BIOACCESSIBILITY OF ENVIRONMENTALLY AGED ^{14}C-ATRAZINE RESIDUES IN AN AGRICULTURALLY USED SOIL AND ITS PARTICLE-SIZE AGGREGATES

NICOLAI D. JABLONOWSKI*, JANETTE MODLER, ANDREAS SCHAEFFER‡, PETER BURAUEL

Forschungszentrum Jülich GmbH, Institute of Chemistry and Dynamics of the Geosphere, ICG-4 – Agrosphere, 52425 Jülich, Germany

‡ University of Technology Aachen - RWTH Aachen Inst. for Environmental Research (Biology V) Environmental Biology and Chemodynamics, 52074 Aachen, Germany

*Corresponding author e-mail: n.d.jablonowski@fz-juelich.de; phone: +49-2461-61-2696; fax: +49-2461-61-2518

Submitted for publication in *Environmental Science & Technology*

Abstract

After 22 years of aging under natural conditions in an outdoor lysimeter the bioaccessibility of ^{14}C-labeled atrazine soil residues to bacteria was tested. Entire soil samples as well as sand-sized, silt-sized and clay-sized aggregates (>20, 20-2, and <2 μm aggregate size, respectively) were investigated under slurried conditions. The mineralization of residual radioactivity in the outdoor lysimeter soil reached up to 4.5 % of the total ^{14}C-activity after 16 days, inoculated with *Pseudomonas* sp. strain ADP. The control samples without inoculated bacteria showed a mineralization maximum of only about 1 % after 44 days of incubation. Mineralization increased in the clay-sized aggregates up to 6.2 % of the total residual ^{14}C-activity within 23 days. With decreasing soil aggregate sizes, residual

^{14}C-activity increased per unit of weight, but only minor differences of the mineralization in the soil and soil size aggregates using mineral-media for incubation was observed. Using additional Na-citrate in the incubation, the extent of mineralization increased to 6.7 % in soil after 23 days following incubation with *Pseudomonas* sp. strain ADP. These results show that long-term aged ^{14}C-atrazine residues are still partly accessible to the atrazine degrading microorganism *Pseudomonas* sp. strain ADP.

Keywords: atrazine, bioaccessibility, *Pseudomonas* sp. strain ADP, aged residues, physical soil fractionation, bio-mineralization, soil aggregates

---

INTRODUCTION

The s-triazine herbicide atrazine [2-chloro-4- (ethylamino)-6-(isopropylamino)-s-triazin] was introduced in 1957 and has been used since 1958 (*1–3*) worldwide for weed control, predominantly in maize cultivation. While the application of atrazine has been forbidden in Germany since March 1991 because of exceedance of permissible concentrations in ground- and drinking water (*4*, *5*), this herbicide is still widely used in agriculture, especially in countries of huge global impact, such as China and USA (*6*, *7*), and industrial purposes (*8*). The reason for atrazine in ground water is due to direct leaching, surface run-off or intrusion via particle binding. A continuous desorption and resulting mobility of atrazine in soils is also under discussion, with focus on the formation of "bound residues" (*9*). Degradation and metabolism of atrazine in soil occurs through microbial activity and non-biotic processes, such as hydrolysis, photo-degradation and oxidation, leading simultaneously to the formation of bound residues (*10*). The half life of atrazine limited by microbial degradation and fixation by binding and sorption ranges from 4-57 weeks (*11–13*). The atrazine metabolites and their transformation rates via biotic and

abiotic processes are slower too, depending on the type of metabolite being chlorinated monoalkylatrazines or hydroxylated (*14*). Numerous investigations have stated that the distribution of atrazine residues in soil is mainly dependent on the soil organic carbon (e.g. humin, humic and fulvic acids) (*15*) and the clay content (*16*), with the majority of the atrazine residues are located in soil particle fractions <20μm (*9*).

Triazines and their environmental behavior have been the subject of numerous investigations (*17–21*). So far, studies on bound residues with labeled triazines have been conducted on short-term (weeks) (*17*) to at most mid-term (months) (*21*) time scales under laboratory conditions. However no studies have been conducted with radioactively labeled triazine residues aged on a long-term time scale (> 20 years) under field conditions. The present study closes this knowledge gap, facilitating a complete assessment of health and environmental risks, and provides data on long-term aged ^{14}C-labeled atrazine residues under environmental conditions. As atrazine has for years been one of the most widely used s-triazine herbicides and as a number of metabolites are also found in the degradation pathways of other triazine herbicides, it can also be seen as a model substance for this class of pesticides.

In this study the application of ^{14}C-labeled atrazine under outdoor conditions is dated back 22 years. "Atrazine residues" in this paper implies the parent compound and all possible metabolites. Due to uniformly triazine ring ^{14}C-labeled applied atrazine, residual atrazine equivalents can be calculated on basis of the specific radioactivity.

The objectives of this study were (i) to localize and quantify the distribution of ^{14}C-labeled atrazine residues in soil; (ii) to analyze the mineralization of the aged ^{14}C-labeled atrazine residues; (iii) to evaluate the bioaccessibility of the naturally aged ^{14}C-labeled atrazine residues in the soil by the specialized bacteria for atrazine degradation *Pseudomonas* sp. strain ADP; (iv) to study the differences in bioaccessibility of ^{14}C-labeled atrazine residues by *Pseudomonas* sp. strain ADP in different soil size aggregates, and (v) to evaluate accelerated bioaccessibility following the addition of carbon sources.

MATERIALS AND METHODS

Soil and Lysimeter Characteristics. The lysimeter soil originated from Puch, Fürstenfeldbruck in Bavaria, Germany. For the lysimeter studies, agriculturally used soil (gleyic cambisol) was taken in 1979. The filling of the lysimeter was in accordance to the natural bedding of the field soil layers. The filled plastic lysimeter with a square surface of dimensions 49x49, and a depth of 73 cm, had a surface area of approximately 0.24 m^2. The soil was utilized for continuous corn production. All lysimeter-soil treatments were performed under field-like conditions in accordance to agricultural practice until August 2005 at the Bavarian Management Agency for Agriculture (LfL), Munich, Germany. Time of application and amount of applied ^{14}C-labeled atrazine and non-labeled atrazine are presented in Table 1. ^{14}C-Labeled atrazine was uniformly ^{14}C ring-labeled. The total atrazine applied within the 3 years are equivalent to 5 kg atrazine per hectare. The new specific activity is calculated from the total amount of applied ^{14}C-radioactivity (56.195 MBq) over the total mass of 133.294 mg of radioactive and non-radioactive atrazine applied and amounts to 421.587 kBq mg^{-1} which is used for all further calculations in this study.

