung des Analyten, was durch akkurate und präzise Stickstoffisotopenwerte für Bentazon gezeigt werden konnte. Im Gegensatz dazu erforderte eine reproduzierbare

Kohlenstoffisotopenanalyse von Bentazone und MCPA mindestens einen 250-fachen Überschuss von TMSH. Entgegen der Erwartungen wurde der δ^{13}C-Wert mit kleiner werdendem TMSH-Überschuss leichter (negativer). Das ist ein Indiz dafür, dass bei zunehmender Konzentration der Methylgruppen im Porenraum des Liners eher eine gleichgewichtsähnliche anstelle einer kinetischen Isotopenfraktionierung auftritt, was letztendlich zu reproduzierbaren δ^{13}C-Werten führt. Vergleicht man die Kohlenstoffisotopenmessungen von GC-IRMS mit der Flüssigchromatographie (LC-) IRMS, so kann man für beide Methoden eine niedrige Standardabweichung (0.3 ‰ für GC- IRMS, 0.1 ‰ für LC- IRMS) und eine vergleichbare Abweichung zum tatsächlichen Wert von -1 ‰ zur Elementaranalysator- IRMS verzeichnen. Schlussfolgerung ist, dass beide Methoden, sowohl GC-IRMS als auch LC-IRMS, einen geeigneten Weg zur online substanzspezifischen Isotopenanalyse anionischer Moleküle darstellen.

In Kapitel 3 werden zwei verschiedene Reaktortypen für die Kohlenstoff- und Stickstoffisotopenanalyse ausgewählter Zielsubstanzen, die zum Teil als schwer analysierbar gelten, getestet. Die substanzspezifische Isotopenanalyse basiert auf der Verbrennung organischer Substanzen zu CO_2 und N_2 in einem Miniaturreaktor. Für viele organische Substanzen ist CSIA bereits etabliert. Im Gegensatz dazu können Heteromoleküle und Substanzen die Halogene beinhalten Probleme bei der Verbrennung darstellen. In diesem Kapitel wird beschrieben, wie die Kohlenstoff- und Stickstoffisotopenanalyse von Atrazin, dessen Metabolit DEA, Dichlobenil und dessen Metabolit BAM und Koffein unter der Verwendung einer Vielzahl von Reaktoren getestet wurde. Einerseits wurden die herkömmlichen Reaktoren, ausgestattet mit CuO/NiO/Pt-Drähten und andererseits, der neu entwickelte Reaktor, der aus einem Ni-Rohr und CuO/NiO-Drähten besteht, getestet. Die Vorteile des neuen Reaktors liegen vor allem darin, dass auf den Reduktionsreaktor bei Stickstoffmessungen verzichtet werden kann, da sowohl die Oxidation von N zu NO_x als auch die Reduktion von NO_x zu N_2 im neuen Reaktor stattfindet. Des Weiteren ermöglicht das neue Design die Reoxidation des Reaktors auch während der Stickstoffanalyse, was die Anzahl der durchführbaren Analysen mit einem Reaktor enorm erhöht. Letztendlich konnten zum ersten Mal mehrere hundert akkurate und präzise Kohlenstoff- und Stickstoffmessungen von Atrazin und Desethylatrazin durchgeführt werden. Trotzdem zeigten beide Reaktortypen starke Unterschiede in der Richtigkeit von Stickstoffmessungen und weniger ausgeprägt bei den Kohlenstoffmessungen. Die Richtigkeit der Isotopenmessungen von verschiedenen Heteromolekülen wurde bezüglich des Reaktordesigns, der Bedingungen während der

Oxidation und der Lebenszeit untersucht. Richtige Isotopenwerte wurden durch Messungen mit externen substanzspezifischen Standards und einer Korrektur der Abweichung zum Sollwert bestimmt. Zusammenfassend ist zu betonen, dass die instrumentell bestimmten Isotopenwerte stets einer Validierung mit substanzspezifischen Isotopenstandards des entsprechenden Analyten bedürfen.

Kapitel 4 demonstriert am Beispiel des biologischen Abbaus des Pestizids Dichlobenil und dessen Metabolit BAM die Anwendbarkeit der substanzspezifischen Isotopenanalyse, die in Kapitel 3 dargestellt wurde. Zum ersten Mal werden Kohlenstoff- und Stickstoffisotopenfraktionierung für Dichlobenil und BAM berichtet, die mit dem biologischen Abbau der Substanzen assoziiert werden können. Der Abbau von BAM, durchgeführt mit den Bakterienstämmen *Aminobacter* sp. ASI1 und MSH1, geht mit einem sehr ähnlichen Fraktionierungsmuster einher. Es wurde gezeigt, dass einerseits die Änderung der Kohlenstoffisotopensignatur während des Abbaus herangezogen werden kann, um Transformationsprozesse qualitativ nachzuweisen und andererseits ist es potentiell möglich, die bemerkenswert robuste Kohlenstoffisotopenfraktionierung für eine quantitative Abschätzung des Abbaus in der Umwelt zu nutzen. Für eine quantitative Abschätzung des Umweltverhaltens, ist dabei aber zu berücksichtigen, dass Dichlobenil auf Grund der guten Sorption im Boden gegenwärtig ist und die Isotopensignatur des entstehenden Metaboliten BAM bestimmt. Erste Abbauexperimente von Dichlobenil mit dem Bakterienstamm *Rhodococcus erythropolis* DSM 9685 zeigten einen kleinen Stickstoff- und kaum einen Kohlenstoffisotopeneffekt. Das entstehende BAM spiegelt die Isotopensignatur von Dichlobenil wieder, solang bis BAM selbst biologisch abgebaut wird und eine Anreicherung von ^{13}C zu beobachten ist. Da eine Vielzahl von Bakterien Dichlobenil abbauen können, und dabei unterschiedliche Fraktionierungsmustern entstehen können, ist es notwendig weitere Abbauuntersuchungen durchzuführen, um eine verlässliche Einschätzung des Umweltverhaltens von Dichlobenil und BAM durchführen zu können. Zusätzlich konnten mit Hilfe der Untersuchungen erste Einblicke in den Reaktionsmechanismus beim enzymatischen BAM-Abbau, katalysiert durch Amidase, erlangt werden. Das gefundene Muster und das Ausmaß der Kohlenstoff- und Stickstofffraktionierung, das beide Bakterienstämme hervorrufen, reflektiert zum einen, dass eine sehr ähnliche Kinetik beider Reaktionen vorliegt und zum anderen, dass der Übergangszustand entweder aus einem tetrahedralen Zwischenprodukt besteht, das durch eine OH^- oder H_3O^+ unterstützte Hydrolyse entsteht, oder aber durch einen konzertierten Reaktionsmechanismus eingeleitet wird.

Insgesamt präsentiert diese Arbeit Methoden, die es ermöglichen präzise und akkurate Kohlenstoff- und Stickstoffisotopenanalysen von einigen der am häufigsten in der Umwelt nachgewiesenen Pestiziden und deren Metaboliten durchzuführen.

Diese Arbeit hat einen wichtigen Schritt geleistet, um das Umweltverhalten von Pestiziden abzuschätzen. Im speziellen, wurde

(i) die Isotopenanalytik für die Pestizide Bentazon, MCPA, Dichlobenil, dessen Metabolit BAM sowie Atrazin und dessen Metabolit DEA entwickelt und validiert. Damit steht ein weiteres Mittel zur Verfügung, um das Abbauverhalten dieser Substanzen im Grundwasser zu beurteilenzum ersten Mal über Anreicherungsfaktoren für Kohlenstoff und Stickstoff während des biologischen Abbaus von Dichlobenil und BAM berichte.

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If we knew what it was we were doing, it would not be called research, would it? (Albert Einstein)

1.

General Introduction

1.1 Pesticides

Pesticides are chemical substances used for pest control of economic plants. They are widely used in agriculture and horticulture. Although pesticides are designed to have a short lifetime, parent substances or metabolites are frequently detected in environmental systems, such as soil and groundwater. For example the ranking of positive pesticide findings (Table 1-1) detected out of approximately 10.000 wells in Germany (UBA, 2006) highlights the importance of the pesticide atrazine and its metabolite desethylatrazine, although the pesticide itself was banned decades ago.

Table 1-1. Ranking of positive findings of pesticides in Germany in near groundwater from 1996 to 2000 and from 1990 to 1995 (after UBA, Daten der Ländergemeinschaft Wasser 2006)

Rank		Agent/Metabolite		detected	
1996-2000	1990-1995		≤0.1 µg/l	>0.1 to ≤1.0 µg/l	>1.0 µg/l
1	1	Desethylatrazine	1715	557	13
2	2	Atrazine	1609	262	10
3	3	Bromacile	144	151	26
4	9	Bentazone	195	61	9
5	6	Diurone	166	50	17
6	4	Simazine	454	62	5
7	5	Hexazinone	119	51	6
8	8	Desisopropylatrazine	216	51	5
9	-	2,6-Dichlorobenzamide (BAM)	98	46	3
10	10	Mecoprop	119	37	5

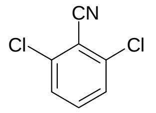
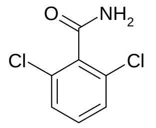
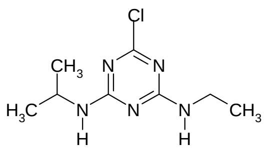
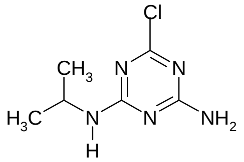
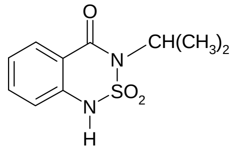
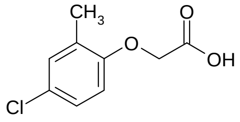
Another pesticide of particular importance is bentazone (3-isopropyl-1H-2,1,3-benzothiadiazine-4(3H)-one-2,2-dioxide), whose incidence in groundwater has increased in the last years. Likewise, a pesticide metabolite with increasingly frequent findings in Germany is 2,6-dichlorobenzamide (BAM), in contrast to its parent compound dichlobenil. In consideration of the fact, that numerous findings axed the concentration limit of 0.1 µg L^{-1} for pesticides in groundwater in the European Union, these data are a telling indication of the increasing importance of the pesticides and even their metabolites as groundwater contaminants. Investigations are necessary to assess the fate of such contaminants in the environment. In the following, the target compounds of this work are shortly introduced, focusing on their chemical properties and the mode of action.

1.1.1 Bentazone and MCPA

Bentazone and (4-chloro-2-methylphenoxy)acetic acid (MCPA) are slightly acidic contact herbicides, which belong to the thiodiazine and phenoxyacetic acid family, respectively. Both compounds are highly mobile due to their high water solubility (Table 1-2).

Bentazone, either in combination with other active ingredients or alone, is used mainly for controlling weeds in corn fields, paddy fields and orchards, but also as algicide in paints and coatings. Bentazone causes damage to the parts of the plant to which it is applied, scion and foliage. The effect is based on inhibition of photosynthesis, which causes the consumption of carbohydrate reserves and loss of chloroplast membrane intactness (Fletscher and Kirkwood, 1982). MCPA is used as post-emergence herbicide for selective control of broadleaf weeds and is immediately absorbed by roots and leaves where it acts as a synthetic growth regulator (Brian and Rideal, 1952).

Table 1-2. Structures, molecular weight, pK_a and solubility of the target compounds

Common name (IUPAC name)	**Chemical structure**	**Molecular weight [g mol^{-1}]**	**pK_a**	**Solubility in water [mg l^{-1}]**
Dichlobenil (2,6-dichlorobenzonitrile)		172	-	18[a] (20 °C)
BAM (2,6-dichlorobenzamid)		190	-	2730 [b] (23 °C)
Atrazine (2-chloro-4-2-isopropylamino-6-ethylamino-s-triazine)		216	-	33[c] (22 °C)
Desethylatrazine - DEA (6-chloro-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine)		188	-	Not found
Bentazone (3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide)		240	2.9[d]	570[e] (22 °C, pH 7)
MCPA (4-Chloro-2-methylphenoxy)acetic acid		207	3.1[f]	274[f] (20 °C, pH 7)

[a] Tomlin (1997), [b] Geyer (1981), [c] Tomlin (1994), [d] IUPAC, 2011, [e] Huber und Otto (1994) [f] Tomlin (2000)

1.1.2 Dichlobenil and its metabolite 2,6-Dichlorobenzamide

The benzonitrile herbicide dichlobenil is a polar compound, having a cyano substituent on the benzene ring (Table 1-2). It is mostly used on non-agricultural areas, in plant nurseries and fruit orchards. Dichlobenil is taken up by the roots and transported throughout the plant, where it inhibits actively dividing cells by disrupting the formation of the cell plate (Vaughan and Vaughn, 1988). Actively growing plants and germinating seedlings are mostly affected by dichlobenil. The metabolite 2,6-dichlorobenzamid (BAM) is produced from incomplete degradation of the pesticide. BAM is more soluble in water than its corresponding parent compound (Table 1-2).

1.1.3 Atrazine and its metabolite Desethylatrazine

2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (Atrazine, Table 1-2) is a triazine herbicide used worldwide to control growth of annual grass and broadleaf weeds during crop production. Its herbicidal function is caused by binding to the quinone-binding protein in photosystem II, thus inhibiting photosynthetic electron transport. Also other photosynthetic processes (such as opening of stomata, transport of ions) as well as the RNA, and protein synthesis are affected. (Huber, 1993; Tappe et al., 2002; Trebst, 2008). Atrazine was banned in the nineties by the European Union, but atrazine and its metabolites are still detected in groundwater and soil systems (see also Table 1-1) (Baran et al., 2007; Sagratini et al., 2007; Tappe et al., 2002). Desethylatrazine is formed by N-dealkylation after preceding oxidation (Ellis et al., 2006; Erickson, 1989).

1.2 Environmental behaviour of pesticides

1.2.1 General

The fate of pesticides in the environment is governed by several processes, which are dependent on (i) substance specific properties (e.g. polarity, water solubility, volatility) and (ii) environmental properties (e.g. soil constituents, soil pH, *in situ* microorganisms, rain events, and climate). The uptake velocity of the pesticides via roots or leafs after the application and the interaction of (i) and (ii) have an impact on the pesticides behaviour and may lead to a penetration through the soil to the groundwater systems. On that way different, often simultaneous processes may take place, like

- chemical and biological transformation reactions (biotic and abiotic degradation)
- retention and/or sorption
- transport (volatilization, run off, leaching).

In general, the more mobile a pesticide is (high water solubility, low sorption potential), the higher is the groundwater contamination potential. The most important environmental processes are transformation reactions, as they may lead to a conversion of contaminants to less harmful substances. Studies of dichlobenil and BAM biodegradation will be presented in this work and therefore we focus in the following section on these particular compounds. The other target substances of the analytical method development will therefore not be discussed with respect to their degradation reactions.

1.2.2 Dichlobenil and BAM

Dichlobenil was banned in Germany and other European Countries during the last decades, due to the frequent findings of its metabolite BAM in the environment (Haarstad and Ludvigsen, 2007; Törnquist et al., 2007; UBA, 2006). It has been shown in numerous studies that dichlobenil itself is biodegraded in topsoil (Briggs and Dawson, 1970; Clausen et al., 2007; Montgomery et al., 1972; Verloop and Nimmo, 1970) and subsurface sediments (Clausen et al., 2007) and additionally that it highly sorbs to soil and sediments (Briggs and Dawson, 1970; Clausen et al., 2004; Jernlås, 1990). Both processes support the low dichlobenil concentration in the environment. In contrast, its degradation product BAM is rapidly transported to groundwater (Holtze et al., 2006; Holtze et al., 2007a; Sorensen et al., 2007) and causes a widespread presence. Figure 1-1 shows the degradation pathway of dichlobenil via enzymatic hydrolysis to the corresponding amide BAM with nitrile hydratase as catalyst and a possible further degradation to the corresponding acid by the amidase enzyme (Banerjee et al., 2002; Kato et al., 2000). Frequent groundwater contamination by BAM indicates that degradation of BAM to 2,6-DCBA and direct degradation of dichlobenil to 2,6-DCBA are of minor importance in the field. BAM, however, has been shown to be the main metabolite in soil occurring by degradation of dichlobenil by the bacteria belonging to the genera *Arthrobacter*, *Rhizobium* and *Rhodococcus* (Jolana Vosáhlová et al., 1997; Miyazaki et al., 1975; Montgomery et al., 1972; Verloop and Nimmo, 1970). In contrast, only few studies reported degradation of BAM in soil samples (Fournier, 1975; Miyazaki et al., 1975; Simonsen et al., 2006) Recently, the further transformation of BAM to the corresponding carboxylic 2,6 DCBA with amidase has been observed in soil previously exposed to dichlobenil (Holtze et al., 2007b; Simonsen et al., 2006; Sorensen et al., 2007) and subsequently resulted in the isolation of the only two BAM-mineralizing bacteria, identified to date, i.e., *Aminobacter* sp. ASI1 and MSH 1.

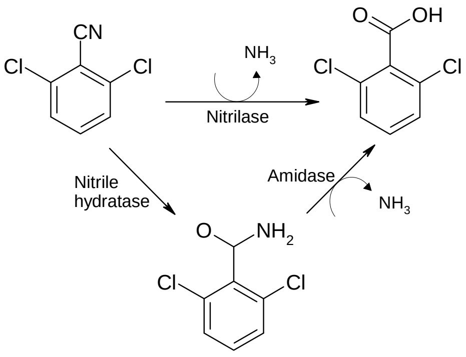


Figure 1-1. Possible enzymatic hydrolysis pathways of dichlobenil degradation catalyzed by different enzymes (adapted from Holtze (2008))

Insight is still needed about the degradation potential of dichlobenil and BAM in the field. As indicator of transformation reactions, the measurements of concentrations of the substances and their metabolites are not fully conclusive, because processes like dilution and sorption may also lead to a considerable concentration decrease. Furthermore the detection and identification of further metabolites may fail if they are rapidly degraded or if they are not accessible to analysis such as in the case of 2,6-dichlorobenzoic acid from BAM. In the absence of complete mass balances, a new, independent approach is therefore needed to assess the fate of pesticides in the environment. This study focuses on compound specific isotope analysis (CSIA) to provide such additional lines of evidence.

1.3 Compound specific isotope analysis

1.3.1 Fundamentals and application

Compound specific isotope analysis (CSIA) can be used to investigate and monitor the fate of organic contaminants in groundwater and soil systems. Isotopes like carbon, nitrogen, oxygen and hydrogen have naturally occurring stable isotopes. CSIA measures the stable isotope composition of a compound at natural abundance. Most applications of CSIA to date refer to volatile compounds such as chlorinated and petroleum hydrocarbons as summarized in various reviews (Hofstetter et al., 2008; Meckenstock et al., 2004; Schmidt et al., 2004). The ratio of the isotopes ($^{13}C/^{12}C$, $^{2}H/^{1}H$, $^{15}N/^{14}N$, $^{18}O/^{16}O$) is given in the δ notation (eq. 1-1) relative to an international standard (Peedee Belemnite for carbon)

$$\delta_x = \left(\frac{R_x}{R_{reference}} - 1 \right) \quad [‰] \qquad \text{(Equation 1-1)}$$

where R_x and $R_{reference}$ are the ratio of the heavy isotope to the light isotope in compound x and an international standard, respectively.

On the one hand, a different isotopic composition of the same chemical substance can be used as fingerprint to determine the source of contamination (Blessing et al., 2009; Hunkeler et al., 2004; Schmidt et al., 2004; Shouakar-Stash et al., 2003). On the other hand, it may serve to verify that biological degradation occurs, because during chemical reactions and biodegradation, molecules containing the light isotope in the reactive position tend to react slightly faster than molecules containing the heavier counterpart in the reactive position (Melander and Saunders, 1980). As a result, the heavier isotope becomes enriched within the remaining substrate and the light isotope becomes enriched in the products. The reason for such an isotope fractionation is the kinetic isotope effect (KIE) and can be described as

$$KIE = \frac{{}^{l}k}{{}^{h}k} \qquad \text{(Equation 1-2)}$$

where ${}^{l}k$ and ${}^{h}k$ are the reaction rate constants of the light isotope and the heavy isotope, respectively. Kinetic isotope effects are called primary if the isotope is located at the position at which the chemical bonding changes during a reaction and is called secondary if the isotope appears at a position next to it (Melander and Saunders, 1980). Compared to kinetic isotope effects during transformation reactions, the isotope fractionation associated with non-degradative processes is much less pronounced (Dempster et al., 1997; Harrington et al., 1999; Slater et al., 1999). Besides the qualitative detection of natural degradation processes, major goals in the use of CSIA are to quantify the extent of *in situ* degradation and to elucidate even degradation pathways. To this end, it needs to be known how large the changes in isotope values are if a compound undergoes a certain degradation reaction.

