Isotope fractionation of micropollutants during large-volume extraction: heads-up from a critical method evaluation for atrazine, desethylatrazine and 2,6-dichlorobenzamide at low ng/L concentrations in groundwater

Aileen Melsbach[a,b], Denis Pittois[c], Michael Bayerle[c], Martina Daubmeier[a], Armin H. Meyer[a,] Kathrin Hölzer[a], Tom Gallé[c], Martin Elsner[a,b]

[a]Helmholtz Zentrum München, Institute of Groundwater Ecology, 85764 Neuherberg, Germany

[b]Technical University of Munich, Chair of Analytical Chemistry and Water Chemistry, 81377 Munich, Germany

[c]Luxembourg Institute of Science and Technology - Environmental Research & Innovation Department, 5, avenue des Hauts-Fourneaux, L-4362 Esch-Sur-Alzette, Luxembourg

Published in "Isotopes in Environmental and Health Studies" 2020, DOI: 10.1080/10256016.2020.1812599

Abstract

Micropollutants are frequently detected in groundwater. Thus, the question arises whether they are eliminated by natural attenuation so that pesticide degradation would be observed with increasing residence time in groundwater. Conventional analytical approaches rely on parent compound/metabolite ratios. These are difficult to interpret if metabolites are sorbed or further transformed. Compound-specific stable isotope analysis (CSIA) presents an alternative for identifying degradation based on the analysis of natural isotope abundances in pesticides and their changes during degradation. However, CSIA by gas chromatography-isotope ratio mass spectrometry is challenged by the low concentrations (ng/L) of micropollutants in groundwater. Consequently, large amounts of water need to be sampled requiring enrichment and clean-up steps from interfering matrix effects that must not introduce artefacts in measured isotope values. The aim of this study was to evaluate the accuracy of isotope ratio measurements of the frequently detected micropollutants atrazine, desethylatrazine and 2,6-dichlorobenzamide after enrichment from large water volumes (up to 100 L) by solid-phase extraction with consecutive clean-up by HPLC. Associated artefacts of isotope discrimination were found to depend on numerous factors including organic matter content and extraction volume. This emphasizes the necessity to perform a careful method evaluation of sample preparation and sample pre-treatment prior reliable CSIA.

Introduction

Pesticides from agricultural applications and their metabolites are frequently detected in surface water and groundwater. Since groundwater is the main source of drinking water in many countries of the European Union, the presence of these compounds is of fundamental social concern [1, 2]. Especially, contaminants such as atrazine (ATZ), its metabolite desethylatrazine (DEA) and 2,6-dichlorobenzamid (BAM) are detected in groundwater in concentrations of tens to hundreds of nanograms per litre [3, 4, 5, 6] even though their application has been legally banned decades ago. They are of major concern as these compounds can have residence times of several decades in groundwater. ATZ is a herbicide used for broadleaf and grassy weed control in the production of corn, sugar cane and crops. Its application was forbidden by the European Union in 2004 [7]. ATZ is known to be either dealkylated to DEA and desisopropylatrazine (DIA) [8], or hydrolyzed to hydroxyatrazine (HAT) [9]. Its degradation depends on the hydraulic and chemical characteristics of the environmental compartment as well as on the pH-value and sorption to organic matter [10]. In the environment, the extent of degradation in the topsoil is much greater than in aquifers [11, 12, 13], as microbial activity is highest in the topsoil. However, since the transit time of pesticides in aquifers can be orders of magnitude greater than in unsaturated soil, the question arises whether on-going attenuation of ATZ is significant, and whether such slow transformation can be observed in groundwater over the time scale of decades. Recent studies based on concentration analysis and modelling show that a comprehensive investigation of such long time scales in the environment is challenging [14, 15, 16].

In a similar case as for ATZ, the application of dichlobenil (DCB, 2,6-dichlorobenzonitrile) was forbidden in 2008, since its main metabolite BAM appeared in groundwater in concentrations of up to 5400 ng/L [17, 18, 19, 20, 21]. DCB had been used mainly in non-agricultural areas such as court yards, private gardens, industrial sites and railway lines to control grasses, broad leaved weeds and to eliminate tree roots [5]. Due to the sorption of DCB to soil, its concentration in groundwater is negligible. Its main metabolite BAM, in contrast, shows a much lower tendency to sorption and a much higher mobility so that it can leach through the vadose zone into groundwater [22]. In the environment, further degradation of BAM does not seem to be prevalent [5] and long-term studies on degradation of BAM in the field are missing.

Conventional approaches to assess degradation of pesticides rely on the measurement of parent

compound concentrations or molar ratios of metabolite-to-parent compounds [16, 23, 24]. Such ratios, however, can be biased if target analytes are subject to sorption and they may show inconsistent trends if parent compounds are repeatedly introduced by fresh mobilization from soil. Further, such assessments are not possible if the transformation product of the pesticide is not known and they give inaccurate estimates if the transformation product is further degraded [25]. To circumvent this problem, compound-specific stable isotope analysis (CSIA) is an alternative analytical method to detect the occurrence of pesticide degradation [25]. Analysis of naturally occurring stable isotopes (e.g., ^{13}C, ^{12}C) and changes in their relative abundance ($^{13}C/^{12}C$) in pesticide target compounds can help to determine the extent of pesticide degradation [25]. Such changes in isotope ratios are caused by kinetic isotope effects occurring during biodegradation of organic compounds. Reactive bonds containing the heavy isotope (e.g., ^{13}C) react more slowly than bonds containing its lighter counterpart (e.g, ^{12}C), leading to an enrichment of heavy isotopes relative to light isotopes in the remaining compound fraction. In contrast to conventional analytical methods, however, it is not the analysis of total concentrations, which determines detection limits, but instead the ability of CSIA by GC-IRMS to analyse also the abundance of rarer isotopes with high precision. For example, as the ^{13}C isotope is rare (about 1.11 % of total carbon) compared to the more abundant ^{12}C isotope, as much as 1 nmol C on column are needed to determine carbon isotopic compositions precisely by GC-IRMS. Micropollutants, however, are often detected in groundwater in the nanograms-per-litre concentration range [26]. As a consequence, extraction from large amounts of water is necessary. Recent method development enabled CSIA of ATZ and BAM within the sub-µg per litre range. Schreglmann et al. [27] presented a non-isotope discriminating enrichment method for accurate CSIA of ATZ and DEA extracted from up to 10 L of water. Besides this method for large-volume water extraction of ATZ and DEA, Torrentó et al. described a method including the extraction of BAM from up to 30 L [28]. However, to assess the long-term environmental fate of a herbicide, methods are needed enabling its detection in groundwater bodies in even lower concentration ranges (low ng per litre range) [6, 27, 29]. Thus, to obtain the amount of target analyte required for accurate CSIA, a large-volume extraction of up to 300 L and therefore a scale up of methods published so far would be necessary. Large-scale SPE, however, does not only enrich the target analyte, but also interferences from the sample matrix and other pollutants. Consequently, an additional sample clean-up, for example using preparative high pressure liquid chromatography (HPLC), is necessary to eliminate interferences.

Therefore, method validation is required to ensure that no method-related isotope fractionation is introduced. The aim of this study was to assess whether the extraction of target compounds from large water sample volumes may compromise accurate CSIA. Thereto, large volumes of spiked and natural groundwater were used to test for the recovery of ATZ, DEA and BAM and to measure the carbon isotopic signature of each target analyte.