TABLE 1. Time of application and applied quantities of non-radioactive and radioactive atrazine as active ingredient as well as the applied ^{14}C-radioactivity.

Time of application	Applied atrazine [mg]	Applied ^{14}C-atrazine [mg]	Specific ^{14}C-activity [kBq mg^{-1}]	Applied ^{14}C-activity [MBq]
1983	38.931	4.382	4271.1	18.716
1984	41.283	4.327	4272.0	18.485
1985	39.924	4.447	4271.2	18.994
Total (133.294 mg)	120.138	13.156		56.195
New specific ^{14}C-activity			421.587 kBq mg^{-1} atrazine	

Soil sampling and treatment. The lysimeter soil containing ^{14}C-labeled atrazine residues was sampled 22 years after the last atrazine application and subdivided in the following layers: 0-10, 10-20, 20-30, 30-40, 40-50, 50-55 cm.

The soil layers were air dried to a water content between 3.5 - 12.9 % (depending on soil layer), pre-sieved (5 mm) and stored in dark glass bottles at 2 °C in the dark until further analysis. For detection of residual ^{14}C-activity the soil samples were freeze dried (Lyovac GT2, Steris), combusted using a Biological Oxidizer OX500 (R.J.Harvey Instrument Corporation) and analyzed by liquid scintillation counter (LSC, 2500 TR, Tri-Carb, Packard Liquid Scintillation Analyzer). For the experiments, the soil was homogenized and sieved ($\leq$2mm). The highest residual ^{14}C-activity was found in the upper soil layer 0-10 cm. This soil, corresponding to 0.3 μg of formerly applied ^{14}C-atrazine g^{-1} dry soil was used for the experiments in this study.

Soil aggregate fractionation. The soil aggregate fractionation was performed in accordance with a previous study by Burauel and Bassman (*22*). For the fractionation, 100 g of dry weight of soil equivalents were placed in 1000 mL Duran glass bottles and 200 g of organic free deionized water (Milli-Q Plus 185, Millipore purification system) was added. The soil-water mixture was shaken for 6 h at 150 rpm (Horizontal Shaker SM 25, Edmund Büler). Thereafter, 600 g of deionized water was added to give a total amount of 800 g. Aggregate size fractions were measured using the sedimentation rate and Stokes's Law. After the first sedimentation of 6 min, the sand-sized aggregates (particle sizes of 20 - 2000 μm) were obtained and the resulting liquid was separated. In the second step, the silt-sized aggregates (particle sizes of 2 - 20 μm) were isolated following a second sedimentation period of 12 h. The resulting supernatant containing the clay-sized aggregates (particle sizes of <2 μm) was centrifuged at 10,000 *g* for 90 min (Beckman J2-21, Rotor JA 14). After centrifugation of the clay-sized aggregates, the resulting supernatant (dissolved organic matter (DOM) fraction, particle size <0.05 μm) was analyzed for desorbed ^{14}C-labeled atrazine residues.

For analysis of the DOM-fractions, 5 mL of the aqueous sample was mixed with 10 mL scintillation cocktail (Instant Scint-Gel Plus^{TM}, Perkin-Elmer) and detection of radioactivity was performed by LSC. The fractionated soil samples were freeze dried until complete dryness and stored for radioactive analysis in the dark. All solid samples containing ^{14}C-labeled atrazine residues were freeze dried and combusted in triplicates via Biological Oxidizer and LSC detected.

Desorption experiments. These experiments were performed to evaluate the influence of the liquid media on the bioaccessibility of the ^{14}C-labeled atrazine residue fraction. To achieve this, 10 g dry soil equivalent (corresponding to a total ^{14}C-activity of 1250 Bq) was treated in accordance to the physical soil fractionation and was mixed with a total amount of 80 g of a) deionized water, b) mineral-media, c) mineral-media containing 2 g L^{-1} Na-citrate and d) mineral-media containing 2 g L^{-1} glucose. To distinguish the desorption potential of mineral-media components, K_2HPO_4, $MgSO_4.7H_2O$, $FeCl_3$ and Na_2EDTA were also tested. The samples were shaken in PE centrifuge tubes for 6 h and centrifuged for 90 min at 10,000 *g* and the ^{14}C-activity in the supernatant was determined via LSC.

Radioactivity in all of the liquid samples of the DOM-fraction were measured by LSC, as described above. A quenching correction generated by external standards was used. The desorption potential of ^{14}C-labeled atrazine residues by using different liquids is presented in Figure 2.

As reported in several previous studies (*17*, *23*, *24*), two extended desorption experiments were conducted to further analyze the desorption potential of the aged ^{14}C-labeled atrazine residues. To achieve this, triplicate samples of 10 g dry weight of soil were mixed with a) 80 g of methanol-water solution (4:1 v/v) and b) with 80 g of $CaCl_2$ solution (0.01 M) in propylene centrifuge tubes, and were shaken for 6 h on a horizontal shaker. In addition, a second set of triplicate samples with methanol-water and $CaCl_2$ solution were also shaken for 24 h, respectively, to measure contact time dependant desorption. The

tubes were centrifuged at 10,000 g for 90 min and residual ^{14}C-activity in the supernatant was LSC detected.

Physico-chemical analysis.

Carbon analysis of the DOM-fraction and desorption liquids. In order to achieve information about an enhanced dissolution of carbon and possibly associated ^{14}C-labeled atrazine residues a range of solvents were used for soil fractionation and desorption studies and were analyzed for total (TC), total organic (TOC) and non-purgeable organic carbon (NPOC) content, using a Shimadzu Total Organic Carbon Analyzer (TOC-5050A, Shimadzu, ASI-5000A Auto Sampler).

Analysis and metabolite detection of the DOM-fraction and desorption liquids. The aqueous DOM-fraction (800 mL) of the physical soil aggregate fractionation was concentrated by freeze drying (Lyovac GT2, Steris) and redissolved in pure methanol (HPLC-grade). Methanol-water solution was used in the desorption experiment for 6 and 24 h, respectively, and concentrated using vacuum evaporation using a Büchi Syncore (50 °C, 150 rpm, variable vacuum between 280 and 30 mbar, Büchi Vakuum Controller V-805). Prior analysis the concentrated samples were transferred to 2 ml Eppendorf® cups and centrifuged for sedimentation of the particles for 5 min at 15,000 g (Hettich Mikro Rapid).