The induced shifts in the isotope composition of organic pollutants during transformation reactions can be investigated in laboratory studies. The decrease in the contaminant concentration and the associated isotope enrichment can be measured simultaneously. These observations may be generally well described by the Rayleigh equation (Hoefs, 1987; Rayleigh, 1896):

$$\ln\frac{R}{R_0} = \ln\frac{R_0 + \Delta R}{R_0} = (\alpha - 1) * \ln f = \frac{\varepsilon}{1000}\ln f \qquad \text{(Equation 1-3)}$$

where R and R_0 are the compound specific isotope ratios of heavy versus light isotopes of an element in the contaminant at a given time and at the beginning of the reaction, respectively. $\Delta R = (R - R_0)$ is the change in the isotope ratio, *f* is the fraction of the remaining contaminant at the given time, α is the fractionation factor between the remaining substrate and the degradation product and ε is the enrichment factor. Evaluation of α and ε from laboratory studies

$$\frac{\varepsilon}{1000} = (\alpha - 1) \qquad \text{(Equation 1-4)}$$

can be achieved by determining the slope ε/1000 from a plot of compound specific isotope ratios (R_t/R_0) versus the concentration of the remaining substrate (C_t/C_0). Once determined, α values can then be used to quantify biodegradation B in percent (%) in samples of contaminated sites based on measured isotope signatures according to:

$$B = 1 - f = \left(\frac{R_t}{R_0}\right)^{\frac{1}{\alpha - 1}} * 100\% \qquad \text{(Equation 1-5)}$$

where R_0 and R are the isotope ratio at the source and at a special location in the field. This approach has repeatedly been used for successful quantification of biodegradation in the field with chlorinated hydrocarbons (Hunkeler et al., 1999; Kuhn et al., 2009; Sherwood Lollar et al., 2001), petroleum hydrocarbons (Beller et al., 2008; Fischer et al., 2006; Griebler et al., 2004; Richnow et al., 2003) or RDX (Bernstein et al., 2010). Additionally, official guidelines are available, which recommend the use of stable isotope fractionation measurements to estimate biodegradation of organic pollutants in the field (Eisenmann and Fischer, 2010; EPA, 2002; Hunkeler et al., 2008). The enrichment factor ε for the degradation of a compound can vary according to the type of the reaction pathway, as, for example demonstrated for aerobic (Gray et al., 2002; McKelvie et al., 2009) and anaerobic (Kuder et al., 2005) MTBE degradation. Likewise, a systematic variation in carbon enrichment factors was shown by Hirschorn et al. (2004) for the oxidative ($\varepsilon_C = -3.9 \pm 0.6$ ‰) and the hydrolytic degradation ($\varepsilon_C = -29.2 \pm 1.9$ ‰) of 1,2-dichlorethane. These cases exemplify the fact that different reaction pathways are associated with different kinetic isotope effects, and therefore show a characteristic pattern of measurable isotope fractionation. A linear relation is expected when plotting the isotope enrichment of e.g. C vs. N (dual isotope plot) during the degradation process. Based on the slopes of such dual isotope plots different degradation pathways may be elucidated (Elsner et al., 2005; Meyer et al., 2009; Penning et al., 2008). This allows the interpretation of degradation processes in the field by comparison with laboratory results, as demonstrated in a MTBE study, where a dual isotope plot constructed from carbon and hydrogen isotope ratios could clearly distinguish between aerobic and anaerobic degradation (Zwank et al., 2005). By identifying the correct enrichment factor, in turn it was possible to attempt a quantification of the extent of biodegradation in each field sample.

1.3.2 Instrumentation

Carbon and nitrogen isotope signatures of the target substances were performed using an isotope ratio mass spectrometer (IRMS) directly connected to a gas chromatograph (GC). The principle of GC-IRMS is well described in Brand (1996). Carbon isotope analysis of bentazone was performed using a high pressure liquid chromatograph (HPLC) coupled to a IRMS via continuous flow (principles are described in Krummen et al. (2004).

1.4 Objectives

This thesis aimed to develop compound specific isotope analysis methods for the pesticides bentazone, MCPA, dichlobenil and its metabolite BAM. A particular challenge for these compounds was to overcome the analytical problems related to the structure of the target compounds and to establish reproducible procedures to determine the carbon and nitrogen isotope ratios. Bentazone and MCPA on one hand are negatively charged molecules and require a derivatization prior to analysis. Dichlobenil and BAM on the other hand are polar compounds containing hetero atoms and chlorine, which might be a challenge for gas chromatographic separation and additionally might show problems related to the combustion process. Once, robust and precise methods are developed they might be used to detect, quantify and even identify transformation reactions. Therefore, degradation experiments with dichlobenil and BAM were performed to demonstrate the applicability of compound specific isotope analysis.

In particular the work focused on the three objectives

(a) To develop a robust method for the determination of C and N isotope ratios of the negatively charged pesticide bentazone by GC-IRMS using trimethylsulfonium hydroxide as derivatization agent. Alternatively, LC-IRMS is used to determine the C isotope ratio of non-derivatized bentazone. Both methods should be assed with respect to precision and accuracy. Furthermore, the derivatization method is applied to determine the C isotope ratio of the pesticide MCPA to demonstrate the applicability of the derivatization method for other negatively charged compounds (Chapter 2).

(b) To investigate the performance of gas chromatograph- isotope ratio mass spectrometry of compounds that contain C, N and Cl atoms in an aromatic structure and are potentially recalcitrant to combustion. Dichlobenil and BAM were used as target substances, complemented by the pesticide atrazine and its metabolite desethylatrazine, as well as caffeine (Chapter 3).

(c) To determine C and N isotope enrichment factors during biotic degradation of the metabolite 2,6-dichlorobenzamid using the two pure bacterial strains *Aminobacter* sp. MSH 1 and *Aminobacter* sp. ASI 1 and to compare both degradation reactions using dual isotope plots (Chapter 4).

Chapter 2 (Reinnicke et al., 2010) is already published in a peer-reviewed environmental journal (Analytical Chemistry). Results of Chapter 3 and Chapter 4 are in preparation to be submitted to Rapid Communication in Mass Spectrometry and Environmental Science and Technology, respectively.

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2.

Small and Reproducible Isotope Effects during Methylation with Trimethylsulfonium Hydroxide (TMSH): A Convenient Derivatization Method for Isotope Analysis of Negatively Charged Pesticides

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2.1 Introduction

Compound specific isotope analysis (CSIA) by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) is a prominent tool in environmental and forensic studies. The isotopic composition of a chemical can be used as a fingerprint to determine its origin (Blessing et al., 2009; Kelly et al., 2005; Shibuya et al., 2006). Additionally, isotope values may be measured to detect, quantify (Abe and Hunkeler, 2006; Meckenstock et al., 2004; Sherwood Lollar et al., 2001), and even identify (Elsner et al., 2007; Fischer et al., 2008) abiotic or biotic transformation reactions of organic compounds based on the kinetic isotope fractionation associated with their (bio)chemical degradation reactions (Elsner et al., 2005; Schmidt et al., 2004).

To date most CSIA measurements of environmental samples have been concerned with low molecular weight hydrocarbons that contain no or few polar functional groups. Since such compounds are volatile, they are amenable to analysis by GC-IRMS. The volatile substances are evaporated in the injector of a gas chromatograph and are subsequently separated on a gas chromatographic column in a He carrier gas stream. The He stream carries the target compounds to a combustion interface, where they are converted online to CO_2, NO_x, etc. with successive nitrogen oxide elimination to N_2 in a reduction oven. Subsequently the He transfers the combusted gases directly into an IRMS (Brand, 1996; Meier-Augenstein, 1999). Compared to conventional dual inlet - IRMS or elemental analyzer (EA) - IRMS instrumentation, GC-IRMS instruments improve lower limits for high precision isotope analysis by several orders of magnitude. Also, the separation capability of gas chromatography makes it possible to single out organic compounds from complex matrices so that CSIA can be conveniently performed on extracts of environmental samples.

A number of studies has been aiming to adapt such measurements also for compounds that have more complex molecular structures and are less amenable to gas chromatography, such as pharmaceuticals (Godin et al., 2007), amino and fatty acids (Godin et al., 2005; Krummen et al., 2004; McCullagh et al., 2006; Meier-Augenstein, 2002; Rieley, 1994), explosives (Bernstein et al., 2008; Hofstetter et al., 2008), and pesticides (Meyer et al., 2008; Penning and Elsner, 2007). Our particular focus is on pesticides, which are widely used in agriculture and horticulture and are repeatedly detected in soil and groundwater systems.

Such compounds are frequently non-volatile or even occur in negatively charged form and therefore cannot be directly analyzed with gas chromatography. For this reason, they either need to be targeted with alternative, liquid chromatography-based methods or be derivatized prior to gas chromatographic separation. Even though both approaches are well-established for conventional analysis of organic compounds, their adjustment to isotope analysis poses unique challenges as discussed shortly (Corr et al., 2007; Smith et al., 2009).

(i) Liquid chromatography-isotope ratio mass spectrometry (LC-IRMS) separates the target compounds by LC, followed by a wet oxidation process at 99.9 °C in the presence of phosphoric acid and sodium peroxodisulfate generating CO_2. The CO_2 is extracted from the eluent through a membrane into a counter stream of helium and carried to an IRMS (Krummen et al., 2004). Such a setup enables compound specific isotope analysis for substances that are not amenable by GC. However, the approach is presently restricted to $^{13}C/^{12}C$ isotope analysis. Also, the sensitivity is an order of magnitude lower than with GC-IRMS, and liquid chromatography must be performed without organic solvents which strictly limits the application to water-soluble substances.

(ii) Alternatively, derivatization can make compounds volatile by protecting their polar groups and compensating for the negative charge with substituents (e.g., methylation, acylation, or silylation) so that they become amenable to gas chromatography. However, this procedure may influence the results of the stable isotope analysis for several reasons. First, the carbon isotopic composition is changed due to additional atoms of the derivatization agent, whose isotope value may differ from the target compound. Second, there is a need for complete conversion of the target compound, which requires that the derivatization agent is used in excess. This means, in turn, that the derivatization agent itself is not completely converted. Therefore, the newly introduced atoms do not only carry the isotope value of the different carbon source but reflect, in addition, the isotope effect of the derivatization (Macko et al., 1998; Meier-Augenstein, 1999; Rieley, 1994; Silfer et al., 1991). Nitrogen isotope ratios, in contrast, are much less affected by derivatization, because no additional N atom is introduced. In theory, if complete conversion is accomplished, N isotope values of the derivatized compounds should be equal to those of the underivatized analyte (Corr et al., 2007; Hofmann et al., 2003; Merritt and Hayes, 1994).

For an appropriate derivatization procedure prior to CSIA, the following criteria are therefore important. (i) The compound must be converted quantitatively in order to avoid kinetic isotope effects in the structure of the target analyte. (ii) A practicable derivatization procedure and an easy-to-handle derivatization agent are expedient. (iii) As few new atoms should be introduced as possible. (iv) The isotopic composition of these newly introduced atoms must be constant and reproducible, meaning that the influence of the isotope effect during the derivatization must be tightly controlled. Depending on the target compound a choice of derivatization agents may fulfil one or more of these criteria.

Silylation. *Trimethylsilyl* and *tert-butyldimethylsilyl* are often used to prepare amino acids derivatives (Hofmann et al., 2003). They have the advantage that a bond is formed to a Si rather than a C atom so that the derivatization reaction shows very little carbon isotope fractionation. On the downside, silylations involve the introduction of many additional carbon atoms and silicon carbide is suspected to be formed in the combustion oven leading to additional bias in isotope values (Shinebarger et al., 2002). The isotope ratio of the target compound is determined with much lower precision (Gross and Glaser, 2004), and the excessive carbon load can lead to incomplete combustion (Gross and Glaser, 2004; Meier-Augenstein, 1999).

Removal of the negatively charged group. Alternative strategies convert organic acids to their corresponding alcohols (Ziadeh et al., 2002) or to decarboxylated compounds (Corso et al., 1998). Since no additional carbon atoms are introduced, isotope values measured by GC-IRMS were not significantly different compared to EA-IRMS (Corso et al., 1998; Zaideh et al., 2001; Ziadeh et al., 2002). The offline manual treatment is time-consuming, however, and the approach was demonstrated for selected substances and may be not universal.

Methylation. An advantage of methylation is that only one carbon atom is introduced to the derivized molecule. Different methylation agents can be used to this end: *Diazomethane* (Tetens et al., 2002) is a universal methylation agent but is explosive and highly toxic, and isotope effects may be not reproducible. *BF_3/methanol*, in contrast, is easy to handle and reproducible isotope values are accomplished (Trust Hammer et al., 1998), but artifacts and side reactions are reported for high BF_3/methanol contents (Knuese Fulk and Shorb, 1970). Both preparations involve labor intensive sample processing prior to GC analysis. *Trimethylsulfonium hydroxide (TMSH) or trimethylanilinium hydroxide*, in contrast, are powerful alternative methylation reagents, which have repeatedly been used in quantitative analysis of acidic compounds with gas chromatography (Diez et al., 2006; Dron et al., 2004; Färber et al., 1991; Ogierman, 1990) but not yet in isotopic measurements. These agents are easy to handle: they simply need to be mixed with the target compound solution, and the derivatization can be directly accomplished in the hot injector of the gas chromatograph eliminating the need for time-consuming off-line pre-treatment (Färber et al., 1991; Ogierman, 1990).

In this study we therefore tested for the first time TMSH as a derivatization agent for compound-specific isotope analysis (CSIA) of pesticides. To this end we chose two frequent pesticides that are negatively charged at circumneutral pH as target compounds, bentazone (3-isopropyl-1H-2.1.3-benzothiadiazone-4(3H)-one 2,2-dioxide) and MCPA (4-chloro-2-methylphenoxy)acetic acid (Scheme 2-1). The goal was to develop a suitable CSIA method using a temperature programmable injector in which online derivatives of negatively charged pesticides with TMSH can be conveniently generated. We further assessed the accuracy and reproducibility of GC-IRMS analysis in dependence on target compound concentrations and derivatization agent-to-analyte-ratios. Our particular focus was on the question whether the analyte was quantitatively converted, on linearity (= amount dependence) of isotope measurements, and on possible isotope effects associated with the derivatization reaction. The performance of carbon isotope analysis using the derivatization technique was compared to LC-IRMS measurements of nonderivatized bentazone.

2.2 Material and Methods

2.2.1 Chemicals

Bentazone (3-isopropyl-1H-2.1.3-benzothiadiazone-4(3H)-one 2,2-dioxide, CAS no.: 25057-89-0) and MCPA ((4-chloro-2-methylphenoxy)acetic acid, CAS no.: 94-74-6) produced by Riedel-de Haën (>98%) were supplied by Sigma Aldrich (Taufkirchen, Germany). Ethyl acetate (99.5%, MERCK) was used as a solvent for the bentazone and MCPA solutions. TMSH (trimethylsulfonium hydroxide, CAS no.: 17287-03-5) produced by Fluka (0.25 Min methanol) was supplied by Sigma Aldrich (Taufkirchen, Germany) and was used as derivatization reagent for bentazone and MCPA.

2.2.2 Derivatization procedure

The derivatization of bentazone and MCPA by trimethylsulfonium hydroxide (TMSH) (Scheme 2-1) was carried out using a temperature programmable GC injector (Optic 3-SC

High Power Injection System, ATAS GL International B.V., Veldhoven, Netherlands) with a packed glassbead liner (PAS Technologie, Magdala, Germany).

Different TMSH-to-analyte ratios were investigated for different bentazone concentrations (3 mg L^{-1}, 30 mg L^{-1}, 60 mg L^{-1}, 120 mg L^{-1}, and 300 mg L^{-1} for carbon isotope analysis and 4500 mg L^{-1} and 5500 mg L^{-1} for nitrogen analysis); for MCPA a 200 mg L^{-1} standard was used. To achieve TMSH-to-analyte ratios of between 0.4 and 800, 8 to 120 µL of 0.25 M TMSH in methanol were added to varying volumes of analyte solutions in ethyl acetate (between 30 and 1000 µL, with analyte concentrations between 3 mg L^{-1} and 5500 mg L^{-1}). Solutions were stable for at least 24 h (data not shown). Between 1 and 30 µL of the premixed solution were then injected into to the glassbead liner (outer diameter 5 mm, inner diameter 3.2 mm, length 81 mm, package length 25 mm) of the injector at 40 °C. A vent time of 300 s (for injected volumes of up to 9 µL) or 720 s (for injected volumes of 10 to 30 µL) with a split flow of 83 mL min^{-1} was used to remove all solvent. Subsequently the split flow was set to 0 mL min^{-1} for 5 min (column flow 1.4 mL min^{-1}), and the injector was heated to 250 °C with a rate of 14 °C/s. This flash heating triggered the derivatization reaction and simultaneously transferred the derivative onto the GC column.

2.2.3 Isotope analysis by GC-IRMS

The GC- 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 set at 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, and liquid samples were injected via a GC Pal autosampler (CTC, Zwingen, Switzerland). The analytical column used was a DB-5 (30 m x 0.25 mm; 1 μm film; J&W Scientific, Folsom; CA, US). The GC oven was programmed from 80 °C (hold: 1 min), ramp 20 °C min^{-1} to 190 °C, ramp 8 °C min^{-1} to 260 °C (hold: 5 min).

2.2.4 Isotope analysis by LC-IRMS

The LC-Isolink system (LC-IRMS) consisted of a Finnigan Surveyor HPLC (LC) including a Surveyor MS Pump and a Surveyor autosampler coupled to a Finnigan MAT 253 isotope ratio mass spectrometer via a Finnigan LC isolink interface (all instruments

Thermo Fisher Scientific, Bremen, Germany). The temperature of the wet combustion oven was set at 99.9 °C, and the helium (grade 5.0) flow rate of the separation unit was set to 1 mL min^{-1}. The oxidation agent used was 10 mM $Na_2S_2O_8$, and the acid was 10 mM H_3PO_4. Both were set to a flow rate of 50 μL min^{-1}. The injection with a Finnigan Surveyor autosampler was performed via a 25 μL loop, and the flow rate was 500 μL min^{-1}. The analytical column was a C16 Acclaim PA2 (3 μm, 3 x 15 mm, Dionex, Idstein, Germany). As liquid chromatographic eluent a 10 mM phosphate puffer (pH 7) was used.

2.2.5 Combustion reactors for GC-IRMS

A commercial ceramic tube filled with CuO/NiO/Pt-wires (Thermo Fisher Scientific, Bremen, Germany) operated at 940 °C was used for C isotope analysis for bentazone and MCPA. Self-made reactors consisting of a ceramic tube (inner diameter 0.8 mm, outer diameter 1.5 mm, length 33 cm, Friatec, Mannheim, Germany) containing one copper, nickel, and platinum wire (diameter 0.1 mm, length 26 cm, purity 99.99 %, Alfa Aesar, Karlsruhe, Germany) operating at 940 °C were used for N isotope analysis of bentazone. Prior to carbon isotope analysis the commercial CuO/NiO/Pt was oxidized for 8 h at 600 °C in a continuous stream of O_2 passing through the reactor tube. The self-made reactor for N measurements was repeatedly reoxidized for 15 min (4 times, after app. 60 runs). For reduction of nitrogen oxides in the He carrier stream a standard reduction reactor from Thermo Fisher Scientific (Bremen, Germany) was operated at 650 °C.

2.2.6 Determination of reference values by EA-IRMS

The carbon and nitrogen isotopic composition of bentazone was also determined by elemental analyzer - isotope ratio mass spectrometry (EA-IRMS) consisting of a EuroEA (EuroVector, Milano, Italy) coupled to a FinniganTM MAT253 IRMS (Thermo Fisher Scientific, Bremen, Germany) by a FinniganTM ConFlow III interface (Thermo Fisher Scientific, Bremen, Germany) and calibration against organic reference materials (USGS 40, USGS 41, IAEA 600) provided by the International Atomic Energy Agency (IAEA, Vienna, Austria). δ^{13}C and δ^{15}N values are reported in permil relative to PeeDee Belemnite (V-PDB) and air, respectively:

$$\delta^{13}C = \frac{\left({}^{13}C/{}^{12}C_{\text{Sample}} - {}^{13}C/{}^{12}C_{\text{Reference}}\right)}{{}^{13}C/{}^{12}C_{\text{Reference}}} \qquad \text{(Equation 2-1)}$$

$$\delta^{15}N = \frac{\left({}^{15}N/{}^{14}N_{\mathrm{Sample}} - {}^{15}N/{}^{14}N_{\mathrm{Reference}}\right)}{{}^{15}N/{}^{14}N_{\mathrm{Reference}}} \qquad \text{(Equation 2-2)}$$

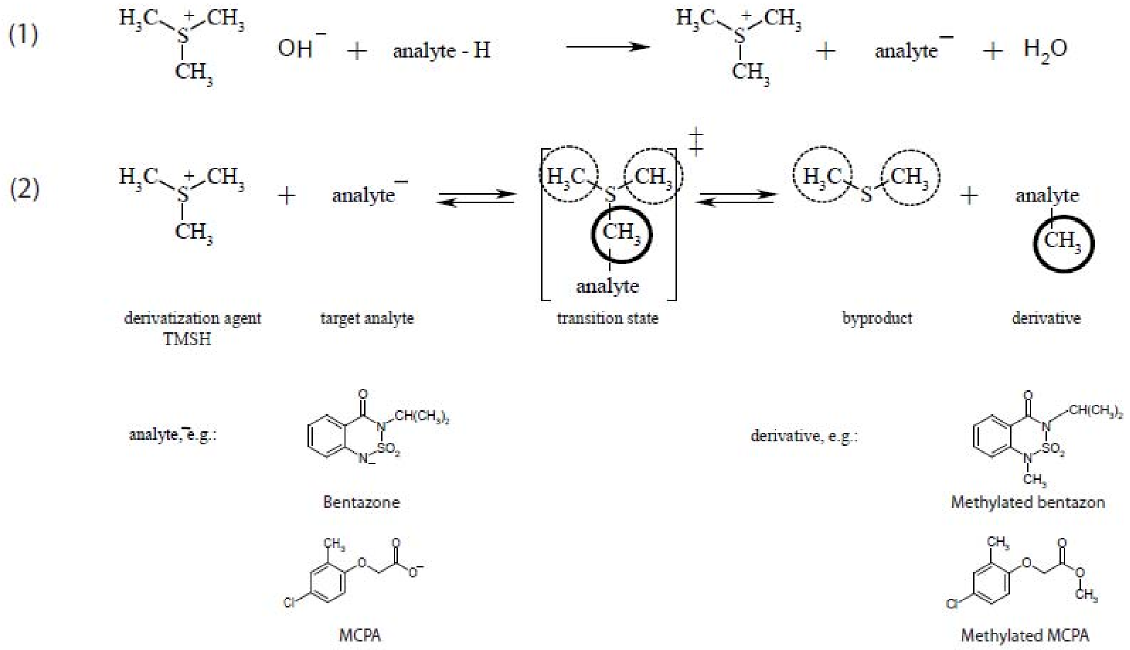
During carbon analysis by GC-IRMS and LC-IRMS, $\delta^{13}C$ values were measured relative to a laboratory CO_2 standard gas that was introduced at the beginning and the end of each run. Linearity of the instrument was checked daily with the laboratory standard and was typically better than -0.05 ‰/V for both nitrogen and carbon for amplitudes between 0.5 V and 10 V. The laboratory standard was calibrated to V-PDB by reference CO_2 standards (RM 8562, RM 8563, RM 8564). In a similar way, an N_2 laboratory standard gas was used for nitrogen GC-IRMS analysis, which had been calibrated to air by reference materials (USGS 40, USGS 41, IAEA 601, USGS 34, and IAEA N-2). All reference standards were provided by the IAEA.

