Aging of ^{14}C-labeled Atrazine Residues in Soil: Location, Characterization and Biological Accessibility

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Aging of ^{14}C-labeled Atrazine Residues in Soil: Location, Characterization and Biological Accessibility

The long-term behavior of the herbicide atrazine and its metabolites in the environment is of continued interest in terms of risk assessment and soil quality monitoring.

The results of this investigation highlight the long-term persistence and environmental behavior of the herbicide atrazine. To date, no comparable results have been published by other parties. Therefore, this study provides important data for the risk assessment of atrazine application areas or atrazine-contaminated sites. It is possible that these findings reported for atrazine presented in this report may also be relevant for other persistent chemicals and pesticides. Clearly, the calculation of predicted environmental concentrations of persistent chemicals based only on laboratory half-life or short-term field dissipation experiments should therefore be reconsidered. Agricultural soils after many years of cultivation may contain multiple aged pesticide residues from applications of various pesticides that become stabilized by binding to the soil matrix. This may challenge the environmental risk assessment of the resulting mixture of long-term available pesticide residues in our agricultural soils. Further, adapted microbial communities with mineralization potential for atrazine [Shaner and Henry, 2007a, Krutz et al., 2008b] or

other pesticides might challenge farmers and industries to reconsider the use and necessity of agrochemicals that lead to environmental pollution and consider the use of alternative techniques in general.

1. Aqueous desorption, detection and quantification of atrazine and its metabolites from an agriculturally used soil was performed 22 years after the last atrazine application. The application of ring-^{14}C-labeled and non-labeled atrazine at the customary rate (1.7 kg ha^{-1}) was performed on an agriculturally used outdoor lysimeter. The lysimeter soil containing long-term aged atrazine for >20 years was subdivided into 10 cm and 5 cm layers (soil: 0-10, 10-20, 20-30, 30-40, 40-50, except at the lysimeter bottom: 50-55 cm; fine gravel: 55-60 cm depth, implemented for drainage purposes) to identify the qualitative and quantitative differences of aged ^{14}C-labeled atrazine residues depending on the soil profile and chemico-physical conditions of the individual soil layers. Deionized water was used for non-exhaustive cold water shaking-extraction of the soil. With increasing soil depth the amount of previously applied ^{14}C-activity decreased significantly from 8.8 % to 0.7 % at 50-55 cm depth, whereas the percentage of desorbed ^{14}C-residues in each soil layer increased from 2 % to 6 % of the total ^{14}C-activity in the sample. The only metabolite detectable by means of LC-MS/MS was 2-hydroxy-atrazine while most of the residual ^{14}C-activity was bound to the soil and was not desorbed. The amount of desorbed 2-hydroxy-atrazine decreased with increasing soil depth from 21 % to 10 % of the total desorbed ^{14}C-residue fraction. The amount of ^{14}C-residues in the soil layers correlated well with the carbon content in the soil and in the aqueous soil extracts (p-value: 0.99 and 0.97, respectively), which may provide evidence of the binding behavior of the aged atrazine residues on soil carbon fractions. The lowest coarse layer (55-60 cm) showed increased residual ^{14}C-activity lead-

ing to the assumption that most ^{14}C-residues were leached from the top soil over time.

2. Further analysis showed that atrazine is still detectable by means of accelerated solvent extraction and LC-MS/MS analysis. Extractions of the 0-10 cm soil layer yielded 60% of the residual ^{14}C-activity. The extracts contained atrazine (1.0 μg kg^{-1}) and 2-hydroxy-atrazine (42.5 μg kg^{-1}). Extractions of the material of the lowest layer 55-60 cm consisting of fine gravel yielded 93% of residual ^{14}C-activity, of which 3.4 μg kg^{-1} was detected as atrazine and 17.7 μg kg^{-1} as 2-hydroxy-atrazine. The atrazine detected in the lowest layer was of almost four times higher mass than in the uppermost soil layer. These findings highlight the fact that atrazine is unexpectedly persistent in soil. The overall persistence of atrazine in the environment might represent a potential risk for successive groundwater contamination by leaching even after 22 years of environmental exposure.

3. In addition, 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 achieved 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 were 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.

4. To gather further information on the bioaccessibility of soil-bound ^{14}C-atrazine residues, soil samples were previously extracted three times with a) mineral media (MM), b) mineral media with 2 gL^{-1} Na-citrate and c) deionized water. Total extractable ^{14}C-activity was 12.1 % for MM, 18.0 % for MM plus citrate and 6.1 % using deionized water, respectively. Extracted soil samples were mixed with 10 ml of the different solutions used for extraction with addition of 1.8-3 $\times$ 10^8 cells ml^{-1} of the atrazine-degrading organism *Pseudomonas* sp. strain ADP. The resultant slurries of extracted soil samples were incubated and the evolved $^{14}CO_2$ was trapped in 0.5 M NaOH solution. Cumulative kinetics showed that 32 d of incubation led to a ^{14}C-atrazine residue mineralization of 2.49 and 2.45 % using MM and MM plus citrate, respectively. Analyzing the supernatant in a similar approach using previously non-extracted soil samples provides evidence that dissolution of atrazine residues in the liquid phase is not a prerequisite for biodegradation. These results indicate that soil-bound ^{14}C-atrazine residues are partly bioaccessible and bioavailable for the specific atrazine-degrading strain *Pseudomonas* sp. strain ADP even after 22 years of natural aging.

Overall, these results represent novel information about the long-term environmental persistence of the still widely used herbicide atrazine. It is noteworthy that atrazine as the parent compound is still detectable in soil and deeper layers even after 22 years of

environmental exposure. This finding indicates a potential long-term risk for soil and groundwater contamination by atrazine. These findings can be useful for environmental and agricultural assessments and environmental policy decisions concerning pest management.

Alterung von ^{14}C-markierten Atrazin-Rückständen im Boden: Lokalisierung, Charakterisierung und Biologische Verfügbarkeit

Herbizide wie Atrazin werden weltweit grossflächig auf landwirtschaftlich genutzten Böden eingesetzt. Im Boden unterliegen Pflanzenschutzmittel mikrobiellen und chemisch-physikalischen Umsatzprozessen oder Verlagerungen durch Auswaschung. Um mögliche langfristige Wirkungen von Atrazin abzuschätzen, bedarf es genauer Informationen über das Langzeitverhalten dieses Pflanzenschutzmittels im Boden. Entscheidend ist dabei, wie fest und in welcher Form die Pflanzenschutzmittel an die Bodenbestandteile gebunden werden. Der genaue Mechanismus dieser Bindungen ist jedoch noch nicht vollständig geklärt. Die vorliegende Arbeit untersucht die Lebensdauer des Herbizids Atrazin im Boden. Es ist zu vermuten, dass die vorliegenden Ergebnisse für Atrazin ebenfalls für andere Chemikalien und Pflanzenschutzmittel in der Umwelt Gültigkeit haben dürften. Aufgrund der langjährigen Applikation von Atrazin beschreiben aktuelle Studien die Anpassung von Bodenmikroorganismen, die den Abbau von Atrazin beschleunigen [Shaner and Henry, 2007a, Krutz et al., 2008b]. Diese Tatsache könnte die Langlebigkeit

von Atrazin im Boden herabsetzen. Kurzzeitexperimente im Labor- oder Feldversuch bilden eine unzureichende Basis für die Berechnungen, wie lange Chemikalien in der Umwelt existieren. Die vorliegende Arbeit ist die erste, die sich im Detail mit den langfristig gealterten Rückständen von Atrazin im Boden beschäftigt. Damit ist diese Arbeit ein erster Schritt zur Bewertung des Risikos, das langfristig von atrazinbelasteten Böden ausgehen kann.

Die Ergebnisse dieser Arbeit lassen sich wie folgt zusammenfassen:

1. Die Applikation von Atrazin erfolgte im Rahmen eines Lysimeter-Langzeitversuchs am Bayerischen Landesamt für Landwirtschaft (LfL). In drei aufeinanderfolgenden Jahren (1983-1985) wurde in praxisüblicher Dosierung (1,7 kg ha^{-1}) ^{14}C-ringmarkiertes und nicht-markiertes Atrazin auf einen landwirtschaftlich genutzten Lysimeterboden aufgetragen. Nach 22 Jahren natürlicher Alterung unter Freilandbedingungen wurde zum Nachweis von ^{14}C-markierten Atrazinrückständen im Bodenprofil der Lysimeterboden in 10 cm und 5 cm Schichten unterteilt (Boden: 0-10, 10-20, 20-30, 30-40, 40-50, ausser am Grund des Lysimeters: 50-55 cm; feiner Kies: 55-60 cm Tiefe, eingefügt zu Drainagezwecken). Unter Berücksichtigung der jeweiligen chemisch-physikalischen Bodeneigenschaften wurde zum Nachweis desorbierbarer ^{14}C-markierter Atrazinrückstände eine schonende Kaltwasserextraktion mit deionisiertem Wasser durchgeführt. Mit zunehmender Bodentiefe nahm die gemessene ^{14}C-Aktivität von 8,8 % (Bodenschicht 0-10 cm) auf 0,7 % (Bodenschicht 50-55 cm) der ursprünglich aufgetragenen ^{14}C-Aktivität deutlich ab. Im Gegensatz dazu stieg die Menge an desorbierter ^{14}C-Aktivität mit zunehmender Bodenschicht von 2 % auf 6 % der Gesamtaktivität in der jeweiligen Bodenschichtprobe an. Mittels LC-MS/MS konnte 2-Hydroxy-Atrazin in der Lösung als einziger Atrazin-Metabolit nachgewiesen werden. Der überwiegende Anteil der gemessenen ^{14}C-Aktivität blieb am Boden gebunden und konnte nicht desorbiert werden. Die Menge an desorbier-

tem 2-Hydroxy-Atrazin fiel bei zunehmender Bodentiefe von 21 % auf 10 % der insgesamt desorbierten ^{14}C-Rückstände ab. Die gemessenen ^{14}C-Rückstände in den Bodenschichten und den wässrigen Bodenextrakten waren positiv korreliert mit dem Kohlenstoffgehalt (p-Werte: 0,99, beziehungsweise 0,97). Die unterste Bodenschicht des Lysimeters (55-60 cm), bestehend aus feinem Kies, zeigte eine erhöhte ^{14}C-Aktivität. Diese Befunde machen deutlich, dass Anteile der ^{14}C-Atrazinrückstände aus den oberen Bodenschichten über die Zeit ausgewaschen wurden.

2. Weitere Analysen zeigten, dass Atrazin nach einer „beschleunigten Flüssigkeitsextraktion"(ASE) der Bodenproben mittels LC-MS/MS in den Extrakten detektiert werden konnte. Durch Extraktion der Bodenproben aus der Schicht 0-10 cm konnten 60% der ^{14}C-Rückständsaktivität extrahiert werden. Die Extrakte enthielten Atrazin (1,0 μg kg^{-1}) und 2-Hydroxy-Atrazin (42,5 μg kg^{-1}). Aus den Proben der untersten Lysimeterschicht 55-60 cm, bestehend aus feinem Kies, ließen sich 93% der ^{14}C-Rückstandsaktivität extrahieren. Davon konnten 3,4 μg kg^{-1} als Atrazin, und 17,7 μg kg^{-1} als 2-Hydroxy-Atrazin nachgewiesen werden. Diese Ergebnisse zeigen, dass die extrahierbare Menge an Atrazin in der untersten Schicht des Lysimeters circa viermal höher ist als in der obersten Bodenschicht. Diese Befunde verdeutlichen, dass Atrazin im Boden verlagert wird und unerwartet langlebig ist.

3. Des weiteren wurde die Bioverfügbarkeit der gealterten ^{14}C-markierten Atrazinrückstände für Mikroorganismen untersucht. Bodenproben, sowie Proben aus der Sand-, Schluff- und Tonfraktion (>20, 20-2, und <2 μm Aggregatgröße) wurden dazu als Schlämme angesetzt. Durch Inokulation der Bodenproben mit dem atrazinabbauenden Mikroorganismus *Pseudomonas* sp. strain ADP erreichte die Mineralisierung der

^{14}C-Rückstandsaktivität in den Bodenproben 4,5 % nach 16 Tagen. Bei der Zugabe von Zitrat konnte eine Steigerung der Mineralisierung der ^{14}C-Rückstände im Boden durch *Pseudomonas* sp. strain ADP auf 6,7 % nach 23 Tagen verzeichnet werden. Kontrollproben ohne Bakterieninokulation zeigten eine maximale Mineralisierung von 1 % nach 44 Tagen Inkubationszeit. In den Tonaggregaten stieg die Mineralisierungsrate der ^{14}C-Rückstandsaktivität nach der Bakterieninokulation auf 6,2 % nach 23 Tagen. Mit abnehmender Bodenaggregatgröße nahm die ^{14}C-Rückstandsaktivität ab, doch wurden nur geringe Differenzen der Mineralisierung im Boden und den Bodenaggregaten während der Inkubation mit Mineralmedium beobachtet. Diese Ergebnisse belegen, dass ^{14}C-markierte Atrazinrückstände für den atrazinabbauenden Mikroorganismus *Pseudomonas* sp. strain ADP zum Teil zugänglich sind.

4. Um Aussagen zur Bioverfügbarkeit von bodengebundenen, ^{14}C-markierten Atrazinrückständen zu treffen, wurden Bodenproben zuvor mit a) Mineralmedium (MM), b) Mineralmedium mit 2 g L^{-1} Na-Zitrat, und c) deionisiertem Wasser dreimal aufeinanderfolgend extrahiert. Die insgesamt extrahierte ^{14}C-Rückstandsaktivität betrug für MM 12,1 %, für MM mit Zitrat 18,0 %, und für deionisiertes Wasser 6,1 %. Die extrahierten Bodenproben wurden mit einer Anzahl von 1,8-3 $\times$ 10^8 Zellen ml^{-1} des atrazinabbauenden Mikroorganismus *Pseudomonas* sp. strain ADP in 10 ml der jeweiligen Flüssigkeit vermengt und inkubiert. Nach 32 Tagen der Inkubation betrug die Mineralisierung der ^{14}C-markierten Atrazinrückstände 2,49 % bei der Verwendung von MM, beziehungsweise 2,45 % im Fall von MM plus Zitratzugabe. Die Untersuchung der Überstände in dem unter Punkt 3 beschriebenen Versuchsansatz mit zuvor nicht-extrahierten Bodenproben lässt vermuten, dass ein Übergang der ^{14}C-markierten Atrazinrückstände in die Lösung keine Voraussetzung zur Bioverfügbarkeit darstellt. Dadurch konnte gezeigt werden, dass bodengebunde-

ne ^{14}C-markierte Atrazinrückstände für den atrazinabbauenden Mikroorganismus *Pseudomonas* sp. strain ADP selbst nach 22 Jahren natürlicher Alterung zum Teil verfügbar sind.

Die vorgestellten Ergebnisse dieser Arbeit liefern neue Informationen zum Langzeitverhalten des Wirkstoffs Atrazin in der Umwelt. Auch nach 22 Jahren konnte Atrazin noch in Bodenproben detektiert werden. Unter Berücksichtigung von in der Literatur angegebenen Halbwertszeiten war dieser Befund nicht zu erwarten. Die Ergebnisse geben Anlass zur Überprüfung der Risikoabschätzung von Pflanzenschutzmitteln nach langjähriger, intensiver Nutzung.

Annotation

The contents and scientific findings of this doctoral thesis have been published and contributed to scientific meetings previously as specified below:

Publications:

1. Jablonowski, N. D., Modler, J., Schäffer, A., Burauel, P. Bioaccessibility of environmentally aged ^{14}C-atrazine residues in an agriculturally used soil and its particle-size aggregates. *Environmental Science and Technology*, **2008**, 42, 5904–5910.

2. Jablonowski, N. D., Koeppchen, S., Hofmann, D., Schäffer, A., Burauel, P. Spatial distribution and characterization of long-term aged ^{14}C-labeled atrazine residues in soil. *Journal of Agricultural and Food Chemistry*, **2008**, 56, 9548–9554.

3. Jablonowski, N. D., Koeppchen, S., Hofmann, D., Schäffer, A., Burauel, P.Persistence of ^{14}C-labeled atrazine and its residues in a field lysimeter soil after 22 years. *Environmental Pollution*, **2009**, 157, 2126–2131.