HPLC/Radio-HPLC-analysis. For the detection of desorbed ^{14}C-labeled atrazine residues in liquid media, UV-HPLC (Dionex, pump M480, sampler Gina 50, UV-detector UVD 3405) and Radio-HPLC (Berthold Radio-Flow Detector LB 590, Jasco UVD 2075 detector, solid scintillation cell YG 150 U4, pump 1580, Gina 50 sampler) was used. For HPLC analysis a mixture of acetonitrile (Biosolve) and acidified water (pH 2.7, 1 ml 25 % H_3PO_4 L^{-1} H_2O) was used. The applied HPLC column was a LiChrospher Select-B (Merck, 250 mm × 4 mm × 5 μm) with an additional pre-column (Merck, Select-B, 4 mm × 4 mm). An isocratic flow of 60 % acidified water (pH 2.7) and 40 % acetonitrile was applied in a linear operating range from 0.1 mg L^{-1} to 100 mg L^{-1}. The injection volume of the

concentrated samples was 20 μL at a flow rate of 1 mL min^{-1} and a wavelength of 223 nm. For Radio-HPLC the same eluent and columns were used. The injection volume of the concentrated samples was 250 μL at a flow rate of 1 mL min^{-1}. The applied gradient conditions at time 0-6 min were 70 % acidified water (pH 2.7) and 30 % acetonitrile, at time 10 min 100 % acetonitrile, and 70 % acidified water and 30 % acetonitrile at time 20 min. The limit of detection was 40 Bq ml^{-1}.

LC-MS/MS-analysis. For LC-MS/MS-analysis a TSQ-Quantum 2002 (Thermo Electron) was used. The applied HPLC column (Perfect Sil Target ODS-3, 125 mm $\times$ 2.1 mm $\times$ 3 μm), used with an additional HPLC pre-column (Perfect Sil Target ODS-3, 1 cm $\times$ 2.1 mm $\times$ 3 μm), was purchased from MZ-Analysentechnik Mainz. The applied HPLC eluents were 0.1 M ammonium acetate solution (eluent A) and acetonitrile (Riedel De Haen, 99.9 % purity, eluent B). The applied gradient conditions were 95 % eluent A and 5 % eluent B at time 0-5 min changing to 100 % eluent B at time 20 min. The flow rate was 0.15 mL min^{-1} and the column temperature was 25 °C. The injection volume of the concentrated samples was 5 μL. The applied MS conditions were positive electrospray ionization (ESI) and multiple reaction monitoring mode (MRM). For atrazine and each of its metabolites the transmission was optimized. As the collision gas, argon 5.0 (purchased from Linde, 99.8 % purity) was used.

Elementary analysis of solid samples. For elementary (Al, Fe, K, Si) analysis of soil and soil size aggregates, 100 mg of freeze dried sample was decomposed with a mixture of 0.25 g of lithium-borate for 30 min at 1000°C. The flux was dissolved in 50 mL HCl (3 %; 0.95 M, respectively) and adjusted to a total volume of 100 mL. The analysis was conducted by inductively coupled plasma with optical emission spectroscopy (ICP-OES; TJA-IRIS-Intrepid spectrometer, Thermo). Determination of carbon was performed by radiofrequency heating in flowing oxygen and following infrared absorption by a Leco RC-412 multiphase carbon determinator. For nitrogen determination 2 mg of sample was

combusted and analyzed by a Leco TCH 600 nitrogen/oxygen/hydrogen determinator and N_2 was determined by thermal conductivity detection.

Microbial cultures and conditions. Cells of *Pseudomonas* sp. strain ADP were grown in Erlenmayer flasks containing 100 mL liquid atrazine culture media prepared in accordance to Mandelbaum et al. (*25*) and incubated on a rotary shaker at 90 rpm and 28 °C. The medium consisted of K_2HPO_4 1.6 g L^{-1}, KH_2PO_4 0.4 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2.2H_2O$ 0.026 g L^{-1}, saccharose 1.0 g L^{-1}, $C_6H_5Na_3O_7.2H_2O$ 1.14 g L^{-1}, basic saline solution 20 mL L^{-1} (consisting of: EDTA 2.5 g L^{-1}, $ZnSO_4.7H_2O$ 19.8 g L^{-1}, $FeSO_4.7H_2O$ 9.14 g L^{-1}, $MnSO_4$ H_2O 1.54 g L^{-1}, $CuSO_4.5H_2O$ 0.4 g L^{-1}, $CoSO_4.7H_2O$ 0.24 g L^{-1}, $Na_2B_4O_7.10H_2O$ 0.18 g L^{-1} or $Na_2B_4O_7$ 0.095 g L^{-1}, H_2SO_4 5.0 mL L^{-1}) and basic vitamin solution 20 mL L^{-1} (consisting of: Thiamin-HCL 5 mg L^{-1}, Biotin 2 mg L^{-1}, folic acid 2 mg L^{-1}, nicotinamide 10 mg L^{-1}, pyridoxine-HCL 10 mg L^{-1}) adjusted to a pH of 7.2. In each flask inoculated with *Pseudomonas* sp. strain ADP, an atrazine solution (stock solution 10 mg atrazine mL^{-1} methanol) was added, with a total inoculum concentration of 50 mg L^{-1}. Successful atrazine degradation was detected via HPLC during the growth of the bacterium. At a sufficient cell density of 1-2 $\times$ 10^8 cells mL^{-1} after 1-3 days of incubation, the cells were separated from the media by centrifugation (3 min at 3000 *g*, Heraeus® Megafuge® 1.0), and harvested. Cells were washed twice in mineral-media and resuspended in mineral-media (after Sambanis, (*26*)) without any atrazine or additional carbon sources; the medium consisted of three components as follows: component I consisting of KH_2PO_4 0.335 g L^{-1}, K_2HPO_4 2.19 g L^{-1}, $(NH_4)_2SO_4$ 0.125 g L^{-1}, was dissolved in 997 mL distilled water. Additionally 1 mL of component II consisting of $MgSO_4.7H_2O$ 10.0 g L^{-1}, $Na_2MoO_4.2H_2O$ 1 g L^{-1} and 1 mL of component III consisting of NaCl 10 g L^{-1}, $CaCl.2H_2O$ 26 g L^{-1}, $Na_2EDTA.2H_2O$ 2.8 g L^{-1}, $FeCl_3.6H_2O$ 2.0 g L^{-1}, as well as 1 mL trace elements solution was added. The use of mineral-media was chosen as pH buffer (pH 7).