Materials and Methods

Chemicals. Atrazine (2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, CAS no.: 1912-24-9), desethylatrazine (2-Amino-4-chloro-6-isopropylamino-1,3,5-triazine, CAS no.: 6190-65-4) and 2,6-dichlorobenzamide (98.5 %, CAS no.: 2008-58-4) were purchased from Dr. Ehrensdorfer GmbH (Augsburg, Germany). Ametryn (2-Ethylamino-4-isopropylamino-6-methylthio-1,3,5-triazine, 98.5 %, CAS no.: 834-12-8) was produced by Fluka, supplied by Sigma Aldrich (Steinheim, Germany). Formic acid (≥98 %, CAS no.: 64-18-6), methanol (≥99.9 %, CAS no.: 67-56-1), acetonitrile (≥99.9 %, CAS no.: 75-05-8) and ethyl acetate (≥99.9 %, CAS no.: 141-78-6) were supplied by Carl Roth (Karlsruhe, Germany). Potassium dihydrogen phosphate was purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany). Ultrapure water was received from a Millipore DirectQ apparatus (Millipore, Bedford, MA, USA).

Environmental Samples. For method evaluation ATZ-, DEA- and BAM-free water samples from the groundwater spring Haertgen 1 (national code: SCC-125-03) in the Luxembourg Sandstone Formation were collected within three different sampling campaigns (12th October 2015, 15th October 2015 and 21st October 2015). The water was collected in 15 L and 2 L Schott bottles (Schott AG, Mainz, Germany). A volume of 185 L per campaign was sampled and pre-treated immediately.

Characterization of the Luxembourg Sandstone Formation. The Luxembourg Sandstone Formation has been described in detail by Farlin et al. [23, 30]. It is a fractured aquifer surrounded by rural areas in Luxembourg. The land is mainly used for agriculture and forested land. For this aquifer, groundwater mean transit times of one to two decades are estimated [30, 31]. This range is comparable to the reported half-lives of ATZ. Consequently, ATZ concentrations of up to 45 ng/L have been measured without showing signs of a decrease, even though the compound was banned 7 years prior to the sampling campaigns of Farlin et al. [30]. Within the same sampling

campaign DEA, the only detectable metabolite of ATZ, was quantified in concentrations between 5 and 110 ng/L.

General Analysis of Environmental Samples – Total Organic Carbon. Total organic carbon (TOC) was measured with a Torch Combustion Analyzer TOC/TN from TELEDYNE TEKMAR. One millilitre of sample was acidified using 1 mL of phosphoric acid (21 %). The inorganic carbon was then removed by purging with synthetic air. The organic carbon in the sample was subsequently converted to carbon dioxide (CO_2) by catalytic combustion using a platinum-based catalyser. Afterwards, the formed CO_2 was transferred into an infrared detector.

Sample Pre-Treatment and Spiking with Target Analytes. Prior to spiking the groundwater with the target analyte, the groundwater was acidified, as pre-tests with spiked tap water had shown an increase of analyte recovery after acidification. To this end, 30 mL of formic acid (≥ 98 %) were added to 2 L of groundwater. Afterwards, the acidified water was transferred into 2 L custom-made Schott-bottles (Schott AG, Mainz, Germany) with a cylindrical rounded bottom. The non-contaminated groundwater was then spiked with different concentrations of ATZ, DEA and BAM (Table S1).

Analyte Enrichment with Solid-Phase Extraction. For solid-phase extraction (SPE), 6 mL CHROMABOND EASY cartridges (Macherey-Nagel GmbH & Co. KG, Düren, Germany) with 200 mg modified polystyrene-divinylbenzene copolymer sorbent were used after pre-tests with ATZ and tap-water (data not shown) resulted in high recoveries (105 % ±34%, 3 replicates). This protocol was established for the purpose of the Luxembourg sampling campaign at a time before alternative SPE methods [27] [28] had been brought forward. As the sorbent mass was designed to work up to a maximum sample volume of 4 L, the total sample was split into aliquots of 4 L, the aliquots were extracted on separate cartridges and the extracts were reunited after the extraction method. Each cartridge was pre-conditioned with 5 mL methanol at a flow rate of 1 mL/min and an equilibration time of 0.1 min using a Gilson GX-274 ASPEC (Gilson Incorporated, Middleton, WI). Subsequently, the samples were loaded by pumping the spiked water from the 2 L Schott-bottles onto the cartridges using a vacuum manifold. After the sample had passed the cartridge, they were kept on the vacuum manifold until the sorbent was dried completely. Then, in contrast to conventional SPE, a subsequent washing step was skipped and the cartridges were transferred directly into the Gilson GX-274 ASPEC instrument, where the elution of the samples was carried

out automatically into 10 mL tubes with 2 mL methanol at a flow rate of 0.5 mL/min and an equilibration time of 0.5 min. After elution with methanol, the samples were transferred into 7 mL clear vials with solid caps (PTFE liner) (Supelco, Bellefonte, PA). Finally, the corresponding volumes of each eluate were combined and evaporated until dryness.

Sample Clean-up with preparative HPLC. Prior to GC analysis, samples were subjected to a clean-up step by preparative HPLC. Thereto, the method described by Schreglmann et al. [27] was modified in order to accommodate analysis of an additional compound (BAM) and to increase the organic phase proportion in response to the high loads of DOM that were brought about by the large extraction volumes of this study. To this end, the sample extracts were dissolved in 800 µL ultrapure water/acetonitrile (70/30). To avoid a blockage of the HPLC column, samples were filtered prior to injection using a 4 mm single use filter device with a 0.2 µm PTFE Membrane (Whatman, Buckinghamshire, United Kingdom). The samples were injected onto a Synergi 4 µm Hydro-RP 80 Å (100 mm x 4.6 mm; Phenomenex, Aschaffenburg, Deutschland) operated in a Shimadzu UHPLC-DAD (Nexera XR, LC-20AD XR). Separation of the analytes was achieved using a gradient of 1 mM phosphate buffer at a pH of 7 and acetonitrile (ACN) at a flow rate of 1.0 mL/min. The method started at 30 % ACN, which was held for 5 min. The proportion of ACN was then increased linearly to 65 % within 7 min. Subsequently, the gradient was increased to 80 % within 0.5 min and held for 1.5 min. At the end of the run, the proportion of ACN was reduced to 30 % again (held for 3 min). The detector was operated at an absorbance of 220 nm. BAM, DEA and ATZ eluted at retention times of 3.5 min, 4.8 min, and 10.5 min, respectively. Thus, the fraction for BAM and DEA was collected from 2.1 to 6.7 min. For ATZ, the fraction from 9.5 to 13.0 min was taken for further analysis. Finally, the samples were freeze-dried and reconstituted in 50 µL ethyl acetate.