Conference contributions:

1. European Geosciences Union, General Assembly 2007, **Poster presentation**: Bioaccessibility of naturally aged ^{14}C-atrazine residues in an agriculturally used soil and

its different soil particle size fractions. 15-20 April **2007**, Vienna, Austria

2. European Geosciences Union, General Assembly 2008, **Oral presentation**: Bioaccessibility of environmentally aged soil-bound ^{14}C-atrazine residues. 13-18 April **2008**, Vienna, Austria

3. SETAC North America, 29th Annual Meeting 2008, **Poster presentation**: Characterization of ^{14}C-labeled atrazine residues after 22 years of aging under outdoor conditions. 16-20 November **2008**, Tampa, USA

4. European Geosciences Union, General Assembly 2009, **Poster presentation**: Sorption and distribution of aged atrazine residues in the drainage system of an outdoor lysimeter experiment. 19-24 April 2009, Vienna, Austria

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List of abbreviations

μ	**m**ikro
°C	temperature in **C**entigrade
%	percent
g	Earth **g**ravity
Si	**si**gnificance of differences between the mean values calculated by the independent two sample t-test
Al	**Al**uminum
ASE	**a**ccelerated **s**olvent **e**xtraction
ATR	**atr**azine
C_{inorg}	**inorg**anic **c**arbon
C_{org}	**org**anic **c**arbon
C_{Total}	**total c**arbon
Ca	**Ca**lcium
cm	**c**enti**m**eter
DOM	**d**issolved **o**rganic **m**atter
ESI+	**p**ositive **e**lectro**s**pray **i**onization
Fe	Iron
g	**g**ram
h	**h**our

ICP-OES ..... **I**nductively **c**oupled **p**lasma **o**ptical **e**mission **s**pectroscopy

K ..... Potassium

kBq ..... **k**ilo **B**equerel

kg ..... **k**ilo**g**ram

L ..... **l**iter

LC-MS/MS ..... **l**iquid **c**hromatography-tandem **m**ass **s**pectrometry

LSC ..... **l**iquid **s**cintillation **c**ounter

MBq ..... **m**ega **B**equerel

Mg ..... **M**a**g**nesium

mg ..... **m**illi**g**ram

min ..... **m**inute

mL ..... **m**illi**l**iter

MRM ..... **m**ultiple **r**eaction **m**onitoring **m**ode

Na ..... Sodium

ng ..... **n**ano**g**ram

NPOC ..... **n**on-**p**urgeable **o**rganic **c**arbon

OH-ATR 2-hydroxy-**atr**azine

P ..... **P**hosphor

rpm ..... **r**ounds **p**er **m**inute

TC ..... **t**otal **c**arbon

TOC ..... **t**otal **o**rganic **c**arbon

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1 Introduction

1.1 History and use of the herbicide atrazine

The s-triazine herbicide atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] (Figure 1.1 A) was introduced in 1957 and has been used since 1958 [Schulte, 2005, Hull, 1967, Jones et al., 1982] worldwide for weed control, predominantly in maize cultivation.

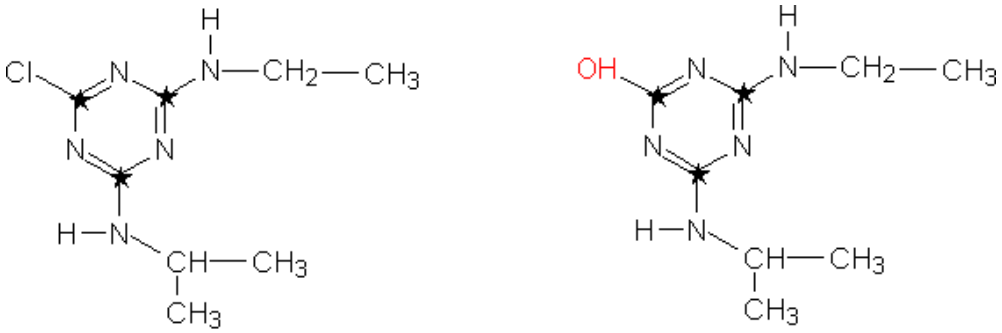

Figure 1.1: Structural formula of atrazine (A) and 2-hydroxy-atrazine (B). ^{14}C-labeled atrazine was uniformly ring-labeled (as indicated).

Atrazine has been one of the largest selling herbicides worldwide for agricultural and industrial purposes. While the application of atrazine has been forbidden in Germany since

March 1991 because of exceedance of permissible concentrations in ground- and drinking water [BGBI, 2001, Tappe et al., 2002], this herbicide is still widely used in agriculture, especially in countries of huge global impact, such as China and USA [Huang et al., 2003, Kolpin et al., 1998], and industrial purposes [Schweinsberg et al., 1999].

Even though its use has been banned in several countries atrazine has been estimated to be the most heavily used pesticide, particularly in the US, where 32,000 to 34,000 metric tons were applied for agricultural purposes in 1993 [Aspelin, 1994] (Figure 1.2 on the next page). In the US, atrazine was applied to 68 % of herbicide-treated acreage in 2003 (Figure 1.3 on page 4) [USEPA, 2004] and has been found in most groundwater supplies from agricultural regions in the US [USEPA, 1990]. Even years after its prohibition in Germany, where it was banned in 1991, it is still found in groundwater. Although atrazine has been the subject of multiple investigations, its long-term environmental behavior is still not entirely clear.

1.2 Atrazine as an environmental hazard

Numerous investigations show that atrazine and/or its metabolites are widely present in ground water, rivers and sediments [Kolpin et al., 1998, Barth et al., 2007]. As reported by USEPA in 1990 [USEPA, 1990], the amount of metabolites detected in well waters was greater than that of the parent atrazine; the amount of metabolites exceeded the US recommended health advisory limit of 3.0 μg L^{-1} in 75 % of documented cases, which is considerably higher than the European advisory limit of 0.1 μg L^{-1} [BGBI, 2001].

Although atrazine was forbidden in several countries because of increased ground and drinking water contamination [Goodrich et al., 1991] it is still widely used for weed control in the US, and particularly in fast developing countries like Brazil, Russia, India and China ("BRIC-countries") and has to be dealt with to secure sustainable soil management

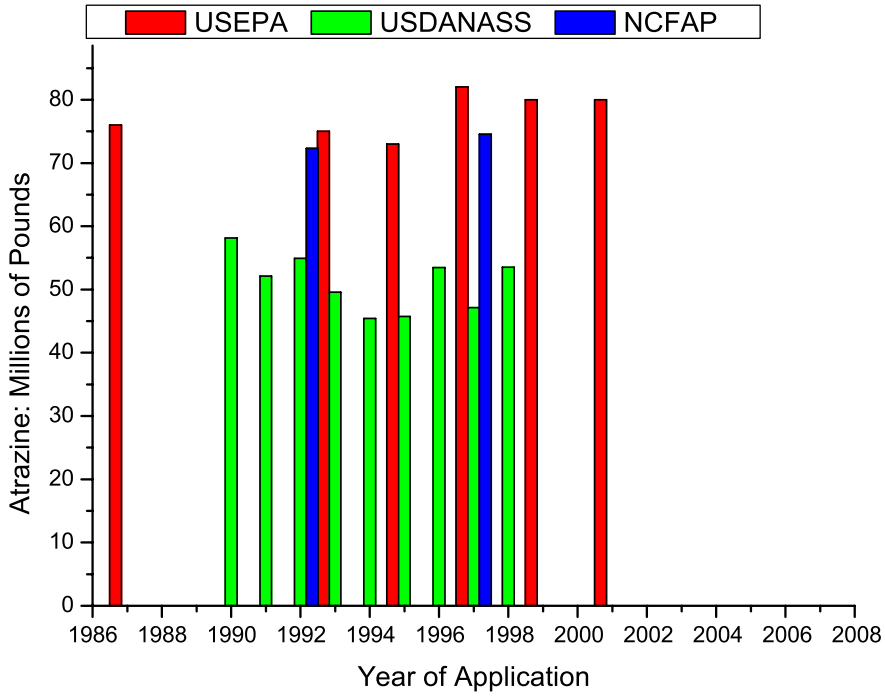

Figure 1.2: Amount of applied atrazine in the US from 1987-2001, where it is still applied to date. Values were taken from online accessible data given in annual reports by the mentioned agencies: USEPA United States Environmental Protection Agency; USDANASS United States Department of Agriculture, National Agricultural Statistics Service; NCFAP National Center for Food and Agricultural Policy. Reliable data on atrazine use and application amount since 2002 could not be found. [USEPA, 1997, USEPA, 1999, USEPA, 2002, Gianessi and Marcelli, 2000, Gianessi and Silvers, 2000, USDA, 1999, USDA, 1998, USDA, 1997, USDA, 1996, USDA, 1995, USDA, 1994, USDA, 1993, USDA, 1992, USDA, 1991].

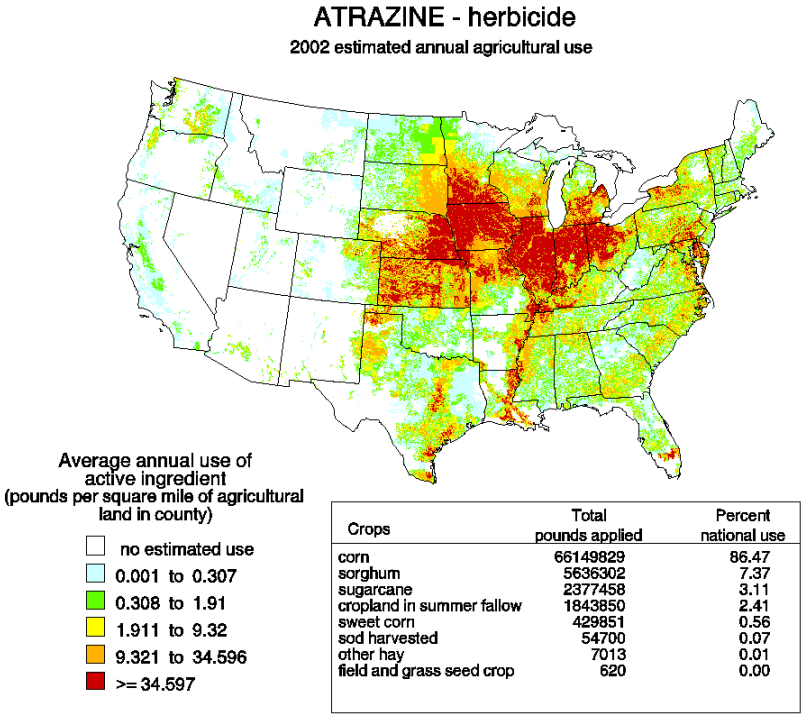

Figure 1.3: Estimated use of the herbicide atrazine in the US [USGS, 2002]. The indicated values of applied atrazine are given in pounds per square mile.

worldwide. Because of its persistence and leaching character in soils it was subject of numerous investigations throughout the world [Tasli et al., 1996, Delphin and Chapot, 2001, Close et al., 1998] and biodegradation of atrazine as a major half life determining process was investigated intensively [Munier-Lamy et al., 2002, Vanderheyden et al., 1997a, Barriuso and Houot, 1996]. The presence of atrazine in soil and water almost world-wide led to approaches to remediate these environments by using atrazine-degrading organisms [Strong et al., 2000, Silva et al., 2004, Mandelbaum et al., 1993, Struthers et al., 1998b, Jones et al., 1982, Topp, 2001, Wackett et al., 2002, Topp et al., 2004].

It has been demonstrated that atrazine has carcinogenic, endocrine, and clastogenic effects [Friedmann, 2002b, Fan et al., 2007, Biradar and Rayburn, 1995]. Atrazine is the subject of controversial discussion due to these potential effects on amphibians, mammals and humans. Numerous investigations have been conducted on amphibians showing endocrine effects, resulting in effects on metamorphosis and in demasculization of these organisms [Freeman and Rayburn, 2005, Hayes et al., 2002b, Sullivan and Spence, 2003]. A number of investigations do not entirely support these findings [Coady et al., 2004, Hecker et al., 2004, Hecker et al., 2005a, Hecker et al., 2005b, Jooste et al., 2005, Oka et al., 2008]

In addition, the potential carcinogenic and endocrine activity of atrazine on mammals is of continued concern. Several studies have been conducted, demonstrating persistent alterations in mammary gland development, and the endocrine-disrupting activity of atrazine on rats [Enoch et al., 2007, Friedmann, 2002a]. Based on observations with mammals, the adverse effects of atrazine on humans was suggested [Greenlee et al., 2004, Hopenhayn-Rich et al., 2002], even though the direct carcinogenic effect of atrazine on humans still remains unclear [Mcelroy et al., 2007].

1.3 Degradation, dissipation and transformation of atrazine

After application, atrazine is subject to several dissipation and physical and chemical degradation pathways. Most losses are due to runoff, infiltration, adsorption and biological degradation [Bacci et al., 1989]. One of the predominant atrazine metabolites is hydroxy-atrazine [2-ethylamino-4-hydroxy-6-(isopropylamino)-*s*-triazine] (Figure 1.1 on page 1 B), resulting from abiotic and/or biotic degradation pathways via hydrolysis and/or microbial degradation in soils [Skipper et al., 1967, Takáts et al., 2001]. Hydroxy-atrazine is thought to be less mobile than atrazine or other atrazine metabolites, and is likely associated as a bound residue to the soil matrix [Russell et al., 1968, Helling, 1971]. As stated by Johnson *et al.*, contaminants like atrazine are not irreversibly bound, resulting in slow leaching of this substance to deeper soil and water layers [Johnson et al., 1999]. However, it is generally accepted that the formation of bound residues, resulting in less mobility or extractability of the pesticide in soil, is influenced by contact time [Lesan and Bhandari, 2004, Sorenson et al., 1993, Barriuso et al., 1991]. Soil constituents, particularly organic carbon, have an important influence on atrazine sorption processes [Blume et al., 2004, Jacques et al., 1999]. 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", representing a persistent pool of contaminants that are not entirely excluded from environmental interaction [Loiseau and Barriuso, 2002].

1.3.1 Bound residues formation

The environmental behavior of atrazine by addition of organic amendments, like plant residues, or its mineralization during bioremediation, field application and agricultural use has been studied intensively [Alvey and Crowley, 1995, Barriuso and Houot, 1996, Silva et al., 2004]. A number of studies have observed a so-called "bound residue" fraction of atrazine in soil [Capriel et al., 1985, Schiavon, 1988, Barriuso et al., 1991, Loiseau and Barriuso, 2002]. So far, studies on bound residues with labeled triazines have been conducted on short-term (weeks) [Barriuso et al., 2004] to at most mid-term (months) [Berns et al., 2005] time scales under laboratory conditions. These bound residues can approach 50 % of the initially applied atrazine even after long-term environmental aging [Capriel et al., 1985], and are mainly located in soil particle size fractions <20 μm [Loiseau and Barriuso, 2002]. Even though atrazine forms soil-bound interfaces it is still unclear whether these bound residues are bioavailable or represent a potential risk for future groundwater contamination. Since atrazine was found in bound forms nine years after its application [Capriel et al., 1985], it can be assumed that this chemical compound is not excluded from environmental interaction even after long-term aging under outdoor conditions. It has been suggested earlier that "wet-dry cycles in the environment may cause pulse inputs to the subsurface from the resistent herbicide pool. During dryer periods, desorption can replenish herbicide in soil solution [...]. If this material [atrazine] is not degraded, irrigation or rainfall can then flush it to the subsurface, where mobility is higher (less organic matter) and microbial numbers are lower" [Pignatello et al., 1993]. This suggestion might become crucial under changing environmental conditions. Soil organic matter is a key factor in the retention of atrazine by soils and the formation of bound residues [Loiseau and Barriuso, 2002], as mentioned above.

The adsorption and binding of pesticides such as atrazine is dependend on several mechnisms. Most important are ionic bonding, ligand exchange, hydrogen bonding, Van der Waals forces, charge transfer interactions, and hydrophobic binding as the main binding mechanisms. Due to its chemical structure the adsorption and binding of atrazine and other s-triazines in soils is mainly related to the soil carbon content, the hydrophobicity, and aromaticity of humic acids in soils [Celano et al., 2008]. As stated, soils with high organic matter and clay content and low pH result in greater atrazine sorption and subsequent hydrolysis [Lerch et al., 1999a]. The sorption of "hydroxylated atrazine degradation products (hydroxyatrazine, deethylhydroxyatrazine, and deisopropylhydroxyatrazine) were found to occur by mixed-mode binding resulting from two simultaneous mechanisms: (1) cation exchange and (2) hydrophobic interaction, whereas cation exchange was a more important binding mechanism to soils than hydrophobic interaction" [Lerch et al., 1997a].

1.3.2 Atrazine degradation in the environment

Triazines and their environmental behavior have been the subject of numerous investigations [Barriuso et al., 2004, Mandelbaum et al., 1995, Cook and Hütter, 1981, Kontchou and Gschwind, 1995, Berns et al., 2005].

According to Anderson, biodegradation of herbicides in soils is generally influenced by three factors: 1. the ability of microorganisms to degrade the herbicide, 2. the quantity of these microorganisms in the soil and 3. the activity of exoenzymes responsible for the degradation of the herbicide [Anderson, 1984]. Most findings regarding fungal or microbial [Kaufman and Blake, 1970, Mandelbaum et al., 1993, Assaf and Turco, 1994a] and chemical [Blumhorst and Weber, 1994] degradation are based on laboratory or short-term field experiments which have limited relevance to long-term outdoor trials. The estimated half-life of atrazine from these short-term tests ranges between a

few days to about one year [Kruger et al., 1993, Accinelli et al., 2001]. The atrazine degradation potential of soil microorganisms is mainly influenced and depending on application history [Shaner and Henry, 2007a], soil depth [Miller et al., 1997], soil moisture content [Kruger et al., 1993], temperature [Dinelli et al., 2000], and soil properties such as soil pH and presence of other nutrients such as nitrogen and/or carbon [Abdelhafid et al., 2000a, Assaf and Turco, 1994b, Gan et al., 1996, Moorman et al., 2001b, Alvey and Crowley, 1995, Mandelbaum et al., 1995, Shapir et al., 1998, Katz et al., 2000, Houot et al., 2000, Abdelhafid et al., 2000b, Moorman et al., 2001a, Guillen Garces et al., 2007]. 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 [Benoit et al., 2000]. The half life of atrazine limited by microbial degradation and fixation by binding and sorption ranges from 4-57 weeks [RSC, 1991, Best and Weber, 1974, Cohen et al., 1984]. 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 [Winkelmann and Klaine, 1991]. 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) [Barriuso et al., 1991] and the clay content [Koskinen and Clay, 1997].