2.3 Result and Discussion

2.3.1 Derivatization of bentazone - Nitrogen isotope analysis

During derivatization of bentazone to form methylated bentazone (Scheme 2-1), no additional nitrogen is introduced to the molecule meaning that no nitrogen isotope effect is expected as long as the derivatization reaction is quantitative. Figure 2-1 shows measured $\delta^{15}N$ values of methylated bentazone when about 5000 mg L^{-1} bentazone were brought to reaction with increasing proportions of the derivatization agent TMSH. Figure 2-1 illustrates that precise and accurate $\delta^{15}N$ values as well as largely constant amplitudes were already achieved at an excess of 20 % derivatization agent (TMSH-to-analyte ratio = 1.2). This result indicates complete conversion of bentazone to methylated bentazone consistent with observations of Yamauchi (1979). In contrast, when TMSH was present in substoichiometric quantities, amplitudes were smaller, and nitrogen isotope values were more negative than the target value. This observation is consistent with incomplete conversion. Due to the nitrogen isotope effect associated with the derivatization reaction, methylated bentazone forms preferentially with ^{14}N in the newly formed bond. When incomplete reaction occurs, the derivative has therefore more negative $\delta^{15}N$ values. We note that this observation does not reflect a decrease in the conversion efficiency but is due to increasing dilution with addition of greater volumes of the derivatizing agent meaning that less target analyte is applied if the same injection volume is used.

Scheme 2-1. Derivatization Reaction of TMSH with negatively charged target compounds



In the tested concentration range we obtained precise $\delta^{15}N$ measurements of -0.6 ± 0.3 ‰ (n = 33) for the bentazone derivatives if TMSH was added in slight excess. This result is in excellent agreement with the value of -0.6 ± 0.2 determined by EA-IRMS, demonstrating a very good accuracy of the method for $\delta^{15}N$ measurements.

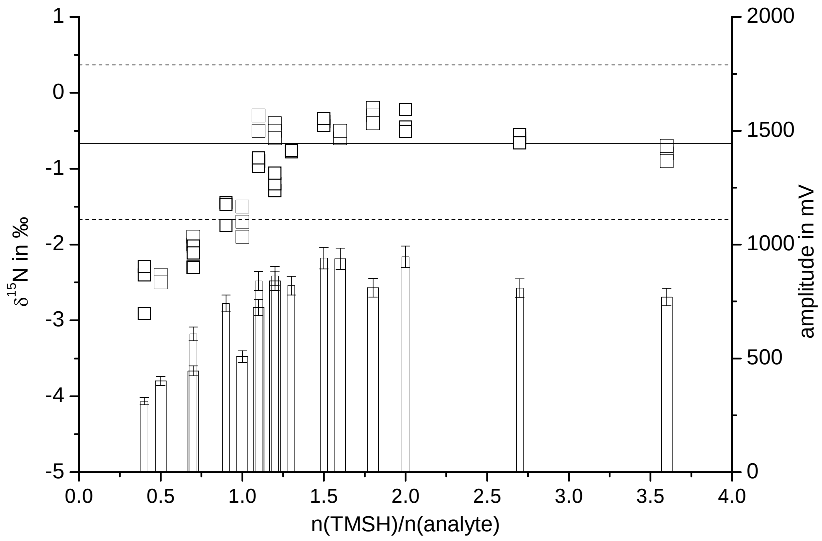


Figure 2-1. Variation in $\delta^{15}N$ values and amplitudes for methylated bentazone (combined data for 4500 and 5000 mg L^{-1}) with increasing TMSH-to-analyte ratios. Bars represent amplitudes and rectangles represent $\delta^{15}N$ values. Error bars represent the standard deviation for triplicate measurements of the amplitudes. The solid line indicates the $\delta^{15}N$ values measured with EA-IRMS. Dashed lines represent the typical total analytical error of ± 1 ‰ for GC-IRMS nitrogen analysis.

2.3.2 Derivatization of bentazone and MCPA - Carbon isotope analysis

Figures 2-2a and 2-3a show carbon isotope values for methylated bentazone (3 to 300 mg L^{-1}) and methylated MCPA (200 mg L^{-1}), respectively, when derivatized at different TMSH-to-analyte ratios. For comparison, EA-IRMS results for bentazone and MCPA are shown as black lines. GC-IRMS measurements did, as expected, not give the target $\delta^{13}C$ value measured with EA-IRMS, since during derivatization one additional carbon atom was introduced into the molecule. However, a surprising result of Figures 2-2a and 2-3a is that for carbon measurements of both compounds a TMSH-to-analyte ratio of at least 250 was necessary to achieve reproducible isotope values of the derivative, even though our results for nitrogen showed that complete conversion occurred already with TMSH in slight excess (see Figure 2-1 and discussion above). Below an excess of 250, $\delta^{13}C$ values of the methylated analyte became increasingly negative when the derivatization agent was added in smaller excess. With TMSH-to-analyte ratios of ≥250, in contrast, the precision of $^{13}C/^{12}C$ isotope measurement showed excellent reproducibility, giving $\delta^{13}C$ values of -30.7 ± 0.3 ‰ (n = 34) for bentazone when using the same batch of derivatization agent and maintaining the same reaction conditions (Figure 2-2b). For MCPA a different batch of TMSH was used giving values of -32.4 ± 0.3‰ (n = 21) (Figure 2-3b).

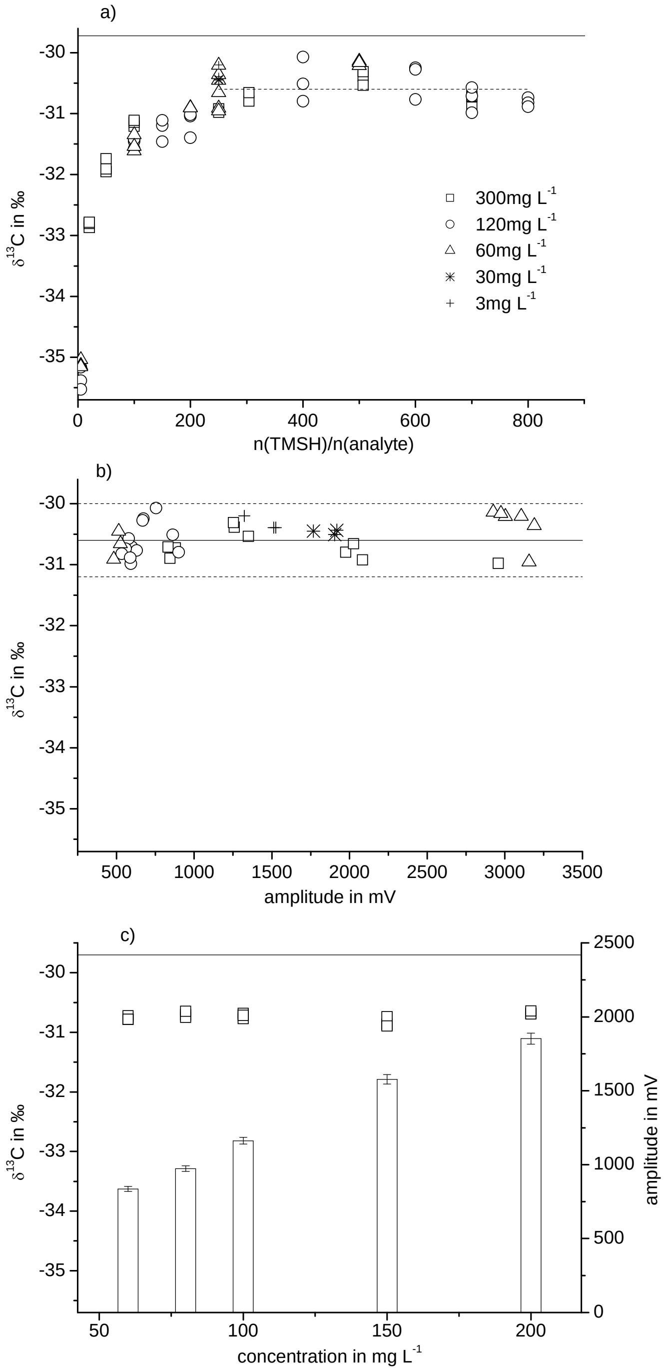


Figure 2-2. (a,b) $\delta^{13}C$ values of bentazone measured with GC-IRMS after derivatization with different TMSH-to-analyte ratios and (c) $\delta^{13}C$ values of nonderivatized bentazone measured with LC-IRMS. Symbols in panels a and b represent different target compound concentrations (squares 300 mg L^{-1}, circles 120 mg L^{-1}, triangles 60 mg L^{-1}, stars 30 mg L^{-1}, crosses 3 mg L^{-1}). Values measured for 60 mg L^{-1}, 30 mg L^{-1}, and 3 mg L^{-1} were acquired with a TMSH excess of 250 and more using large volume injection; for 120 mg L^{-1} and 300 mg L^{-1} 1 µl injections were used. Solid lines in panels a and c represent the $\delta^{13}C$ value determined with EA-IRMS. Dashed line in (a) and the black line in (b) give the mean $\delta^{13}C$ value calculated for all measurements at a TMSH excess of 250 to 800 with an uncertainty of 0.6 ‰ (2σ). The columns in (c) represent amplitudes, and the black rectangles represent $\delta^{13}C$ values of measurements by LC-IRMS.

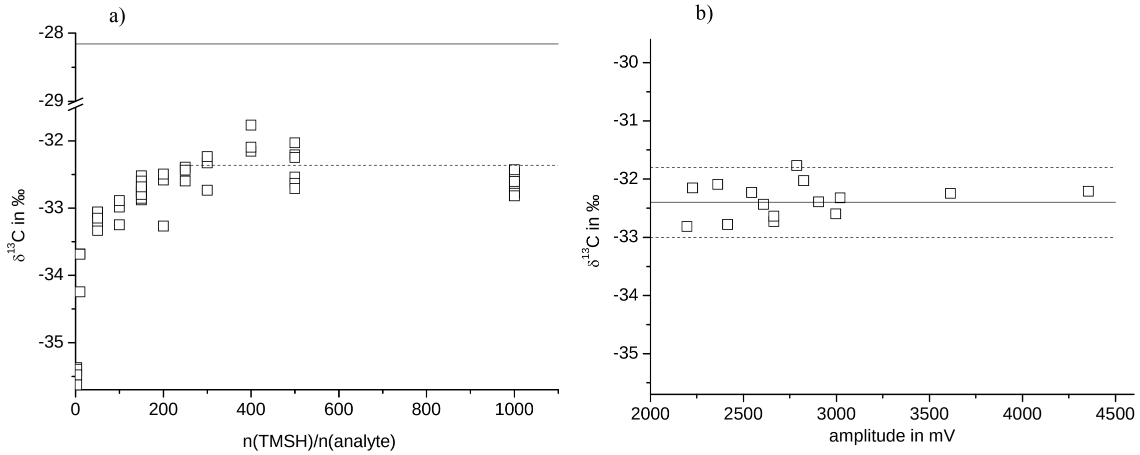


Figure 2-3. Variation in $\delta^{13}C$ values of 200 mg L^{-1} MCPA after derivatization with different TMSH-to-analyte ratios. The solid line in (a) represent the $\delta^{13}C$ values measured with EA-IRMS. The dashed line in (a) and the black line in (b) give the mean $\delta^{13}C$ value calculated for all measurements at a n(TMSH)/n(analyte) ratio of at least 250 with an uncertainty better than 0.6 ‰ (2σ).

This reproducibility was independent of concentrations, with good linearity of bentazone isotope measurements in the concentration range of 3 to 300 mg L^{-1}. Because the target compounds bentazone and MCPA were completely converted in the range of TMSH tested, no isotope fractionation is expected from atoms in their molecular structure. The isotope shifts observed in the derivative must therefore be attributable solely to the methyl group introduced by TMSH. The following paragraph discusses what kinetic isotope effects are expected for such a reaction.

2.3.3 Expected trend for a kinetic isotope effect

Scheme 2-1 shows the reaction of one of three chemically equivalent methyl groups of TMSH with a negatively charged analyte (e.g., anionic form of bentazone or MCPA). Such a reaction may be expected to occur according to an S_N2 mechanism leading to large carbon kinetic isotope effects (typically $^{12}k/^{13}k$ = 1.03 to 1.09 (Elsner et al., 2005)). Figure 2-4a illustrates that the kinetic isotope effect is expressed only in the methyl group that is transferred to the analyte, because only this carbon atom reacts. The other two, nonreacting methyl groups in CH_3-S-CH_3 have simply the isotopic composition of the TMSH from which they are formed. The figure illustrates the resulting enrichment of ^{13}C in the remaining TMSH, the trend of the byproduct CH_3-S-CH_3, and the depletion in the methyl group that is transferred to the analyte (for explanation of the underlying trends see, e.g., Hunkeler et al. (2009)). The derivatization reaction with respect to TMSH may be thought to proceed from the left (very beginning of reaction) to the right of Figure 2-4a, where the observable end point is determined by the TMSH-to-analyte ratio (being to the left at a large excess of TMSH and to the right at stoichiometric proportions). Therefore, isotope values in the methyl group of the derivative are expected to be strongly depleted in ^{13}C compared to original TMSH, where the depletion becomes stronger at greater TMSH-to-analyte ratios.

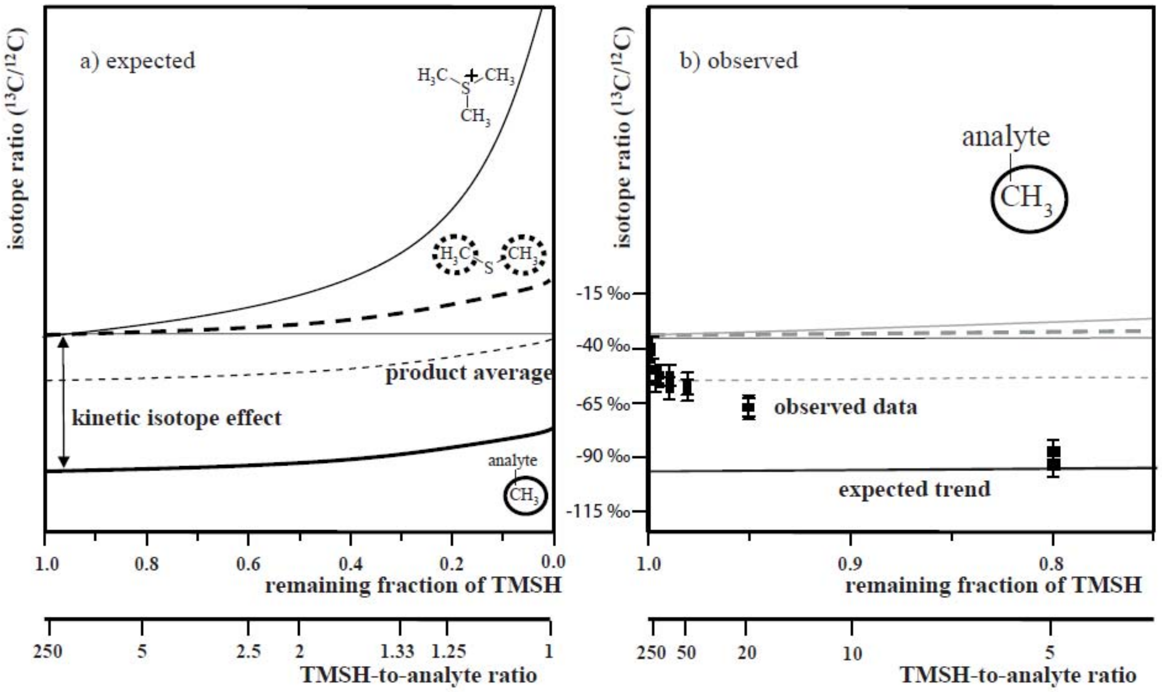


Figure 2-4. Expected (a) and observed (b) trends in isotope ratios in the reaction of TMSH to dimethylsulfide and CH3 during the derivatization reaction. In panel b the scale of the *y*-axis applies to the experimental data points only; the underlying qualitative sketch was added to better illustrate the difference between expected and observed trends.

2.3.4 Observed trend in the methyl group of the bentazone derivative.

To compare this expected trend with experimental observations, the isotope value of the transferred methyl group to bentazone was calculated according to

$$\delta^{13}C_{CH_3} = (n+1)*\delta^{13}C_{CH_3-analyte} - n\delta^{13}C_{analyte} \qquad \text{(Equation 2-3)}$$

where *n* is the number of carbon atoms in the analyte, $\delta^{13}C_{analyte}$ is the analyte isotope value determined with EA-IRMS, and $\delta^{13}C_{CH_3-analyte}$ is the value of the derivative measured with GC-IRMS. Figure 2-4b shows calculated $\delta^{13}C_{CH_3}$ values with the expected qualitative trend of Figure 2-4a underlain. Even though error propagation gives an uncertainty of ±5 ‰ for these calculated $\delta^{13}C_{CH_3}$ values, Figure 2-4b clearly shows that the observed trend is contrary to our expectations: the methyl group of the derivative becomes more enriched in ^{13}C rather than depleted at greater TMSH-to-analyte ratios. At a small TMSH excess (TMSH-to-analyte ratio of 5, fraction of remaining TMSH = 0.8) the isotope ratio of the methyl group is still consistent with the characteristics of a kinetic isotope effect. Values of -90.6 ‰ ± 4‰ (n = 5) calculated according to eq 2-3 give evidence of a methyl substituent that is strongly depleted in ^{13}C. As illustrated in Figure 4a, the kinetic isotope effect can be approximately estimated as the difference between $\delta^{13}C_{CH_3}$ and the starting TMSH δ^{13}C value:

$$\varepsilon_{reaction} \approx \delta^{13}C_{CH_3-analyte} - \delta^{13}C_{TMSH} \qquad \text{(Equation 2-4)}$$

Even though the initial δ^{13}C value of TMSH was not analyzed, values of between -20 ‰ and -60 ‰ may typically be expected for organic materials (Coplen et al., 2002). This would result in estimates of $\varepsilon_{reaction}$ between -30 ‰ and -70 ‰ or $^{12}k/^{13}k = 1.03$ to 1.07 for the kinetic isotope effect. These numbers are indeed highly indicative of an S_N2 reaction where simultaneous bond cleavage and bond formation results in such large isotope fractionation (see above Elsner et al. (2005)). The isotopic discrimination in the methyl group became less pronounced at greater TMSH-to-analyte ratios, however, being only -39.5 ‰ ± 6.2 ‰ ($n = 34$) for TMSH/analyte ≤ 250 (see also Figure 2-2a). This observation cannot be explained purely by the model of a kinetic isotope effect during a one-way (forward) derivatization reaction.

2.3.5 Mechanistic hypothesis: Close-to-equilibrium isotope fractionation

The only possible explanation is, therefore, that at higher TMSH-to-analyte ratios a backward reaction with appreciable reverse isotope fractionation occurred. This hypothesis is consistent with earlier studies which report transmethylation products in TMSH derivatives meaning that the alkylation reaction is to some extent reversible (Diez et al., 2006). If forward and backward reactions are equally fast, equilibrium isotope fractionation occurs. Equilibrium isotope effects usually reflect relative differences in bond stiffness where the heavy isotope tends to concentrate in the component with stiffer bonds (=molecular vibrations of higher energy (Hartshorn and Shiner, 1972)). In organic molecules such differences are usually smaller between reactant and product (reaction energy) than between reactant and transition state (activation energy). Equilibrium isotope effects therefore tend to be smaller than kinetic isotope effects explaining the trend observed in Figure 2-4b. Accordingly, our mechanistic picture is that in the pore space of the glass beads in the packed liner a certain concentration of methyl groups (TMSH-to-analyte ratio ≥250) was necessary to achieve the situation under which close-to-equilibrium conditions could be achieved. Consistent with this hypothesis, we observed a decrease in the reproducibility of δ^{13}C values when deliberately reducing the injection depth so that the injection was performed into the upper, headspace part of the packed liner where no pores were present (results not shown).