Quantification with GC-qMS. The recovery of the target analytes from groundwater samples was determined with gas chromatography – quadrupole mass spectrometry (GC-qMS). Thereto, a 7890A GC was hyphenated with a 5975C qMS (Agilent, Santa Clara, CA, US). For peak separation, a DB-5 column (60 m × 0.25 mm × 1 µm) (J&W Scientific, Santa Clara, CA) was used. The helium carrier gas (grade 5.0) was set to a flow of 1.4 mL/min. Using a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland), three microliters of each sample were injected in splitless mode at a temperature of 250 °C. The GC program started at a temperature of

70 °C, which was held for 1 min. Afterwards, the temperature was ramped with 18 °C/min to 155 °C. Then, the temperature was increased with 2 °C/min to 240 °C and finally with 30 °C/min to 260 °C (held for 5 min) before the column was baked for 11 min at a temperature of 280 °C. The target analytes were introduced into the MS via a heated transfer line (250 °C). Subsequently, the ions were created with electron impact ionization at an electron-accelerating voltage of 70 V. The MS was run in scan mode (from mass-to-charge ratio (m/z) 40 to 550). ChemStation E.02.02.1431 was used as software to control the instrument and to evaluate the data. To determine the recovery, the target analytes were quantified with ametryn (20 mg/L) dissolved in ethyl acetate as an internal standard using its m/z 227 as a quantifier ion. Data evaluation for DEA and BAM was carried out using m/z 172 and 173, respectively, as quantifier ions. ATZ was quantified using the mass-to-charge ratio 200. All analytes were identified by their mass spectrum and by comparison of their retention times with those of an authentic standard.

EA-IRMS Measurement for Determination of Reference Values. The carbon isotope composition of the reference standards of ATZ, DEA and BAM were determined by elemental analyser-isotope ratio mass spectrometry (EA-IRMS) [32]. Thereto, an EuroEA (Euro Vector, Milano, Italy) was coupled to a Finnigan MAT 253 IRMS via a FinniganTM ConFlow III interface (Thermo Fisher Scientific, Bremen, Germany). The reference standards were calibrated against the organic referencing materials L-glutamic acid (USG 40 and USG 41) and caffeine (IAEA 600) supplied by the International Atomic Agency (Vienna, Austria). The carbon isotope ratios are reported as isotope values ($\delta^{13}C$) in per mil relative to the international reference PeeDee Belemnite (V-PDB) according to equation 1:

$$\delta^{13}C = \frac{^{13}C/^{12}C_{Sample} - ^{13}C/^{12}C_{Reference}}{^{13}C/^{12}C_{Reference}} \quad (1)$$

The isotope values were measured relative to our laboratory CO_2 monitoring gas. The gas had been previously calibrated against RM8563 (CO_2) purchased from the International Atomic Energy Agency (IAEA). The reference gas was introduced into the system three times at the beginning and at the end of each analysis run.

GC-IRMS Conditions for Carbon Isotope Analysis. Carbon isotope analysis was conducted with a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific, Milan, Italy) hyphenated via a Finnigan Combustion III interface (Thermo Fisher Scientific) with a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS) (Thermo Fisher Scentific, Bremen, Germany). For the analysis, the IRMS parameters were kept at a vacuum of 2.1×10^{-6} mbar, an accelerating potential of 9 kV and an emission energy of 2 mA. Target analytes were transformed to CO_2 at a temperature of 1030 °C using a NiO tube/CuO-NiO reactor (Thermo Fisher Scientific). The GC was equipped with a 60 m Rxi-5ms column (Restek GmbH, Bad Homburg, Germany) with an inner diameter of 0.25 mm and a film thickness of 1 µm. At the beginning of the GC temperature program, the oven was set to a temperature of 50 °C, which was held for 1 min. Afterwards, the oven temperature was increased linearly with 18 °C/min to 155 °C, followed by 5 °C/min to 240 °C (held for 0 min). Subsequently, the temperature was increased with 30 °C/min to 260 °C, held for 5 min. Finally, a ramp of 15 °C/min heated the column to a temperature of 280 °C, which was held for 9 min. A volume of between 1 µL and 3 µL of sample extract was injected on-column using a PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Helium (grade 5.0) carrier gas flow and injector temperature program were controlled by an Optic 3 device (ATAS, GL Science, Eindhoven, Netherlands). The ATAS injector was equipped with a custom-made glass on-column liner. It had an initial temperature of 50 °C (held for 300 s). Subsequently, the injector temperature was increased with 4°C/s to 250 °C. Meanwhile, the split flow, which started at 14 mL/min, was set to 0 ml/min for 120 s and then back to its initial flow of 14 mL/min. At the beginning of each run, the flow rate was set to 0.3 mL/min, held for 2 min and then increased linearly to 1.4 mL/min within 120 s. To control and verify the instrument performance, reference standards of ATZ, DEA and BAM were used to monitor retention time and shifts in $\delta^{13}C$ values during the sequence.

Correction Procedure applied for Isotope Analysis. For each sample we aimed to measure the isotope value three times. All reported isotope ratios are expressed as arithmetic means of samples collected in different sampling campaigns with the respective estimates of their standard deviations ($\pm \sigma$). The calibration was performed by bracketing the samples with authentic reference standards to apply the principle of identical treatment by Werner and Brand [33]. With this method, drifts that occurred within a sequence as well as differences in the combustion efficiency were corrected directly.

Results and Discussion

Recovery of ATZ, DEA and BAM from groundwater. The extraction of different water volumes resulted in unsatisfactory and non-reproducible recoveries for all three target analytes (Figure 1). Considering the differences between the mass spiked and the mass recovered, the applied method seems to be non-quantitative when extracting the large volumes of groundwater targeted in this study.

Non-quantative SPE may potentially be caused by a breakthrough of the target analyte. This can occur due to high mass loadings or due to high sample volumes, where the target analyte already sorbed to the cartridge material is washed off. For the method presented in this study, the latter reason is unlikely, as a maximum volume of 4 L groundwater was loaded onto the SPE column. Alternatively, minor losses might occur during transformation steps. Another reason for low recoveries may be the interaction of analytes with components present in the water matrix. Indeed, pre-tests with tap water, where matrix components are minimized, gave much greater ATZ recoveries of 105 ± 17 % (V = 4 L; n = 3) applying the same extraction method at a pH of 2. Similar discrepancies in analyte recoveries between tap and environmental water were observed in our recent study for other polar micropollutants such as desphenylchloridazon (DPC), where recoveries of DPC in tap water were measured at approximately 100 %, while desphenylchloridazon (DPC) from environmental water samples showed a recovery of 30 % [28]. Comparably low recoveries have been measured for ATZ in soil [34]. Commonly detected matrix components, which are present in soil and groundwater but not in tap water, are humic substances (HS) like fluvic and humic acids. Even though previously published studies of ATZ extraction methods from groundwater showed higher recoveries of up to 100 % [35, 36], there are only few studies validating the extraction of atrazine from large volumes of water rich in humic substances, and at trace concentrations of micropollutants [37]. On the other hand, the interaction of ATZ with HS, in particular with humic acids and fulvic acids has been studied before [38, 39, 40]. It has been observed that, depending on the pH-value of the water and the amount of HS present, ATZ-HS complexes may be formed. For a pH smaller than 5, complexes between ATZ and the HS are formed by either a proton-transfer bonding or a hydrogen bonding between N-H in the secondary amino groups or the triazine ring of ATZ with the oxygen containing functional groups within the

humic acids [41] (Scheme S1). The water in the Luxembourg Sandstone Formation is reported to have a pH of approximately 7 [23, 42, 43]. Consequently, due to the acidification of the water prior SPE, the pH of the water was decreased to approximately 4, which is expected to facilitate the ionic interaction between ATZ and HS. A TOC content of the extracted groundwater of 1.64 ± 0.02 mg/L was measured for the water sampled on for the third sampling campaign (21st October). While data from the precise other sampling dates are not available, they can be estimated from long-term measurements of the total organic carbon of the spring from where the groundwater for spiked samples was taken (Figure S1). Here, values range between 0.6 mg/L and 2 mg/L. Hence, differences in the total organic carbon (TOC) content of the water due to changes in flow components by surficial water intrusion during rain periods are a likely hypothesis to explain the low recoveries and the inter-day differences in the recovery observed in our study.