For the experimental set-up, 10 g dry soil weight or soil aggregates was placed in hermetic 250 mL Schott-Duran bottles and received 10 mL of mineral-media, including *Pseudomonas* sp. strain ADP, at a concentration of 1-2 $\times$ 10^8 cells mL^{-1}. The cell number was determined by a Beckman Multisizer 3 (Coulter Counter, Beckman & Coulter). Due to small quantities of clay-sized aggregates these studies were conducted with 1 g dry weight clay-sized aggregates, inoculated with 1 mL of mineral-media containing equal bacteria concentration, respectively. These incubation conditions favored the activity of the *Pseudomonas* inoculum and the indigenous soil microflora. As a control, soil incubation was prepared, which did not contain the pseudomonad inoculum. The microbial incubations were shaken daily for 120 min at 100 rpm on a rotary shaker to promote aeration of the slurry.

The inoculated soil and particle size aggregates were incubated for 1, 2, 3, 9, 16, 23, 30 and 44 days at room temperature (RT, 22°C $\pm$ 2°C) in the dark. All experiments were performed in triplicates.

The accelerated mineralization of aged ^{14}C-labeled atrazine residues was measured using a method employed by Mandelbaum et al. (*18*) with the addition of other carbon sources. For this purpose 2 g L^{-1} of a) Na-citrate and b) glucose were added to the mineral-media, as described above, and the media was added to soil samples as described previously. The samples were taken at the same time intervals as those without additional carbon sources.

At the end of each incubation period the soil inoculum was sacrificed following the acidification of the medium with 100 μl of 11 M HCl (resulted in a pH of 2-3), 5 min of sonication, shaken for 120 min and subsequently incubated for 24 h at 22°C $\pm$ 2°C in the dark. This was necessary to achieve complete gaseous release of dissolved or bound $^{14}CO_2$ in the soil-slurry approach. Afterwards, the trapped $^{14}CO_2$ was measured by LSC. The efficiency of 100 μl of 11 M HCl for the gaseous release of dissolved or sorbed $^{14}CO_2$ from the soil slurry and the trapping efficiency of 0.5 M NaOH was previously tested with

$NaH^{14}CO_3$. The trapping efficiency of released $^{14}CO_2$ in 1.5 ml of 0.5 M NaOH was found to be 100 % after 11 h.

To determine ^{14}C-labeled atrazine residue mineralization after each incubation period, $^{14}CO_2$ evolution was measured after trapping in NaOH. A glass vial containing 1.5 mL of 0.5 M NaOH was placed in a cap-holder inside the sealed 250 mL Duran bottles. For determination of released $^{14}CO_2$ the 1.5 mL aliquot of NaOH was transferred into a 20 mL LSC-vial and the trapping vial was washed with 5 mL of deionized water. The liquids were mixed with 10 mL scintillation cocktail and measured by LSC. The amount of $^{14}CO_2$-radioactivity was determined by LCS for 15 min using a quenching correction by internal standard.

RESULTS AND DISCUSSION

Soil and soil-aggregates analysis. Even 22 years after the last application, sorption of ^{14}C-labeled atrazine residues was significantly greater in the surface layer 0-10 cm than in the lower soil layers. Measurements gave a clearly decreasing profile of residual ^{14}C-activity from 8.8 % in the 0-10 cm soil layer to 0.7 % in the 50-55 cm soil layer of the initially applied ^{14}C-atrazine radioactivity, corresponding to the equivalent of 0.3 and 0.05 μg of the formerly applied ^{14}C-labeled atrazine g^{-1} dry soil, respectively (Figure 1). However, it must be mentioned that the amount of detected ^{14}C-activity is not necessarily related to ^{14}C-labeled atrazine or metabolite molecules. Association of ^{14}C-activity with microbial biomass, soil organic matter or even an inorganic form of carbon has to be considered.

Table 2 presents the measured residual ^{14}C-labeled atrazine in the different soil size aggregates. The aqueous DOM-fraction (particle sizes <0.05 μm; pH 6.1 $\pm$ 0.2) showed only minor desorbed ^{14}C-activity of 0.38 Bq mL^{-1} ($\pm$ 0.01). By analyzing the concentrated DOM-fraction and used methanol-water solution from desorption study via Radio-HPLC (limit of quantification, LOQ: 40 Bq mL^{-1}), UV-HPLC (LOQ: 0.1 mg L^{-1}) and LC-MS/MS (limit of detection, LOD: 0.125 ng mL^{-1} for atrazine and 2-hydroxy-atrazine

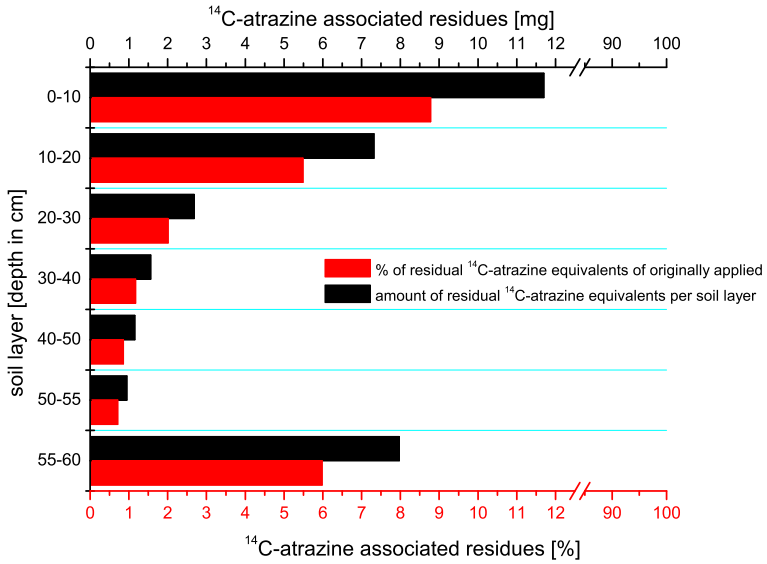

FIGURE 1. Measured and calculated ^{14}C-labeled atrazine residues of each soil layer in average. The percentage indicates the ^{14}C-labeled atrazine associated residues of initially applied total ^{14}C-labeled atrazine.

standard methanol solution) respectively, no atrazine as active compound was detected. As described in previous studies, the main binding mechanisms of non-extractable residual atrazine are due to charge transfer (*27*, *28*), hydrogen bridges (*29*, *30*) and hydrophobic exchange reactions (*30*, *31*). While HPLC and radio-HPLC analysis were not sensitive enough to detect the ^{14}C-atrazine associated residues, LC-MS/MS analysis clearly detected 2-hydroxy-atrazine (0.25 ng mL^{-1}) as the only metabolite to be desorbed or extracted, respectively. This leads to the conclusion that atrazine as the primary compound is either degraded or irreversibly sequestered into the soil matrix and may therefore be considered to be a bound residue (*32*).