A number of soil microorganisms such as fungi and bacteria capable of the biotransformation and mineralization of atrazine were described and isolated from different soils, mostly related to atrazine application history, as is the case in agriculturally used soils or pesticide spill sites [Levanon, 1993, Mirgain et al., 1993, Mougin et al., 1994, Mandelbaum et al., 1995, Fadullon et al., 1998, Struthers et al., 1998a]. In recent years, accelerated atrazine mineralization in the field has been observed in several places

[Vanderheyden et al., 1997b, Pussemier et al., 1997, Shaner and Henry, 2007b]. This finding is clearly correlated to the atrazine application history in these soils [Yassir et al., 1999, Bridges et al., 2008]. This observation led to the conclusion that soil microflora have become adapted to atrazine, using this chemical as a nitrogen and carbon source to sustain microbial growth [Houot et al., 2000, Abdelhafid et al., 2000b].

As a consequence of this, the effect of atrazine as a herbicide is modified by the reduced persistence of this chemical in atrazine-adapted soils [Krutz et al., 2007]. As recently stated, "the potential for cross-adaptation among s-triazine herbicides and the subsequent reduction in the control of otherwise sensitive weed species" is possible [Krutz et al., 2008a]. Time will show the extent to which this ecological adaption will sustainably affect the crop yield in these regions. However, the potential of an enhanced contamination of soils and water due to higher application amounts in order to achieve the required herbicidal effect of atrazine should be considered. However, the search for new solutions and alternatives will challenge pesticide-producing companies. In non-atrazine-adapted soils the mineralization and dissipation due to microbial activity will remain unpredictably small, also influenced by abiotic factors such as temperature, soil pH, "soil nutrients" like organic carbon or nitrogen, and soil moisture content. Therefore, the half-life calculation of atrazine in the field on long time scales must be seen critically.

1.4 Intention and goals of the presented doctoral research

A noticeable number of investigations concerning atrazine and its metabolites have been undertaken, but as yet little is known about the long-term behavior of this widely applied

herbicide. Therefore, investigations of the long-term behavior of atrazine are of continued importance because this herbicide is still widely used throughout the world.

No studies have been conducted with radioactively labeled triazine residues aged on a long time scale (> 20 years) under field conditions. The present study closes this knowledge gap, facilitating a further assessment of environmental and potentially also health risks, and provides data on long-term aged ^{14}C-labeled atrazine and its 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 dates back 22 years. "Atrazine residues" in this doctoral thesis refers to the parent compound and all possible metabolites. Since uniformly triazine ring ^{14}C-labeled atrazine was applied, residual atrazine equivalents can be calculated on the basis of the specific radioactivity.

This doctoral research was focused on the key aspects as follows.

1.4.1 Location, desorption and characterization of aged ^{14}C-labeled atrazine residues

The first part of the study was conducted to localize and quantify the distribution of ^{14}C-labeled atrazine residues in soil. Further, this research deals with the desorption capability of ^{14}C-labeled atrazine residues naturally aged for a long period (>20 years). The overall objective of this research was to detect, characterize and quantify water-desorbable atrazine residues in different layers of an agriculturally used lysimeter soil after 22 years of natural aging. A further aim was to develop a simple analytical methodology to de-

tect these residues after non-exhaustive cold water shaking extraction as the experimental part of a soil aggregate fractionation via liquid chromatography tandem mass spectrometry (LC-MS/MS). Characteristics of ^{14}C-labeled atrazine residues in soil aged under outdoor agricultural conditions have not been reported to date. These data may provide important additional information concerning soil and potentially also groundwater risk assessment.

1.4.2 Extraction and characterization of aged ^{14}C-labeled atrazine residues

The objective of this study was to quantify and characterize the atrazine residues still present in the surface soil of the outdoor lysimeter soil (0-10 cm) and in the lowest lysimeter increment (55-60 cm) consisting of fine gravel. Since most residual ^{14}C-activity was detected in these layers, the aim was to evaluate whether the detected ^{14}C-activity was associated with the parent compound atrazine and/or with other atrazine metabolites.

1.4.3 Evaluating bioaccessibility of aged ^{14}C-labeled atrazine residues in soil and soil size aggregates

This part of the study was performed to analyze the mineralization of the aged ^{14}C-labeled atrazine residues and 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. A further aim was to study the differences in bioaccessibility of ^{14}C-labeled atrazine residues by *Pseudomonas* sp. strain ADP in different soil size aggregates, and to evaluate accelerated bioaccessibility following the addition of carbon sources.

1.4.4 Evaluating bioaccessibility of aged, soil bound ^{14}C-labeled atrazine residues

Numerous investigations have been undertaken concerning the extraction and estimation of the bioavailability of atrazine and atrazine residues. Barriuso *et al.* determined the potential bioavailability of atrazine on the basis of laboratory experiments using a solvent extraction [Barriuso et al., 2004]. In the literature, most extractions were performed using organic solvents or liquids which influence the soil matrix and soil biology dramatically. In this study, a water-based gentle shaking extraction using mineral medium or just deionized water was applied. A similar approach to this study was performed by Kristensen *et al.* describing the successful mineralization of short-term aged atrazine and mecoprop by the specific triazine mineralizing strain *Pseudomonas* sp. strain ADP [Kristensen et al., 2001]. The purpose of this study was to gather information on the bioaccessibility and degradation of these long-term aged soil-bound atrazine residues in an agriculturally used soil.

1.4.4.1 Introductory comments on the biodegradation and bioaccessibility studies of soil and soil-bound ^{14}C-labeled atrazine residues

Further, a wide range of data about the biodegradation of atrazine and its residues are available using different kinds of bacteria or fungi [Topp et al., 2000, Radosevich et al., 1995, Assaf and Turco, 1994a, Kaufman and Blake, 1970]. The most competent microorganism *Pseudomonas* sp. strain ADP was used for the experiments in this study and was first described by Mandelbaum *et al.* [Mandelbaum et al., 1995]. Since most studies were performed under laboratory conditions or with atrazine aged naturally for only a short time little is known about the biodegradation of long-term aged atrazine "bound residues" in cultivated soil under outdoor conditions. As already stated in a study

by Regitano *et al.* on simazine [Regitano et al., 2006], strongly sorbed herbicides are slowly available over time even though aging of pesticides in soil decreases bioavailability [Park et al., 2003, Barriuso et al., 2004]. While many soils still contain atrazine and its residues from former or current applications particularly in the vadose zone, influences of soil use and soil management are of further interest concerning risk assessment, environmental fate, and remediation processes.

2 Material and Methods

2.1 Soil characteristics and atrazine application

The experimental soil originated from a long-term lysimeter study used for maize conducted at the Bavarian State Research Center for Agriculture (LfL), Munich, Germany. The soil was a gleyic cambisol (18 % sand, 64.4 % silt, 17.6 % clay; 1.45 % organic carbon content (0-10 cm soil layer); for details see Table 3.1 on page 34) originating from Puch, Fürstenfeldbruck in Bavaria, Germany. The plastic lysimeter (dimensions: 49x49x73 cm, with a surface area of 0.24 m^2) was filled in 1979 in accordance with the natural soil layers of the field of origin. Subsequently, the lysimeter was part of a long-term study of corn production in a field plot. Corn was planted annually and the lysimeter-soil treatments under outdoor conditions were in accordance with agricultural practice until the end of the experiment in 2005. Since 1982 three consecutive applications of uniformly ^{14}C-ring-labeled atrazine and non-labeled atrazine were performed. The time and amount of application is presented in Table 2.1 on the next page. The amount of atrazine at each application was approximately 1.7 kg ha^{-1}, corresponding to the recommended agricultural application dose of 1.7 - 2.8 kg ha^{-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. The atrazine and ^{14}C-ring-labeled atrazine with a chemical purity of 99.7 % were purchased from the former Ciba-Geigy. The lysimeter soil was solely used for annual maize cultivation with minimum tillage until August 2005. No plowing simulation was performed. After maize harvesting crop residues were removed manually.

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	

Table 2.1: Time of application and applied quantities of non-radioactive and radioactive atrazine as active ingredient as well as the applied ^{14}C-radioactivity.

For the statistical analysis the independent two sample t-test was applied in order to determine the significance of differences between the mean values. Significance values are given in figures and tables as Si in %.

2.2 Physico-chemical analysis

2.2.1 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, 55-60 cm (55-60 cm consisted of fine gravel).

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) and pulverized in a mortar. The gravel samples were sieved (5 mm) dry to separate gravel from intruded soil and clay particles. Penetrated roots were removed by hand. Prior to combustion and extraction, a subsample of 125 g of oven dried (105°C) fine gravel was crushed and homogenized using a Planetary Mill (350 rpm, 45min; Planetary Mill PM 400, Retsch). Additional drying experiments showed no significant differences of residual ^{14}C-activity when the samples were dried at 105°C or dried via freeze drying. Calculations of residual ^{14}C-activity and atrazine residues in the lysimeter as a whole were based on estimated soil bulk density of 1.5 g cm^{-3} and gravel of 1.8 g cm^{-3}. For quantification of residual ^{14}C-activity, freeze dried and homogenized subsamples of soil or gravel (nine replicates, each 1-2 g dry-weight) were weighed into porcelain vials for combustion using a Biological Oxidizer OX500 (R.J.Harvey Instrument Corporation). Emerging $^{14}CO_2$ was trapped in Oxysolve C-400 scintillation cocktail (Zinser Analytik). Radioactivity was detected by liquid scintillation counter (LSC) using a 2500 TR, Tri-Carb, Packard Liquid Scintillation Analyzer by internal standard.

2.2.1.1 Soil aggregate fractionation

The soil aggregate fractionation was performed in accordance with a previous study by Burauel and Bassman [Burauel and Bassmann, 2005]. 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 described by the 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.

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.

2.2.2 Liquid sample preparation and analysis

Subsamples of the aqueous DOM-fraction were analyzed for desorbed residual ^{14}C-activity in triplicates. Five 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 using an external standard (2500 TR, Tri-Carb, Packard Liquid Scintillation Analyzer).

The dissolved total organic carbon (TOC) and non-purgeable organic carbon (NPOC) content was measured prior to further treatments (Shimadzu Total Organic Carbon Analyzer, TOC-5050A, Shimadzu, ASI-5000A Auto Sampler). Results for desorbed ^{14}C-activity, pH and carbon content are given in Table 3.5 on page 46. The remaining DOM-fraction was concentrated by freeze drying to a residual volume of 1 - 3 mL prior to further LC-MS/MS

analysis. Samples were transferred into 2 mL Eppendorf tubes and centrifuged for sedimentation of the particles for 5 min at 15,000 *g* (Hettich Mikro Rapid). Residual particles of the concentrated DOM-fractions in the bottle used for freeze-drying were dissolved in 2 mL of pure methanol (HPLC-grade) and treated as above.

2.2.2.1 Desorption/extraction 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 containing ^{14}C-atrazine residues 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 (the composition of the mineral-media is described in detail in chapter "Microbial cultures and conditions", 2.3 on page 26), 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, $MgSO_4 \cdot 7H_2O$, $FeCl_3$ 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 3.2 on page 42.

As reported in several previous studies [Barriuso et al., 2004, Eric et al., 2003, Blume et al., 2004], 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.

2.2.2.2 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 as described above (see Chapter 2.2.2.1 on the previous page), which were used for soil fractionation and desorption studies 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).

2.2.2.3 Preparation of the DOM-fraction and desorption/extraction liquids prior further analysis and metabolite detection

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 extraction 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 tubes and centrifuged for sedimentation of the particles for 5 min at 15,000 g (Hettich Mikro Rapid).

2.2.2.4 Accelerated solvent extraction of the soil and gravel samples

An accelerated solvent extraction (ASE) device (ASE 200, Dionex) was used to extract the soils. The ASE-extraction was similar to the extraction method previously described by Gan *et al.* [Gan et al., 1999]. In this study, a methanol-water solution (4:1 v:v) was used for extraction since results showed slightly higher residual ^{14}C-activity in the extracts than when using methanol alone, consistent with previous findings [Huang and Pignatello, 1990]. For extraction, triplicates of 10 g freeze dried (Lyovac GT2, Steris) and homogenized soil or 10 g of powdered and homogenized gravel samples were weighed into 11 mL stainless steel ASE cells. The remaining space above the samples was filled with fine, annealed sand (Merck) to reduce the extract volume and to avoid clogging of the ASE steel filter lid. The extraction temperature was 135°C at 100 bar (1500 psi) with a flush volume of 60 % of extraction cell volume. The heat-up time was 5 min, static time 15 min and the total extraction time 15-18 min. Each sample was extracted eight consecutive times under the same ASE conditions to determine extraction efficiency and to recover most of the extractable fraction. To determine ASE-extracted residual ^{14}C-activity, a triplicate of 0.5 mL of each extract sample was mixed with 3.5 mL scintillation cocktail (Instant Scint-Gel Plus^{TM}, Perkin-Elmer) and detection of radioactivity was performed by LSC. An external standard was used for quenching correction.

2.2.2.5 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. 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 and quantification for radio-HPLC was 40 Bq mL^{-1}, and for UV-HPLC 0.1 mg L^{-1}, respectively.

2.2.3 LC-MS/MS-analysis

LC-MS/MS was the method of choice for characterizing and quantifying the nature of the desorbed and extracted atrazine residues since previous UV-HPLC and radio-HPLC (as described above, 2.2.2.5 on the preceding page) analysis showed no results.

For LC-MS/MS-analysis a TSQ-Quantum 2002 (Thermo Electron) equipped with CTC-HTC-PAL sampler, and HPLC (Agilent) with binary pump and thermostatted column compartment (Agilent Serie 1100) was used. The applied HPLC column (Perfect Sil Target ODS-3, 125 mm × 2.1 mm × 3 μm), used with an additional HPLC pre-column (Perfect Sil Target ODS-3, 1 cm × 2.1 mm × 3 μm), was purchased from MZ-Analysentechnik Mainz. The applied HPLC eluents, in accordance with Takáts *et al.* [Takáts et al., 2001] (Table 2.2 on the next page), were 0.1 M ammonium acetate solution (eluent A) and acetonitrile (Riedel De Haen, 99.9 % purity, eluent B). The applied gradient conditions were 83 % eluent A and 17 % eluent B at time 0-5 min changing to 100 % eluent B at

time 20 min (Table 2.2). The flow rate was 0.15 mL min^{-1} and the column temperature was 25 °C. The injection was performed in triplicate and the total injection volume of each sample 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.

Atrazine (chemical purity: 97.4 %) and its metabolites such as desethyldesisopropyl-2-hydroxy-atrazine (98.5 %), desisopropyl-2-hydroxy-atrazine (99.0 %), desethyldesisopropyl-atrazine (98.0 %), desethyl-2-hydroxy-atrazine (99.0 %), desethyl-atrazine (99.9 %), desisopropyl-atrazine (96.1 %) and 2-hydroxy-atrazine (96.0 %) were purchased from Riedel-de Haën.

First, a compound separation was obtained by HPLC (Table 2.2). For lower detection limits the fragmentation of atrazine and its metabolites was studied and the most intensive fragmentation in each case was selected. All the transitions were measured in parallel, which is known as the above mentioned MRM.

	Liquid gradient	
	Eluent A	Eluent B
Time	0.1 M ammonium-acetate solution	acetonitrile
[min]	[%]	[%]
0	83	17
5	83	17
20	0	100
25	0	100
30	83	17
40	83	17

Table 2.2: Gradient program conditions applied for the HPLC associated with LC-MS/MS.

Since 2-hydroxy-atrazine was the only metabolite to be detected, deuterated (D_5)-atrazine

and (D_5)-2-hydroxy-atrazine (Dr. Ehrenstorfer GmbH, Germany) with a concentration of 0.01 μg mL^{-1} was used as internal standard for quantification. From each liquid sample 100 μL was mixed with 100 μL of D_5 STD standard solution resulting in 0.001 μg 100μL^{-1} of injected sample.

All the settings for the analysis and the mass transfer of all possible atrazine metabolites are given in Table 2.3 on the facing page. The analytical detection limit using LC-MS/MS for atrazine and 2-hydroxy-atrazine was 0.125 ng mL^{-1} liquid. The method detection limit was 0.188 ng g^{-1} soil extracted.