Isotope fractionation after large volume extraction. Figure 2 shows the differences in isotope values of extracts from spiked groundwater samples compared to the reference value of the standard with which they were spiked.

For ATZ, no isotopic discrimination was observed for extraction volumes smaller than 10 L (Figure 2a). This corresponds to the results of an alternative method presented by Torrentó et al. [28], where the isotope fractionation of ATZ was measured for extraction volumes of up to 10 L using a different sorbent material and sorbent mass (combination of 8 g Sepra ZT and 8 g SDB-1. Contrary to expectations, however, the presented SPE method introduced a significant isotope fractionation of up to 6 ‰ for extraction volumes greater than 10 L. As described by Bakkour et al. [44] solid phase extraction of a target analyte from large samples volumes implies that concomitantly also matrix constituents become enriched including TOC of groundwater and even oligomers of the SPE material (Figure 3). In the SPE extraction protocol described here, the high background may be a particular result of the missing washing step within the SPE. This step is not mandatory but results in cleaner samples, as it is usually applied to remove interfering undesirable compounds without eluting the target analyte [45]. It is accomplished by choosing a washing solvent similar or slightly stronger than the solvent used within the sample load step. Thus, matrix compounds, which are not as strongly retained by the sorbent as the target analyte, will be washed out, while the compound of interest remains within the sorbent [45]. Therefore, by omitting this

step within the procedure, after sample loading not only the target analytes were eluted but also components of the groundwater matrix. DEA showed no isotope fractionation for extraction volumes lower than 10 L. As shown in Figure 2b, isotope fractionation was also not observed for BAM. However, neither DEA, nor BAM could be measured for extraction volumes greater than 10 L, as background interferences were too high compared to the low signals for peak detection (Figure S2).

If in the case of a high background, the $\delta^{13}C$ value of GC-IRMS measurements does not represent the isotopic ratio of the target analyte, this may have several reasons. *(1) Difficulty of background correction.* After peak separation target compounds are combusted to CO_2 prior to IRMS analysis. Thus, peak separation and background correction are critical since otherwise it would not be possible to distinguish whether CO_2 origins from the target analyte or from interfering matrix components. If the TOC is so high that an adequate background correction is no longer feasible, peaks would represent a mean of the $\delta^{13}C$ of the background and the target analyte. As the background has an isotopic signature different from the target analyte, determination of analyte-specific isotope ratios and, hence, identification of shifts in $\delta^{13}C$ values of ATZ that would demonstrate natural degradation in the field would be biased. *(2) Compromised oxidation efficiency.* Such an elevated background may in addition compromise the oxidation efficiency of the oxidation reactor which is positioned after the GC column to convert target compounds to CO_2 prior to isotope analysis. This combustion of target analytes to CO_2 is achieved by oxygen stored in the form of CuO and NiO in the reactor. When feeding a too high carbon load onto the reactor, the combustion capacity of the reactor may be exhausted. As a result, target analytes are only partially combusted and measured $\delta^{13}C$ values are more negative ('lighter') than the true isotope value. In the case of the data presented in Figure 2, this reason, however, can be excluded because the isotope values $\delta^{13}C$ of both ATZ and DEA become more positive ('heavier') with an increasing extraction volume. High variations in the standard deviations (SD) of different sampling dates suggest that the observed deviations from true values are not reproducible so that the bias introduced by the method cannot be corrected. *(3) Compromised chromatographic performance.* Additionally, the high content of dissolved organic carbon posed chromatographic challenges, as the matrix and the target analyte were sorbed to the pre-column of the on-column injection, as identified during regular shortening. Such desorption creates active spots, which were found to result in reduced amplitudes of the target analyte in samples, and even in standards measured after

a sample. These spots were enhanced by the injection technique even though cold-on-column injection was initially used to increase the sensitivity. By decreasing the injector temperature, carrier gas flow and the split flow during sample injection, this procedure also increases matrix effects. The matrix residues sorbed to the pre-column could not be removed by heating of the GC oven and caused either partial or complete blockages of the system, which needed to be removed by shortening the corresponding parts of the pre-column. Thus, the high matrix background did not only affect the sample itself, but there was carry-over of the matrix affecting also isotope analysis of atrazine standards introduced after samples of large volume water extraction. An additional effect observed were isotope effects during chromatography leading to double peaks (Figure 4). This influence on the isotope value of the target analyte needed to be eliminated by either automatic or by manual integration of both peaks.

Suggestions for Improvement. The results of our evaluation illustrate the case of an extraction method which needs to be further optimized in order to achieve sensitive, accurate and robust isotope analysis. It appears advisable to omit the acidification as a sample preparation step for SPE and rather add a buffer to in order to reduce possible interactions of the target analytes with humic substances. We hypothesize that the method tested may be significantly improved for robust and accurate carbon isotope analysis if measures are taken to eliminate interferences from DOC of groundwater and, potentially, from soluble SPE cartridge material. Further sample clean-up may be achieved by different approaches, most prominently by including a washing step within the SPE, after the sample has been loaded and prior to its elution.

Environmental Significance and Outlook

Hence, the SPE method of this study represents a case which necessitates further optimization. The results are presented in order to emphasize the necessity of a careful method validation of large volume extraction. They show that the hyphenation of many analytical procedures may not only have an influence on the recovery, but also on the integrity and reproducibility of isotope values and thus on the interpretation of field data. Consequently, prior to the extraction and measurement of environmental samples, methods need to be carefully validated, such as in

Torrentó et al. [28]. The following case of an environmental study illustrates that otherwise inaccurate conclusions may be drawn.

Possible Bias in the Interpretation of Environmental Data. Figure 5a shows isotope values of ATZ from different natural atrazine-containing groundwater samples that were analysed in parallel to the method evaluation presented in this manuscript. This comparison includes samples deriving from an aquifer of the Luxemburg Sandstone Formation, which have all been prepared for CSIA according to the protocol described in this chapter. In contrast, for the other samples (Franconia and Austria), sample pre-treatment and extraction was carried out according to the methods of Schreglmann et al. [27].

Groundwater samples analysed from Franconian and Austrian aquifers according to Schreglmann et al. [27] showed isotope values of atrazine close to commercially available products, which usually fall within a range of $\delta^{13}C$ values between -26 ‰ to -33 ‰ [32, 46, 47, 48]. In contrast, there is a correlation between $\delta^{13}C$ and the ATZ concentration detected in the Luxembourg Sandstone Formation (Figure 5b and c).

Carbon isotope values of samples collected in the Luxembourg Sandstone Formation (Figure 5b), show a significant enrichment in ^{13}C of up to -16 ‰ within the low ng/L concentration range. Comparing the measured $\delta^{13}C$ values from Luxembourg to the carbon isotope values of ATZ measured in other field samples and to the isotope signatures of commercially available products, the detected ^{13}C enrichment would be interpreted as an indication of extensive degradation. According to laboratory degradation experiments of Meyer et al.[25] and Lihl et al.[49], where degradation of ATZ by *Arthrobacter aurencens* TC1 and *Rhodococcus* sp. NI 86/21 was quantified, a $\delta^{13}C$ (ATZ) of -16 ‰ would indicate that 80 to 90 % of the ATZ has already been degraded presuming an ATZ source signature of -26 ‰.