2.3.6 Calculation of accurate isotope values of the nonderivatized target compound.

The knowledge of $\delta^{13}C_{CH_3}$ also makes it possible to accurately calculate the isotope values of nonderivatized target analytes. We found that $\delta^{13}C_{CH_3}$ was constant for the same lot of derivatization agent (solution of TMSH in methanol) but changed significantly if a new batch of commercial TMSH product was used. For routine analyses we therefore suggest that samples be bracketed by standards with known δ^{13}C composition that can be used to derive the appropriate $\delta^{13}C_{CH_3}$ value. Subsequently, this number is used to calculate the δ^{13}C values of nonderivatized target analytes according to

$$\delta^{13}C_{analyte} = (n+1)/n * \delta^{13}C_{CH_3-analyte} - \delta^{13}C_{CH_3}/n \qquad \text{(Equation 2-5)}$$

2.3.7 Comparison of GC-IRMS and LC-IRMS measurements of bentazone

LC-IRMS measurements of bentazone are shown in Figure 2-2c. The black line indicates the bentazone value determined with EA-IRMS. δ^{13}C values of bentazone (concentration range 60 to 200 mg L^{-1}) show excellent reproducibility with -30.7 ± 0.1 ‰ (n = 15), but they have an offset of 1 ‰ compared to the EA-value of bentazone (-29.7 ‰). This observation is consistent with other LC-IRMS studies on amino acids, which have also observed offsets for isotope values of some target compounds (Smith et al., 2009) suggesting incomplete wet oxidation of some analytes.

2.4 Conclusions

This study addresses different analytical approaches (GC-IRMS and LC-IRMS) for isotope analysis of negatively charged target compounds with a careful view on accuracy and reproducibility. In this context, TMSH was tested for the first time as a methylation agent prior to GC-IRMS analysis, with the two negatively charged pesticides bentazone and MCPA as target analytes. Derivatization with TMSH showed the following advantageous properties. (i) *Minimum handling effort*: The derivatization reaction could be performed fully automated by flash heating in the porous liner of a temperature programmable GC injector. (ii) *Quantitative analyte conversion at stoichiometric addition of TMSH* as reflected in excellent accuracy and reproducibility of nitrogen isotope values. (iii) *Equilibrium-like rather than kinetic carbon isotope effects when TMSH was added in ≥250-fold excess,* as reflected in very good reproducibility of carbon isotope values indicating robust derivatization conditions. The reproducibility and accuracy of GC-IRMS for carbon isotope analysis of methylated bentazone under optimized conditions compared even favourably with LC-IRMS measurements of the nonderivatized target compound. Both methods showed low standard deviations (0.3 ‰ for GC-IRMS, 0.1 ‰ for LC-IRMS), and both showed an offset of about 1 ‰ compared to the reference value of EA-IRMS. In both cases a correction is therefore necessary, albeit in different ways. In the case of GC-IRMS the isotope value of the newly added methyl group must be specifically corrected for (eq 2-5), whereas for LC-IRMS the correction involves a constant offset. This study therefore highlights two independent, convenient approaches with GC-IRMS and LC-IRMS for compound specific isotope analysis of negatively charged target analytes. Both solutions are fully automated and involve little more handling than conventional GC-IRMS. Depending on the type of sample, the matrix present and the peak separation required, either of the two methods may be preferable for a given application. In principle both delineate expedient ways, however, for compound specific isotope investigations of negatively charged target compounds in future studies.

2.5 References

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3.

Gas chromatography-isotope ratio mass spectrometry (GC-IRMS) of recalcitrant compounds: Performance of commercial combustion reactors and strategies for referencing

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3.1 Introduction

Compound-specific isotope analysis (CSIA) is routinely used to monitor the carbon and nitrogen isotope composition of a multiplicity of organic compounds to assess their origin and fate in the environment (Schmidt et al., 2004; Shouakar-Stash et al., 2003). Automated online measurement using a gas chromatograph-combustion-isotope ratio mass spectrometer (GC-IRMS) is a valuable technique for volatile organic compounds. Once separated from a compound mixture, organic substances are converted online to CO_2, N_2, and NO_X in a combustion reactor consisting of a ceramic tube, conventionally filled with CuO/NiO/Pt wires operating at 900 to 1000 °C (Brand, 1996; Meier-Augenstein, 1999). For nitrogen measurements, in addition a reduction reactor is required which operates at 600 - 650 °C and contains Cu wires to reduce NO_X to N_2 (Merritt and Hayes, 1994). Isotope values are typically determined in comparison to peaks of a monitoring gas, which are introduced in the beginning and the end of each chromatographic run (Meier-Augenstein, 1997; Werner and Brand, 2001).

Most compound-specific isotope methods in environmental applications to date refer to volatile compounds such as low molecular weight chlorinated (Hunkeler and Aravena, 2000; Jochmann et al., 2006; SherwoodLollar et al., 2007; Zwank et al., 2003) or petroleum hydrocarbons (Gray et al., 2002; Jochmann et al., 2006; Mancini et al., 2003; Pond et al., 2002; Zwank et al., 2003). These substances have relatively simple structures and their isotope analysis is well established (Hunkeler et al., 2008). Studies frequently report excellent accuracy meaning that (i) uncorrected GC-IRMS instrument values of underivatized target compounds coincide with those from offline analysis or elemental analyzer-IRMS; and (ii) reported standard deviations are typically ± 0.3 ‰ for carbon and ± 1 ‰ for nitrogen corresponding to the specification of the manufacturer (Hunkeler et al., 2008; Sessions, 2006; SherwoodLollar et al., 2007). Such results may seem to suggest that complete conversion and accurate isotope results can be taken for granted when addressing new target compounds by GC-IRMS.

Superb method performance also has consequences for the strategy of standard bracketing. If careful analysis of isotope standards can clearly establish the trueness and precision of GC-IRMS isotope analysis for a given target compound (Jochmann et al., 2006; SherwoodLollar et al., 2007), some laboratories measure standards only at the beginning and at the end of daily measurement series. In other protocols more frequent standard bracketing is applied. Presently, no clear guidelines exist about which strategy to adopt.

In recent years, more complex substances such as pesticides (Meyer et al., 2008; Penning and Elsner, 2007; Reinnicke et al., 2010), amino acids (Corr et al., 2007; Krummen et al., 2004; Sauheitl et al., 2009) and steroids (Baume et al., 2006; Becchi et al., 1994) have come into the focus of compound-specific isotope investigations. Due to their polarity, molecules tend to be less amenable to GC influencing the peak shape and separation. In addition, they have more complex chemical structures including hetero and halogen atoms. Potentially, this can make the combustion more difficult leading to incomplete conversion and entailing systematic isotope fractionation. A recent study in our lab showed that $\delta^{13}C$ isotope values of atrazine – a heteroaromatic compound containing C, N and Cl – were not reproducible and showed poor accuracy when determined with a conventional reactor incorporating NiO-CuO-Pt wires typically operated at the recommended temperature of 940 °C. When the operating temperature was reduced to 800°C, precision improved, but $\delta^{13}C$ values still showed an offset of 1.2 ‰ compared to EA-IRMS (Hartenbach et al., 2008; Meyer et al., 2008). Better accuracy, but higher limits for precise isotope analysis were obtained with a reactor containing only NiO wires operated at 1030°C (Meyer et al., 2008). This can be problematic because (i) many applications require low limits for precise isotope analysis while (ii) reproducibility and trueness of isotope values are crucial for any isotope investigation, particularly in forensic studies (Benson et al., 2006; Blessing et al., 2009).

These recent results emphasize the need for further investigations addressing cases of recalcitrant target compounds. Promising in this context is the introduction of a new combustion reactor (Thermo Scientific, Bremen, Germany). Compared to the conventional model, the new reactor contains a NiO-tube that displays a greater surface area, and it is operated at 1030 °C instead of 940°C (Fig. 3-1). The greater active surface results in higher capacity, which is particularly important for nitrogen isotope analysis. In addition, the new design integrates the reduction process in the combustion tube eliminating the need for a separate reduction reactor. Most importantly, the new reactor design relies on high temperature conversion on a NiO surface. Potentially, this may lead to better conversion of target compounds that are difficult to convert with the conventional design.

The objective of this study was, therefore, to test the two different reactors for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope analysis of selected target compounds that we identified as particularly difficult to analyze. Specifically, we chose atrazine, its metabolite desethylatrazine (DEA), 2,6-dichlorobenzonitrile (dichlobenil) and its main metabolite 2,6-dichlorobenzamide (BAM). These compounds are on the one hand environmental pollutants which are frequently detected in groundwater. On the other hand, they are challenging to analyze due to their complex structure including C, N and Cl (Table 3-1). The performance of the reactors was carefully tested for accuracy (= trueness and precision) of carbon and nitrogen isotope values and, in a second part of this study, with respect to peak response as a lumped indicator for chromatographic performance and conversion efficiency. The investigations were conducted at the Helmholtz Zentrum Muenchen (Germany), and selected measurements were repeated in the application laboratory of Thermo Fisher Scientific (Bremen, Germany). The implications of our results are discussed for the study of new target compounds, with a particular focus on standard bracketing strategies.

3.2 Experimental

3.2.1 Instrumentation

A GC-IRMS system consisting 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) was used for measurements. The emission energy was set to 1.5 mA for carbon isotope analyses and 2.0 mA for nitrogen isotope analyses. He grade 5.0 was used as carrier gas and liquid samples were injected *via* a GC Pal autosampler (CTC, Zwingen, Switzerland). All injections were conducted splitless (2 min) into a hot injector, held at 250 °C. Flow rate was set to 1.4 mL/min and the analytical column was a DB 5 (30 m × 0.25 mm; 1 μm film; J&W Scientific, Folsom; CA). Standard solutions containing either atrazine and DEA or dichlobenil and BAM or caffeine were analysed using following temperature programmes. Atrazine and DEA measurements started at 150 °C (hold 1 min), ramped with 10 °C/min to 190 °C (hold 1 min), followed by a ramp of 3 °C/min to 230 °C (hold 2 min) and finally ramped with 30°C/min to

280°C (hold 3 min). For dichlobenil and BAM the GC program was set to 120 °C (hold 1 min), ramp with 8 °C/min to 200 °C (hold 1 min) and ramp 15 °C/min to a final temperature of 280 °C (hold for 2 min). For caffeine the GC oven was set to 70 °C (hold for 1 min), ramp with 25 °C/min to 180 °C and ramp with 5 °C/min to 240 °C (hold 1 min) and finally ramp with 25 °C/min to 280 °C (hold 2 min).

Target values for carbon and nitrogen isotope ratios were determined in a complementary approach by elemental analyzer isotope ratio mass spectrometry (EA-IRMS) consisting of an EuroEA (EuroVector, Milano, Italy) coupled to a Finnigan TM MAT253 IRMS (Thermo Fisher Scientific, Bremen, Germany) by a ConFlow III interface (Thermo Fisher Scientific, Bremen, Germany). Calibration was performed against organic reference materials (USGS 40, USGS 41, IAEA 600) provided by the International Atomic Energy Agency (IAEA, Vienna, Ausria).

δ^{13}C and δ^{15}N values are reported in per mill relative to Vienna PeeDee Belemnite (VPDB) and air, respectively:

$$\delta^{13}C = \frac{\left({}^{13}C/{}^{12}C_{Sample} - {}^{13}C/{}^{12}C_{Standard}\right)}{{}^{13}C/{}^{12}C_{Standard}} \qquad \text{(Equation 3-1)}$$

$$\delta^{15}N = \frac{\left({}^{15}N/{}^{14}N_{Sample} - {}^{15}N/{}^{14}N_{Standard}\right)}{{}^{15}N/{}^{14}N_{Standard}} \qquad \text{(Equation 3-2)}$$

δ^{13}C and δ^{15}N values were reported relative to a laboratory standard gas (CO_2 and N_2, respectively) that was introduced at the beginning and the end of each run. The standard gases were calibrated against V-PDB and air, respectively, by reference standards (RM 8562, RM 8563, RM 8564, USGS 40, USGS 41, IAEA 601, USGS 34, and IAEA N-2). All reference standards were provided by the IAEA.

3.2.2 Oxidation reactors

A commercial oxidation reactor (Fig. 3-1a), consisting of a ceramic tube filled with CuO/NiO/Pt-wires (operated at 800 °C, 940 °C and 980°C, respectively) was used for carbon and nitrogen measurements. The reactor was hyphenated to a standard reduction reactor (650 °C) for subsequent NO_x reduction to N_2 (all reactors from Thermo Scientific, Bremen, Germany). For nitrogen isotope measurements the CuO/NiO/Pt reactor was used without initial oxidation. For carbon isotope measurements it was initially oxidized for 4 h and the conditioning was routinely repeated after 40 to 60 measurements to ensure sufficient oxidation capacity.

Alternatively, a recently introduced NiO tube/ CuO-NiO reactor (Thermo Scientific, Bremen, Germany) was used, consisting of a ceramic tube filled with a Ni-tube and NiO/CuO wires (Fig. 3-1b). Since this reactor contains both, oxidation and reduction unit, no extra reduction reactor was necessary for nitrogen measurements. In contrast to the conventional oxidation reactor, the NiO tube/ CuO-NiO reactor was coupled to the separation column via a silcosteel capillary. The operating temperature of the new reactor was set to 1030 °C. The reactor was initially oxidized for 2 hours at 600 °C, and 12 h at 1030 °C. Repeated conditioning (oxidation of 30 minutes to 1 hour) was routinely performed after 30 to 50 measurements.

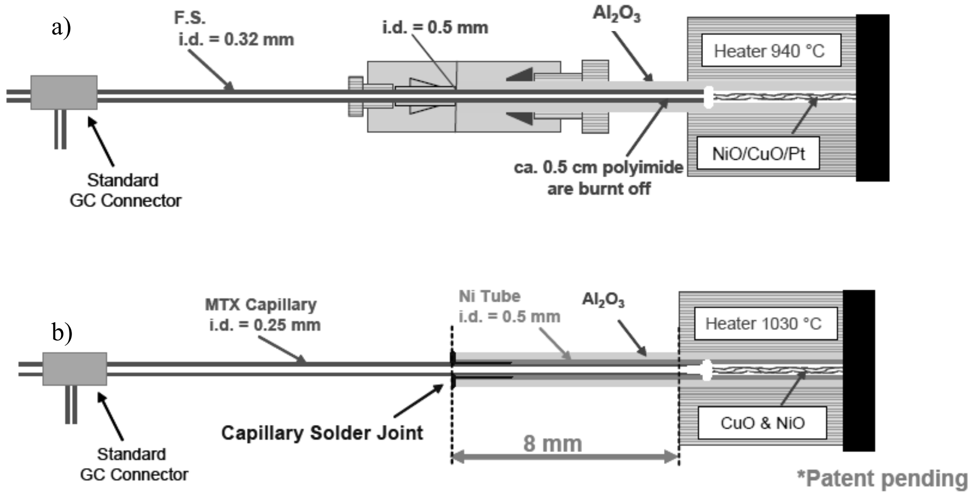


Figure 3-1. Different tested oxidation reactors of the manufacturer Thermo Scientific (Bremen, Germany) with (a) the conventional NiO/CuO/Pt reactor operated at 940 °C and (b) the new NiO tube/ CuO-NiO reactor operated at 1030 °C.

3.2.3 Chemicals

Atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine, CAS: 1912-24-9) was supplied by Tropitzsch (97.7%), Desethylatrazine (>99.9 %, CAS No.: 6190-65-4), Dichlobenil (2,6-dichlorobenzonitrile, >98 %, CAS No.: 1194-65-6) and BAM (2,6-dichlorobanzamid, >98 %, CAS No.: 2008-58-4) were supplied by Riedel-de Haën distributed by Sigma Aldrich (Taufkirchen, Germany). Caffeine (CAS No.: 158-08-2) provided by the

IAEA was used as reference standard to probe for quantitative conversion. Methanol (pestanal, 99.9 %, MERCK, Germany) was used as solvent.

3.3 Results and Discussion

3.3.1 Testing different reactor types for trueness and precision of isotope values

$^{13}C/^{12}C$ analysis of dichlobenil and BAM with the conventional reactor design

In a recent study (Meyer et al., 2008) we observed that the conventional reactor design – i.e., CuO/NiO/Pt-wires threaded through a ceramic tube – could not accomplish precise carbon isotope analysis of atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine) when operated at 940 °C.

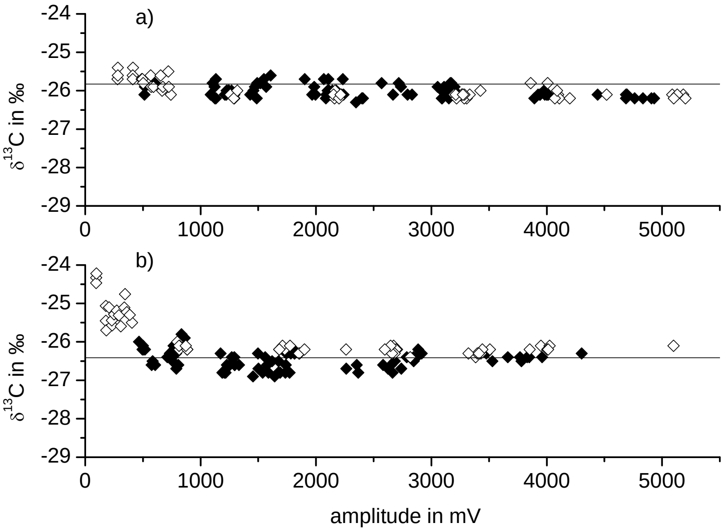


Figure 3-2. $\delta^{13}C$ values of dichlobenil and BAM standards using the conventional oxidation set up using NiO/CuO/Pt reactor operated at 940 °C (open diamonds) and 800 °C (filled diamonds), respectively. Values for (a) dichlobenil and (b) BAM. Black lines represent the $\delta^{13}C$ values determined by EA-IRMS.

Precision improved, but values remained systematically 1.2‰ off when the reactor was operated at 800°C indicating incomplete conversion (Hartenbach et al., 2008; Meyer et al., 2008). We therefore tested the conventional reactor design for carbon isotope analysis of two other relevant organic substances that also have an aromatic structure and contain both nitrogen and chlorine inside their molecular structure, that is, the herbicide dichlobenil and its metabolite BAM. In contrast to the results for atrazine, the conventional NiO/CuO/Pt reactor gave true and precise $\delta^{13}C$ values for dichlobenil (-26.4 ‰ ± 0.3 ‰, n = 80) and BAM (-26.2 ‰ ± 0.1 ‰, n = 34) over a broad concentration range (Fig. 3-2), as represented by the corresponding range of amplitudes. Minimum amplitudes (m/z 44) required for precise $^{13}C/^{12}C$ analysis of dichlobenil were 250 mV corresponding to 1.6 nmol C on column (Fig. 3-2a) and 500 mV for BAM corresponding to 2.6 nmol C on column (Fig. 3-2b). Different temperatures (800°C and 940°C) of the oxidation reactor caused no discernible differences in isotope values. These result correspond to typical textbook examples of compound-specific carbon isotope analysis (SherwoodLollar et al., 2007).

$^{15}N/^{14}N$ analysis of dichlobenil and BAM with the conventional reactor

Analyzing $^{15}N/^{14}N$ values of dichlobenil and BAM using an unoxidized NiO/CuO/Pt reactor at 980°C gave precise values for dichlobenil (Fig. 3-3a) and BAM (Fig. 3-3b) with $\delta^{15}N$ = -0.9 ± 0.5 ‰ (n = 43) and $\delta^{15}N$ = -12.6 ± 0.4 ‰ (n = 34), respectively. However, both compounds showed an offset compared to the EA value of -2 % for dichlobenil and -1.2 ‰ for BAM (Fig. 3-3). In contrast to carbon isotope analyses, we analyzed constant compound concentrations (400 ng N on column) so that a decrease in the corresponding amplitudes directly indicates a decrease in conversion efficiency. Figure 3-3b illustrates that after 34 measurements, nitrogen isotope values of BAM started to drift by a total of 5‰ towards more negative values indicating a decrease in the system performance. Surprisingly, amplitudes remained constant (543 mV ± 3 %, n = 43) demonstrating that the accuracy of isotope analysis did not necessarily correlate with, and could not be detected from a decrease in conversion efficiency. Reactor conditioning for 10 minutes extended the reactor lifetime in the long run, as already observed for bentazone nitrogen isotope analyses (Reinnicke et al., 2010). Initially, however, a conditioning period was required before precise $^{15}N/^{14}N$ analysis of dichlobenil and BAM was reestablished (Fig. 3-3, injection 44 to 60, 109 to 115). During the conditioning phases, amplitudes were at first small, but noticeably recovered indicating that conversion efficiency was initially decreased. After the conditioning phases, $\delta^{15}N$ values of dichlobenil and BAM stabilized, but higher standard deviations and an offset in the isotope values compared to EA values had to be accepted for both compounds.