The distribution of ^{14}C-activity in the differently sized aggregates show highest ^{14}C-labeled atrazine residues in the clay-sized aggregates, corresponding to results reported by Barriuso et al. (*15*), with 0.5 μg atrazine residues g^{-1} dried aggregates. Due to the high content of C, Al, Fe and Si (Table 2) as binding agents it could be assumed that atrazine residues are directly associated with clay minerals or indirectly via C-, Al-, Fe-

and Si-binding mechanisms on clay particles (*33*). As sorption of aged atrazine residues is very likely due to C-binding mechanisms, Al- and Fe-oxides may play a relatively minor role, according to Clausen et al. (*34*) and Kovaios et al. (*35*) in model studies; whereas, Si-binding using silica gel is reversible (*35*).

TABLE 2. Residual ^{14}C-atrazine activity in the soil and different soil size aggregates. ± mean standard deviation of nine replicates. Values for carbon, nitrogen, aluminium, iron, potassium and silicon are in %. ± indicate the mean standard deviation of three (C) and five replicates (N). The mean standard deviation of three replicates for Al, Fe, K and Si is ± 3%.

	Soil	Sand-sized aggregates 20 - 2000μm	Silt-sized aggregates 2 - 20μm	Clay-sized aggregates <2μm
^{14}C-activity [Bq g^{-1}]	125 ±2.9	103 ±1.2	177 ±3.0	196 ±7.5
^{14}C-atrazine associated residues [μg g^{-1}]	0.3	0.2	0.4	0.5
Element [%]				
N	0.217 ±0.004	0.150 ±0.005	0.295 ±0.004	0.380 ±0.009
C_{org}	1.450 ±0.010	1.230 ±0.020	2.080 ±0.010	2.530 ±0.010
C_{anorg}	0.052 ±0.017	0.039 ±0.005	0.060 ±0.001	0.062 ±0.001
C_{Total}	1.502	1.269	2.14	2.592
Al	5.9	5.1	8.8	11.4
Fe	2.1	1.6	3.8	5.6
K	3.1	2.4	4.3	5.3
Si	35.0	37.8	28.5	23.0

Desorption experiments. Results of the desorption study showed that mineral-media, including Na-citrate or glucose enhanced the desorption for aged ^{14}C-labeled atrazine residues in the soil (Figure 2). Since in the published studies (*9*, *17*) 0.01 M $CaCl_2$-solution has been used for desorption and extraction of aged atrazine residues, the results of this study show that the desorption potential of water is only slightly lower than that for 0.01 M $CaCl_2$-solution (2.8 versus 3.3 % desorbed ^{14}C-activity of total experimental setup ^{14}C-activity). Mineral-media, with citrate or glucose, respectively, were found to be 8.7 % and were comparable with methanol-water solution at 8.6 % after 6 h. Analysis

with selected mineral-media components showed highest desorption potential of 8.8 ± 0.1 % for K_2HPO_4 (Table 3). Desorption of ^{14}C-labeled atrazine residues released by these minerals may play an important role in dissociation of prior non-bioaccessible, particle bound atrazine residues leading to an enhanced microbial degradation.

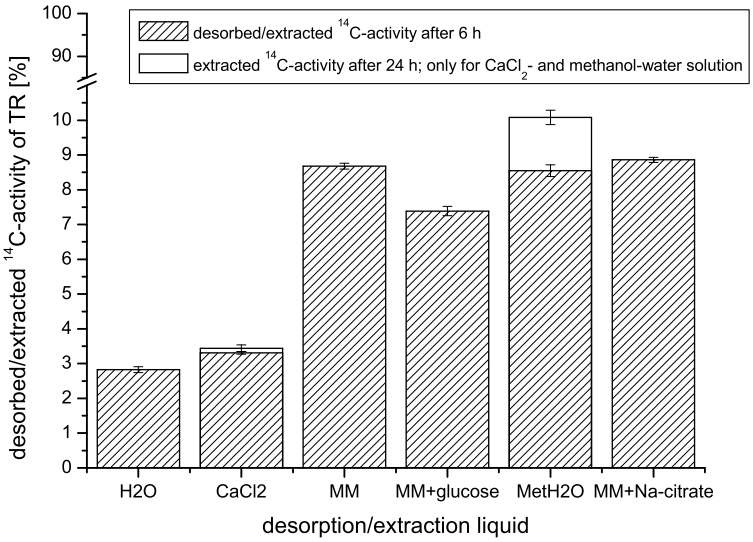

FIGURE 2. Desorption/extraction of ^{14}C-labeled atrazine residues in the soil with different solutions. Bars indicate desorbed/extracted ^{14}C-activity in % of total ^{14}C-activity in 10 g of soil dry weight. Indications on the X-axis: H2O: deionized organic free water; CaCl2: 0.01 M $CaCl_2$-solution; MM: mineral-media only; MM+glucose: 2 g L^{-1} dissolved in mineral-media; MetH2O: methanol-water solution (4:1 v/v); MM+Na-citrate: 2 g L^{-1} dissolved in mineral-media. TR total ^{14}C-activity in the sample. Error bars indicate mean standard deviation of nine replicates.