When relating the measured carbon isotope value of ATZ to the extracted water volume, however (Figure 5c) a correlation of ^{13}C enrichment with the extraction volume becomes apparent, in a similar manner as observed in Figure 2. This trend is not observed for the samples collected in Austria and Franconia. Hence, based on our observations in the present study, isotopic shifts induced by extensive sample extraction would be a likely explanation of these results.

Consequently, the application of the SPE protocol described may lead to potentially inaccurate conclusions about degradation in natural samples if it is not accompanied by a careful method evaluation as presented here. This emphasizes the general need of method validation.

Opportunities for Further Analytical Developments. To further decrease limits of sensitive CSIA, different opportunities exist. First, fractionative HPLC clean-up can be repeated using a different gradient method in order to separate target analytes from additional remaining interferences. Second, Molecularly Imprinted Solid Phase Extraction (MISPE), a selective enrichment method to remove matrix in organic solvents by specific intramolecular interactions, can be applied after fractionative HPLC. Here, materials have been developed for organic micropollutants such as triazines [50, 51] but not yet for BAM. Both approaches, however - fractionative HPLC and MISPE - are highly labour intensive. Hence, the application of comprehensive GC×GC-IRMS represents a further attractive option to enable carbon isotope analysis of analytes in complex matrices. Here, resolution and separation capacity are increased by adding a second dimension to the system and thus separating the background from the target analyte [52]. Hence, dedicated analytical efforts are necessary, and bear potential, to further push the limits for analysis of organic trace compounds in groundwater.

ASSOCIATED CONTENT

Supporting Information

Further details on volumes and concentrations of groundwater used for this study, hypothesized chemical structure of ATZ-Humic substance-complex and TOC analysis.

AUTHOR INFORMATION

Corresponding Author

*Phone: + 49 89 2180 78232; fax: + 49 89 2180 78255; e-mail: m.elsner@tum.de

Author's Note

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by the Luxembourg National Research Fund under the project number C09/SR/02. The authors would like to thank Dr. Julien Farlin (Luxembourg Institute of Science and Technology) for his help and support during the sampling campaigns as well as his comments during data evaluation that improved the quality of the manuscript.

References

1. Fenner K, Canonica S, Wackett LP, et al. Evaluating Pesticide Degradation in the Environment: Blind Spots and Emerging Opportunities. Science. 2013;341:752-8.
2. Schwarzenbach RP, Escher BI, Fenner K, et al. The Challenge of Micropollutants in Aquatic Systems. Science. 2006;313:1072-7.
3. Bohn T, Cocco E, Gourdol L, et al. Determination of atrazine and degradation products in Luxembourgish drinking water: origin and fate of potential endocrine-disrupting pesticides. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment. 2011;28:1041-54.
4. Baran N, Mouvet C, Negrel P. Hydrodynamic and geochemical constraints on pesticide concentrations in the groundwater of an agricultural catchment (Brevilles, France). Environ Pollut. 2007;148:729-38.
5. Björklund E, Anskjær GG, Hansen M, et al. Analysis and environmental concentrations of the herbicide dichlobenil and its main metabolite 2,6-dichlorobenzamide (BAM): A review. Sci Total Environ. 2011;409:2343-56.
6. Tappe W, Groeneweg J, Jantsch B. Diffuse atrazine pollution in German aquifers. Biodegradation. 2002;13:3-10.
7. COMMISSION DECISION of 10 March 2004concerning the non-inclusion of atrazine in Annex I to Council Directive 91/414/EEC and thewithdrawal of authorisations for plant protection products containing this active substance, (2004).
8. Barbash JE, Resek EA. Pesticides in ground water: distribution, trends, and governing factors. Chelsea, MI: Ann Arbor Press; 1996.
9. Meyer AH, Penning H, Elsner M. C and N isotope fractionation suggests similar mechanisms of microbial atrazine transformation despite involvement of different Enzymes (AtzA and TrzN). Environ Sci Technol. 2009;43:8079-85.
10. Tomlin CSD. The Pesticide Manual: A World Compendium of Pesticides. 13th ed. Hampshire, UK: British Crop Protection Council; 2003.
11. Johnson AC, Hughes CD, Williams RJ, et al. Potential for aerobic isoproturon biodegradation and sorption in the unsaturated and saturated zones of a chalk aquifer. Journal of Contaminant Hydrology. 1998;30:281-97.

12. Tuxen N, Tuchsen PL, Rugge K, et al. Fate of seven pesticides in an aerobic aquifer studied in column experiments. Chemosphere. 2000;41:1485-94.
13. van der Pas LJT, Leistra M, Boesten J. Rate of transformation of atrazine and bentazone in water-saturated sandy subsoils. J Pestic Sci. 1998;53:223-32.
14. Farlin J, Drouet L, Gallé T, et al. Delineating spring recharge areas in a fractured sandstone aquifer (Luxembourg) based on pesticide mass balance. Hydrogeol J. 2013;21:799-812.
15. Farlin J, Gallé T, Bayerle M, et al. Using the long-term memory effect of pesticide and metabolite soil residues to estimate field degradation half-life and test leaching predictions. Geoderma. 2013;207–208:15-24.
16. Adams CD, Thurmann EM. Formation and transport of deethylatrazine in the soil and vadose zone. J Environ Qual. 1991;20:540-7.
17. Auersperger P, Lah K, Kus J, et al. High precision procedure for determination of selected herbicides and their degradation products in drinking water by solid-phase extraction and gas chromatography-mass spectrometry. J Chromatogr A. 2005;1088:234-41.
18. Törnquist M, Kreuger J, Adielsson S. Occuring of pesticides in Swedish water resources against a background of national risk-reduction programms - results from 20 years of monitoring. XIII Symposium Pesticide Chemistry - Environmental Fate and Human Health; 2007 September 3-6, 2007.
19. Porazzi E, Pardo Martinez M, Fanelli R, et al. GC-MS analysis of dichlobenil and its metabolites in groundwater. Talanta. 2005;68:146-54.
20. Schipper PNM, Vissers MJM, van der Linden AMA. Pesticides in groundwater and drinking water wells: overview of the situation in the Netherlands. Water Sci Technol. 2008;57:1277-86.
21. COMMISSION DECISION of 18 September 2008concerning the non-inclusion of dichlobenil in Annex I to Council Directive 91/414/EEC and thewithdrawal of authorisations for plant protection products containing that substance, (2008).
22. Reinnicke S, Simonsen A, Sørensen SR, et al. C and N Isotope Fractionation during Biodegradation of the Pesticide Metabolite 2,6-Dichlorobenzamide (BAM): Potential for Environmental Assessments. Environ Sci Technol. 2012;46:1447-54.
23. Farlin J, Bayerle M, Pittois D, et al. Estimating Pesticide Attenuation From Water Dating and the Ratio of Metabolite to Parent Compound. Groundwater. 2017;55:550-7.
24. Spalding RF, Snow DD, Cassada DA, et al. Study of pesticide occurence in two closely spaced lakes in northeastern Nebraska. J Environ Qual. 1994;23:571-8.
25. Meyer AH, Elsner M. 13C/12C and 15N/14N Isotope Analysis To Characterize Degradation of Atrazine: Evidence from Parent and Daughter Compound Values. Environ Sci Technol. 2013;47:6884-91.
26. Elsner M, Jochmann MA, Hofstetter TB, et al. Current challenges in compound-specific stable isotope analysis of environmental organic contaminants. Anal Bioanal Chem. 2012;403:2471-91.
27. Schreglmann K, Hoeche M, Steinbeiss S, et al. Carbon and nitrogen isotope analysis of atrazine and desethylatrazine at sub-microgram per liter concentrations in groundwater. Anal Bioanal Chem. 2013;405:2857-67.
28. Torrentó C, Bakkour R, Gaétan G, et al. Solid-phase extraction method for stable isotope analysis of pesticides from large volume environmental water samples. Analyst. 2019;144:2898-908