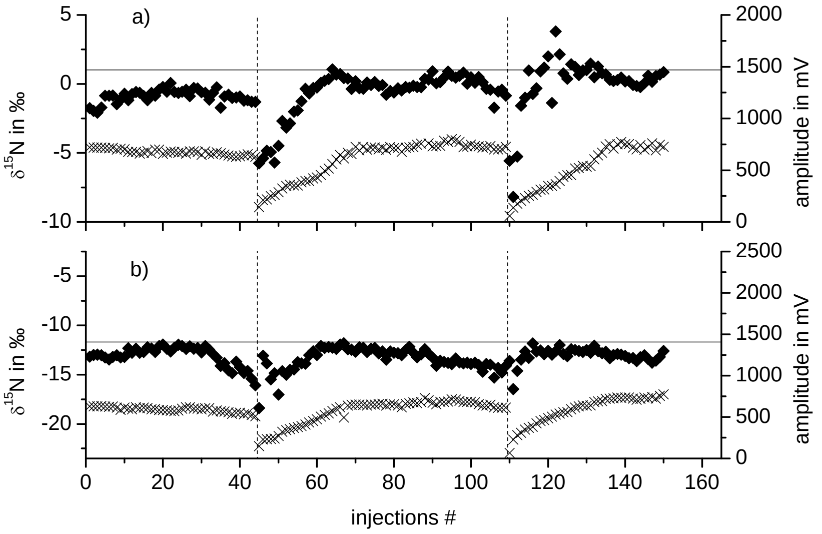
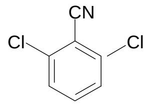
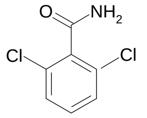
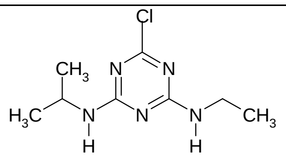
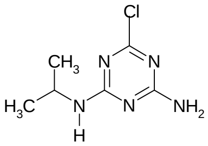


Figure 3-3. $\delta^{15}N$ values (filled diamonds) and amplitudes (crosses) of (a) dichlobenil and (b) BAM using the conventional NiO/CuO/Pt reactor at 980 °C. Black lines give the $\delta^{15}N$ values determined by EA-IRMS. Dashed lines represent reactor conditioning.

$^{13}C/^{12}C$ and $^{15}N/^{14}N$ analysis of atrazine and desethylatrazine (DEA) with the new NiO tube/ CuO-NiO reactor

For those cases where the conventional reactor showed unsatisfactory performance (i.e., $\delta^{13}C$ analysis of atrazine (Meyer et al., 2008) and $\delta^{15}N$ analysis of BAM and dichlobenil) the newly introduced NiO tube/ CuO-NiO reactor from Thermo Fisher was tested. In addition, we extended the spectrum of analyses including $\delta^{15}N$ analysis of atrazine as well as $\delta^{13}C$ and $\delta^{15}N$ analysis of desethylatrazine (DEA), the main metabolite of atrazine. Besides analyses in the Institute of Groundwater Ecology at Helmholtz Zentrum Muenchen (Germany), independent nitrogen and carbon isotope measurements with the NiO tube/ CuO-NiO reactor were conducted for all target compounds in the IRMS application laboratory of Thermo Scientific (Bremen, Germany). All measurements with the NiO tube/ CuO-NiO reactor setup that were conducted in 3.3.1 of this study are summarized in Table 3-1. The initial performance of the new NiO tube/ CuO-NiO reactor was excellent for carbon and nitrogen isotope measurements of atrazine standards over more than 300 measurements showing an average of -28.5 ± 0.5 ‰ (n = 95) for $\delta^{13}C$ and -2.1 ± 0.4 (n = 122) for $\delta^{15}N$ (Fig. 3-4, injection 1 to 350).

Table 3-1. Structures of investigated compounds, carbon and nitrogen isotope values determined by EA-IRMS and GC-IRMS (Stable isotope Laboratory, Muenchen, Germany) and GC-IRMS measurements from Thermo IRMS application Laboratory, Bremen, Germany)

Common name	**Dichlobenil**	**2,6-dichlorobenzamid (BAM)**	**Atrazine**	**Desethylatrzine (DEA)**
	herbicide	metabolite of dichlobenil	herbicide	Metabolite of atrazine
structure				
EA-IRMS				
$\delta^{13}C$ [‰]	-25.8 ± 0.1 (n = 4)	-26.4 ± 0.1 (n = 4)	-28.6 ± 0.1 (n = 4)	-29.2 ± 0.1 (n = 4)
$\delta^{15}N$ [‰]	1.1 ± 0.2 (n = 4)	-11.4 ± 0.2 (n = 4)	-1.3 ± 0.2 (n = 4)	-4.2 ± 0.2 (n = 4)
Stable Isotope Laboratory IGÖ, NiO tube/ CuO-NiO reactor				
$\delta^{13}C$ [‰]	-25.4 ± 0.3 (n = 3)	-26.4 ± 0.1 (n = 4)	-28.5 ± 0.2 (n = 95)	-30.0 ± 0.6 (n = 94)
Thermo IRMS Application Laboratory, NiO tube/ CuO-NiO				
$\delta^{13}C$ [‰]	-26.0 ± 0.1 (n = 6)	-26.3 ± 0.2 (n = 6)	-28.7 ± 0.2 (n = 5)	n.a.
$\delta^{15}N$ [‰]	0.5 ± 0.1 (n = 10)	-14.4 ± 0.7 (n = 10)	-1.5 ± 0.1 (n = 10)	-4.5 ± 0.6 (n = 10)

[3] Mean values represent data of the NiO/CuO/Pt reactor without oxidation (Fig. 3-2, injection 1 to 43 for dichlobenil and 1 to 34 for BAM).

Simultaneous analysis of DEA isotope ratios gave average values of -30.0 ± 0.6 (n = 94) for $\delta^{13}C$ and -4.8 ± 0.5 (n = 122) for $\delta^{15}N$ (Fig. 3-4, injection 1 to 350). Concentrations varied between 20 mg L^{-1} and 500 mg L^{-1}, and injection volumes between 1 and 9 µl. Observed limits for precise nitrogen isotope analysis were amplitudes of ≥ 100 mV, corresponding to 5.6 nmol N of atrazine and 6.4 N nmol of DEA on column, respectively. Highest loadings tested were 23.1 nmol N and 26.6 nmol N, respectively, corresponding to amplitudes of 556 mV ± 83 mV and 544 mV ± 82 mV, respectively.

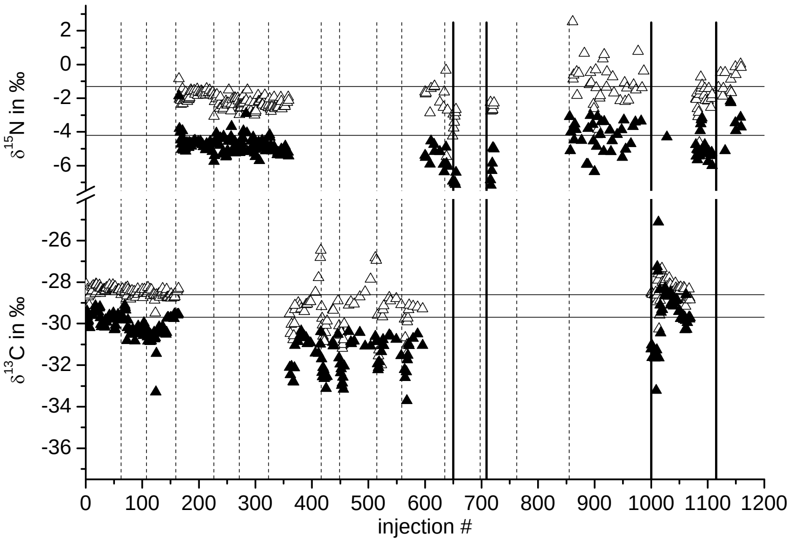


Figure 3-4. δ^{13}C and δ^{15}N measurements of atrazine (open triangle) and desethylatrazine (filled triangles) using a total of five NiO tube/ CuO-NiO reactors. Injections 0 to 650 represent data determined with NiO tube/ CuO-NiO reactor I. Solid vertical lines indicate the change to a new NiO tube/ CuO-NiO reactor, and dashed vertical lines show reactor conditioning. Horizontal black lines give the isotope values of atrazine and desethylatrazine determined by EA-IRMS, respectively.

Isotope values determined in the Bremen laboratory were in perfect agreement with measurements conducted on GC-IRMS and EA-IRMS in Munich. In Bremen, for nitrogen isotope measurements 1 µl of 750 mg L^{-1} atrazine and DEA solutions were injected (17.4 nmol N atrazine and 19.9 nmol N of DEA on column) giving amplitudes of 2400 mV ± 72 mV and 2100 mV ± 63 mV. For carbon isotope measurements 1 µl of 50 mg L^{-1} (1.9 nmol C of atrazine on column) were injected producing amplitudes of 3500 mV ± 42 mV. After 350 injections (corresponding to a cumulative loading of roughly 800 nmol atrazine as well as 800 nmol DEA on column) the precision of isotope values deteriorated in the long term series in Muenchen, as represented by higher standard deviations (Fig. 3-4). Lower helium gas flow through the reactor indicated clogging, thus impairing the performance of the reactor (data not shown). Subsequently, four additional NiO tube/CuO-NiO reactors were tested (Fig. 3-4, injection 650 to 1160). They still showed good trueness, but significantly lower precision compared to the first NiO tube/ CuO-NiO reactor. Nonetheless, the overall performance was greatly improved in comparison with the conventional NiO/CuO/Pt reactor. Besides the obvious advantages – i.e., the ability to operate without additional reduction reactor and to oxidize the combustion reactor for extended series of nitrogen isotope measurements – with the new NiO tube/ CuO-NiO reactor we obtained for the first time true and precise atrazine and DEA analysis over sequences of several hundred measurements in our laboratory.

$^{13}C/^{12}C$ and $^{15}N/^{14}N$ analysis of dichlobenil and BAM with the new NiO tube/ CuO-NiO reactor

Analogous to the results with the conventional NiO/CuO/Pt reactor (Figure 3-2), also the new NiO tube/ CuO-NiO reactor gave true and precise values for $\delta^{13}C$ isotope analysis of dichlobenil and BAM (Table 3-1). This is in contrast with the performance of the NiO tube/ CuO-NiO reactor for $\delta^{15}N$ isotope analysis for BAM as shown in Figure 3-5a (for comparison, also data of the conventional reactor is shown again, Fig. 3-5b).

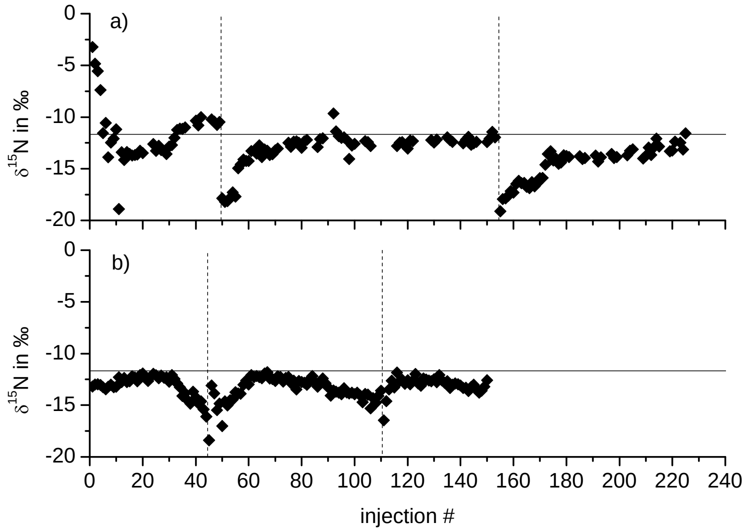
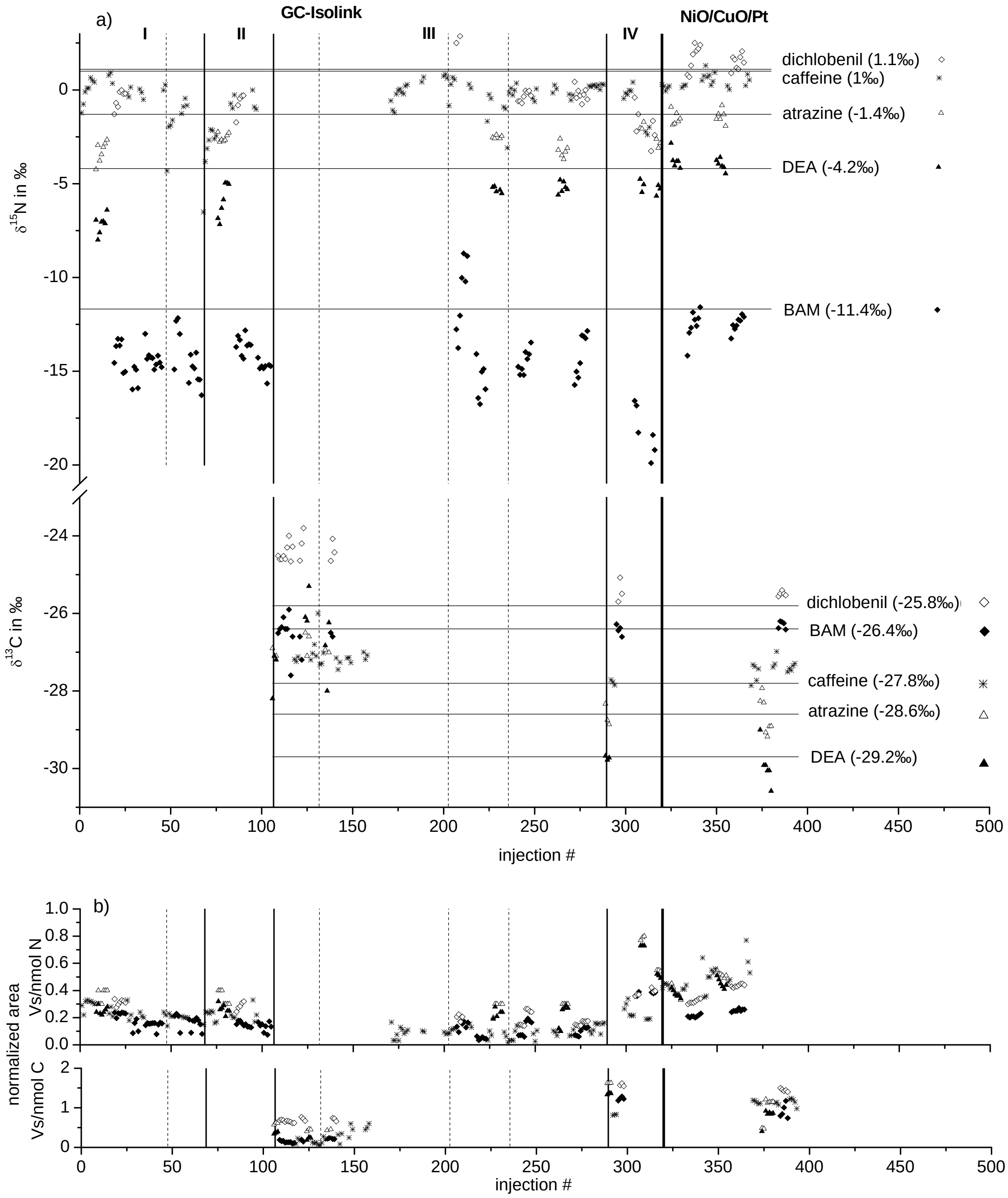


Figure 3-5. $\delta^{15}N$ measurements of BAM using the two different combustion reactors. Panel (a) represents data obtained with the NiO tube/ CuO-NiO reactor at 1030°C, panel (b) shows in comparison again the data obtained with the conventional NiO/CuO/Pt reactor at 980°C (same data as in Figure 3-2b).

A long term series of BAM measurements in Muenchen showed an offset of $\delta^{15}N$ compared to the EA value (Fig. 3-5), which varied depending on the life time of the reactor. Although the NiO tube/ CuO-NiO reactor seemed to have a longer life time compared to the conventional NiO/CuO/Pt reactor, both reactors had a similar performance indicated by a conditioning phase after reactor conditioning and a drift in offsets. Also results obtained in Bremen (Thermo Scientific laboratory) showed $\delta^{15}N$ isotope values of BAM with a systematic drift and an offset of -3 ‰ (n = 10) compared to EA-IRMS (Table 3-1). Different substance amounts on column ware required for both laboratories, depending on the instrument sensitivity. Minimum amplitudes around 300 mV in Muenchen were obtained with 2 µl of a 500 mg L^{-1} BAM solution (5.3 nmol N on column), and in contrast, in Bremen with 0.5 µl of 750 mg L^{-1} BAM solution (2 nmol N on column) amplitudes of 270 mV. Contrasting with BAM, nitrogen isotope values of dichlobenil ($\delta^{15}N$ of 0.8 ± 0.4 ‰, measurements from Bremen, Table 3-1) showed a good trueness, agreeing well with values determined by EA-IRMS. The discrepancy between carbon and nitrogen data is surprising because the NiO tube/ CuO-NiO reactor is operated in the same way (temperature, reactor conditioning frequency) in C and N mode. Therefore, for both elements the same degree of conversion and a similar trueness would be expected.

3.3.2 Testing NiO tube/ CuO-NiO reactors on a daily basis: monitoring peak response and accuracy of isotope measurements

The measurements in 3.3.1 were motivated by the objective to establish a robust reactor performance for our target compounds, in a manner similar to Sherwood Lollar et al. (2007) or Jochmann et al. (2006). However, the results in 3.3.1 are not yet conclusive. Contrasting with good performance for some compounds (Table 3-1), precision for atrazine was found to vary between reactors of the same type (Figure 3-4), EA-IRMS nitrogen target values of BAM were not always reproduced (Figure 3-3) and nitrogen isotope values of BAM even showed a drift over time which would prevent systematic offset corrections (Figure 3-5). This part of this study was, therefore, motivated by the aim to obtain better insight into the factors causing these inconsistencies and to develop strategies to deal with them on a daily basis.


a)
GC-Isolink
NiO/CuO/Pt
I
II
III
IV
dichlobenil (1.1‰)
caffeine (1‰)
atrazine (-1.4‰)
DEA (-4.2‰)
BAM (-11.4‰)
δ^{15}N in ‰
0
-5
-10
-15
-20
-24
-26
-28
-30
δ^{13}C in ‰
dichlobenil (-25.8‰)
BAM (-26.4‰)
caffeine (-27.8‰)
atrazine (-28.6‰)
DEA (-29.2‰)
0
50
100
150
200
250
300
350
400
450
500
injection #
b)
1.0
0.8
0.6
0.4
0.2
0.0
Vs/nmol N
normalized area
2
1
0
Vs/nmol C
injection #

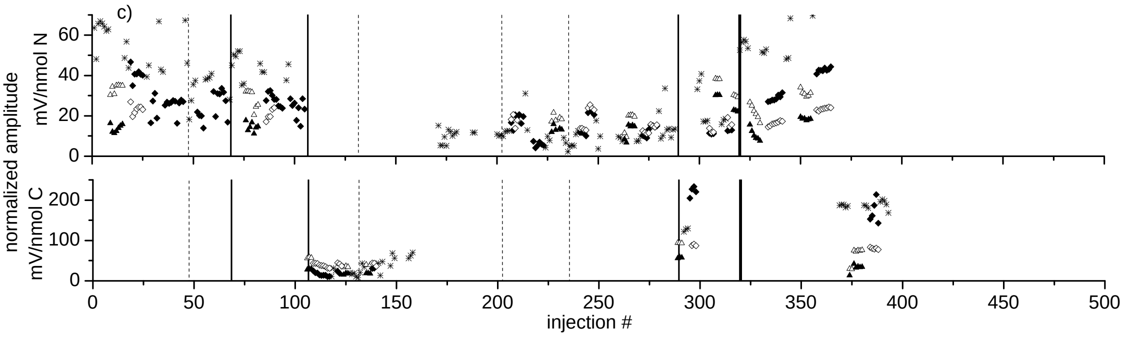


Figure 3-6. $\delta^{15}N$ and $\delta^{13}C$ values (a) for caffeine (crosses), atrazine (open triangles), desethylatrazine (filled triangles), dichlobenil (open diamonds) and BAM (filled diamonds) using a total of four NiO tube/ CuO-NiO reactors (injection 1 to 319) and a CuO/NiO/Pt reactor (injection 320 to 393). Solid horizontal lines in (a) represent the target isotope value of each compound determined by EA-IRMS. Normalized areas (b) and amplitudes (c) represent the conversion efficiency of dichlobenil, BAM, atrazine, DEA and caffeine of each reactor. Dashed vertical lines represent reactor conditionings, and solid vertical lines indicate the use of a different reactor.

Isotope shifts often are explained by incomplete conversion of target compounds (Merritt et al., 1995). To systematically monitor this effect, and to test the performance of different reactors, this part of this study therefore integrated measurements of (i) our target compounds and (ii) caffeine into daily measurement sequences on a regular basis. As shown in Figure 3-6, besides the trueness and accuracy of isotope values (Fig. 3-6a), also the peak response was monitored. It is stated both as area in Vs/nmol (to indicate the integrated response, Fig. 3-6b) and as amplitude in mV/nmol (to indicate the respective signal-to-background ratio, Fig. 3-6c). A total of five different reactor tubes (one conventional, four NiO tube/ CuO-NiO reactors) were tested on two identical GC-IRMS instruments (Fig. 3-6). Table 3-2 summarizes the amplitudes that were obtained for each reactor, together with the respective amounts of nitrogen and carbon on-column. The Table also illustrates that amplitudes during our long-term monitoring were affected by changes in sensitivity on the different instruments (due to enforced instrument shutdown during power failure, filament changes, re-focusing, etc.) Our instruments are not equipped with a dual inlet system by which the absolute yield in molecules per ion can directly be measured. In an alternative approach we therefore determined a *relative* sensitivity on a daily basis by monitoring the amplitude of reference gas CO_2 (m/z = 44) and N_2 (m/z = 28), respectively, when it was introduced through the open split at a pressure of 2.5 bar.