Carbon bound desorbed residual ^{14}C-activity in liquid fractions. As expected, the use of mineral-media extraction liquid resulted in a much higher total carbon content (31.2 mg L^{-1}) than using water (5.9 mg L^{-1}) after soil desorption (data not shown). These results lead to the conclusion that the detected ^{14}C-activity in mineral-media after soil desorption (Figure 2) can be attributed to the ring ^{14}C-components of the originally applied ^{14}C-labeled atrazine and therefor most likely associated with the extractable carbon fraction. It may be assumed that dissolved carbon associated with residual ring

TABLE 3. Desorption potential of mineral-media components used in bioaccessibility studies. The used amounts of the components are equal to the amounts used for the liquid media for the bioaccessibility studies, calculated for 80 mL water per 10 g dry soil sample for extraction. ± indicate the mean standard deviation of three replicates (in %). 100 % equals the total amount of ^{14}C-activity in the soil sample; n.a. no analysis.

compound	% of total desorbed ^{14}C-atrazine residual activity		
	1. extraction	2. extraction	3. extraction
K_2HPO_4 (0.1752 g)	8.83 ±0.11	5.40 ±0.07	2.24 ±0.05
$MgSO_4.7H_2O$ (0.8 g)	3.79 ±0.10	2.10 ±0.06	0.92 ±0.07
$FeCl_3.6H_2O$ (0.16 g)	3.18 ±0.09	0.92 ±0.08	n.a.
Na_2EDTA (0.0028 g)	3.09 ±0.02	n.a.	n.a.

^{14}C-components favors higher bioaccessibility, thus resulting in enhanced mineralization rates.

Mineralization of ^{14}C-labeled atrazine associated residues. The control samples analyzed for soil showed a very small mineralization, consisting of less than 1 % of the residual ^{14}C-activity present in the samples. This mineralization comes from indigenous soil bacteria present in the soil samples mineralizing ^{14}C-residues originating from formerly applied ^{14}C-ring labeled atrazine (Figure 3 A).

The samples inoculated with *Pseudomonas* sp. strain ADP increases in mineralization extents of 2.5 % of the total ^{14}C-activity in the setup after three days, increasing to 5 % after 44 days of incubation (Figure 3 A). These results indicate that mineralization of aged atrazine residues was facilitated by the specialized atrazine degrading strain *Pseudomonas* sp. strain ADP. The mineralization by *Pseudomonas* sp. strain ADP in the sand- and silt-sized aggregates reached maxima of 5.5 and 4.6 %, respectively, after an incubation period of 44 days (Figure 3 B). In both cases, mineralization took place more slowly than in soil. Slower degradation in comparison to the soil might be related to missing nutrient components, which are important for microbial activity and which are not sufficiently present in the sand- and silt-sized aggregates. In addition, there was considerable mineralization of the ^{14}C-activity in the clay-sized aggregates of 6.2 % after

23 d although the high standard deviation caused by natural heterogeneities in the small amounts of initial sample associated with high organic carbon and ^{14}C-labeled atrazine residues (Table 2) has to be considered critically.

Taking into consideration the differing bacteria/residue ratios, the averaged mineralization of the soil-size aggregates shows a similar behavior to that of soil incubated with *Pseudomonas* sp. strain ADP (Figure 3 A and B). This result proves that the physical aggregate fractionation is gentle. The similar mineralization behavior indicates that the residual atrazine ring carbon is partially bioaccessible, irrespective of its spatial configuration, that is, independently of the scale of the particles or aggregates present in the soil.

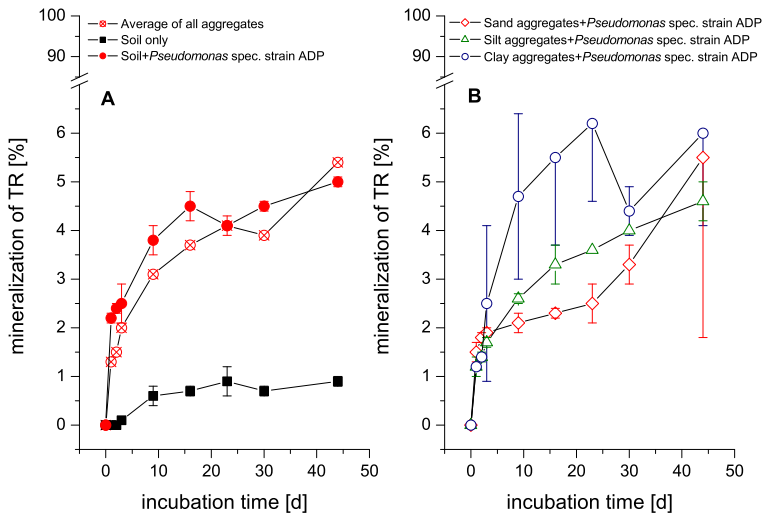

FIGURE 3. Mineralization of ^{14}C-labeled atrazine residues in the soil incubated without *Pseudomonas* sp. strain ADP, soil with addition of *Pseudomonas* sp. strain ADP, and mineralization curve of averaged mineralization of sized aggregates (A). Mineralization of ^{14}C-labeled atrazine residues in the soil size aggregates: sand-sized aggregates (2000 - 20μm), silt-sized aggregates (20 - 2μm) and clay-sized aggregates (<2μm) all incubated with *Pseudomonas* sp. strain ADP (B). TR total ^{14}C-radioactivity of the sample. Error bars indicate mean standard deviation of three replicates.

The amounts of $^{14}CO_2$ produced from microbial activity following the addition of citrate or glucose are presented in Figure 4 B. Figure 4 A represents the mineralization kinetics

of ^{14}C-labeled atrazine residues in total soil with and without addition of *Pseudomonas* sp. strain ADP as described above. The addition of citrate to soil only resulted in an enhanced mineralization of 6.7 % after 23 d of the ^{14}C-atrazine associated residues by *Pseudomonas* sp. strain ADP. However, the addition of glucose led to a mineralization extent of just 3.6 % after 23 d, which was less than the mineralization of the atrazine ring carbon with no glucose amendment (Figure 4 A).

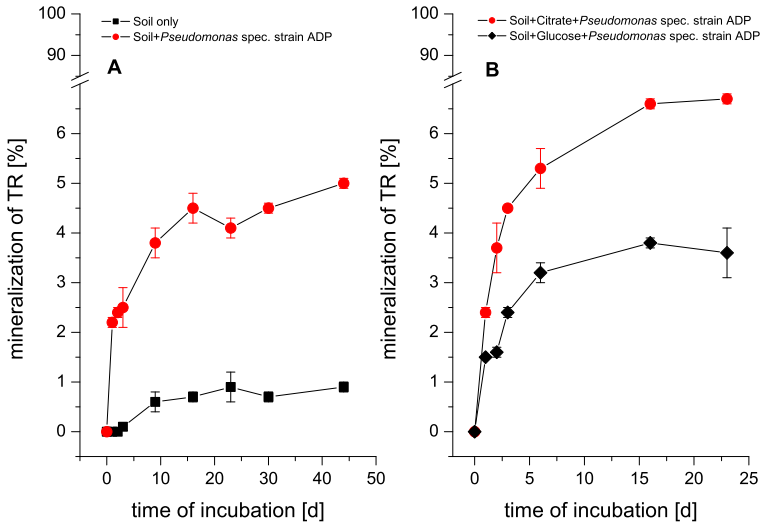

FIGURE 4. Mineralization of ^{14}C-labeled atrazine residues in the soil incubated with and without addition of *Pseudomonas* sp. strain ADP (A). Mineralization of ^{14}C-atrazine-residues in the soil including Na-citrate or glucose incubated with *Pseudomonas* sp. strain ADP (B). TR total ^{14}C-activity in the sample. Error bars indicate mean standard deviation of three replicates.