29. Dörfler U, Feicht EA, Scheunert I. S-triazine residues in groundwater. Chemosphere. 1997;35:99-106.
30. Farlin J, Gallé T, Bayerle M, et al. Predicting Pesticide Attenuation in a Fractured Aquifer Using Lumped-Parameter Models. Groundwater. 2013;51:276-85.
31. Farlin J, Maloszewski P. On the use of spring baseflow recession for a more accurate parameterization of aquifer transit time distribution functions. Hydrology and Earth System Sciences. 2013;17.
32. Meyer AH, Penning H, Lowag H, et al. Precise and accurate compound specific carbon and nitrogen isotope analysis of atrazine: critical role of combustion oven conditions. Environ Sci Technol. 2008;42:7757-63.
33. Werner RA, Brand WA. Referencing strategies and techniques in stable isotope ratio analysis. Rapid Commun Mass Spectrom. 2001;15:501-19.
34. Olness A, Basta NT, Rinke J. Redox effects on resin extraction of herbicides from soil. Talanta. 2002;57:383-91.
35. Carabias-Martinez R, Rodriguez-Gonzalo E, Herrero-Hernandez E, et al. Determination of herbicides and metabolites by solid-phase extraction and liquid chromatography Evaluation of pollution due to herbicides in surface and groundwaters. J Chromatogr A. 2002;950:157-66.
36. Turiel E, Fernández P, Pérez-Conde C, et al. Trace-level determination of triazines and several degradation products in environmental waters by disk solid-phase extraction and micellar electrokinetic chromatography. J Chromatogr A. 2000;872:299-307.
37. Hogendoorn E, van Zoonen P. Recent and future developments of liquid chromatography in pesticide trace analysis. J Chromatogr A. 2000;892:435-53.
38. Prosen H, Zupančič-Kralj L. The interaction of triazine herbicides with humic acids. Chromatographia Supplement. 2000;51:155-64.
39. Wang Z, Gamble DS, Langford CH. Interaction of atrazine with Laurentian humic acid. Anal Chim Acta. 1991;244:135-43.
40. Wang X, Guo X, Yang Y, et al. Sorption Mechanisms of Phenanthrene, Lindane, and Atrazine with Various Humic Acid Fractions from a Single Soil Sample. Environ Sci Technol. 2011;45:2124-30.
41. Sposito G, Martin-Neto L, Yang A. Atrazine complexation by soil humic acids. J Environ Qual. 1996;25:1203-9.
42. Tosheva Z, Kies A, Hofmann H. Radon in potable waters in Luxembourg: Nukleonika; 2010.
43. Umsetzung der europäischen Wasserrahmenrichtlinie (2000/60/EG) - Bericht zur Bestandsauf-nahme für Luxemburg. In: Infrastructures MdDded, editor. Esch-sur-Alzette: Le Gouvernement Du Grand-Duché de Luxembourg; 2014. p. 193.
44. Bakkour R, Bolotin J, Sellergren B, et al. Molecularly Imprinted Polymers for Compound-Specific Isotope Analysis of Polar Organic Micropollutants in Aquatic Environments. Anal Chem. 2018;90:7292-301.
45. Subramanian G. Quality assurance in environmental monitoring: Instrumental methods: VCH; 1995.
46. Annable WK, Frape SK, Shouakar-Stash O, Set al. 37Cl, 15N, 13C isotopic analysis of common agro-chemicals for identifying non-point source agricultural contaminants. Appl Geochem. 2007;22:1530-6.

47. Hartenbach AE, Hofstetter TB, Tentscher PR, et al. Carbon, hydrogen, and nitrogen isotope fractionation during light-Induced transformations of atrazine. Environ Sci Technol. 2008;42:7751-6.
48. Grundwasserökologie HZM-If. Carbon and nitrogen isotope analysis of commercially available atrazine in-house standards over the last two decades. in-house databaseunpublished.
49. Lihl C, Heckel B, Grzybkowska A, et al. Compound-specific chlorine isotope fractionation in biodegradation of atrazine. Environ Sci: Processes Impacts. 2020;22:792-801.
50. Kueseng P, Noir ML, Mattiasson B, et al. Molecularly imprinted polymer for analysis of trace atrazine herbicide in water. J Environ Sci Health, Part B. 2009;44:772-80.
51. Turiel E, Martín-Esteban A, Fernández P, et al. Molecular Recognition in a Propazine-imprinted Polymer and Its Application to the Determination of Triazines in Environmental Samples. Anal Chem. 2001;73:5133-41.
52. Tobias HJ, Sacks GL, Zhang Y, et al. Comprehensive Two-Dimensional Gas Chromatography Combustion Isotope Ratio Mass Spectrometry. Anal Chem. 2008;80:8613-21.