MS conditions:					
ESI	polarity ESI positive	MRM mode	scan width 0.2 [Da]	scan time 0.4 sec	peak width (FWHM) 0.7
Tune file parameters:					
spray voltage 4500 [V]	sheath gas pressure 45 [psi]	aux gas pressure 10 [arbitrary units]	ion transfer capillary 230 [°C]	collision cell pressure 1 [mTorr]	
parent mass [Da]	product mass [Da]	collision energy [V]	tube lens [V]	molecule	retention time [min]
128	86.2	20	155	desethyldesisopropyl- 2-hydroxy-atrazine	4.46
156	69.1	36	209	desisopropyl- 2-hydroxy-atrazine	4.75
146	104.0	24	186	desethyldesiso- propyl-atrazine	5.78
170	128.1	22	207	desethyl- 2-hydroxy-atrazine	5.45
188	146.1	24	180	desethyl- atrazine	17.19
174	104.1	28	178	desisopropyl- atrazine	10.46
198	156.1	24	198	2-hydroxy- atrazine	15.48
216	174.1	24	189	atrazine	21.8
203	161.2	26	192	D_5-standard 2-hydroxy-atrazine	15.21

Table 2.3: Conditions applied for LC-MS/MS analysis of associated ^{14}C-labeled atrazine residues in liquid samples. Abbreviations: ESI electrospray ionization; MRM multiple reaction monitoring; Da Dalton; FWHM full width at half maximum; V voltage

2.2.3.1 Elementary analysis of solid samples

Before analysis, homogenized subsamples were dried for 3 h at 105°C. For elementary (Al, Ca, Fe, K, Mg, Na, Si) analysis of soil, soil size aggregates and gravel, 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 30-50 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.

2.3 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.* and incubated on a rotary shaker at 90 rpm and 28 °C [Mandelbaum et al., 1993]. The medium consisted of K_2HPO_4 1.6 g L^{-1}, KH_2PO_4 0.4 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.026 g L^{-1}, saccharose 1.0 g L^{-1}, $C_6H_5Na_3O_7{\cdot}2H_2O$ 1.14 g L^{-1}, basic saline solution 20 mL L^{-1} (consisting of: EDTA 2.5 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 19.8 g L^{-1}, $FeSO_4{\cdot}7H_2O$ 9.14 g L^{-1}, $MnSO_4$ H_2O 1.54 g L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.4 g L^{-1}, $CoSO_4{\cdot}7H_2O$ 0.24 g L^{-1}, $Na_2B_4O_7{\cdot}10H_2O$ 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 [Sambanis, 1985], 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 $MgSO_4 \cdot 7H_2O$ 10.0 g L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 1 g L^{-1} and 1 mL of component III consisting of NaCl 10 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 26 g L^{-1}, $Na_2EDTA \cdot 2H_2O$ 2.8 g L^{-1}, $FeCl_3 \cdot 6H_2O$ 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 ± 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.* with the addition of other carbon sources [Mandelbaum et al., 1995]. 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 ± 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.

2.3.1 Biodegradation study of soil-bound ^{14}C-atrazine residues: soil sample extraction

For extraction, 10 g of dry soil sample equivalents were shaken with 80 ml of a) mineral media, b) mineral media plus citrate and c) deionized water. These extraction liquids were chosen since the impact on soil structure is supposed to be smaller when compared to solvent extraction. Mineral media (MM), as described above in chapter 2.3 on page 26, was consecutively used for bioaccessibility studies of the bound-residues. For the soil-slurry setup with additional citrate as a carbon source 2 gL^{-1} of Na-citrate was added. Samples were shaken in Beckman PE-centrifuge bottles for 6 h at 150 rpm on a horizontal shaker (SM 25, Edmund Büler). After extraction the extracted soils were centrifuged at 10,000 *g* for 90 min (Beckman J2-21, Rotor JA 14). The supernatant was separated and the volume quantified. Triplicates of 1 ml of the supernatant liquid were analyzed for extracted ^{14}C-activity via LSC, using 3.5 ml of Insta-Gel Plus (Perkin Elmer) as liquid scintillation cocktail. The quantified volume and the detected ^{14}C-activity of the extracts was used for calculation of the remaining soil-bound ^{14}C-residues in the samples. The remaining ^{14}C-activity was equaled to 100 % for the biodegradation study on soil-bound residues, detected as evolved $^{14}CO_2$.

2.3.2 Biodegradation study of soil-bound ^{14}C-atrazine residues: soil-slurry inoculation

Soil-slurry setups of the previously extracted soil were performed in triplicates. To each setup 10 ml of a) MM, b) MM plus citrate and c) deionized water with cells of *Pseudomonas* sp. strain ADP and a cell density of 1.8-3 $\times$ 10^8 cells per ml were added. Extracted soil samples and liquid bacteria suspension were mixed by vigorous shaking. A glass vial

containing 1.5 ml 0.5 M NaOH solution was placed inside the PE bottles containing the extracted soil samples and bacteria inoculum to trap evolved $^{14}CO_2$. Hermetically closed samples were incubated on a rotary shaker (125 rpm) at room temperature (22 $\pm$2 °C).

3 Results and Discussion

3.1 General comments

After more than 20 years of aging under outdoor conditions, atrazine as the parent compound is still present in the soil (refer to Chapter 3.3.5 on page 50; results see Table 3.6 on page 52). Besides atrazine, the metabolite 2-hydroxy-atrazine represents the major identifiable and quantifiable component in the soil extracts; these findings are consistent with the results of Capriel and Haisch, however, after a much shorter incubation time [Capriel and Haisch, 1983]. As presented below, the residual ^{14}C-activity in the complete lysimeter soil corresponds to 25 % of the total initially applied ^{14}C-atrazine activity. This finding gives important information about the general turnover of pesticide-associated carbon in the soil. Even though a considerable portion of ^{14}C-activity could not be extracted and analyzed it is to be assumed that ^{14}C-activity is associated within the *s*-triazine ring structure. The percentage of residual ^{14}C-activity in the top soil layer (0-10 cm) is equal to 8 % of the total mass of atrazine initially applied to the lysimeter. In the fine gravel layer (55-60 cm), 4 % of the initially applied ^{14}C-activity was detected after separating the gravel from soil particles and intruded root detritus (Table 3.6 on page 52).

The presence of organic carbon sources stimulating atrazine degradation by microbial activity has been studied using citrate amendment and other carbon compounds

[Silva et al., 2004, Assaf and Turco, 1994b]. Neither soil-intruded nutrients, such as plant detritus and root exudates of the annual corn plantations, nor regular fertilizer application, could promote complete atrazine degradation via biological or physico-chemical processes during more than 20 years under environmental influences in the soil of this investigation. Although a lysimeter study might have limited direct interaction with the surrounding field soil, it does provide relevant data on pesticide behavior *in situ*, under real environmental conditions. *In situ* lysimeter studies provide a realistic and comparable system to investigate chemical processes and can often readily be expanded to large-scale calculations.

3.2 Chemico-physical and ^{14}C-activity analysis of solid samples

3.2.1 Soil and soil-aggregates

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 3.1 on the next page).

These results correspond to previous studies where most residual atrazine was found in the upper soil layer, but after much shorter periods of aging time [Wagner and Chahal, 1966, Capriel et al., 1985, Kruger et al., 1993]. Results of the originally applied ^{14}C-ring-labeled atrazine and the physico-chemical characteristics of each soil layer are given in Table 3.1 on page 34.

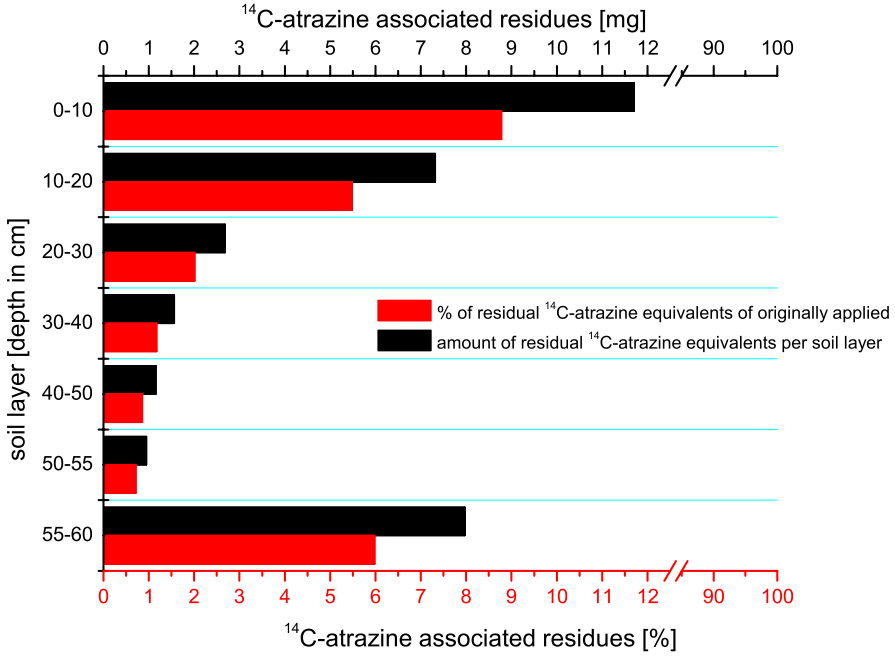

Figure 3.1: Measured and calculated ^{14}C-labeled atrazine residues of each soil layer in average. The percentage indicates the ^{14}C-labeled atrazine associated residues of initially applied total ^{14}C-labeled atrazine.

Soil-layer [depth in cm]	^{14}C-activity [Bq g^{-1}]	C_{org} [%]	C_{inorg} [%]	C_{Total} [%]	Al [%]	Fe [%]	K [%]	Mg [%]	Ca [%]	Na [%]
0-10	136.95 ±5.31	1.450 ±0.008	0.032 ±0.001	1.482	4.68	2.26	1.44	0.49	0.45	0.70
10-20	85.63 ±3.96	0.924 ±0.004	0.023 ±0.005	0.947	4.75	2.28	1.41	0.50	0.45	0.69
20-30	31.42 ±1.55	0.626 ±0.005	0.010 ±0.002	0.635	5.44	2.68	1.51	0.60	0.50	0.70
30-40	18.27 ±0.87	0.424 ±0.005	0.006 ±0.001	0.430	6.20	3.26	1.59	0.72	0.49	0.66
40-50	13.42 ±0.79	0.357 ±0.002	0.007 ±0.001	0.364	6.23	3.29	1.69	0.82	0.89	0.73
50-55	11.14 ±0.78	0.350 ±0.002	0.020 ±0.002	0.371	6.23	3.27	1.65	0.74	0.53	0.72
55-60	93.25 ±5.61	1.137 ±0.012	7.017 ±0.005	8.153	2.12	1.63	0.56	4.02	16.18	0.25

Table 3.1: Residual ^{14}C-atrazine activity in the soil. ± mean standard deviation (n = 3). Values for carbon, aluminium, iron, potassium, magnesium, calcium and sodium are in %. ± mean standard deviation (n = 3). The mean standard deviation of three replicates for Al, Fe, K, Mg, Ca and Na is ± 3%.

In total, 25 % of the applied ^{14}C-activity is still present in the lysimeter soil. The overall loss of ^{14}C-activity must be attributed to a combination of the processes of plant uptake, mineralization, volatilization and leaching. 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. The deepest layer (55-60 cm), consisting of fine gravel (drainage purposes) and intruded soil and root detritus, showed an increased ^{14}C-activity of 6 % of the initially applied amount (Figure 3.1 on page 33). The results of residual ^{14}C-activity are highly correlated with the organic carbon content in the corresponding soil layer (p-value: 0.99, for data see Table 3.1 on the facing page). These results correspond to previous studies giving clear indications that organic carbon in soil is a long-term sink for atrazine and/or its metabolites [Lesan and Bhandari, 2004]. Capriel *et al.* demonstrated that most of the herbicide residues were associated with soil organic matter [Capriel et al., 1985]. It can be assumed that >20 years after the last application of ^{14}C-labeled atrazine the soil-associated ^{14}C-labeled atrazine residues have reached a distribution equilibrium. Therefore, these results identify the long-term environmental behavior of the herbicide atrazine and/or its metabolites in an agricultural soil, indicating that most residues are still located in the upper soil layer, when minimum- or no-tillage soil treatment was applied. As reported by Pignatello *et al.*, environmental dry-wet cycles may cause pulse inputs to the subsurface from the resistant herbicide pool [Pignatello et al., 1993]. Less organic matter in the subsurface may subsequently cause higher mobility of the residues, and microbial degradation is lower due to limited microbial populations. Since the lowest soil layer functioning as a drainage layer showed similar residual ^{14}C-activity compared to the first 10 cm, it is likely that the major fraction of previously applied atrazine disappeared by leaching. The accumulation of the residual atrazine-^{14}C-activity in this soil layer was mainly associated with organic plant detritus and an accumulation of clay-sized aggregates.

Table 3.2 presents the measured residual ^{14}C-labeled atrazine activity in the different soil size aggregates. The given difference for ^{14}C-activity in the total soil when compared to the values given in Table 3.1 on page 34 (125 ±2.9 vs 136.95 ±5.31 Bq g^{-1}) must be attributed to irregularities during the oxidation process. Further ^{14}C-analysis of the total soil resulted in well corresponding results to the values of 125 Bq g^{-1} as given in Table 3.2. All further ^{14}C-activity calculations were based on an average ^{14}C-activity of 125 Bq g^{-1}.

	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_{inorg}	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

Table 3.2: 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%.

The aqueous DOM-fraction (particle sizes <0.05 μm; pH 6.1 ± 0.2) showed only minor desorbed ^{14}C-activity of 0.38 Bq mL^{-1} (± 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 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 [Piccolo et al., 1992, Sposito et al., 1996], hydrogen bridges [Li and Feldbeck, 1972, Martin-Neto et al., 1994] and hydrophobic exchange reactions [Lerch et al., 1997b, Martin-Neto et al., 1994]. 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 by the applied shaking extraction, respectively.

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.*, with 0.5 μg atrazine residues g^{-1} dried aggregates [Barriuso et al., 1991]. Due to the high content of C, Al, Fe and Si (Table 3.2 on the facing page) 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 [Herwig et al., 2001]. 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.* and Kovaios *et al.* in model studies [Clausen and Fabricius, 2001, Kovaios et al., 2006]; whereas, Si-binding using silica gel is reversible [Kovaios et al., 2006].

3.2.2 Analysis of solid samples after AS-extraction

Even after eight consecutive extraction steps, approximately 40 % and 7 % of the ^{14}C-activity could not be extracted from the soil and gravel, respectively. It remains unclear whether the soil-bound residues are the parent compound atrazine or its metabolites. Regardless, the residues are sequestered into soil organic matter compounds or entrapped within nanostructures of other organic soil compounds such as humic acids. The major

role of humic substances in the sorption of hydrophobic organic substances has long been known. Abate *et al.* found an increased adsorption of hydroxy-atrazine and atrazine onto humic acid enriched soil that might support this suggestion [Abate et al., 2004]. Obviously, unspecific ^{14}C-activity might also be part of the soil carbon pool as a result of microbial or chemicophysical degradation processes. Further investigations are in progress to determine the nature of the soil-bound atrazine residues. However, the results of combusted soil and gravel samples after consecutive ASE-extractions gave overall recoveries of about 100 % indicating adequate analytical preparation of the samples and detection of residual ^{14}C-activity by the used methods. It can be estimated that increased extraction efficiency of soil from the top layer by different extraction setups utilizing other solvents, chemical derivatization by silylation [Haider et al., 2000], pH or temperature, might result in a higher quantifiable yield of atrazine. As found previously, up to 50 % of bound residues were associated with the parent compound atrazine and could be released by vigorous extraction [Loiseau and Barriuso, 2002]. The fact that atrazine is still detectable provides evidence that soil-bound ^{14}C-activity in the upper soil layer is at least partly associated with the parent compound atrazine. Thus, further continuous leaching into deeper soil horizons must be considered.

As indicated earlier, the estimated half-life of atrazine ranges between days to months in previous short-term studies; clearly, these data must be reconsidered. Assuming a first-order decay of the parent compound atrazine with an expected environmental half-life of 1 year (approximately the longest half-life reported in the literature, e.g. [Jones et al., 1982]) and a total amount applied of 133.3 mg, the residual atrazine in the lysimeter is calculated to be as little as 0.2 μg in total. In clear contrast to that the amount detected is 110 μg, taking only the extracted atrazine from soil layer 0-10 cm and 55-60 cm into consideration. This is approximately 550 times higher than expected according to the calculation. Estimating the persistence of atrazine or likely other triazine pesticide compounds in soils under environmental conditions by calculating the half-life from short-term experiments is

highly problematic. Despite several chemical and biological pathways of atrazine degradation, as well as plant uptake and sequestration over time, the presented environmental long-term persistence is unexpectedly high and is crucial for accurately describing triazine herbicide fate in soils.