Table 3-2. Typical amplitudes and corresponding C and N amounts of dichlobenil, BAM, atrazine, DEA and caffeine on column using different types of oxidation reactors and two equal GC-IRMS instruments with different sensitivity

	NiO tube/ CuO-NiO reactor I+II		**NiO tube/ CuO-NiO reactor III**		**NiO tube/ CuO-NiO reactor IV**		**NiO/CuO/Pt**	
	Amplitudes (nmol N or C on column)	**Relative sensitivity for N_2 at m/z = 28[a] CO_2 at m/z = 44**	**Amplitudes (nmol N or C on column)**	**Relative sensitivity for N_2 at m/z = 28[a] CO_2 at m/z = 44**	**Amplitudes (nmol N or C on column)**	**Relative sensitivity for N_2 at m/z = 28[a] CO_2 at m/z = 44**	**Amplitudes (nmol N or C on column)**	**Relative sensitivity for N_2 at m/z = 28[a] CO_2 at m/z = 44**
				N_2				
dichlobenil	(304 ± 50) mV (6.3 nmol N)	2.3	(307 ± 49) mV (17.4 nmol N)	1	(147 ± 30) mV (2.7 nmol N)	3.7	(258 ± 7) mV (3.1 nmol N)	3.5
BAM	(333 ± 84) mV (5.3 nmol N)	2.3	(266 ± 43) mV (14.8 nmol N)	1	(406 ± 32) mV (2.5 nmol N)	3.7	400 ± 10 mV (2.7 nmol N)	3.5
atrazine	(826 ± 126) mV (11.6 nmol N)	2.3	(262 ± 33) mV (13.9 nmol N)	1	(291 ± 40) mV (3.7 nmol N)	3.7	632 ± 32 mV (5.8 nmol N)	3.5
DEA	(429 ± 61) mV (13.3 nmol N)	2.3	(218 ± 32) mV (16 nmol N)	1	(260 ± 42) mV (2.7 nmol N)	3.7	430 ± 13 mV (6.6 nmol N)	3.5
caffeine	(648 ± 190) mV (5.8 nmol N)	2.3	(345 ± 38) mV (29.2 nmol N)	1	(622 ± 33) mV (9.7 nmol N)	3.7	773 ± 57 mV (2.9 nmol N)	3.5
				CO_2				
dichlobenil	-	-	(479 ± 55) mV (8.3 nmol C)	1.5	1355 ± 31 mV (3.8 nmol C)	4	879 ± 24 mV (11 nmol C)	1
BAM	-	-	(210 ± 78) mV (7.4 nmol C)	1.5	3067 ± 169 mV (3.5 nmol C)	4	1605 ± 270 m V (9.4 nmol) C	1
atrazine	-	-	(493 ± 118) mV (7.4 nmol C)	1.5	831 ± 3 mV (2.2 nmol C	4	693 ± 12 mV (9.3 nmol C)	1
DEA	-	-	(207 ± 55) mV (6.4 nmol C)	1.5	440 ± 11 mV (1.9 nmol C)	4	271 ± 10 mV (8 nmol C)	1
caffeine	-	-	(1019 ± 593) mV (19.4 nmol C)	1.5	1960 ± 65 mV (3.9 nmol C)	4	1098 ± 49 mV (5.9 nmol C)	1

In Table 3-2 we attributed a relative value of "1" to the poorest sensitivity observed with each gas, whereas better sensitivities are expressed by correspondingly higher numbers. The normalized areas and amplitudes of Figure 3-6 and 3-7 are calculated according to

$$[\text{normalized area and amplitude, respectively}]/[\text{nmol N (or C)}]$$
$$= \frac{[\text{peak area and amplitude, respectively}]}{[\text{relative daily sensitivity for N (or C) of the instrument}]}/[\text{nmol N (or C) on column}] \qquad \text{(Equation 3-3)}$$

These values therefore represent lower boundaries for the sensitivity of the analysis corresponding to days of the worst instrument performance.

Analyte loading and relative peak response

Based on experiences in the Thermo application laboratory, minimum amplitudes around 300 mV (NiO tube/ CuO-NiO reactor) and 500 mV (NiO/CuO/Pt reactor) are recommended for carbon and nitrogen isotope measurements. Due to different sensitivities of the instruments, different substance amounts on column were required to obtain these amplitudes (Table 3-2). For the NiO tube/ CuO-NiO reactor IV only 2.7 to 3.7 nmol N on column were needed to achieve amplitudes between 147 and 406 mV, due to the high sensitivity of the respective instrument at that week. In contrast, measurements with the NiO tube/ CuO-NiO reactor III required 13.9 to 17.4 nmol N on column for comparable amplitudes due to the poor sensitivity on another instrument at another time. To assess to which extent an increased substance amount on column can influence the peak response, normalized areas and amplitudes (according to eq. 3-3) were plotted versus the corresponding substance amount on column. As shown in Figure 3-7, peak areas and amplitudes decreased for both, nitrogen (Fig. 3-7a) as well as carbon (Fig. 3-7b) measurements when the amount of substance on column was increased. These observations support the hypothesis that conversion efficiency is better at smaller substance loadings. Conversely, they suggest that incomplete conversion and / or poor chromatographic performance occurs at higher loadings. These results emphasize the need for maximum instrument sensitivity and indicate that amplitudes around 300 mV rather than 500 mV may be advantageous with the new NiO tube/ CuO-NiO reactor, since they can be reached at smaller substance loadings.

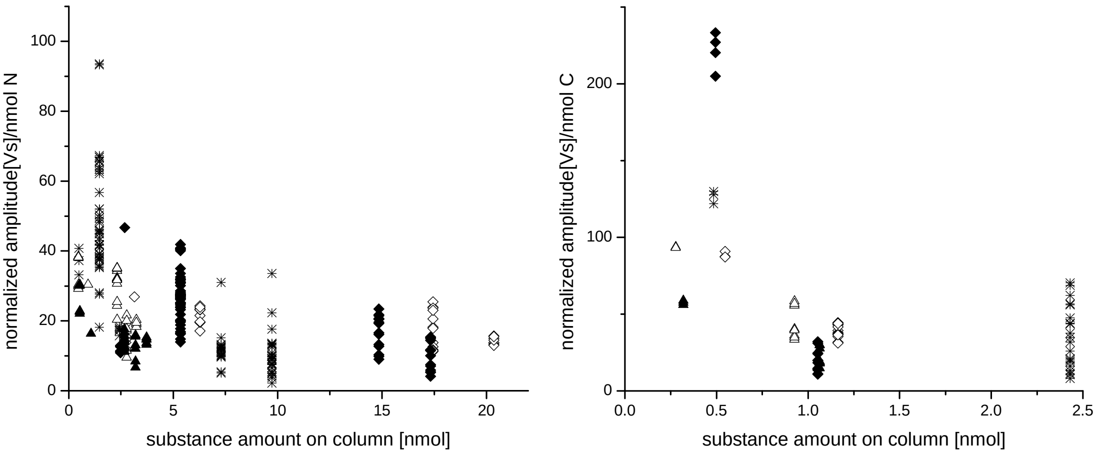


Figure 3-7. Dependency of substance amount on column and conversion efficiency represented by normalized amplitudes per nmol N (a) and C (b), respectively of dichlobenil (empty diamond), BAM (black diamond), atrazine (empty triangle), DEA (black triangle) and caffeine (cross).

Analyte loading and trueness of isotope values

From these findings it would be expected that a more sensitive instrument which allows for smaller analyte loadings would lead to better conversion and therefore most reproducible isotope values. Isotope measurements of reactor IV represent data obtained with the highest nitrogen instrument sensitivity. The substance amount on column was kept low (between 2.5 and 9.7 nmol N on column) and amplitudes were between 150 and 622 mV (Table 3-2). Carbon isotope values were accurate and precise (Fig. 3-6, injection 289 to 298). Nevertheless, nitrogen isotope analysis showed a poor precision for dichlobenil, BAM, atrazine and caffeine (Fig. 3-6a, injection 299 to 319). Combining the result of all tested reactors we observed no evident correlation between conversion efficiency and trueness of isotope values between the tested reactors (Fig. 3-6). In the most indicative measurements with reactor I and II, atrazine showed reproducible peak response with both reactors (Figure 3-7b and 3-7c, injection 9 to 15 and 76 to 82), but only reactor II generated precise nitrogen isotope values, whereas the values of reactor I drifted. Likewise, DEA, which was analyzed simultaneously with atrazine, showed a different behavior (reactor II) and resulted in a shift of nitrogen isotope values to less negative numbers. To our surprise we therefore found that atrazine and DEA nitrogen analysis could actually not reproduce the true carbon and nitrogen values obtained in Part I of this work (Fig. 3-4). Reactor I, II and reactor III (after the third reactor conditioning) were comparable and resulted in precise nitrogen isotope values, but showed a notable offset of -1.5 ± 0.4 ‰

(dichlobenil), -1.7 ± 0.5 ‰ (atrazine), -1.9 ± 1 ‰ (DEA), -1.6 ± 1.3 ‰ (caffeine), and even -2.9 ± 0.9 ‰ or BAM. Notably, nitrogen isotope values of caffeine, a substance commonly used as reference compound for instrument installation, resulted in nitrogen isotope values with a poor accuracy for all tested NiO tube/ CuO-NiO reactors. In contrast, carbon isotope analysis showed accurate and precise values for all compounds (Fig. 3-6a, injection 289 to 298 and 369 to 393), except for reactor III (Fig. 3-7, injection 106 to 160).

In conclusion, we could not establish a clear correlation between peak response – taken as an indicator of incomplete conversion – and the trueness of isotope values (Fig. 3-6). Rather, our results suggest that the trueness of nitrogen isotope values varied to a great extent from reactor to reactor, and even during the lifetime of the same reactor (expressed in different offsets). Taken together, these observations imply that the well-established approach to aim for (i) complete conversion and (ii) an absolute trueness of isotope values in GC-IRMS (Jochmann et al., 2006; SherwoodLollar et al., 2007) is not pragmatic for some target compounds. This raises the question about analytical protocols and referencing strategies by which accurate isotope analysis of such compounds may nonetheless be achieved.

3.3.3 Strategies for referencing

Our data demonstrate the need for a referencing strategy which corrects for (a) substance-specific and (b) reactor- and lifetime-dependent offsets of isotope values. Figure 3-6 clearly shows that it would not be possible, for example, to correct an offset in DEA nitrogen isotope values with the corresponding offset of atrazine (injection 220 to 320), or to correct measurements at the end of a series with an offset determined at the beginning (data throughout Figure 3-6). Therefore, to implement such a correction into daily analysis, we implemented the following referencing strategy: (i) to measure standards before and after the sample to correct for a certain offset and (ii) to use a reference material which is identical to the target substances. In the following we present the application and the practicability of such a referencing strategy for BAM as example. Figure 3-8 shows data from a biodegradation experiment of BAM, where samples (white circles) showed more positive $\delta^{15}N$ values than the original reactant. The shift was different between samples, but well reproducible for each sample, as indicated by the agreement of triplicates (clusters of three points in Figure 3-8).

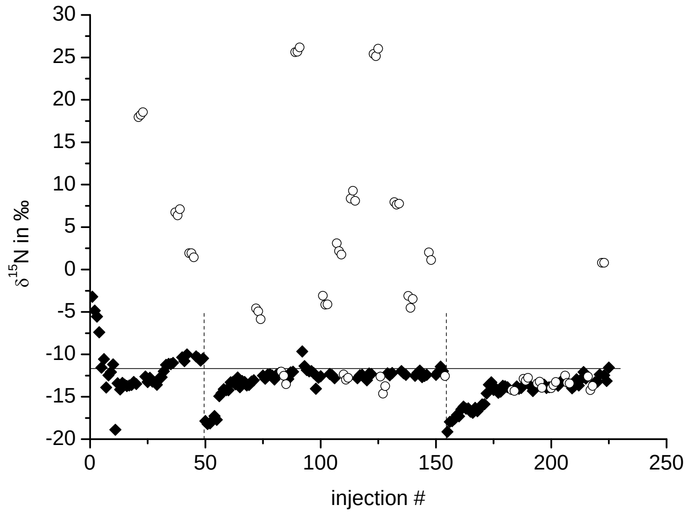


Figure 3-8. δ^{15}N values of BAM standards (filled diamonds) and different BAM samples from a degradation experiment corresponding to different degrees of degradation (empty circles) obtained with a NiO tube/ CuO-NiO reactor. Black line represents the BAM standard value determined by EA-IRMS and the dashed lines indicate reactor conditionings.

Samples were bracketed by measurements of our compound-specific BAM isotope standard (black diamonds) at a similar concentration as the samples.

Sample measurements were not conducted during the unsystematic drift of the nitrogen isotope values that was observed in the conditioning phase after each conditioning of the NiO tube/ CuO-NiO reactor (Fig. 3-8, injections 1 to 15, 49 to 64 and 155 to 175), but started only when the standard deviation of at least five measurements of the BAM standard was within the analytical error (1 ‰ for nitrogen analyses). Triplicate sample measurements were subsequently bracketed by triplicate standard measurements, and sample isotope values were corrected with the offset of the mean value of standards measured before and after the sample. Figure 3-8 illustrates that with this procedure precise and true values can be obtained.

3.4 Conclusion

The combustion of organic target compounds in a continuous He flow inside a miniature reactor tube is the key step in compound-specific isotope analysis by GC-IRMS and has led to a boost of new applications in many disciplines. Contrasting with the excess oxidation power that can be applied in offline analysis or elemental analyzer reactors, however, this process is fueled by just a few miniature CuO / NiO wires. Nonetheless, previous studies have reported excellent precision and trueness suggesting that complete conversion and true isotope values can be taken for granted in GC-IRMS measurements. This study addressed organic target compounds that contain nitrogen and chlorine inside an aromatic structure and may, therefore, be more recalcitrant to complete conversion than the typical spectrum of substances. It comprises a representative number of combustion reactors and tests two different reactor types (conventional NiO/CuO/Pt versus NiO tube/ CuO-NiO wires). The NiO tube/ CuO-NiO reactor showed compelling advantages compared to the conventional one: no additional reduction reactor was necessary, reactor conditioning could be routinely performed for nitrogen isotope measurements, and reliable atrazine and DEA analysis was for the first time obtained over several hundred measurements.

Overall, however, both reactor types showed notable variations in the accuracy of nitrogen and, to a lesser extent, also of carbon isotope measurements. Rather than giving a clear correlation with peak areas as an indicator of conversion efficiency, our results suggest that the trueness of isotope values is substance-, reactor batch- and even reactor lifetime-dependent. Our data demonstrate that (i) complete conversion and (ii) an absolute trueness of isotope values in GC-IRMS may not be achievable for some target compounds. Instrument data should, therefore, never be taken as “face value”, but measurements must continuously be validated with compound-specific isotope standards of the respective analyte. Nonetheless, accurate isotope analysis may be achieved. In an exemplary case we demonstrate that stringent bracketing with a compound-specific isotope standard allows for a continuous drift correction so that accurate isotope values can be obtained. In future studies it is, therefore recommended to routinely test the performance of reactor batches for a given target compound, and to adapt the referencing strategy accordingly.

3.4 References

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4.

Carbon and Nitrogen Isotope Fractionation Associated with Biotic Degradation of the Pesticide Metabolite 2,6-Dichlorobenzamide (BAM) by *Aminobacter* spp. ASI1 and MSH1

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4.1 Introduction

The herbicide 2,6-dichlorobenzonitrile (dichlobenil) is used in many countries and causes a widespread presence of its metabolite 2,6-dichlorobenzamid (BAM) in groundwater systems (Haarstad and Ludvigsen, 2007; Törnquist et al., 2007; UBA, 2006). Dichlobenil is only applied at non-agricultural areas such as court yards, in plant nurseries and fruit orchards. It is immobile in the soil where it sorbs; BAM on the other hand, leaches into groundwater due to its high water solubility and low sorption affinity (Clausen et al., 2004). Although dichlobenil-containing herbicides were banned in EU in 2008 (EU, 2008), BAM concentrations in groundwater are still increasing indicating a considerable persistence of the metabolite in the environment.

Degradation of dichlobenil to BAM is catalysed by nitrile hydratases (Fig. 4-1) (Holtze et al., 2006). Further transformation of BAM, in contrast, has been observed only recently in soil previously exposed to dichlobenil (Simonsen et al., 2006). So far, only two BAM-mineralising bacteria (*Aminobacter* sp. strains ASI1 and MSH1) have been isolated and characterised (Simonsen et al., 2011a; Simonsen et al., 2006; Sorensen et al., 2007). In aquatic systems BAM degradation seems to be limited (Clausen et al., 2007), although *Aminobacter* spp. are ubiquitous in the environment (Rousseaux et al., 2001).

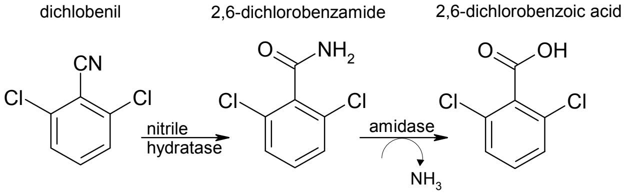


Figure 4-1. Hydrolysis of dichlobenil to 2,6-dichlorobenzamid (BAM) catalysed by nitrile hydratase and the further degradation of BAM to 2,6-dichlorobenzoic acid catalysed by amidase.

It is therefore of particular interest to evaluate the fate of BAM in the environment, focusing on degradation processes. Here, the classical approach of identifying and quantifying the pollutant and its metabolite(s) is not sufficient for a complete risk assessment. Processes like dilution, sorption and/or volatilization lead to a considerable concentration decrease but not to pollutant elimination. Furthermore the detection and identification of further metabolites such as 2,6-dichlorobenzoic acid (Fig. 4-1) fails if they are rapidly degraded or if they are not accessible to analysis. Consequently, it is 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), molecules containing the light isotope in the reactive position react slightly faster than molecules containing a heavy isotope in the reactive bond. As a consequence the heavier isotopes become enriched in the remaining substrate. This isotopic enrichment offers considerable potential to detect (Elsner et al., 2005; Hirschorn et al., 2004; Zwank et al., 2005) and even quantify (Abe and Hunkeler, 2006; Sherwood Lollar et al., 2001) degradation of organic contaminants in the environment (Elsner et al., 2005; Hunkeler et al., 2008; Schmidt et al., 2004) – even when no further metabolites can be detected (Elsner, 2010) such as in the case of 2,6-dichlorobenzoic acid formation from BAM. The KIE is position-specific and depends on the type of reaction mechanism (Elsner et al., 2005; Schmidt et al., 2004). A linear relation is expected when plotting changes in isotope values of C vs. N (dual isotope plot) during the degradation process. Based on the slopes of such dual isotope plots different degradation pathways may be elucidated (Elsner, 2010; Elsner et al., 2004; Meyer et al., 2009; Penning et al., 2008; Schmidt et al., 2004).

This study investigates for the first time carbon and nitrogen isotope fractionation associated with BAM degradation, using the only bacterial strains (*Aminobacter* sp. strains MSH1 and ASI1) known to degrade BAM to date (Simonsen et al., 2006; Sørensen et al., 2007). Isotope enrichment factors and dual isotope plots of the degradation reactions were determined in order to obtain insight into the underlying reaction mechanisms. In addition, carbon and nitrogen isotope fractionation were also investigated during BAM formation studying dichlobenil degradation with the bacterial strain *Rhodococcus erythropolis* DSM 9685.

4.2 Materials and Methods

4.2.1 Chemicals

Dichlobenil (2,6-dichlorobenzonitrile, CAS no.: 1194-65-6) was purchased from Fluka and BAM (2,6-dichlorobenzamid, CAS no.: 2008-58-4) was purchased from Riedel-de Haën (>98 %) 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.

4.2.2 Degradation studies

Degradation of 2,6-dichlorobenzamid (BAM) was studied using two different bacterial strains, *Aminobacter* sp. ASI1 and MSH1. Degradation studies of dichlobenil were performed using the pure culture *Rhodococcus erythropolis* DSM 9685. Freeze stock of MSH1 and DSM 9685 was pre-grown in R2B medium (Difco, Detroit, Michigan, USA) and freeze stock of ASI1 was pre-grown in MS (Sørensen and Aamand, 2003) 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 pre-cultures 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 (OD_{600nm}) of 0.8 for MSH1, 0.9 for ASI1 and 0.6 for DMS 9685 determined in a Varian Cary 50 Bio UV-Visible Spectrophotometer. Triplicates of the dichlobenil degradation experiment were conducted in 1000 mL sterilized Erlenmeyer flasks which were closed with a viton seal. Triplicates of BAM and dichlobenil degradation experiments contained 500 mL MS each. Initial cell density of MSH1 was 10^6 cells ml^{-1}, ASI1 and DMS 9685 were adjusted to 10^7 cells ml^{-1}. The microcosms were spiked with BAM dissolved in sterilized MilliQ water in a stock solution of 1200 mg L^{-1} to yield an initial concentration of 12 mg L^{-1}. BAM provided the only carbon and nitrogen source for MSH1 and ASI1 (Simonsen et al., 2006; Sørensen et al., 2007). The initial concentration of dichlobenil was 9 mg L^{-1}. The degradation experiments were performed in triplicates with orbital shaking (125 rpm) incubated at room temperature in the dark. The concentrations of BAM and dichlobenil in non-sterile experiments were compared to sterile controls without cells. For quantification purposes 0.5 mL aliquots were centrifuged 2 min at 14000 rpm (Centrifuge 5417R, Eppendorf, Hamburg, Germany). 200 µl of the supernatant were used for quantification of BAM and dichlobenil and 200 µl were stored at -18 °C.

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 lyophilised. The remains (salts and BAM) were resubstituted 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}. In contrast to the procedure for BAM, isotope analysis of dichlobenil was performed using direct immersion solid phase microextraction (SPME) from 10 to 20 ml aliquots using a PDMS/DVB fibre from Supelco analytical supplied by Sigma Aldrich (Taufkirchen, Germany). Until SPME extraction aliquots were stored frozen.