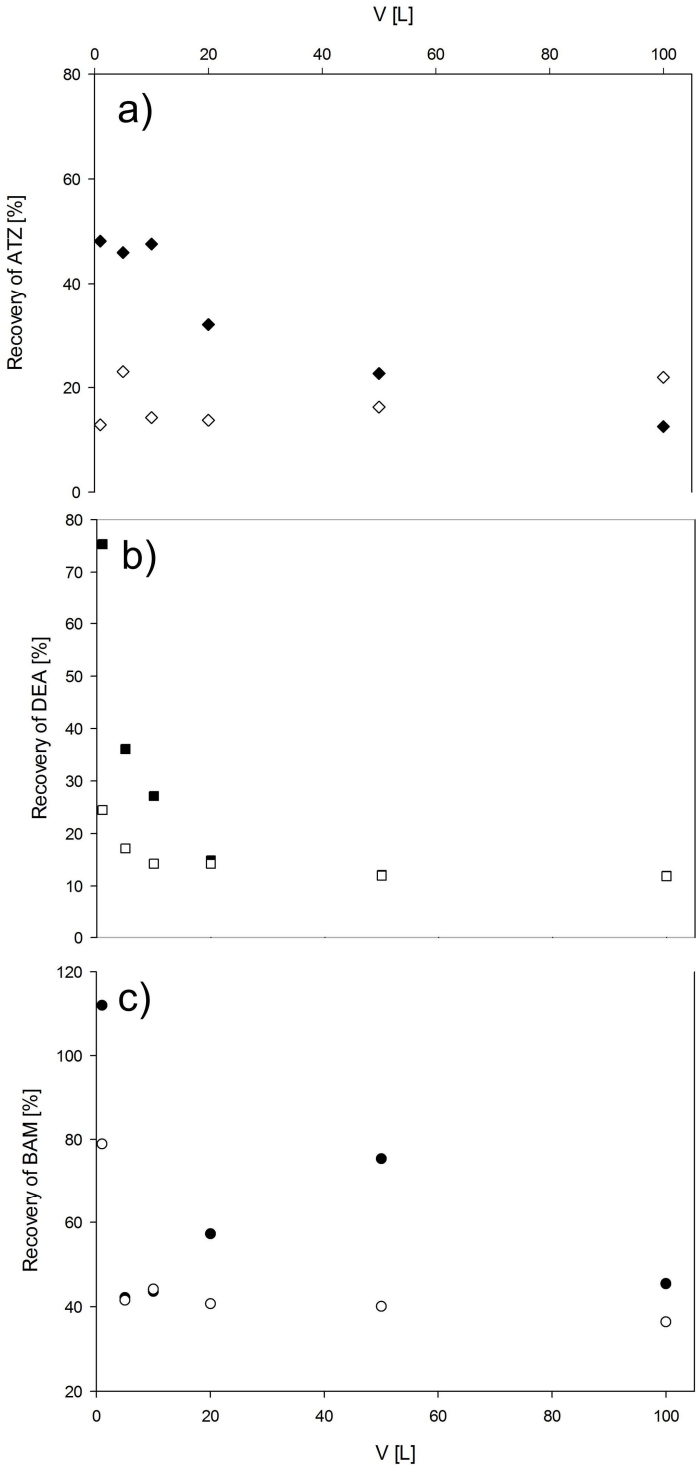


Figure 1: Recovery in percent of a) ATZ (diamonds) b) DEA (squares) and c) BAM (circles) in relation to water volumes extracted; Extracts from spiked samples for which the original groundwater was collected on 15th October 2015 are represented as black symbols, while those for which the groundwater was collected on 21st October 2015 are represented as white shapes.

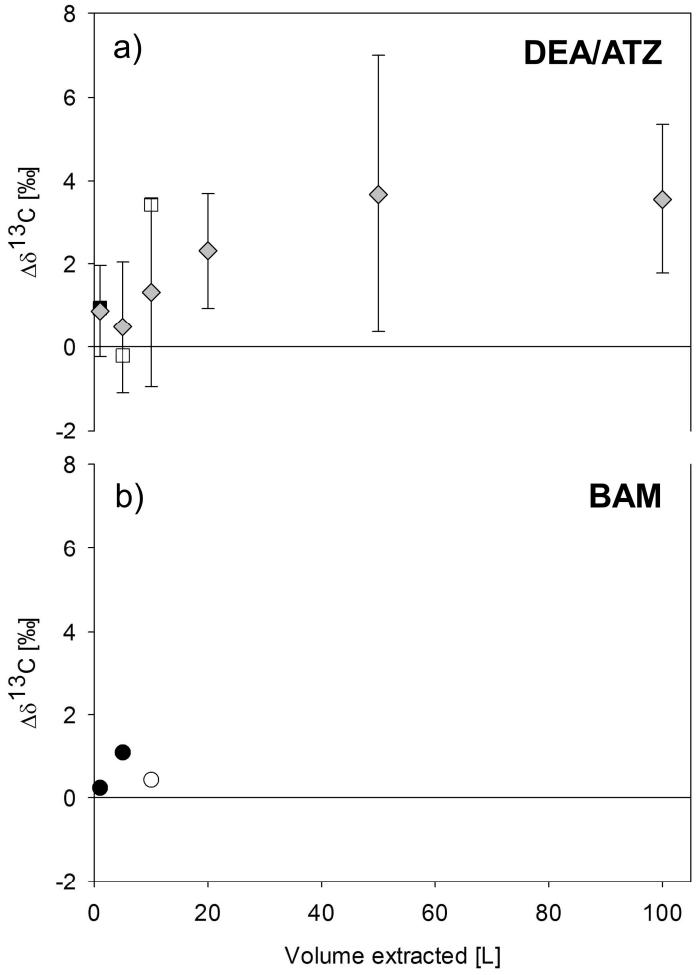


Figure 2: Deviation of carbon isotope values of standard measurements induced by large volume water extraction of a) ATZ (grey diamonds), DEA (black squares); error bars show the standard deviation of the isotope fractionation between the 3 sampling campaigns. b) BAM (black circles); the y-axis represents the deviation from the true value, where the black line indicates the absence of isotope fractionation; white symbols correspond to samples for which only a single measurement was achieved; note: for BAM and DEA no standard deviation was calculated as isotope fractionation of only two sampling campaigns (15th October 2015 and 21st October 2015) was measured and standard deviation of each measured replicate was < 0.6 ‰.

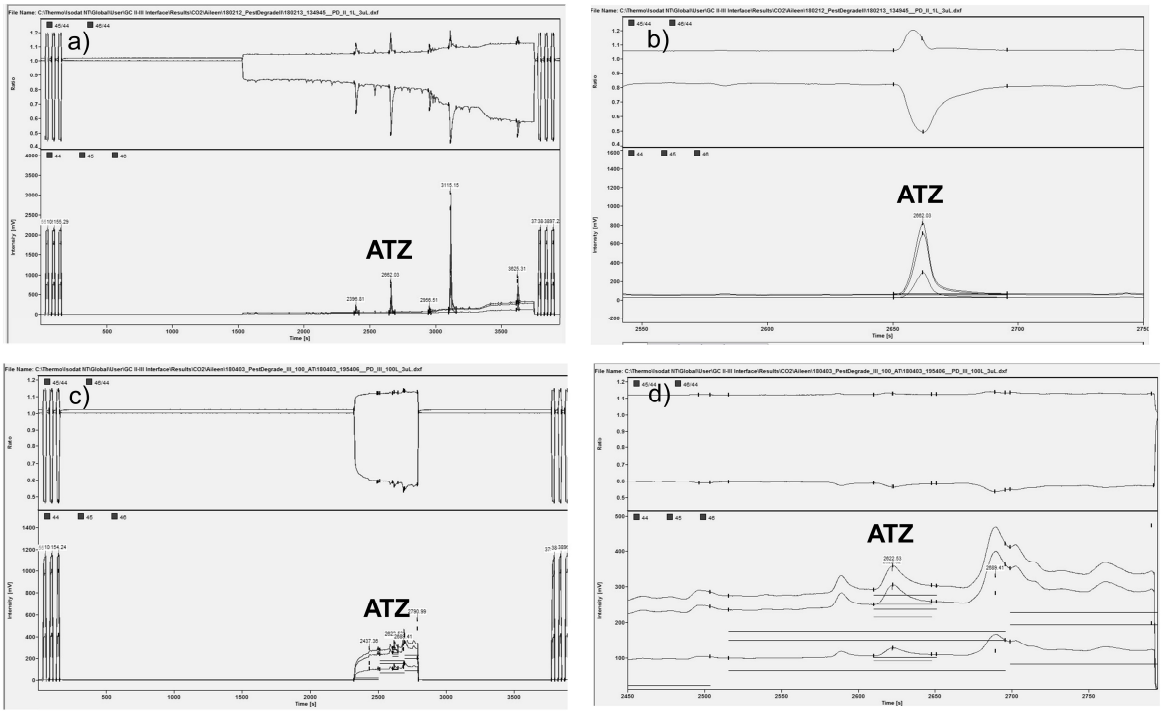


Figure 3: Chromatograms of ATZ after HPLC-cleanup and on-column injection of a) and b) 1 L of water extraction and c) and d) 100 L of water extraction