3.2.3 Elementary analysis of solid samples prior to and after AS-extraction

Results for elementary analysis prior to and after AS-extraction are given in Table 3.3 on the next page. A considerably higher amount of Al and Fe, known for the adsorption affinity of various organic and inorganic compounds [Sawhney and Singh, 1997, Clausen and Fabricius, 2001], can be found in the surface soil layer. Nevertheless, the residual ^{14}C-activity is more likely associated with the organic carbon fraction, being almost equal in the upper soil and gravel layers. This assumption is in accordance with previous studies suggesting that retention of atrazine is mainly due to soil organic matter [Laird et al., 1994]. As shown in Table 3.3 on the following page, harsh extraction of soil and gravel samples did not noticeably change the amounts of the analyzed soil elements. It could be observed that the first extracts were slightly clear to yellow. It is likely that some of the extracted ^{14}C-activity was incorporated into humic substances that were subsequently extracted. The minor decrease of organic carbon content after extraction is likely the result of extracted humic substances from the soil; sequential extracts of the soils showed decreasing coloration with extraction number. As observed, single particles consisting of black porous cinder or intruded root detritus found in the gravel layer might have retained most of the residual ^{14}C-activity and associated atrazine residues due to their high surface area and organic carbon content. Further research concerning this matter is in progress.

element	C_{org}	C_{inorg}	N	Al	Ca	Fe	K	Mg	Na	P
sample	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
prior to extraction										
soil	1.42 $\pm$0.05*	0.07 $\pm$0.03**	0.19 $\pm$0.01***	4.75	0.44	2.23	1.43	0.49	0.89	0.12
gravel	1.38 $\pm$0.03$^{\diamond}$	8.99 $\pm$0.01$^{\diamond\diamond}$	0.05 $\pm$0.00$^{\diamond\diamond\diamond}$	1.54	20.1	0.99	0.4	4.87	0.2	0.02
after extraction										
soil	1.17 $\pm$0.31*	0.08 $\pm$0.07**	0.16 $\pm$0.01***	4.74	0.41	2.28	1.40	0.49	0.68	0.12
gravel	1.29 $\pm$0.01$^{\diamond}$	8.78 $\pm$0.03$^{\diamond\diamond}$	0.05 $\pm$0.00$^{\diamond\diamond\diamond}$	1.51	19.77	0.94	0.37	4.77	0.19	0.02

Table 3.3: Elements in soil and gravel prior to extraction and after extraction (weight percent). Prior to AS-extraction: $\pm$ standard deviation of $n = 3$. After AS-extraction: $\pm$ standard deviation of $n = 9$. Mean variation for Al, Ca, Fe, K, Mg, Na, P: for concentration >1%: $\pm$ 3%; for concentration <1% and >0.1%: $\pm$ 10%, and for concentration <0.1%: $\pm$ 20%. Significance (Si) is given for the differences within the soil (0-10 cm) and the gravel (55-60 cm) fraction before and after extraction. Significance for soil at: *>99.95% for C_{org}; **<75% for C_{inorg}; and ***>99.5% for N. Significance for gravel at: $^{\diamond}$>99.5% for C_{org}; $^{\diamond\diamond}$>90% for C_{inorg}; and $^{\diamond\diamond\diamond}$>99% for N.

3.3 Chemico-physical and ^{14}C-activity analysis of liquid samples

3.3.1 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 3.2 on the next page).

Since in the published studies [Loiseau and Barriuso, 2002, Barriuso et al., 2004] 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). Desorption in mineral-medium, with citrate or glucose, respectively, was found to be 8.7 % and was 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 3.4 on page 43). 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.

3.3.2 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. These results lead to the conclusion that the detected ^{14}C-activity in mineral-media after soil des-

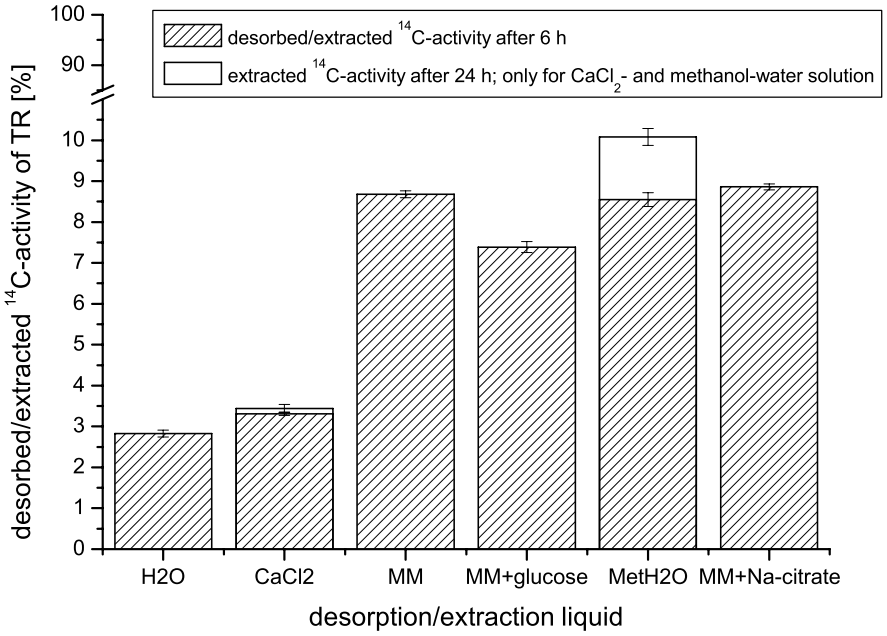

Figure 3.2: Desorption/extraction of ^{14}C-labeled atrazine residues in the soil with different solutions. Bars indicate desorbed/extracted ^{14}C-activity in % of total ^{14}C-activity in 10 g of soil dry weight. Indications on the X-axis: H2O: deionized organic free water; CaCl2: 0.01 M $CaCl_2$-solution; 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.

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
$MgSO_4 \cdot 7H_2O$ (0.8 g)	3.79 ±0.10	2.10 ±0.06	0.92 ±0.07
$FeCl_3 \cdot 6H_2O$ (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.

Table 3.4: 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.

orption (Chapter 3.3.1 on page 41, Figure 3.2 on the facing page) can be attributed to the ring ^{14}C-components of the originally applied ^{14}C-labeled atrazine and is therefore most likely associated with the extractable carbon fraction. It may be assumed that dissolved carbon associated with residual ring ^{14}C-components favors higher bioaccessibility, thus resulting in enhanced mineralization rates. However, taking the results of Chapter 3.5 on page 60 into consideration, this general assumption does not exclude potential bioaccessibility of soil-bound atrazine residues.

3.3.3 DOM-fractions from various soil depths

The liquid soil-water fraction or DOM-fraction resulted from a previously applied gentle physical soil aggregate fractionation. The total soil-water contact time was 24 h, including 6 h of rigorous shaking of the mixture. The ^{14}C-activity of desorbed ^{14}C-residues in the liquid was determined via LSC. The results of desorbed ^{14}C-activity are given in Figure 3.3 on the following page.

Results were calculated for one liter. The pattern of desorbed ^{14}C-activity corresponds to the residual activity in the soil matrix, shown in Figure 3.1 on page 33. The amount

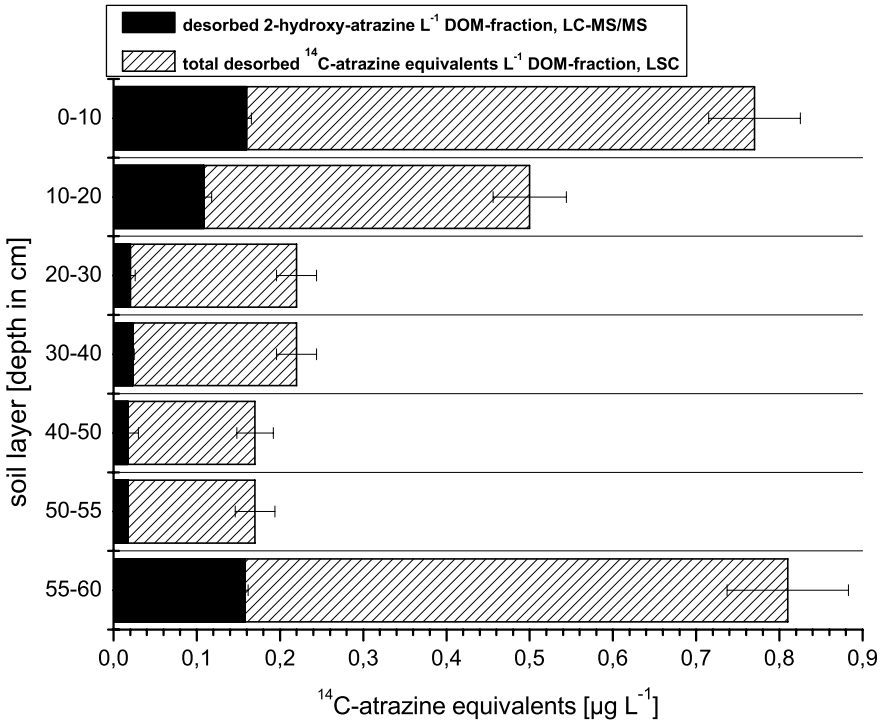

Figure 3.3: Amounts of total desorbed ^{14}C-atrazine equivalents in μg L^{-1} DOM-fraction, measured via LSC detection and desorbed 2-hydroxy-atrazine in μg L^{-1} DOM-fraction, detected via LC-MS/MS. Error bars indicate mean standard deviation of n = 9.

of detected ^{14}C-labeled residues decreases from 0.8 to 0.2 μg L^{-1} of sample liquid. This corresponds to 2 and 6 % of the ^{14}C-activity present in the soil sample, respectively (Figure 3.4).

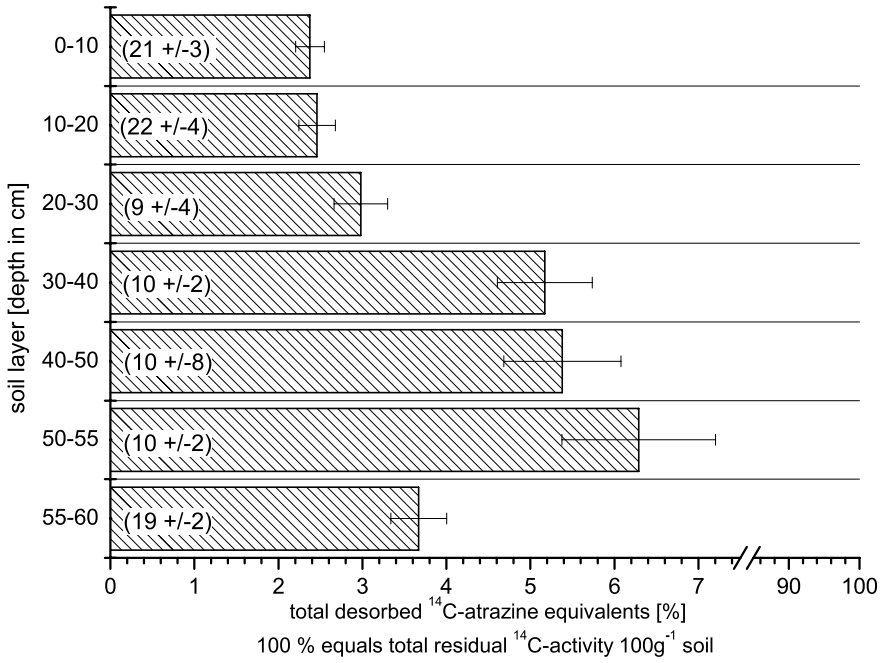

Figure 3.4: Total desorbed ^{14}C-atrazine equivalents in % of total ^{14}C-activity in the sample. 100 % equals total residual ^{14}C-activity 100g^{-1} soil of the individual soil layer. Numbers in parentheses indicate the percentage of 2-hydroxy-atrazine of total desorbed. Error bars indicate mean standard deviation of n = 9.

With increasing soil depth the desorbable fraction of ^{14}C-residues increased clearly. These results may provide evidence that ^{14}C-labeled atrazine residues in deeper soil layers are potentially less tightly bound and hence more mobile, reaching deeper soil layers by continuous leaching. Herewith naturally occurring desorption processes of atrazine residues from soil and mobilization processes can be assessed. The increase of the desorption ca-

pability of ^{14}C-labeled atrazine residues with increasing soil depth is positively correlated with the soil organic carbon content (p-value: 0.95) as well as with the TOC content in the DOM-fraction (p-value: 0.97; Table 3.5). This shows the overall relevance of organic carbon as a binding reactant in solid and liquid phases for atrazine and/or its metabolites.

DOM-fraction of soil-layer [depth in cm]	^{14}C-activity [Bq mL^{-1}]	pH	TOC [mg L^{-1}]	NPOC [mg L^{-1}]
0-10	0.33 ±0.011	5.89 ±0.04	4.80 ±0.11	4.46 ±0.23
10-20	0.21 ±0.009	6.56 ±0.37	3.30 ±0.17	2.55 ±0.17
20-30	0.09 ±0.005	6.99 ±0.06	2.56 ±0.88	2.20 ±0.79
30-40	0.09 ±0.006	7.25 ±0.11	2.67 ±0.28	2.94 ±0.21
40-50	0.07 ±0.005	7.43 ±0.00	1.90 ±0.29	1.69 ±0.12
50-55	0.07 ±0.005	7.79 ±0.01	2.68 ±0.12	3.72 ±1.21
55-60 soil particles	0.34 ±0.010	8.88 ±0.00	4.98 ±0.47	1.93 ±0.14
55-60 gravel	0.20 ±0.009	8.69 ±0.19	3.70 ±0.39	1.14 ±0.09

Table 3.5: Residual ^{14}C-atrazine activity, pH, total organic carbon (TOC) and non-purgeable organic carbon (NPOC) content in the DOM-fraction. ± mean standard deviation of nine replicates.

3.3.4 LC-MS/MS analysis of desorption liquids

Since the results given in Figure 3.3 on page 44 and Figure 3.4 on the previous page represent the quantity of desorbed ^{14}C-activity detected via LSC, LC-MS/MS analysis was applied in order to characterize the nature of the desorbed ^{14}C-labeled atrazine residues. LC-MS/MS had been successfully applied in previous studies to detect and characterize atrazine and its metabolites [Takáts et al., 2001, Abián et al., 1993, Nélieu et al., 1994]. Liquid samples were analyzed for all MRM transitions.

LC-MS/MS chromatograms for atrazine, 2-hydroxy-atrazine and other atrazine metabolites are shown in Figure 3.5 on page 48, with retention times of 20.99 and 16.67 min for atrazine and 2-hydroxy-atrazine, respectively. Atrazine and atrazine-metabolite standard solution

was added to non-contaminated soil prior to aqueous shaking extraction. As a control this figure demonstrates the clear determination of added atrazine and its metabolites via LC-MS/MS analysis applied as a standard mixture in the aqueous fraction.

The environmental behavior of hydroxy-atrazine is a matter of controversy. As reported by Brouwer *et al.* and Sorenson *et al.*, hydroxy-atrazine shows high adsorption and low desorption in soil suggesting that at depths greater than 20 cm hydroxy-atrazine is due to *in situ* degradation of atrazine previously translocated to those depths [Brouwer et al., 1990, Sorenson et al., 1993]. However, the amount of desorbed 2-hydroxy-atrazine from subsamples of each soil layer given in Figure 3.3 on page 44 ranges from 0.16 in the upper layer to 0.017 μg L^{-1} DOM-fraction in the 50-55 cm soil layer. From the lowest, coarse layer at 55-60 cm the amount desorbed was 0.16 μg L^{-1} DOM-fraction, and was therefore similar to soil from the top layer. As given in Figure 3.4 on page 45 the amounts of desorbed 2-hydroxy-atrazine represent 21 % of the total desorbed ^{14}C-activity in the two upper soil layers and 10 % in the 50-55 cm soil layer, respectively. The amount of 2-hydroxy-atrazine with respect to the total desorbed ^{14}C-activity from samples of the intermediate soil layers (20 to 55 cm of depth) was around 10 %. These results are in accordance with those obtained by Mahía *et al.* and Lerch *et al.* showing that hydroxy-atrazine was the main long-term metabolite found in soil [Mahía et al., 2007, Lerch et al., 1999b]. It is unclear whether these results are due to the previously mentioned *in situ* transformation of atrazine in deeper soil layers or due to direct leaching of hydroxy-atrazine from the surface layer. But the accumulation in the 55-60 cm layer underlines the leaching character of 2-hydroxy-atrazine. The major fraction of non-characterized ^{14}C-activity in the liquid is due to non-detectable quantities of other atrazine metabolites and/or represents non-substance-specific ^{14}C. These ^{14}C-compounds resulting from the degradation of the ^{14}C-labeled atrazine are likely associated with the dissolved organic carbon pool, since the amount of ^{14}C-activity and DOM concentration is very well correlated (p-value: 0.97).