4.2.3 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 x 2.00 mm, 5 µm (Phenomenex, Aschaffenburg, Germany) and a UV detector. The eluents were a buffer solution (KH_2PO_4 0.001 mol L^{-1}, pH 7.0) and acetonitrile (Berg et al., 1995). 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.

4.2.4 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, and liquid samples were injected via GC Pal autosampler (CTC, Zwingen, Switzerland). The split/splitless injector was held at 250 °C and operated for 2 min in splitless mode after which it was switched to split mode (split ratio1:10), with a flow rate of 1.4 mL min^{-1}. 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 a 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 operated at 1030 °C was used for carbon and nitrogen isotope analysis, respectively (Reinnicke et al., in prep., see Chapter III).

The carbon and nitrogen isotopic composition of dichlobenil and BAM in-house standards was determined by elemental analyzer-isotope ratio mass spectrometry (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, Austria).

$\delta^{13}C$- and $\delta^{15}N$-values are reported in permil relative to Vienna PeeDee Belemnite (VPDB) and air, respectively:

$$\delta^{13}C = \frac{\left({}^{13}C/{}^{12}C_{\mathrm{Sample}} - {}^{13}C/{}^{12}C_{\mathrm{Srandard}}\right)}{{}^{13}C/{}^{12}C_{\mathrm{Standard}}} \qquad \text{(Equation 4-1)}$$

$$\delta^{15}N = \frac{\left({}^{15}N/{}^{14}N_{\mathrm{Sample}} - {}^{15}N/{}^{14}N_{\mathrm{Standard}}\right)}{{}^{15}N/{}^{14}N_{\mathrm{Standard}}} \qquad \text{(Equation 4-2)}$$

Carbon and nitrogen isotope analysis via GC-IRMS of BAM and dichlobenil have been validated as described in chapter III of this work.

4.2.5 Carbon and nitrogen isotope enrichment factors for biodegradation of BAM

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

$$\ln\frac{R_t}{R_0} = \ln\left(\frac{1+\delta^h E}{1+\delta^h E_0}\right) = \varepsilon * \ln f \qquad \text{(Equation 4-3)}$$

R and R_0 are the compound specific isotope ratios of heavy versus light isotopes of an element in the contaminant at a given time and at the beginning of the reaction. $\delta^h E$ and $\delta^h E_0$ are the corresponding isotopic signatures 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 (KIE) 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 values of ε whereas inverse isotope effects (i.e., preference for heavy isotopes) are expressed as positive ε-values. Dilution of observable isotope fractionation due to non-reactive atoms of the same element can be taken into account to estimate an apparent position-specific kinetic isotope effect (AKIE) according to

$$AKIE \approx \frac{1}{(n * \varepsilon)} \qquad \text{(Equation 4-4)}$$

(Elsner et al., 2005) where n is the number of atoms of the element present in the molecule. The term “apparent” accounts for the fact that - in addition to the “dilution” by non-reacting atoms - observable isotope fractionation is occasionally smaller than expected because the intrinsic kinetic isotope effect is masked in the AKIE value. Masking can occur if the bond-changing step is not the rate limiting one. In contrast to AKIE values, dual isotope slopes are much less affected by masking, since the KIE of both elements are typically affected to the same extent (Elsner et al., 2007; Fischer et al., 2008; Vogt et al., 2008).

4.3 Result and Discussion

4.3.1 Degradation of BAM

The strains *Aminobacter* sp. MSH1 as well as ASI1 effectively degraded BAM, consistent with previous studies (Simonsen et al., 2006; Sørensen et al., 2007). With ASI1 BAM was degraded rapidly within 20 hours and with MSH1 the compound was degraded within 7 days, whereas no BAM degradation was observed in sterile controls (Fig. 4-2). The different degradation times may be explained by different initial cell densities of ASI1 and MSH1 (10^7 and 10^6 cells ml^{-1}, respectively). Degradation with MSH1 showed a lag phase of less than 30 hours, whereas no lag phase was observed with ASI1. As shown in Fig. 4-3, degradation of BAM by both bacterial strains induced an enrichment of ^{13}C (Fig. 4-3a) and ^{15}N (Fig. 4-3b) in the remaining BAM. No isotope fractionation was observed in the sterile control confirming that no isotope fractionation was associated with lyophilisation of BAM and subsequent extraction with dichloromethane.

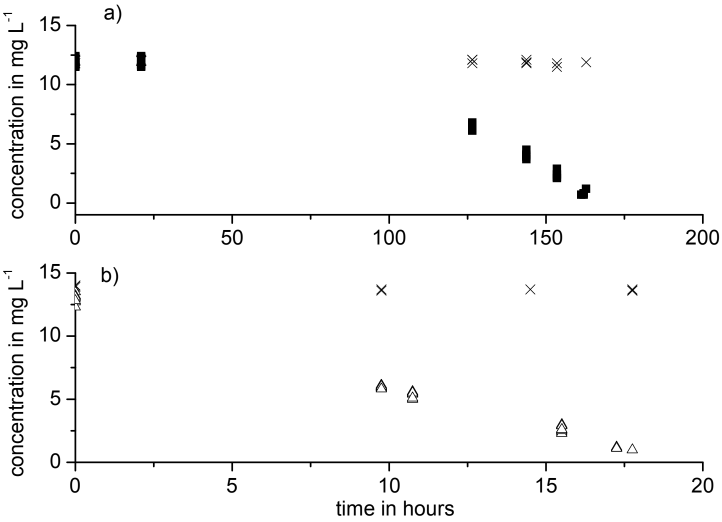


Figure 4-2. Degradation of BAM (triplicates) using the pure bacterial strains (a) *Aminobacter* sp. MSH1 (black rectangle) and (b) *Aminobacter* sp. ASI1 (open tringel). Crosses in panel a and b represent the concentration of BAM in the sterile controls.

Nitrogen isotope fractionation of BAM was slightly stronger with *Aminobacter* sp. MSH1 than with ASI1, showing an enrichment of ^{15}N by 38 ‰ at 94 % conversion, compared to a corresponding enrichment of 28 ‰ at 93 % conversion with ASI1. Carbon isotope fractionation, in contrast, was identical with both strains (Fig. 4-4a). Isotope fractionation was quantified in Rayleigh plots and given in terms of the corresponding enrichment factors (Figure 4-4a,b, Table 4-1).

Table 4-1. Bulk carbon and nitrogen isotope enrichment factors (ε_{carbon}, $\varepsilon_{nitrogen}$). Apparent kinetic isotope effect (AKIE) for carbon and nitrogen and Δ values of dual isotope plots for biotic hydrolysis of BAM. Uncertainties given are 95 % confidence intervals of linear regression.

	enzyme	ε_{carbon} **[‰]**	$\varepsilon_{nitrogen}$ **[‰]**	$\Delta=\delta^{15}N/\delta^{13}C = \varepsilon_{nitrogen}/\varepsilon_{carbon}$	**AKIE**$_{carbon}$	**AKIE**$_{nitrogen}$
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

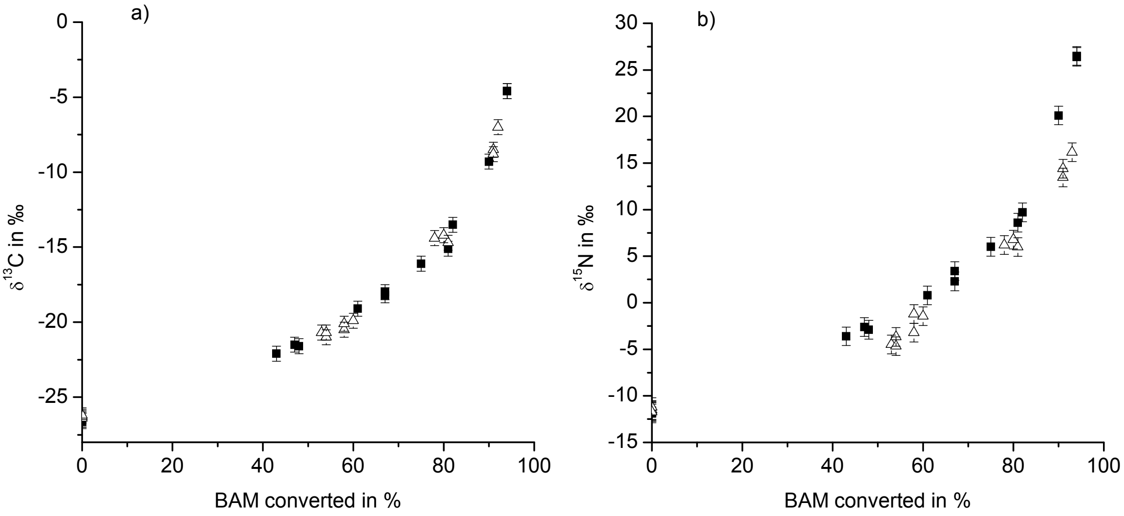


Figure 4-3. Changes in isotope ratios of carbon (a) and nitrogen (b) during biotic degradation of BAM using two bacterial strains, *Aminobacter* sp. MSH1 (black rectangle) and *Aminobacter* sp. ASI1 (open triangle). Error bars represent total uncertainty of carbon (± 0.5 ‰) and nitrogen (± 1 ‰).

Fig. 4-4c compares the dual isotope plot of both BAM degraders. The slopes of δ^{15}N against δ^{13}C ($\Delta \approx \varepsilon_{\text{nitrogen}}/\varepsilon_{\text{carbon}}$) are distinguishable for ASI1 and MSH1 (Table 4-1) suggesting subtle differences in the initial degradation mechanisms despite the same net reaction (amide hydrolysis). The microbes are phylogentically closely related, however showing differences in physiology (Sorensen et al., 2007). 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. 4-4 using the enrichment factors of both reactions (Table 4-1). AKIEs obtained for carbon are remarkably large, of comparable magnitude as the theoretical maximum KIE values for C-N bond cleavage given by the Streitwieser limit of 1.06 (Elsner et al., 2005). These pronounced isotope effects suggest that the reaction was not masked and therefore isotope fractionation represents the enzymatic reaction. Possible mechanistic hypotheses for amide hydrolysis involve a sequence of reaction steps, involving either a tetrahedral intermediate, through an OH^- and H_3O^+ catalysis (Fig. 4-5a,b) or a concerted mechanism (Fig. 4-5c).

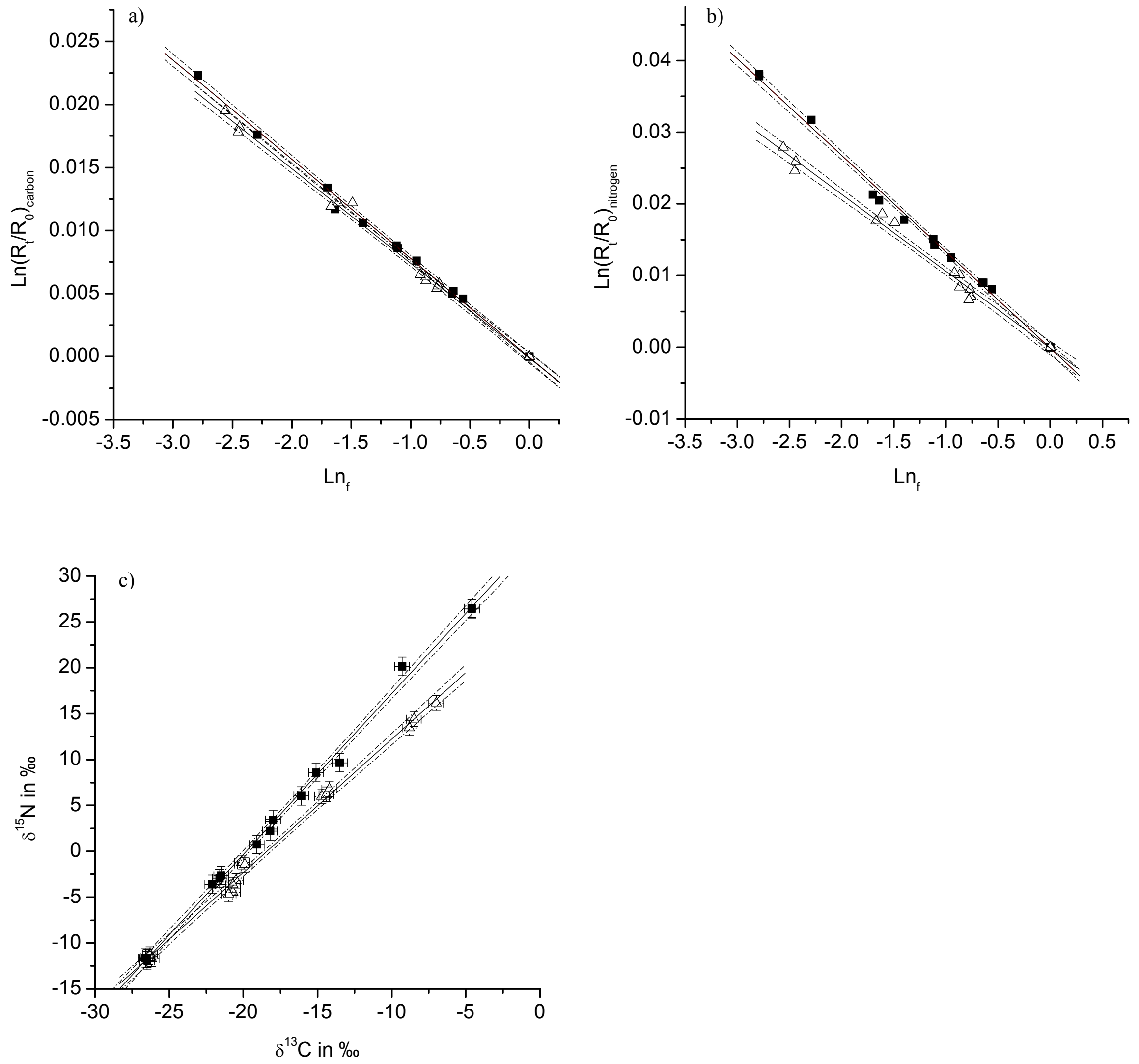
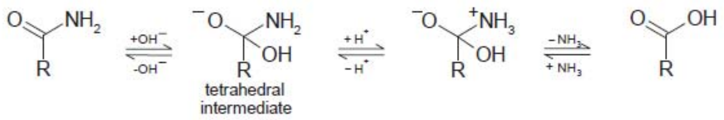


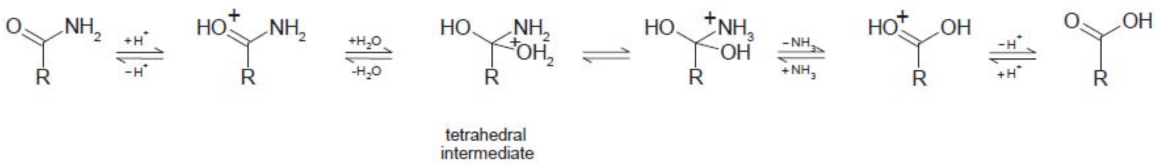
Figure 4-4. Logarithmic plots according to Rayleigh equation of carbon (a) and nitrogen (b) isotope ratios. (c) two dimensional isotope plot of carbon and nitrogen of BAM isotope ratios for *Aminobacter* sp. ASI 1 (open triangel) and *Aminobacter* sp. MSH 1 (black rectangle). The slope of regression is determined using bulk data of all strains and is given as Δ. Error bars indicate total uncertainty of carbon isotope (± 0.5 ‰) and nitrogen isotopes (± 1 ‰). Dotted lines represent 95% confidence interval of linear regression.

A nucleophilic attack on the carbonyl carbon atom next to an amide bond (Bakowies and Kollman, 1999; Marlier, 2001; Schwarzenbach et al., 2003) is well established to show a rather large primary carbon isotope effect and a small nitrogen isotope effect (Marlier et al., 1999; O'Leary et al., 1974), as observed in our study. Also the slight variation in dual isotope plots may be attributable to the occurrence of two or more quick successive transformation steps, consistent with previous studies (Mancini et al., 2008; Penning et al., 2008a).

a) OH^- -promoted amide hydrolysis



b) H_3O^+ -promoted amide hydrolysis



c) concerted mechanism

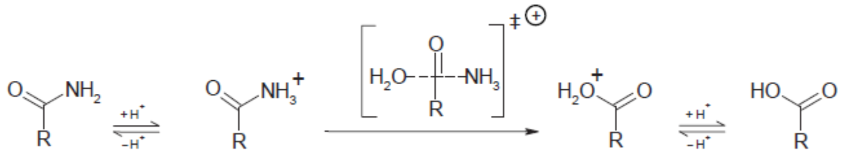
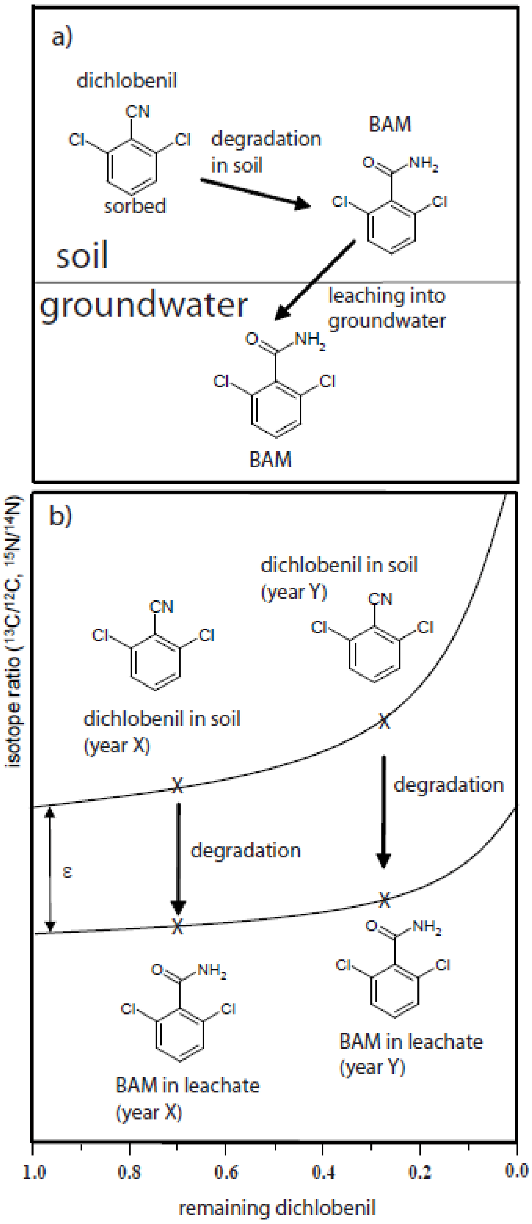


Figure 4-5. Schematic illustration of possible BAM hydrolysis to 2,6-dichlorobenzoic acid with a nucleophilic attack on the carbonyl carbon.

4.3.2 Degradation of dichlobenil to BAM

In natural samples, isotope values of BAM may not only be affected by its degradation, but also by its formation as a metabolite of the pesticide dichlobenil. Monitoring studies in European groundwater systems indicate that concentrations of dichlobenil are low, or even negligible in the presence of elevated BAM concentrations (Haarstad and Ludvigsen, 2007; Törnquist et al., 2007; UBA, 2006). Dichlobenil has a high sorption affinity to soil (Briggs and Dawson, 1970; Clausen et al., 2004; Jernlås, 1990). On the one hand, this sorption is thought to have a buffer effect leading to low dichlobenil concentrations in groundwater. On the other hand, a pool of sorbed dichlobenil resides in soil from which BAM can be formed through degradation and subsequently leach into groundwater (Scheme 4-1a). Recent results in batch cultures have shown that *Aminobacter* sp. MSH1 degrades dichlobenil and BAM at the same time (Simonsen et al., 2011b). Such a scenario of simultaneous BAM degradation and formation can complicate the process assessment using CSIA, as illustrated in Scheme 4-1b. (For isotope effects during sequential reactions see, for example, Hunkeler et al. (1999; 2002), Slater et al. (2001), Bloom et al. (2000), Van Breukelen et al. (2005) and Morill et al. (2005; 2009).) On the one hand, biodegradation of dichlobenil is expected to lead to isotope fractionation as illustrated in Scheme 4-1b. The remaining dichlobenil becomes enriched in ^{13}C, while BAM is initially depleted in ^{13}C compared to dichlobenil and shows subsequently a shift towards more ^{13}C reflecting the trend of its parent compound from which it is formed. Such a relative enrichment of ^{13}C in BAM over time can, therefore, have two reasons: (i) formation from dichlobenil which contains more ^{13}C (Scheme 4-1b); (ii) enrichment of ^{13}C as a result of the isotope effect during BAM degradation (Figure 4-3).

Scheme 4-1. (a) Environmental behaviour of dichlobenil and BAM. Dichlobenil is sorbed to soil constituents, from where it is degraded to BAM. (b) Expected isotope fractionation during dichlobenil degradation and the resulting isotope signature of BAM during its formation only (in this scenario no BAM degradation is assumed yet).



Since this may introduce bias into interpretations of results from CSIA, for a reliable risk assessment it is important to investigate the isotope fractionation of dichlobenil as well. We conducted a degradation experiment with dichlobenil using *Rhodococcus erythropolis* DSM 9685 having nitrile hydratase enzyme activity (Holtze et al., 2006) to get first insight into the magnitude of isotope fractionation related to dichlobenil degradation. The transformation was followed up to 61 % of dichlobenil degradation. Only very small carbon isotope fractionation was observed, with an overall enrichment of ^{13}C by 0.7 ‰. This value is hardly significant within the analytical uncertainty of the method (Table 4-2). Likewise, very small nitrogen isotope fractionation was observed, with a depletion of ^{15}N ($\Delta\delta^{15}N$ of -2.3 ‰) after 61 % dichlobenil degradation. This is consistent with other studies reporting an inverse isotope effect if in the transition state an additional bond is formed to the element of concern (Meyer et al., 2009; Schramm, 1998).