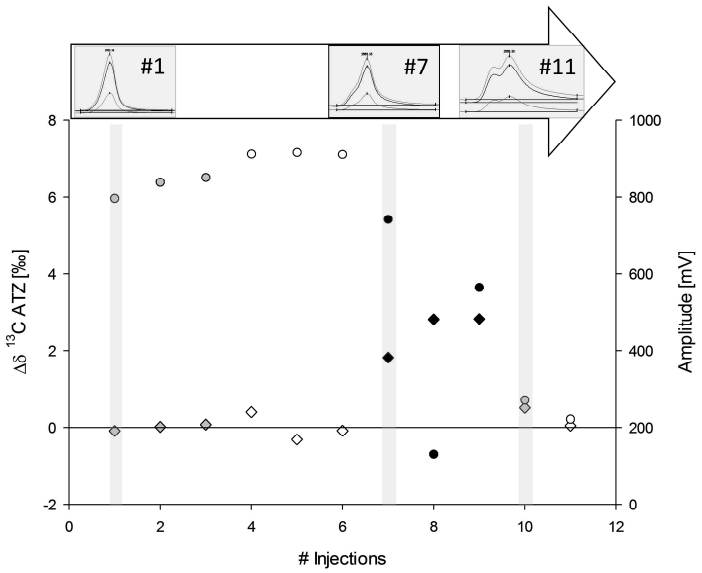
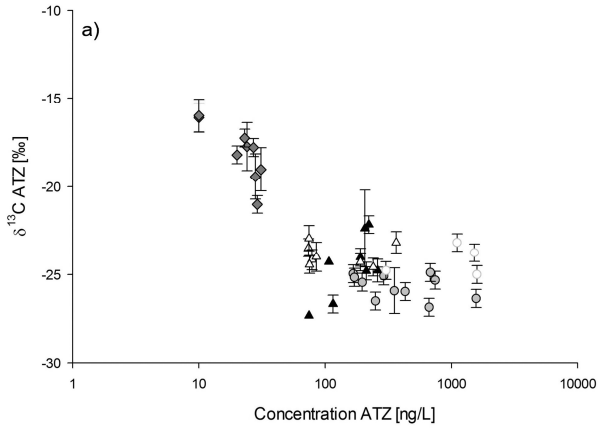
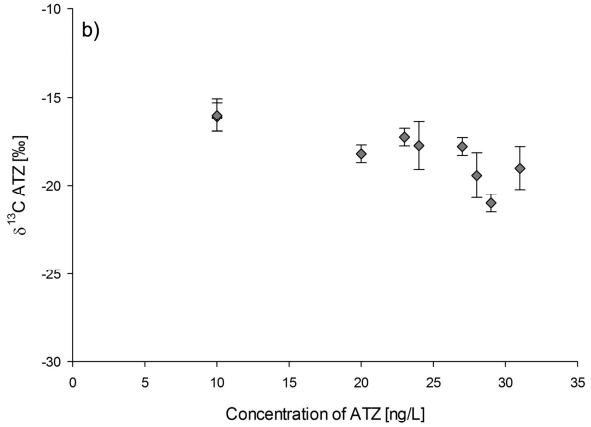
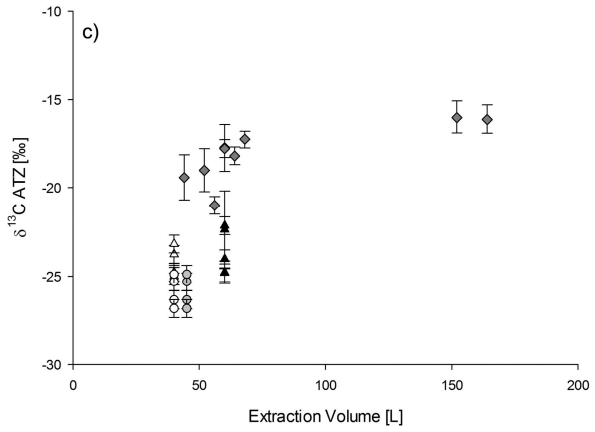


Figure 4: Deviation of atrazine carbon isotope values of standard measurements and spiked groundwater samples used for method validation observed within a sequence; circles represent the amplitude and diamonds the deviation from target carbon isotope values of standard 1 (grey), standard 2 (white) and spiked groundwater samples (10 L sample extraction, black).


a)
-10
-15
-20
-25
-30
δ 13C ATZ [‰]
1
10
100
1000
10000
Concentration ATZ [ng/L]


b)
-10
-15
-20
-25
-30
δ 13C ATZ [‰]
0
5
10
15
20
25
30
35
Concentration of ATZ [ng/L]


c)
-10
-15
-20
-25
-30
δ 13C ATZ [‰]
0
50
100
150
200
Extraction Volume [L]

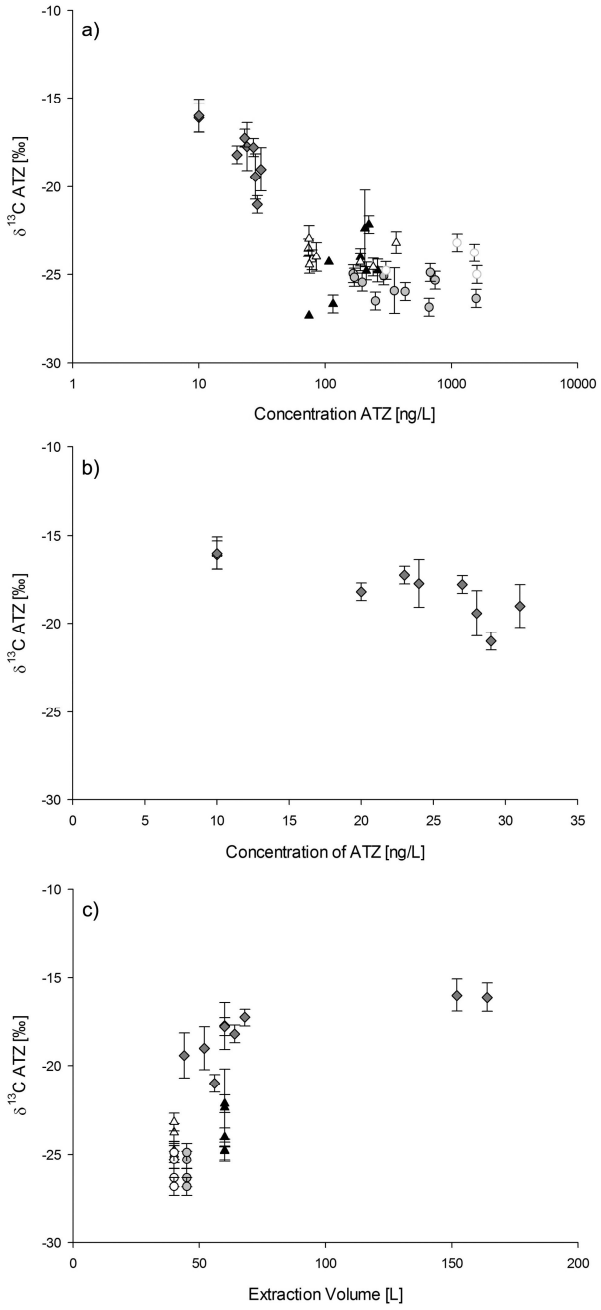


Figure 5: a) and b) Carbon isotope values of ATZ versus analyte concentration in natural groundwater samples.; c) carbon isotope values of ATZ in relation to the volume of water extracted for CSIA; dark grey diamonds represent samples collected in the aquifer of the Luxembourg Sandstone Formation and extracted according to the method evaluated here. In contrast, circles (ATZ samples from two different collection sites in Austria) and triangles (ATZ samples from two different collection sites in Franconia) were extracted according to the method of Schreglmann et al. [27].