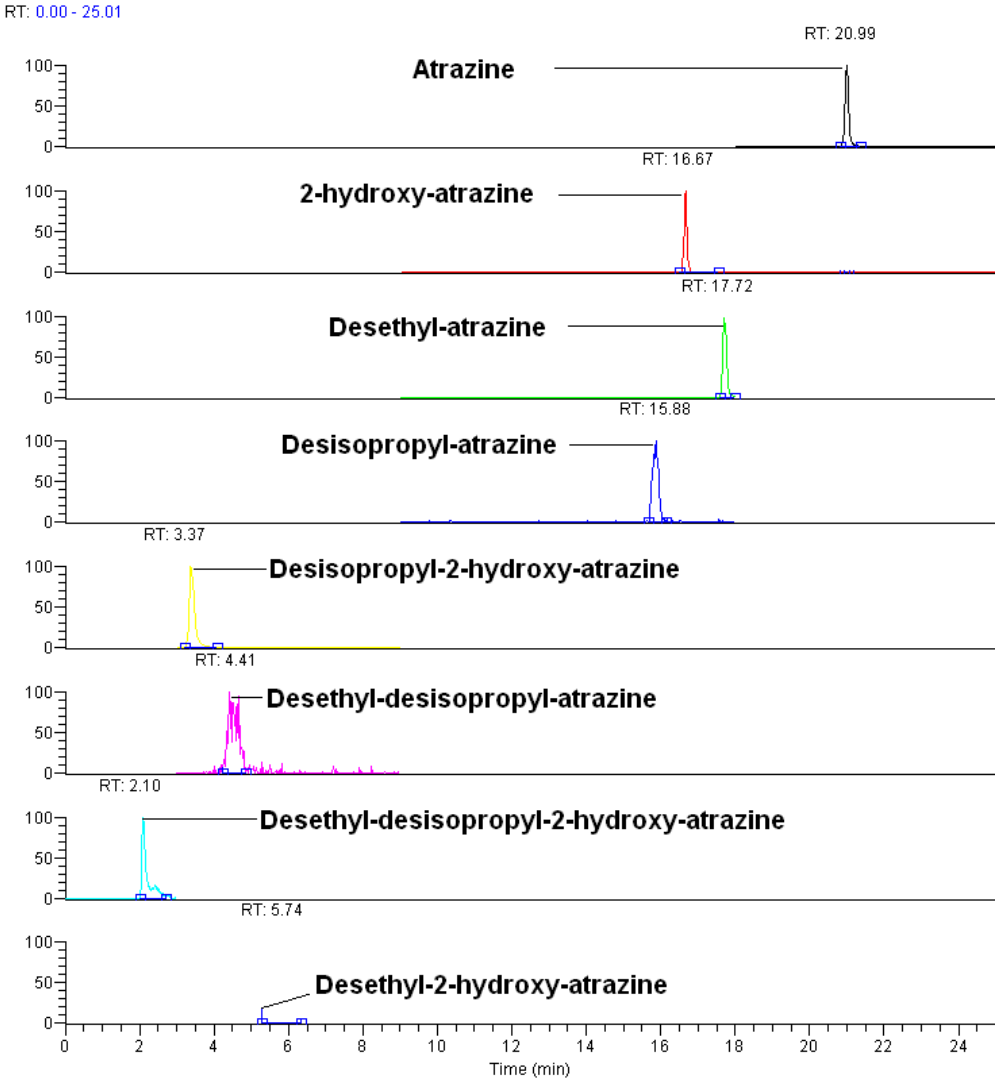

Figure 3.5: LC-MS/MS chromatograms showing the mass scale of all metabolite fragments.

2-Hydroxy-atrazine is considered to be the main atrazine metabolite occurring in a first degradation step [Takáts et al., 2001], due to biotic and/or abiotic factors. Kruger and Coats [Kruger and Coats, 1996] showed that hydroxy-atrazine was equally persistent in surface and subsurface soils. Under conditions of ground water contamination the order of leaching potential determined by Schiavon [Schiavon, 1988]: desethyl-atrazine > atrazine > desethyldesisopropyl-atrazine > deisopropyl-atrazine > hydroxy-atrazine might be questionable since in the study presented here only water was used to desorb the aged atrazine residues; these residues resulted from the originally applied atrazine as the only parent compound. Since 2-hydroxy-atrazine was found to be the only water-desorbable metabolite, with a high concentration detected in the lowest layer (Figure 3.3 on page 44 and Figure 3.4 on page 45), this indicates a considerable leaching potential in soil. This assumption contradicts the results presented by Sorenson *et al.* and Clay and Koskinen where hydroxy-atrazine is tightly adsorbed to the soil and could not be easily leached [Sorenson et al., 1993, Clay and Koskinen, 1990a].

Non-desorbable ^{14}C-activity originating from ^{14}C-labeled atrazine residues may be due to the parent compound or metabolites, corresponding to the assumption by Clay and Koskinen [Clay and Koskinen, 1990b] after a desorption study of atrazine from soil. Since Capriel *et al.* demonstrated the long-term persistence of atrazine in soil it could be assumed that these compounds might represent a long-term source of soil pollutants and might be leached to deeper soil layers by DOM- or soil-particle-associated transport processes [Capriel et al., 1985, Barriuso et al., 1991]. However, the fate and leaching of atrazine and/or its metabolites is mainly due to soil conditions and carbon contents less than the water solubility of these pesticide compounds [Ivey and Andrews, 1965, Rodgers, 1968, Burnside et al., 1969, Alhajjar et al., 1990]. In conclusion, atrazine and/or hydroxy-atrazine as its main metabolite are persistent in soil, even in trials being managed similar to agricultural practice under outdoor conditions.

These data derived from such a long-term experiment will be very valuable for future hazard assessment strategies.

3.3.5 Analysis of ASE extracts

Table 3.6 on page 52 presents the analytical results of the ASE extracts and LSC measurements of oxidized samples. As given in Figure 3.6 on the facing page, most residual ^{14}C-activity was extracted in the first extraction step. The applied ASE-settings in accordance with Gan *et al.* were highly effective for the extraction of aged atrazine residues from soil and homogenized gravel using a methanol-water solution, as previously suggested [Gan et al., 1999, Huang and Pignatello, 1990]. In comparison to previous extraction studies using vigorous shaking with water or Soxhlet extraction, the ASE yielded a considerably greater amount of aged atrazine residues; approximately 60 % of the total residues in the respective layer in the case of soil and 93 % in the case of homogenized gravel (Table 3.6 on page 52). However, the extractable amount of ^{14}C-activity is still measurable after 8 extractions, leveling off at 1.59 % for soil and 0.41 % for gravel.

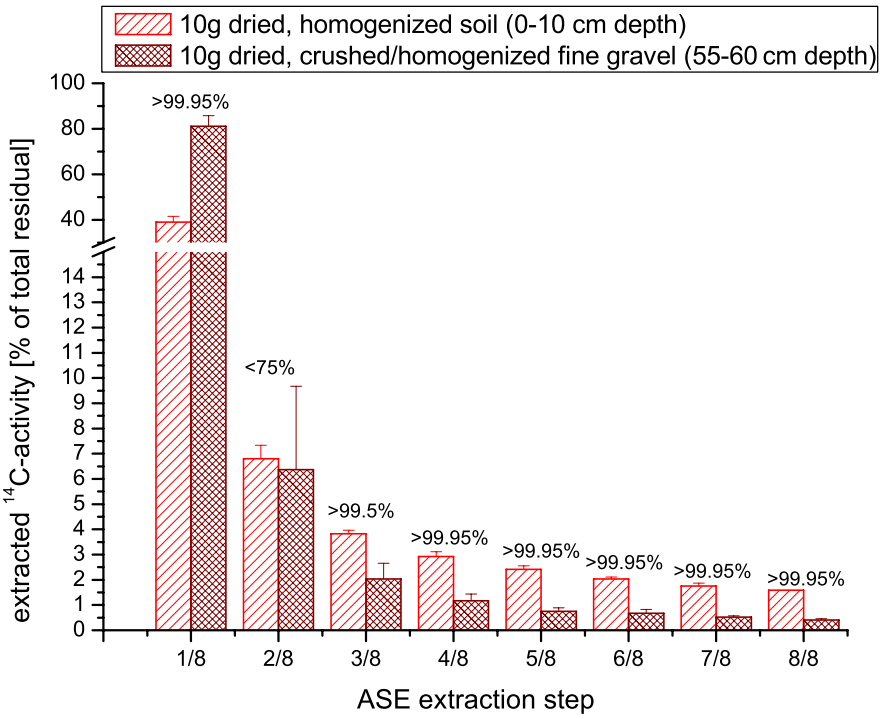

Figure 3.6: Residual ^{14}C-activity in each extract from soil and fine gravel in eight consecutive extraction steps using methanol-water solution (4:1 v:v) by means of accelerated solvent extraction. Standard deviation of $n = 9$. Values above the bars indicate statistical differences in the soil and gravel layer (Si = % of significance).

		% extractable of total residual ^{14}C [%]	% of total applied ^{14}C [%]	concentration [μg kg^{-1}]	amount of total residual [μg layer^{-1}]
Top layer	ATR	0.35	0.03	0.99 $\pm$0.09*	35.56
soil	OH-ATR	14.90	1.15	42.45 $\pm$2.25**	1 528.81
(0-10 cm)	^{14}C	60.37 $\pm$1.86***	7.70	284.90	10 300.00
Bottom layer	ATR	1.40	0.06	3.44 $\pm$0.41*	74.37
gravel	OH-ATR	7.22	0.29	17.69 $\pm$0.53**	382.33
(55-60 cm)	^{14}C	93.09 $\pm$0.75***	4.00	147.00	5 300.00

Table 3.6: Detected atrazine and 2-hydroxy-atrazine by means of LC-MS/MS as percentage of total remaining residues per layer, calculated per kg sample and depth increment. ^{14}C-activity of ASE extracts after eight consecutive extraction steps of 10 g dry soil or gravel using methanol-water solution (4:1, v:v) at 135°C, 1500 psi. ATR atrazine; OH-ATR 2-hydroxy-atrazine; ^{14}C-activity detected by means of LSC (% extractable; % of total applied) and by Oxidizer. Characterization and quantification by means of LC-MS/MS (atrazine concentration detectable; amount of total residual detected as atrazine). ($\pm$ standard deviation of $n = 3$). Significance (Si) is given for the different concentrations obtained between the soil (0-10 cm) and the gravel layer (55-60 cm) for extracted ATR at *>99.95%; and OH-ATR at **>99.95%; ^{14}C-activity at ***>99.95%.

As shown in Figure 3.7 on the following page A and B, atrazine was detected only in the first extract by means of LC-MS/MS for both soil and gravel. As described previously, all the extracts were analyzed for the following metabolites, among the parent compound atrazine: desethyldesisopropyl-2-hydroxy-atrazine, desisopropyl-2-hydroxy-atrazine, desethyldesisopropyl-atrazine, desethyl-2-hydroxy-atrazine, desethyl-atrazine, desisopropyl-atrazine, and 2-hydroxy-atrazine. The only detectable triazine metabolite was 2-hydroxy-atrazine in each of the eight consecutive ASE extracts. It is noteworthy that the amount of extractable atrazine as the parent compound was approximately four times higher in the lower gravel layer than from the topsoil layer, as given in Table 3.6 on the preceding page: 1.4 % (0.06 % of total applied) versus 0.4 % (0.02 % of total applied). This value equals 3.44 μg kg^{-1} of atrazine in the gravel layer versus 0.99 μg kg^{-1} of atrazine extractable in the topsoil layer. These values show statistically significant differences, at a significance of $Si = 99.95$ % (Table 3.6 on the facing page). It should be noted that the lowest gravel layer was artificial and did not represent a natural soil layer. The time course of atrazine leaching to the lower gravel layer remains unclear. However, these findings suggest the leaching character and long-term persistence of atrazine as well as its main metabolite 2-hydroxy-atrazine, particularly in lower soil increments. The long-term persistence of atrazine in the gravel layer might also be attributed to reduced microbial and chemico-physical degradation processes. This can be supported by the fact that only half the amount of unspecific residues expressed as atrazine equivalents were found in the lower gravel layer compared to the top-soil layer as given in Table 3.6 on the preceding page: 5.3 mg atrazine equivalents in gravel layer 55-60 cm versus 10.3 mg in soil layer 0-10 cm.

The overall recovery of 2-hydroxy-atrazine in all extracts from soil was 14.9 % of residual ^{14}C-activity (1.15 % of total applied) and 7.22 % of residual ^{14}C-activity (0.29 % of total applied) for gravel. Lerch and Li [Lerch and Li, 2001] found that the content of hydroxy-atrazine in agricultural soil is frequently higher than the chloro-derivatives, with a higher

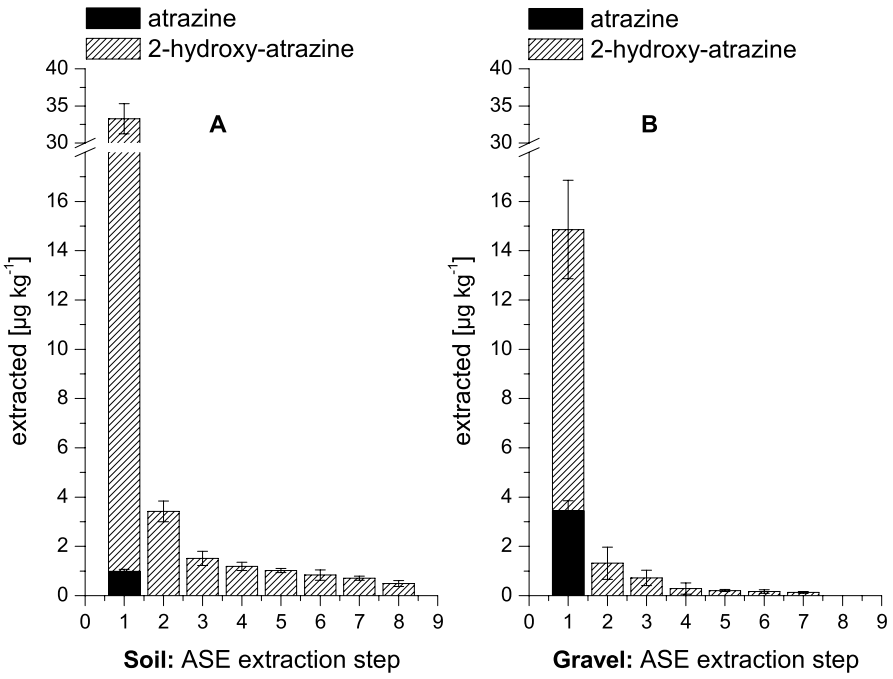

Figure 3.7: Extracted atrazine and 2-hydroxy-atrazine in soil (A) and gravel (B) each triplicates of 10 g sample, after 8 consecutive extraction steps using ASE, quantified by means of LC-MS/MS; calculated per kg sample. Standard deviation of $n = 9$.

concentration in the top 10 cm layers [Sorenson et al., 1993].

Although the amount of parent herbicide and metabolites appear low when related to the total initially applied atrazine, the absolute amounts need to be considered: atrazine at 0.03 % of total applied atrazine (0-10 cm) and 0.06 % of total applied atrazine (55-60 cm) correspond to 0.11 mg atrazine within the two soil layers of the lysimeter. Di-hydroxy-atrazine amounts to 1.15 % of total applied atrazine (0-10 cm) and 0.29 % (55-60 cm) and totals 1.9 mg. Adjusting these amounts to the soil weights of both layers leads to atrazine concentrations of 0.99 μg kg^{-1} soil (0-10 cm) and 3.44 μg kg^{-1} gravel (55-60 cm), respectively. In the case of 2-hydroxy-atrazine this amounts to 42.5 μg kg^{-1} soil (0-10 cm) and 17.7 μg kg^{-1} gravel (55-60 cm; Table 3.6 on page 52 and Figure 3.7 on the preceding page).

3.4 Mineralization of ^{14}C-labeled atrazine associated residues in soil and soil-size aggregates

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 3.8 on page 57 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 3.8 on page 57 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 3.8 on the facing page 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 (Chapter 3.2.1 on page 32, Table 3.2 on page 36) 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 3.8 on the next page 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.

The amounts of $^{14}CO_2$ produced from microbial activity following the addition of citrate or glucose are presented in Figure 3.9 B. Figure 3.9 on page 58 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

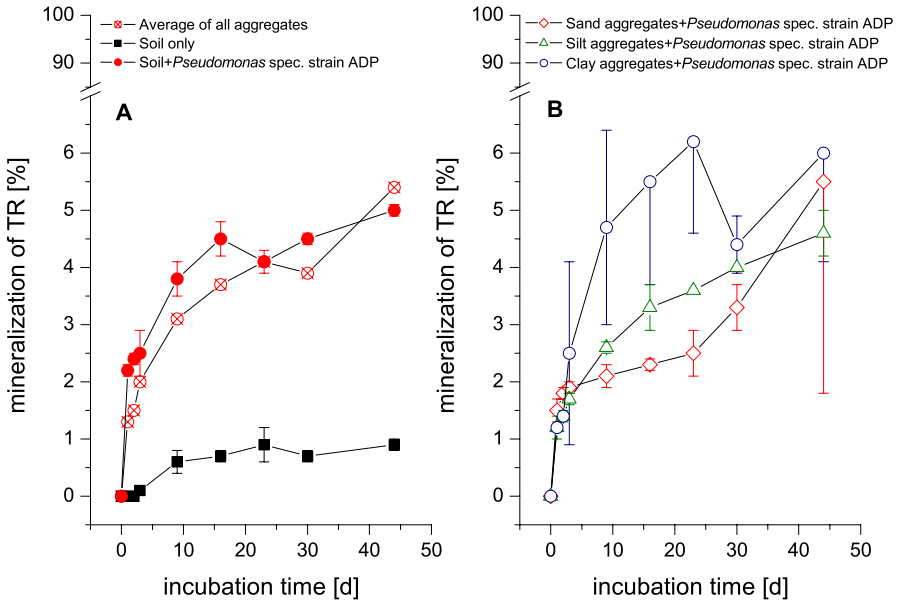

Figure 3.8: Mineralization of ^{14}C-labeled atrazine residues in the soil incubated without *Pseudomonas* sp. strain ADP, soil with addition of *Pseudomonas* sp. strain ADP, and mineralization curve of averaged mineralization of sized aggregates (A). Mineralization of ^{14}C-labeled atrazine residues 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.

carbon with no glucose amendment (Figure 3.9 A).