Table 4-2. First carbon and nitrogen isotope values of a dichlobenil degradation experiments using *Rhodococcus erythropolis* DSM 9685.

Concentration of dichlobenil [mg L^{-1}]	dichlobenil converted [%]	$\delta^{13}C$ [‰]	$\delta^{15}N$ [‰]
8.3 ± 0.2	0	-25.8	1.1 ± 0.2
5.7 ± 0.1	32	-25.8 ± 0.4	-1.1 ± 0.2
3.3 ± 0.1	61	-25.1 ± 0.1	-1.2 ± 0.1

The resulting picture is that BAM would in fact show 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 in BAM take place. If BAM is simultaneously formed from dichlobenil degradation, this enrichment may be smaller than expected, since it would be diluted from the continuous input of freshly formed BAM. When assessing the biodegradation of BAM using nitrogen isotope analysis one has to consider that as long as dichlobenil is still present, an enrichment of ^{15}N in BAM compared to the original dichlobenil can be attributable to two reasons: (i) enrichment in ^{15}N due to the preceding inverse isotope fractionation during dichlobenil degradation (i.e., molecules containing ^{15}N are more quickly transformed to BAM). (ii) enrichment of ^{15}N in BAM due to its own degradation. Consequently, interpretations could potentially overestimate BAM degradation. The prediction and quantification of such a scenario in the environment is not easily performed and requires caution.

4.4 Environmental significance and outlook

In this study we report for the first time carbon and nitrogen isotope fractionation associated with biotic degradation of the herbicide dichlobenil and its important metabolite BAM. Carbon isotope fractionation determined with *Aminobacter* sp. ASI1 and MSH1 was strikingly similar. Therefore, this work did not only demonstrate that changes in carbon isotope ratios of BAM can be used for a qualitative detection of its natural biotransformation. Due to the remarkably robust carbon isotope fractionation observed, isotope data may potentially be even used for quantitative estimates of BAM degradation in the environment. As soon as dichlobenil is completely degraded, isotope values of BAM can be uniquely linked to its own biodegradation. Consequently, in such situations it will be possible to quantify BAM biodegradation in the environment using the enrichment factors determined in this study.

Since dichlobenil is present in the soil as recalcitrant pool, due to its high sorption affinity, isotope fractionation associated to dichlobenil biodegradation has to be investigated as well. First experiments showed a small carbon isotope effect, which is hardly significant and a small nitrogen isotope effect. Since plenty of dichlobenil degraders are known, isotope enrichment factors associated to different degraders should be determined to get a complete picture of dichlobenil and BAM degradation in the environment.

Furthermore we got a first clue on the reaction chemistry of enzymatic BAM transformation and observed that the amidase enzyme, produced by the two bacteria to catalyse BAM degradation, gave similar dual isotope plots in the different microorganisms (Fig. 4-4c), likely reflecting similar kinetics and transition states corresponding to one of the mechanisms of Fig. 4-5.

4.5 References

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5. Conclusion and Outlook

Pesticides are applied in many areas (e.g. agriculture, private households, forestry and road industry) for weed control. Depending on their chemical und physical properties and the conditions in the environment, they can be degraded, as well as their metabolites, sorb to soil particles or they may leach to groundwater from where they can further spread. Substances of particular importance due to frequent findings in German groundwater are the herbicide bentazone and the metabolite 2,6-dichlorobenzamide (BAM) (UBA, 2006), which is formed by incomplete degradation of the herbicide dichlobenil a benzonitrile. The scientific question in the environmental aspect is focused on degradation, because it is the only process, which leads to a transformation of the pollutant into non-toxic compounds. The assessment of the degradation in the aquatic environment is not conclusive by using only concentration measurements of the contaminants and their metabolites. Dilution and sorption as well as fast transformation of degradation products can lead to misinterpretations of the environmental fate. Alternatively, compound specific isotope analysis (CSIA) is applied as a new approach to investigate the fate of organic contaminants in the environment. CSIA measures the isotope composition of a compound at natural abundance and is already well established for a multiplicity of substances (e.g. low molecular hydrocarbons). The advantages of this method compared to concentration measurements are on one hand, different isotopic compositions of the same chemical substance can be linked to different source of contamination and on the other hand, CSIA may serve to assess, verify, quantify and even elucidate biological degradation. However, the lack of CSIA methods for frequently detected pesticides was noticeable. Pesticides are commonly polar compounds with a complex chemical structure including hetero atoms, which make them less volatile and the required combustion during analysis might be incomplete, respectively. Meyer et al. (2008) and Penning and Elsner (2007) presented the isotope analysis of the pesticide atrazine and isoproturon, respectively. Both substances showed restrictions related to their isotope analysis, indicating the need for a detailed method development regarding further pesticides. To ass the spectrum of compounds to be analysed, I evaluated in this thesis carbon and nitrogen isotope analysis of the slight acidic pesticides bentazone and MCPA as well as dichlobenil and its metabolite BAM. The target compounds were complemented with the pesticide atrazine and its metabolite desethylatrazine to test a new set up for isotope measurements using GC-IRMS. Bentazone and MCPA are negatively charged at circumneutral pH and require therefore a derivatization prior to gas chromatographic separation or may alternatively be analysed using a liquid chromatography (LC). I tested for the first time the promising derivatization agent trimethylsulfonium hydroxide for isotope analysis of the methylated pesticides (chapter 2) using GC coupled to an isotope ratio mass spectrometer (IRMS). The handling effort, the analyte conversion at certain TMSH addition and the isotope effect related to the introduction of an additional carbon of the methyl group was assessed and investigated, respectively. Additionally I compared carbon isotope analysis derived form GC-IRMS with the alternative isotope analysis using LC-IRMS. The derivatization can be performed fully automated in a porous liner within a temperature programmable injector. By flash heating the analyte is methylated and is then analysed by gas-chromatography. Nitrogen isotope analysis resulted in excellent accurate and reproducible isotope values at stoichiometric addition of TMSH, which is an indicator for a quantitative analyte conversion. Carbon isotope values showed an isotope effect when TMSH was added in ≤250-fold excess. Consequently, using a sufficient excess of TMSH reproducible carbon and nitrogen isotope analysis was achieved. Comparison of carbon isotope analysis using LC-IRMS and GC-IRMS resulted in low standard deviations for methylated bentazone and bentazone, respectively, but showed an offset. In both cases a correction is possible, although in different ways. For GC-IRMS the value of the newly introduced carbon must be corrected using a mass balance (eq 2-5, Chapter 2), whereas for LC-IRMS a constant offset correction is applied.

In contrast to bentazone and MCPA, dichlobenil and BAM isotope analysis using GC-IRMS does not need derivatization, although a derivatization procedure of BAM was successfully tested for quantification purposes (Porazzi et al., 2005). In this work I present carbon and nitrogen isotope analysis of the pesticide dichlobenil and its metabolite BAM using GC-IRMS (chapter 3). Carbon isotope analysis with a conventional oxidation reactor was precise and accurate over a broad concentration range for dichlobenil (-26.4 ‰ ± 0.3 ‰) and BAM (-26.2 ‰ ± 0.1 ‰). Surprisingly, nitrogen isotope analysis of the target compounds, including caffeine, atrazine and desethylatrazine showed a reactor, a lifetime and even a substance dependent offset compared to the elemental analyser values when using newly introduced Ni tube/NiO-CuO reactors. Subsequently, a standard bracketing is required for nitrogen isotope analysis using compound-specific isotope standards, allowing a continuous drift correction. To test the applicability of the developed method for dichlobenil and BAM carbon and nitrogen isotope analysis a degradation experiment was carried out (chapter 4). Besides the analytical implementation, the goal was to investigate the carbon and nitrogen isotope fractionation of BAM associated to biodegradation. A biotic degradation experiment was performed using the pure bacterial strains *Aminobacter* sp. MSH1 and ASI1. Both are to date the only known isolated strains capable to degrade BAM. The degradation by the both strains was investigated up to 95 % degradation and resulted in a remarkable carbon and nitrogen isotope fractionation. The carbon isotope enrichment was consistent for MSH1 (ε = -7.8 ‰ ± 0.2 ‰) and ASI1 (ε = -7.5 ‰ ± 0.2 ‰). In contrast, slight differences in nitrogen isotope fractionation were observed for MSH1 (ε = -13.5 ‰ ± 0.2 ‰) and ASI1 (ε = -10.7 ‰ ± 0.3 ‰). This is also illustrated in the logarithmic plots according to the Rayleigh equation and the two dimensional isotope plot (Figure 4-4, chapter 4). Comparison of the AKIE values of nitrogen and carbon to values form the literature (Elsner et al., 2005) suggest that the small differences in nitrogen isotope fractionation are related to the chemical reaction steps during the amide hydrolysis of BAM (Figure 4-5, chapter 4) rather than specific uptake mechanism of MSH1 and ASI1, respectively. However, to assess the environmental fate of BAM it is additionally needed to investigate the isotope fractionation related to dichlobenil degradation, because dichlobenil can still be present in nature and therefore affect the isotope composition of BAM. In an experiment using *Rhodococcus erythropolis* DSM 9685 for dichlobenil degradation, after 61 % only a very small carbon isotope enrichment of 0.7 ‰ was detected, which is hardly significant when considering the analytical uncertainty of the method. Nitrogen isotope fractionation was more prominent and showed a depletion of -2.3 ‰. Figure 6-1 illustrates the environmental behaviour of dichlobenil and BAM and the expected isotopic changes. Dichlobenil highly sorbs to soil (Briggs and Dawson, 1970; Clausen et al., 2004; Jernlås, 1990), which on one hand is thought to have a buffer effect leading to low dichlobenil concentrations in groundwater, on the other hand, a pool of sorbed dichlobenil is expected to reside in soil, from which BAM can be formed through degradation and subsequently leach into groundwater (Figure 6-1, left panel). Two fundamental information were gained from my investigation with respect to the isotope values of BAM in the environment. First, BAM would largely show the same $\delta^{13}C$ value as the dichlobenil from where it was formed (Figure 6-1, upper right panel) due to a non-carbon fractionating degradation reaction of dichlobenil. Second, if BAM is degraded, and at the same time formed from dichlobenil, the degradation-induced enrichment of ^{13}C in BAM may be smaller than expected, since it would be “diluted” from the continuous input of freshly formed BAM (see Figure 6-1, lower right panel). The situation changes as soon as dichlobenil has been completely degraded in a BAM-containing water parcel. From then, isotope shifts of BAM would correspond to the trend of Figure 4-3 (chapter 4) and can be uniquely linked to BAM biodegradation. Consequently, BAM biodegradation can even be quantified in aqueous environments using the enrichment factors determined in this study. Which of the scenarios prevails would have to be assessed on a case by case basis.

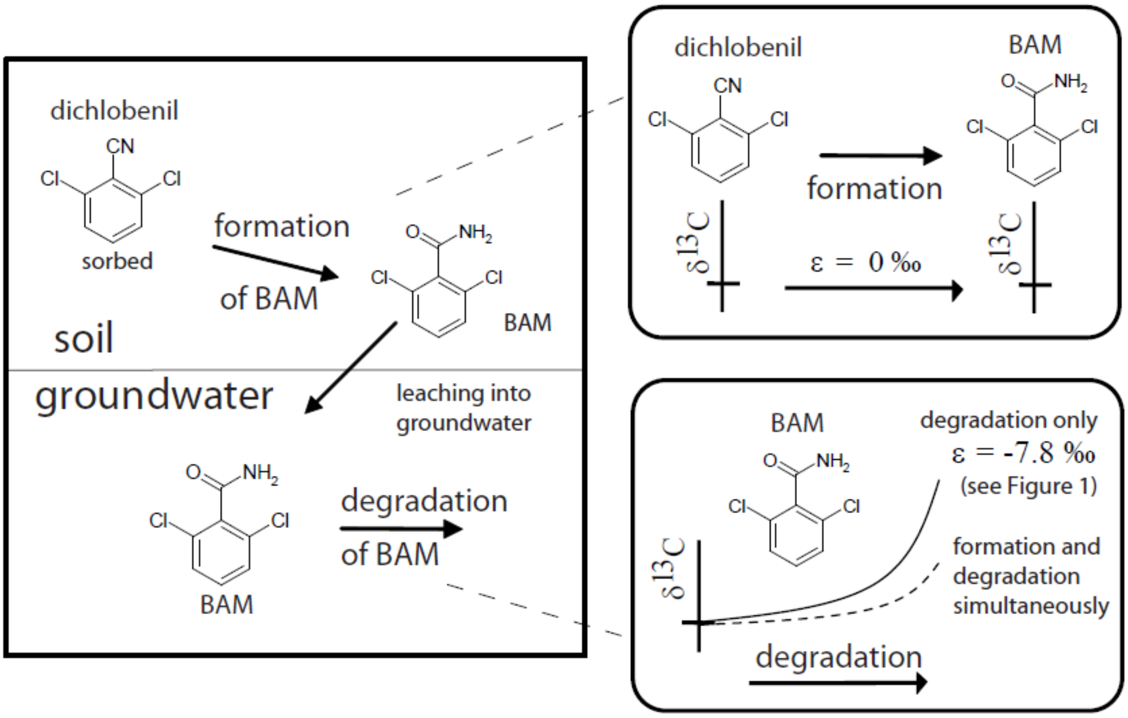

Figure 6-1 (adapted from Scheme 4-1, chapter 4). Environmental behaviour of dichlobenil and BAM and associated isotope fractionation. Dichlobenil is sorbed to soil, where it can be transformed to BAM. BAM leaches into groundwater and may be further degraded (left box). Since degradation of dichlobenil shows negligible isotope fractionation, freshly formed BAM is expected to have the same isotopic signature as dichlobenil (right upper box). In contrast, during BAM degradation isotope fractionation is observed (right lower box). Isotopic enrichment is diluted (dashed line) if fresh BAM is delivered from ongoing dichlobenil degradation. As soon as dichlobenil has been completely converted, isotope fractionation of BAM is expected to show an enrichment trend as observed in this study (solid line).

In summary, this work demonstrates on one hand how important it is to validate compound specific isotope analysis of complex compounds, such as the target compounds bentazone, dichlobenil and its metabolite BAM. Even standardization is required for dichlobenil and BAM to obtain accurate and precise nitrogen isotope analysis. On the other hand, once robust CSIA methods are available, isotope fractionation can be linked to degradation processes and even help to elucidate reaction mechanism as shown for BAM.

Next work should be directed to the application of the developed methods to field samples. So far and to my knowledge no pesticide isotope values from environmental samples are published. Concentrations in a ppb range are challenging with respect to isotope analysis.

A strategy to deal with the low concentration is for example solid phase extraction. Thereby, specific sorbent materials are used to separate the target compounds from the water matrix. To elute the substances from the sorbent material appropriate organic solvents are used. For a proper interpretation of field data it is necessary to exclude isotope fractionation during the extraction process or, if isotope fractionation takes place, to prove its systematic behaviour. However, for reliable assessment of environmental samples it is furthermore indispensable to push future research towards investigations, whether non-degradative processes cause isotope fractionation as well, as this would interfere with interpretations based on enrichment factors determined in biodegradation experiments. The focus in this context should therefore be directed to sorption processes of the contaminants to soil constituents. To my knowledge, no data are available with respect to isotope fractionation associated to sorption of the target pesticides. The target compounds of the study have the potential to sorb to specific soil constituents. Bentazone and MCPA, for example, might sorb to protonated iron oxides (e.g. goethide), due to ionic interactions through negatively charged bentazone (deprotonated at nitrogen) and MCPA (deprotonated at oxygen) and positively charged iron oxides. Another possibility for bentazone adsorption is by interaction of the aromatic ring of bentazone with a positively charged surface (Ania and Beguin, 2007) and in such a case carbon isotope effects might occur. The pesticide dichlobenil as well as BAM have a sorption affinity to topsoils and clayey till sediments, whereby dichlobenil sorption is much higher (Clausen et al., 2004). Kopinke (2005) and Caimi and Brenna (1997) showed that during sorption of organic substances a non-negligible carbon and hydrogen isotope fractionation occur, whereby Meckenstock et *al.*(1999) could not observe isotope effects when column experiments with aromatic hydrocarbons were performed. Nevertheless, sorption has to be considered as a potential fractionation process and depending on the magnitude this has to be implied in interpretations of isotope data from field samples. Therefore, it is highly recommended to perform batch experiments, using potential sorbents to investigate isotope fractionation of the target compounds associated to sorption. Only then a reliable assessment of the fate of pesticides in the environment using CSIA is possible.

Nevertheless, this work contributes robust GC-IRMS methods, which allow the determination of the carbon and nitrogen isotope composition of the target compounds. This opens the field for further investigations concerning degradation (abiotic and biotic) experiments using bentazone and MCPA, to investigate isotope fractionation associated to non-degradative environmental processes and to finally apply CSIA to field samples to assess the fate of the frequently detected pesticides and metabolites in the environment.

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Clarification

Dr. Martin Elsner: Supervision, proof-reading

Prof. Dr. Rainer Meckenstock: Proof-reading of parts of the thesis, discussion

Dr. Sibylle Steinbeiss: Proof-reading of parts of the thesis

Harald Lowag: Performed analysis by EA-IRMS (Elemental Analyzer – Isotope Ratio Mass Spectrometry) to obtain reference isotope values for caffeine, bentazone, MCPA, 2,6-dichlorobenzonitrile, 2,6-dichlorobenzamide, atrazine and desethylatrazine

The PhD candidate was the leading author of the complete work. The thesis was written by the PhD candidate. Comments of co-authors were included in the revised thesis version by the PhD candidate. The detailed contributions in each publication were the following:

Chapter 1: "General Introduction", Chapter 5: "Conclusion and outlook" and Chapter: "Recapitulate Discussion"

These chapters were written by the PhD candidate. Comments from Dr. Martin Elsner were included in the revised version.

Chapter 2: "Small and reproducible isotope effects during methylation with trimethylsulfonium hydroxide (TMSH): A convenient derivatization method for isotope analysis of negatively charged molecules."

This chapter was written by the PhD candidate. The concept of this work was developed by the PhD candidate together with Dr. Martin Elsner. The sample preparation, GC-IRMS measurements of methylated bentazone and LC-IRMS measurements of bentazone were entirely done by the PhD candidate. The GC-IRMS measurements of MCPA derivates were assisted by Dr. Anat Bernstein. The evaluation, statistical data analyses, their graphical illustration and the interpretation were done by the PhD candidate. Dr. Martin Elsner, Dr. Anat Bernstein and the PhD candidate discussed the results together. Comments of Dr. Martin Elsner and Dr. Anat Bernstein were included into the revised version by the PhD candidate. The work was published in the ACS Journal *Analytical Chemistry*:

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Chapter 3: “Gas chromatography-isotope ratio mass spectrometry (GC-IRMS) of recalcitrant target compounds: performance of different combustion reactors and strategies for standardization.”

The PhD candidate was the leading author of this chapter. The manuscript was written by the PhD candidate independently and comments of Dr. Martin Elsner, Dr. Andreas Hilkert, Dr. Dieter Juchelka were included by thy PhD candidate afterwards. The ideas and concept of the work was developed by the PhD candidate together with Dr. Martin Elsner, Dr. Dieter Juchelka and Dr. Andreas Hilkert. The GC-IRMS measurements of 2,6-dichlorobenzonitrile and 2,6-dichlorobenzamide were performed by the PhD candidate. The GC-IRMS measurements of atrazine, desethylatrazine and caffeine were performed by the Ph.D. candidate together with Dr. Armin Meyer and Dr. Sibylle Steinbeiss. Reference GC-IRMS measurements of 2,6-dichlorobenzonitrile, 2,6-dichlorobenzamide, atrazine and desethylatrazine were repeated in the IRMS application laboratory of Thermo Fisher Scientific (Bremen, Germany) and were performed by Dr. Dieter Juchelka and Giovanni Calderone. The evaluation, the graphical illustration and the interpretation were accomplished by the PhD candidate. The PhD candidate, Dr. Martin Elsner, Dr. Andreas Hilkert and Dr. Dieter Juchelka discussed the results together. The manuscript is in preparation to be submitted to the Journal *Rapid Communication in Mass Spectrometry.*

Chapter 4: “Carbon and nitrogen isotope fractionation associated with biotic degradation of the pesticides metabolite 2,6-dichlorobenzamide (BAM) by *Aminobacter* spp. ASI1 and MSH1.”

The manuscript of the chapter was written by the PhD candidate. Comments of Dr. Martin Elsner, Dr. Allan Simonsen, Dr. Sebastian R. Sørensen as well as Dr. Jens Aamand were included in the revised version by the PhD candidate. The ideas and the concept of the work were developed by the PhD candidate together with Dr. Sebastian R. Sørensen and Dr. Martin Elsner. The experimental design was conceived by the PhD candidate in together with Dr. Allan Simonsen, Dr. Sebastian R. Sørensen, Dr. Jens Aamand and complemented by Dr. Martin Elsner. Cultivation of bacteria, sample preparation was done by the PhD candidate assisted by Laurentius Sauer (internship). The GC-IRMS measurements of 2,6-dichlorobenzonitrile and 2,6-dichlorobenzamide were done by the PhD candidate. The evaluation and the graphical illustration were done by the PhD candidate independently. The PhD candidate and Dr. Martin Elsner interpreted and discussed the results together. The manuscript is in review at the ACS Journal *Environmental Science and Technology* (corresponding author Dr. Martin Elsner)

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Conference contributions

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