It is likely that citrate increases co-metabolic degradation pathways of atrazine residues or leads to priming-effects. Further, Piccolo et al. (*36*) found that an addition of small organic acids, e.g. citric acid to humic substances lead to a dispersion of humic material into small submicelles. It is to assume that increased mineralization by glucose and citrate amendment is due to priming-effects since these additional carbon sources are easily degradable by microorganisms. This effect might be supported by dispersion of

large humic associates with incorporated ^{14}C-labeled atrazine residues by citrate amendment, making these freed residues more accessible for the microorganisms. However, the influence of citrate amendment on soil carbon structures and resulting changes in aged ^{14}C-labeled atrazine residues on bioaccessibility is under current investigation.

Further, stimulated bacterial growth resulting in increased metabolism of the aged ^{14}C-labeled atrazine residues is possible. Laboratory analysis by HPLC showed that atrazine in liquid media (50 mg L^{-1}) could be completely mineralized within 24 h by *Pseudomonas* sp. strain ADP, while using mineral-media with additional citrate or glucose. In the absence of these carbon sources, 50 % of applied atrazine was mineralized within the same time interval, whereas no degradation took place using deionized water only.

These results correspond to those achieved by Silva et al. (*37*) where biostimulation of *Pseudomonas* sp. strain ADP to degrade atrazine in soil with citrate amendment was significant. However, it must be pointed out that mineralization in the presented study originated from long-term aged ^{14}C-labeled atrazine residual fractions in soil.

Analysis of the supernatants of all incubations showed no obvious changes in dissolved ^{14}C-activity in comparison to evolved $^{14}CO_2$. This indicates that evolved $^{14}CO_2$ likely originates from mineralization of particle bound ^{14}C-atrazine residues. As Park et al. (*38*) demonstrated *Pseudomonas* sp. strain ADP is chemotactic towards atrazine and the authors showed clear evidence of access to atrazine sorbed to soil, which may support the presented observations. While Capriel et al. (*39*) found parent atrazine in soil and in humic acids nine years after natural aging, it may be assumed that evolved $^{14}CO_2$ could be due to bound ^{14}C-labeled atrazine. Since small amounts of one main atrazine-metabolite (2-hydroxy-atrazine) could be detected with the applied LC-MS/MS methods it can be expected that the bound ^{14}C-residues fraction comprises of atrazine metabolites or even atrazine as the parent compound.

The presented results have implications on the assessment of the long-term fate and bioaccessibility of aged atrazine and its residues. The data highlight that atrazine and/or

its metabolites are biologically accessible even after 22 years of aging. This is an important information to be used in the field of environmental management and bioremediation as well as in environmental risk assessment of persistent pesticides.

ACKNOWLEDGMENTS

We acknowledge thankfully the help and assistance of the laboratory co-workers, namely Thorsten Wachtmeister for microbiological skills, Stephan Koeppchen for HPLC-/LC-MSMS-analysis and Mr. Günter Henkelmann, LfL Munich for the lysimeter soil. Special thanks are dedicated to Mr. M. Michulitz, Ms. H. Lippert and Ms. N. Merki, ZCH, for physico-chemical analysis. Sincere thanks are given to Mr. Morton Canty for revision of this manuscript.

References

(1) Schulte, M. Transgene herbizidresistente Kulturen - Rückblicke und Ausblicke nach 8 Jahren internationaler Anbaupraxis. *Gesunde Pflanzen.* **2005**, *57*, 37–46.

(2) Hull, H. M. *Herbicide handbook of the Weed Society of America*; W. F. Humphrey Press: Geneva, New York, 1967.

(3) Jones, T.W.; Kemp, W.M.; Stevenson, J.C.; Means, J.C. Degradation of atrazine in estuarine water/sediment systems and soils. *J. Environ. Qual.* **1982**, *11*, 632–638.

(4) Trinkwasserverordnung *(BGBl. I 2001, 971–972, Anlage 2 (zu §6 Abs. 2) Chemischer Parameter)*, 2001.

(5) Tappe, W.; Groeneweg, J.; Jansch, B. Diffuse atrazine pollution in German aquifers. *Biodegradation.* **2002**, *13*, 3–10.

(6) Huang, G.; Li, Q.; Zhang, X. Adsorption and desorption of atrazine by three soils. *Bull. Environ. Contam. Toxicol.* **2003**, *71*, 655–661.

(7) Kolpin, D.W.; Thurman, E. M.; Linhart, S. M. The environmental occurrence of herbicides: the importance of degradates in ground water. *Arch. Environ. Contam. Toxicol.* **1998**, *35*, 385-390.

(8) Schweinsberg, F.; Abke, W.; Rieth, K.; Rohmann, U.; Zullei-Seibert, N. Herbicide use on railway tracks for safety reasons in Germany?. *Toxicology Letters.* **1999**, *107*, 201-205.

(9) Loiseau, L.; Barriuso, E. Characterization of the atrazine's bound (nonextractable) residues using fractionation techniques for soil organic matter. *Environ. Sci. Technol.* **2002**, *36* (4), 683–689.

(10) Benoit, P.; Barriuso, E.; Bergheaud V.; Etiévant, V. Binding capacities of different soil size fractions in the formation of herbicide-bound residues. *Agronomie.* **2000**, *20*, 505–512.

(11) The Royal Society of Chemistry. *The Agrochemical Handbook*, 3rd ed.; Cambridge, UK, 1991.

(12) Best, J.A.; Weber, J.B. Disappearance of s-triazines as effected by soil pH using a balance-sheet approach. *Weed Sci.* **1974**, *22*, 364–373.

(13) Cohen, S.; Creager, S.; Carsel, R.; Enfield, C. Potential pesticide contamination of groundwater from agricultural uses. In *Treatment and Disposal of Pesticide Waste*;

Krueger, R.F., Seiber, J.N., Eds.; American Chemical Society: Washington, DC, 1984.