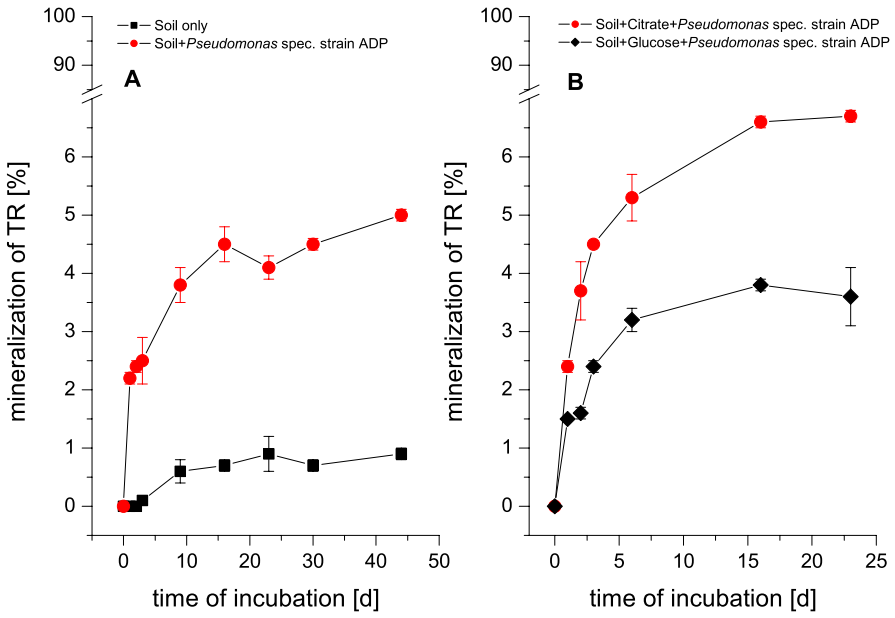

Figure 3.9: Mineralization of ^{14}C-labeled atrazine residues in the soil incubated with and without addition of *Pseudomonas* sp. strain ADP (A). Mineralization of ^{14}C-atrazine-residues in the soil including Na-citrate or glucose incubated with *Pseudomonas* sp. strain ADP (B). TR total ^{14}C-activity 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.* 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 [Piccolo et al., 1996]. 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 microbial attack. 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 analyses 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.* where biostimulation of *Pseudomonas* sp. strain ADP to degrade atrazine in soil with citrate amendment was significant [Silva et al., 2004]. 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.

Analyses 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.* 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 [Park et al., 2003]. Since atrazine was detected in this investigation after long-term aging under outdoor conditions (Chapter 3.3.5 on page 50) and Capriel *et al.* 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 [Capriel et al., 1985]. Since small amounts of the previously described 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 com-

pound.

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.

3.5 Evaluating bioaccessibility of naturally aged soil-bound ^{14}C-atrazine residues

3.5.1 Soil sample extraction/desorption

Extracted and desorbed ^{14}C-activity originating from ^{14}C-atrazine residues is shown in Figure 3.10 on the next page. The mineral medium used for extraction was chosen to reduce soil aggregate disruption and soil biological activity as can be assumed while using organic solvents.

As shown in Figure 3.10 on the facing page, 2.7 % of total residual ^{14}C-activity could be extracted in a first extraction step using water only. This corresponds to the dissolved ^{14}C-activity in the supernatant of all previous degradation studies (Figure 3.12 on page 66), which is likely due to shearing forces of the soil-slurry setups. In non-extracted soil, the mineralization increased to 5 % after 44 days of incubation with the atrazine-degrading organism *Pseudomonas* sp. strain ADP (Figure 3.12 on page 66 B).

The mineral media used for extraction containing the components K_2HPO_4/KH_2PO_4 influenced the extraction ability, which corresponds to the results obtained by

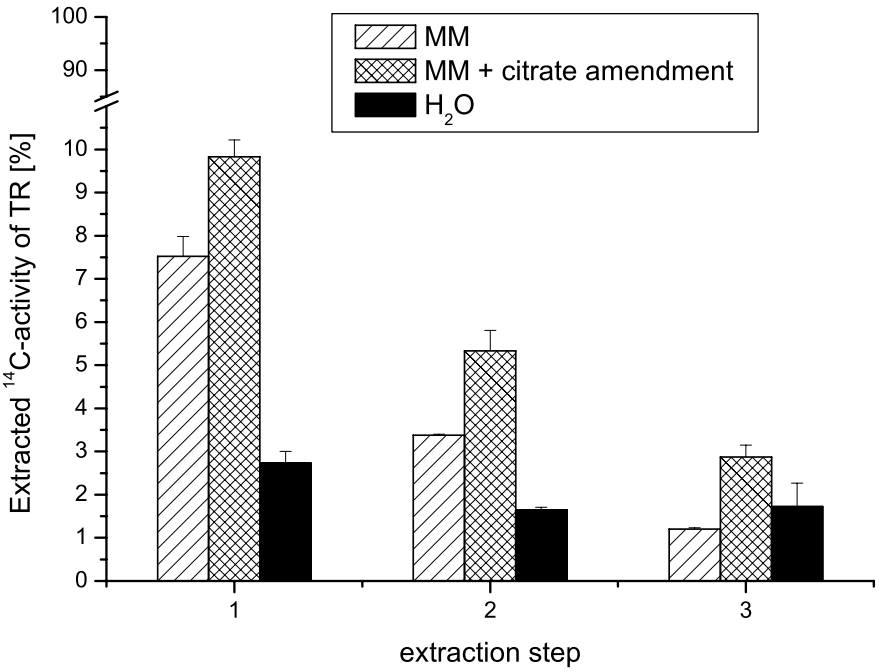

Figure 3.10: Extraction of ^{14}C-atrazine residues from the soil with indicated liquids: MM: mineral-media; MM+Citrate: mineral-media plus 2 gL^{-1} citrate amendment; H_2O: deionized organic free water. Bars indicate extracted ^{14}C-activity in % of total ^{14}C-activity in 10 g of soil. TR total ^{14}C-activity in the sample. Error bars indicate mean standard deviation of three replicates.

[Lerch et al., 1997b]. These results indicated that the binding mechanisms of aged atrazine residues are due to cation exchange rather than hydrophobic interaction. Using MM for extraction, 12.1 % of the residual ^{14}C-activity could be extracted. While using MM plus additional citrate amendment, 18.03 % could be extracted after consecutive extraction. A possible disaggregation of the macromolecular structure of soil humic substances hosting ^{14}C-atrazine residues during the extraction process is assumed to result in higher residual ^{14}C-activity in the liquid phase using MM plus additional citrate. This finding can be explained by the results obtained by Piccolo *et al.* evaluating the behavior of organic acids on the disaggregation of complex soil humic structures [Piccolo et al., 1996]. Further, it could be postulated that MM plus additional citrate extraction interrupts the weak bonding mechanisms such as H-, π-bonding and hydrophobic interactions as described by Wershaw *et al.* [Wershaw, 1986]. Such a process might result in a higher dissolution and extraction of residual ^{14}C-activity.

3.5.2 Microbial mineralization of soil bound ^{14}C-atrazine residues

As described above, mineralization of long-term aged ^{14}C-atrazine residues by *Pseudomonas* sp. strain ADP is possible. Ongoing research gives evidence that residual ^{14}C-activity is also associated with 2-hydroxy-atrazine and even the parent compound atrazine. Based on the results discussed above (Chapter 3.3.5 on page 50), it is to be assumed that evolved $^{14}CO_2$ as a result of microbial degradation is generated from atrazine and/or its metabolites. As Alvey and Crowley suggested, atrazine degraders as *Pseudomonas* and *Rhodococcus* have a broad substrate range capable of degrading atrazine and its assorted metabolites [Alvey and Crowley, 1995]. Early studies by Behki and Kahn showed that n-dealkylation and dehalogenation of atrazine and its metabolites by *Pseudomonas* species is possible [Behki and Kahn, 1986]; a complete mineralization of the triazine metabolite deethylsi-

mazine was successful by a combination of the organisms *Pseudomonas* and *Rhodococcus* [Cook and Hütter, 1984]. A complete mineralization of triazine substituents was demonstrated using different pseudomonad isolates from soil and sewage [Cook and Hütter, 1981].

As shown in Figure 3.11 on page 65, increasing incubation time of the soil-slurry setups resulted in an increase in the mineralization kinetics of the aged ^{14}C-atrazine residues from the previously extracted soil. Park *et al.* demonstrated that *Pseudomonas* sp. strain ADP showed chemotactic behavior towards atrazine giving clear evidence of access to atrazine sorbed by soil, as mentioned before [Park et al., 2003]. Therefore it may be assumed that evolved $^{14}CO_2$ originates from soil-bound atrazine or likely associated residues.

Using MM with additional organisms of the species *Pseudomonas* sp. strain ADP a total of 2.49 % of residual ^{14}C-activity in the soil-slurry was trapped as $^{14}CO_2$ after 32 days of incubation. Additional citrate showed similar results in the total $^{14}CO_2$-evolution during the incubation period of 2.45 %, both achieved with previously extracted soil samples. However, using water only with additional *Pseudomonas* sp. strain ADP total $^{14}CO_2$-evolution was smaller (1.54 %). This observation could be explained by a reduction of cell activity caused by osmotic stress of the organisms since the water used for the previous cell washing was deionized water. The previously non-extracted soil in Figure 3.12 on page 66 C showed a ^{14}C-residue mineralization of 1.74 % after 44 days of incubation. This result is similar to the inoculated sample with *Pseudomonas* sp. strain ADP in the extracted soil (Figure 3.11 on page 65) indicating that evolved $^{14}CO_2$ must be due to microbial activity of *Pseudomonas* sp. strain ADP mineralizing soil-bound atrazine residues. The high standard deviation in Figure 3.12 on page 66, B, C, D, must be explained by experimental artifacts due to impurities in the sample measured by LSC.

These results show that even after long-term environmental aging soil-bound atrazine residues are still partly accessible.

As the incubation progressed, it became clear that in all setups mineralization increased whereas ^{14}C-activity in the supernatant stayed more or less constant in the range of 1 to 4.5 % during the incubation period (Figure 3.12 on page 66). Even though Figure 3.12 D shows a high standard deviation with increasing incubation time the results of residual ^{14}C-activity in the supernatant increased with increasing mineralization rate. In comparison to the kinetics of Figure 3.12 on page 66 A, B, C, additional citrate accelerated the extractability of ^{14}C-activity, which underlines the results of the soil extraction given in Figure 3.10 on page 61 A.

Further, this outcome indicates that mineralization of ^{14}C-atrazine residues is not entirely dependend on the desorbable amount. Dissolution or desorption potential of ^{14}C-atrazine residues from the soil matrix is therefore not a predetermining condition for bioavailability. These results do not correspond to the results obtained by Johnson *et al.* due to the bioavailability of nonextracable atrazine in soil after three months of aging, suggesting that all biodegradation occurred in the dissolved phase [Johnson et al., 1999]. The present study focused the long-term behavior and the bioaccessibility of aged atrazine residues, likely present as metabolites in the dissolved phase and in the soil-bound phase. These results are of specific interest concerning bioremediation processes and microbial-pesticide residue interactions in soils, since bound atrazine and pesticide residues represent a "potentially large contaminant fraction in agricultural soils" [Johnson et al., 1999].

It is to be concluded that mineralized ^{14}C-atrazine residues detected as trapped $^{14}CO_2$ mainly originate from soil-bound ^{14}C-atrazine-residues present in the aged soil samples. Even though the values for mineralization of soil-bound atrazine residues are small it becomes clear that they are not entirely excluded from environmental interactions.

The influence of microbial activity on the mineralization of naturally long-term aged soil and soil-bound ^{14}C-atrazine residues was assessed by a comparison of soil samples previ-

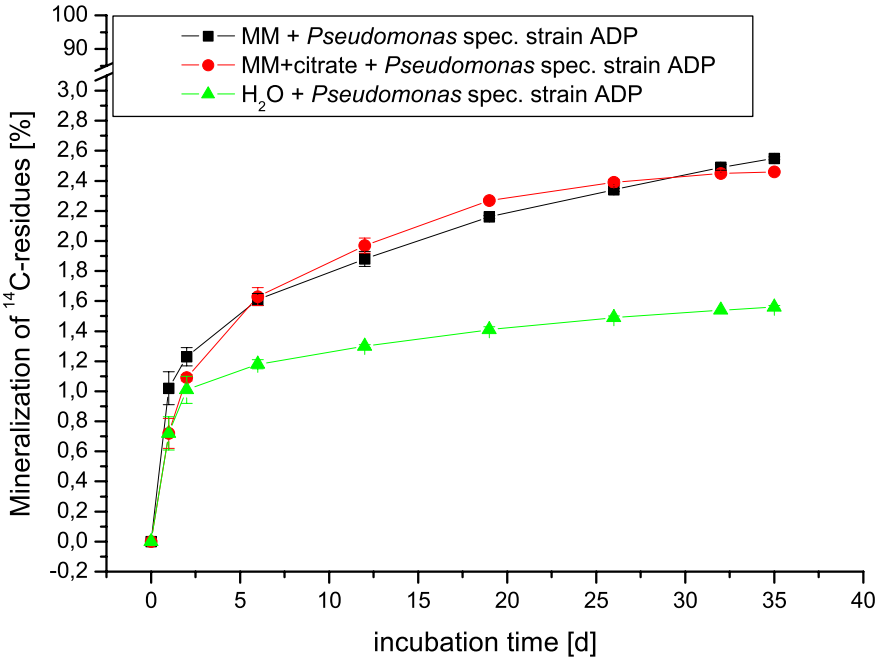

Figure 3.11: Mineralization of soil bound ^{14}C-atrazine-residues in the extracted soil samples incubated with mineral medium (MM) or deionized water (H_2O) with the addition of 10 ml of *Pseudomonas* sp. strain ADP (*P.*ADP), and a cell density of 1.8-3 $\times$ 10^8 cells ml^{-1}. Error bars indicate mean standard deviation of three replicates.

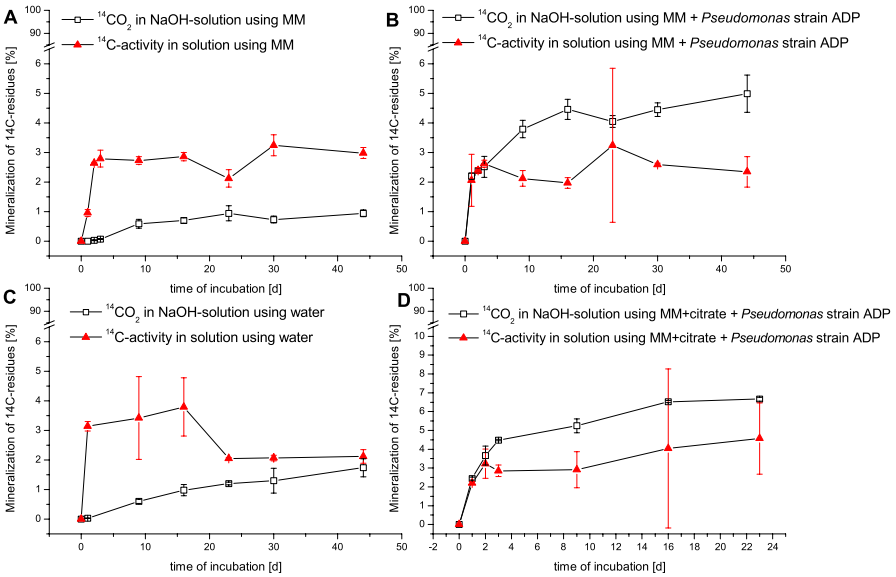

Figure 3.12: Desorption of ^{14}C-atrazine residues in previously non-extracted soil samples incubated with mineral media (MM) or deionized water (H_2O) with (Figure B and D) or without (Figure A and C) addition of 10 ml of *Pseudomonas* sp. strain ADP (*P.*ADP), and a cell density of 1.8 -3 $\times$ 10^8 cells ml^{-1}. Open triangles (SN) represent percentage of ^{14}C-activity in the supernatant. Red squares (CO_2) indicate percentage of mineralized ^{14}C-atrazine residues as $^{14}CO_2$. Error bars indicate mean standard deviation of three replicates.

ously extracted by gentle means and non-extracted soil samples incubated as soil-slurry setups with the atrazine degrading organism *Pseudomonas* sp. strain ADP. Previous gentle extraction using mineral media containing K_2HPO_4/KH_2PO_4 showed an effective extraction capacity which was even increased by citrate amendment. It has been demonstrated that mineralization of ^{14}C-atrazine residues was possible in extracted and non-extracted soil samples. As observed from the results obtained from previously extracted soil aged, soil-bound ^{14}C-atrazine-residues are partly bioaccessible for *Pseudomonas* sp. strain ADP. Additional citrate in the soil slurry setups could stimulate the mineralization rate by *Pseudomonas* sp. strain ADP as well as the extraction of ^{14}C-atrazine residues during the incubation period. Increased mineralization of the aged ^{14}C-atrazine residues detected as evolved $^{14}CO_2$ was not dependent on desorbed or extracted ^{14}C-atrazine residues in the soil-slurry setup. These results indicate that it was possible to assess the bioaccessibility of aged soil-bound ^{14}C-atrazine residues by *Pseudomonas* sp. strain ADP.