(14) Winkelmann, D.A.; Klaine, S.J. Atrazine metabolite behavior in soil-core microcosms: formation, disappearance, and bound residues. In *Pesticide Transformation Products: Fate and Significance in the Environment*; ACS Symposium Series 459; American Chemical Society: Washington, DC, 1991.

(15) Barriuso, E.; Schiavon, M.; Andreux, F.; Portal, J.M. Localization of atrazine non-extractable (bound) residues in soil size fractions. *Chemosphere.* **1991**, *22* (12), 1131–1140.

(16) Koskinen, W.C.; Clay, S.A. Factors affecting atrazine fate in north central U.S. soils. *Rev. Environ. Contam. Toxicol.* **1997**, *151*, 117–165.

(17) Barriuso, E.; Koskinen, W.C.; Sadowski, M.J. Solvent extraction characterization of bioavailability of atrazine residues in soils. *J. Agric. Food Chem.* **2004**, *52*, 6552–6556.

(18) Mandelbaum, R.T.; Allan, D.L.; Wackett, L.P. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. *Appl. Environ. Microbiol.* **1995**, *61* (4), 1451–1457.

(19) Cook, A.M.; Hütter, R. S-triazines as nitrogen sources for bacteria. *J. Agric. Food Chem.* **1981**, *29*, 1135-1143.

(20) Kontchou, C.Y.; Gschwind, N. Mineralization of the herbicide atrazine in soil inoculated with a *Pseudomonas* strain. *J. Agric. Food Chem.* **1995**, *43*, 2291–2294.

(21) Berns, A.; Vinken, R.; Bertmer M.; Breitschwerdt, A.; Schäffer, A. Use of N-15-depleted artificial compost in bound residue studies. *Chemosphere.* **2005**, *59*, 649–658.

(22) Burauel, P.; Bassmann, F. Soils as filter and buffer for pesticides - experimental concepts to understand soil functions. *Environ. Pollut.* **2005**, *133*, 11–16.

(23) Eric, J.; Davies, D.; Nusrat, J. The adsorption of herbicides and pesticides on clay minerals and soils. Part 2. Atrazine. *J. Incl. Phenom. Macrocycl. Chem.* **2003**, *46*, 57–64.

(24) Blume, E.; Bischoff, M.; Moorman, T.B.; Turco, R.F. Degradation and binding of atrazine in surface and subsurface soils. *J. Agric. Food Chem.* **2004**, *52*, 7382–7388.

(25) Mandelbaum, R.T.; Wackett, L.P.; Allan, D.L. Mineralization of the s-triazine ring of atrazine by stable bacterial mixed cultures. *Appl. Environ. Microbiol.* **1993**, *59* (6), 1695–1701.

(26) Sambanis, A. *Experimental and modeling studies on the dynamics of cultures of the dilate Tetrahymena pyriformis grown on several bacterial species*; PhD. Thesis, University of Minnesota: Minneapolis, 1985.

(27) Piccolo, A.; Celano, G.; de Simone, C. Interaction of atrazine with humic substances of different origins and their hydrolysed products. *Sci. Tot. Environ.* **1992**, *117/118*, 403–412.

(28) Sposito, G.; Martin-Neto, L.; Yang, A. Atrazine complexation by soil humic acids. *J. Environ. Qual.* **1996**, *25*, 1203–1209.

(29) Li, G.-C.; Feldbeck, G.T. A study of the mechanism of atrazine adsorption by humic acid from muck soil. *Soil Sci.* **1972**, *113*, 140–148.

(30) Martin-Neto, L.; Vieira, E.M.; Sposito, G. Mechanism of atrazine sorption by humic acid: A spectroscopic study. *Environ. Sci. Technol.* **1994**, *28*, 1867–1873.

(31) Lerch, R.N.; Thurman, E.M.; Kruger, E.L. Mixed mode sorption of hydroxylated atrazine degradation products to soil: A mechanism for bound residue. *Environ. Sci. Technol.* **1997**, *31*, 1539–1546.

(32) Führ, F. Non-extractable pesticide residues in soil. In *IUPAC - Pesticide Science and Biotechnology*; Greenhalgh, R., Roberts, T.R., Eds; Blackwell Scientific Publications: London, 1987.

(33) Herwig, U.; Klumpp, E.; Narres, H.-D.; Schwuger, M. J. Physicochemical interactions between atrazine and clay minerals. *Appl. Clay Sci.* **2001**, *18*, 211-222.

(34) Clausen, L.; Fabricius, I. Atrazine, isoproturon, mecoprop, 2,4-D, and bentazone adsorption onto iron oxides. *J. Environ. Qual.* **2001**, *30*, 858-869.

(35) Kovaios, I.D.; Paraskeva, C.A.; Koutsoukos, P.G.; Payatakes, A.C. Adsorption of atrazine on soils: model study. *J. Colloid Interface Sci.* **2006**, *299*, 88-94.

(36) Piccolo, A.; Nardi, S.; Concheri, G. Macromolecular changes of humic substances induced by interaction with organic acids. *Europ. J. Soil Sci.* **1996**, *47*, 319–328.

(37) Silva, E.; Fialho, A.M.; Sa-Correia, I.; Burns, R.G.; Shaw, L.J. Combined bioaugmentation and biostimulation to cleanup soil contaminated with high concentrations of atrazine. *Environ. Sci. Technol.* **2004**, *38*, 632–637.

(38) Park, J.-H.; Feng, Y.; Ji, P.; Voice, T.C.; Boyd, S.A. Assessment of bioavailability of soil-sorbed atrazine. *Appl. Env. Microbiol.* **2003**, *69* (6), 3288-3298.

(39) Capriel, P.; Haisch, A.; Khan, S.U. Distribution and nature of bound (nonextractable) residues of atrazine in a mineral soil nine years after the herbicide application. *J. Agric. Food Chem.* **1985**, *33*, 567–569.

(40) Liebich, J.; Schloter, M.; Schäffer, A.; Vereecken, H.; Burauel, P. Degradation and humification of maize straw in soil microcosms inoculated with simple and complex microbial communities. *European J. Soil Sci.* **2007**, *58*, 141–151.

Brief

After an aging process of 22 years under outdoor conditions residues of the originally applied ^{14}C-labeled atrazine are still biologically accessible which has implications for the environmental management of soils.