4 Epilogue

Since its introduction in the late 1950s the pre-emergent herbicide atrazine has been among the most heavily used pesticides in the world. In the US, it has been ranked at or near the top of all agricultural pesticides in terms of quantity, with 76.5 million of pounds, worth about $2 billion, applied annually since 1990, mainly for corn production [USEPA, 2006b]. Atrazine is also heavily used in Canada, Africa, and the Asia-Pacific region [Kegley et al., 2008]. In Europe, atrazine was heavily used until the early 1990's when it was banned or severely restricted in most EU countries because drinking water concentrations exceeded generic limits. Atrazine is still being applied and is readily detected in water streams and wells in considerable amounts, ranging from 0.12 μg L^{-1} up to 7.0 μg L^{-1} [USEPA, 2004, USEPA, 1990]. In addition to parent pesticides, their degradation products or metabolites were also detected in generally higher amounts [Kolpin et al., 1998], representing a potential risk for soil and water contamination. In view of the widespread use of atrazine, its ready dispersal and persistence in the environment, and strong indications of adverse ecological effects, there is reason to suggest that the use of atrazine should be curtailed.

Atrazine use has resulted in widespread contamination of ground and surface waters and its dispersal in the atmosphere. Atrazine "is the second most frequently detected pesticide" in freshwater sources in the US [USEPA, 2006a]. Even more than 18 years after

it was banned in Germany, atrazine remains the most abundant pesticide in groundwater samples [LAWA, 2003]. It is difficult to imagine a site not impacted by agrochemicals worldwide [Nations and Hallberg, 1992, Thurman and Cromwell, 2000] since atrazine has been detected in rainwater frequently and in different places [Brun et al., 2008, Goolsby et al., 1997, Bossi et al., 2002, Sanusi et al., 2000], and also in fog, ambient air, arctic ice and seawater, all at great distances from urban and agricultural areas [Glotfelty et al., 1987, Chernyak et al., 1996].

The presented findings prove long-held suspicions that atrazine and its metabolites can persist in water and soil for decades. The persistence of atrazine in groundwater for at least 18 years after application stopped has already been mentioned. In the presented investigation, results of a 22 years long-term study are referred to. In 1983 atrazine ^{14}C-labeled in the triazine ring was applied to a lysimeter in a corn field over three successive years at customary application rates; 22 years later, residues of labeled atrazine and hydroxy-atrazine, the initial hydrolysis product and microbial metabolite, were detected in the topsoil of the lysimeter, as well as at deeper levels. Moreover, 25% of the applied ^{14}C was still present in the lysimeter soil-most of it extractable with water-methanol solution. Based on the analytical findings 0.1% of total applied atrazine is still detectable after 22 years. In an earlier study on atrazine that had been aged in soil for nine years, 83% of initially applied ^{14}C-activity remained in the soil [Capriel et al., 1985]. Both of these aging experiments involved limited applications of the herbicide: however, when applied annually over decades in normal agricultural use, the accumulated burden of residues in soil could be substantial and the associated risk significant. The atrazine burden in soil may represent a long-term source of dissolved or colloid-bound atrazine and its metabolites to ground water or surface waters.

That, after decades of weathering, atrazine is still detectable, and metabolites (apparently

with triazine ring intact) are still abundant in soil, underscores our lack of understanding of the environmental behavior of this herbicide. The concept of a dissipation "half-life" (DT50) has been questioned before. Nevertheless, the lysimeter study clearly demonstrates the invalidity of applying a DT50 value obtained from short-term experiments to long timescales: after 22 years a 550-fold greater concentration of atrazine was found than predicted by a DT50 of one year - the longest among several reported values as short as a few days (Figure 4.1). Whether such residues in-place represent a risk remains an area of active debate. Periodic rainfall or irrigation events may result in pulse inputs of leachate from the surface into deeper soil or ground water layers [Pignatello et al., 1993] where they may impact underground or surface drinking water sources.

Exposure to atrazine represents a potential threat to human and ecological health. Potentially the most serious threat is endocrine disruption (ED). The maximum allowed contaminant level for atrazine in drinking water is 0.1 μg L^{-1} (EU), 2 μg L^{-1} (WHO), or 3 μg L^{-1} (USEPA). At doses as low as 0.1 μg L^{-1}, atrazine was found to cause dramatic changes in sexual differentiation in amphibians [Hayes et al., 2002a, Tavera-Mendoza et al., 2002, Hayes et al., 2002b]. Other studies show atrazine ED effects as an increased percentage of apoptosis and reduced blastocyst development of murine preimplantation embryos [Greenlee et al., 2004], and mammary growth and development in the offspring of rats chronically exposed into adulthood [Enoch et al., 2007]. Some new work, however, has found no ED in amphibians or carcinogenic effects of atrazine on mammals [Jooste et al., 2005, Mcelroy et al., 2007, Trantacoste et al., 2001].

Given the widespread dispersal and persistence of atrazine in the environment, the validation of the initial claims of ED effects among amphibians and mammals is cause for serious alarm. A recent USEPA re-registration decision [USEPA, 2006b], citing the contradicting studies on atrazine ED, states that the environmental ED of atrazine remains merely

"hypothetical" at this time.

Clearly, the available information justifies concern about the continued widespread use of atrazine. The first reaction is dismay at the paucity of data and lack of understanding of the fate and effects of atrazine. Obviously, a priority should be to resolve the ED controversy surrounding atrazine. Studies with this goal in mind should be conducted by independent parties without a stake in the outcome, include all relevant test organisms, take into account the different reproductive cycles of individual organisms, and should be performed with field-relevant dose regimes. Unfortunately, there are in general no official standards for evaluating the ED potential of anthropogenic chemicals as yet. EPA has missed multiple deadlines for issuing guidelines [Erickson, 2008], no doubt encumbered by the experimental challenges inherent in ED. Long-term effects on wildlife, particularly aquatic organisms, are difficult to simulate in the laboratory, both as a result of potential time limitations due to the organisms' seasonal reproductive cycles and the complex interactions and food-chain dependencies that affect the system-wide movement of pollutants in the environment. Effects on humans remain a possibility but are even more difficult to evaluate. While atrazine metabolites are typically less acutely toxic than atrazine itself, virtually nothing is known about their ED effects. Many of them are more persistent than atrazine.

The data clearly suggest that it will take decades for atrazine to be degraded in the environment. In the meantime, ongoing surveillance of atrazine and its metabolites is essential in ground/surface waters and soils, not only in areas of direct application but also in remote areas. Further research on the environmental behavior of atrazine and its degradation products should be supported independently. Since soil is a long-term sink for many chemical pollutants, the data obtained for atrazine are likely to have broad relevance. A concerted effort is needed to obtain more conclusive data on the ED effects of atrazine and its metabolites on amphibians and mammals, including humans. A considerable reduction

of pesticide use through the adoption of alternative techniques, without crop yield reduction, has been suggested earlier [Pimentel, 1997]. Due to limited natural resources and a growing demand for clean food and water the overall intensive use of pesticides gives reason for alarm. Finally, the use of atrazine, as a highly recalcitrant substance in soils and as a suspect for ED and carcinogenic effects, should be reconsidered, particularly in sensitive areas like near-surface groundwater reservoirs and areas susceptible to soil erosion.

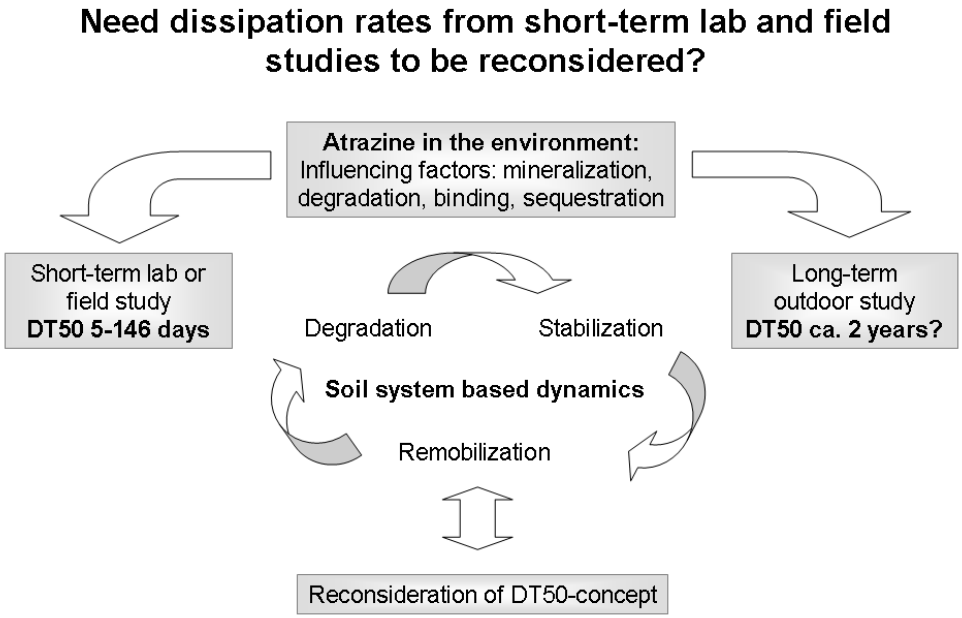

Figure 4.1: Schematic overview of dissipation times (DT50) of pesticides in soil, based on the quantification of mineralization, degradation and binding rates

Dissipation times (DT50) of pesticides in soil are based on the quantification of mineralization, degradation and binding rates (laboratory studies) or the latter two (field studies; Figure 4.1). Corresponding DT50 values for atrazine were reported in the range of 5 to

146 days with a median of 37 days for laboratory studies, and 61 days for field studies, respectively [Haider and Schäffer, 2006]. In the lysimeter study presented, after 22 years incubation of atrazine in soil under field conditions, 0.1% of the applied parent compound is still detectable. At least two mechanisms could explain the significantly increased persistence of atrazine in soil. (i) The herbicide and its metabolites readily bind to the soil matrix forming non-extractable residues (NER) due to irreversible binding to humic matter and clay-humus complexes either by physical entrapment or covalent binding [Capriel et al., 1985, Haider et al., 2000]. NER represent a long-term stabilized soil pool of the residues due to the reduced bioavailability for microorganisms. However, NER may slowly become released from such a pool [Barriuso et al., 2008] leading to low concentrations of pesticide residues in the soil water after long incubation times that may leach to the groundwater. (ii) In addition, the microbial degradation of atrazine will become slower and slower after a certain low level of the parent compound is achieved, such as the μg kg^{-1} concentration of this investigation in soil aged for 22 years. This mechanism is known for a large variety of organic chemicals in natural environments [Boethling and Alexander, 1979]. Ten subsequent half-lives will reduce the amount of any parent compound to about 0.1% irrespective of the mechanism of degradation, following for example simple first order, di- or multi-compartment kinetics. Since the analysis of the soil after 22 years of incubation still revealed the presence of about 0.1% atrazine, the DT50 can be roughly estimated to have a maximum of about 2 years. Since atrazine degradation was analyzed only at a single long-term point of incubation (22 years), its degradation half-life may, however, be shorter than 2 years and can only be confirmed by more long-term incubations with intermittent chemical analysis.

5 Summary

The long-term behavior of the herbicide atrazine and its metabolites in the environment is of continued interest in terms of risk assessment and soil quality monitoring.

The results of this investigation highlight the long-term persistence and environmental behavior of the herbicide atrazine. To date, no comparable results have been published by other parties. Therefore, this study provides important data for the risk assessment of atrazine application areas or atrazine-contaminated sites. It is possible that these findings reported for atrazine presented in this report may also be relevant for other persistent chemicals and pesticides. Clearly, the calculation of predicted environmental concentrations of persistent chemicals based only on laboratory half-life or short-term field dissipation experiments should therefore be reconsidered. Agricultural soils after many years of cultivation may contain multiple aged pesticide residues from applications of various pesticides that become stabilized by binding to the soil matrix. This may challenge the environmental risk assessment of the resulting mixture of long-term available pesticide residues in our agricultural soils. Further, adapted microbial communities with mineralization potential for atrazine [Shaner and Henry, 2007a, Krutz et al., 2008b] or other pesticides might challenge farmers and industries to reconsider the use and necessity of agrochemicals that lead to environmental pollution and consider the use of alternative techniques in general.

1. Aqueous desorption, detection and quantification of atrazine and its metabolites from an agriculturally used soil was performed 22 years after the last atrazine application. The application of ring-^{14}C-labeled and non-labeled atrazine at the customary rate (1.7 kg ha^{-1}) was performed on an agriculturally used outdoor lysimeter. The lysimeter soil containing long-term aged atrazine for >20 years was subdivided into 10 cm and 5 cm layers (soil: 0-10, 10-20, 20-30, 30-40, 40-50, except at the lysimeter bottom: 50-55 cm; fine gravel: 55-60 cm depth, implemented for drainage purposes) to identify the qualitative and quantitative differences of aged ^{14}C-labeled atrazine residues depending on the soil profile and chemico-physical conditions of the individual soil layers. Deionized water was used for non-exhaustive cold water shaking-extraction of the soil. With increasing soil depth the amount of previously applied ^{14}C-activity decreased significantly from 8.8 % to 0.7 % at 50-55 cm depth, whereas the percentage of desorbed ^{14}C-residues in each soil layer increased from 2 % to 6 % of the total ^{14}C-activity in the sample. The only metabolite detectable by means of LC-MS/MS was 2-hydroxy-atrazine while most of the residual ^{14}C-activity was bound to the soil and was not desorbed. The amount of desorbed 2-hydroxy-atrazine decreased with increasing soil depth from 21 % to 10 % of the total desorbed ^{14}C-residue fraction. The amount of ^{14}C-residues in the soil layers correlated well with the carbon content in the soil and in the aqueous soil extracts (p-value: 0.99 and 0.97, respectively), which may provide evidence of the binding behavior of the aged atrazine residues on soil carbon fractions. The lowest coarse layer (55-60 cm) showed increased residual ^{14}C-activity leading to the assumption that most ^{14}C-residues were leached from the top soil over time.

2. Further analysis showed that atrazine is still detectable by means of accelerated solvent extraction and LC-MS/MS analysis. Extractions of the 0-10 cm soil layer yielded 60% of the residual ^{14}C-activity. The extracts contained atrazine (1.0 μg kg^{-1}) and 2-hydroxy-atrazine (42.5 μg kg^{-1}). Extractions of the material of the lowest layer 55-60 cm consisting of fine gravel yielded 93% of residual ^{14}C-activity, of which 3.4 μg kg^{-1} was detected as atrazine and 17.7 μg kg^{-1} as 2-hydroxy-atrazine. The atrazine detected in the lowest layer was of almost four times higher mass than in the uppermost soil layer. These findings highlight the fact that atrazine is unexpectedly persistent in soil. The overall persistence of atrazine in the environment might represent a potential risk for successive groundwater contamination by leaching even after 22 years of environmental exposure.

3. In addition, 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 achieved 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 were 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.

4. To gather further information on the bioaccessibility of soil-bound ^{14}C-atrazine residues, soil samples were previously extracted three times with a) mineral media (MM), b) mineral media with 2 gL^{-1} Na-citrate and c) deionized water. Total extractable ^{14}C-activity was 12.1 % for MM, 18.0 % for MM plus citrate and 6.1 % using deionized water, respectively. Extracted soil samples were mixed with 10 ml of the different solutions used for extraction with addition of 1.8-3 $\times$ 10^8 cells ml^{-1} of the atrazine-degrading organism *Pseudomonas* sp. strain ADP. The resultant slurries of extracted soil samples were incubated and the evolved $^{14}CO_2$ was trapped in 0.5 M NaOH solution. Cumulative kinetics showed that 32 d of incubation led to a ^{14}C-atrazine residue mineralization of 2.49 and 2.45 % using MM and MM plus citrate, respectively. Analyzing the supernatant in a similar approach using previously non-extracted soil samples provides evidence that dissolution of atrazine residues in the liquid phase is not a prerequisite for biodegradation. These results indicate that soil-bound ^{14}C-atrazine residues are partly bioaccessible and bioavailable for the specific atrazine-degrading strain *Pseudomonas* sp. strain ADP even after 22 years of natural aging.

Overall, these results represent novel information about the long-term environmental persistence of the still widely used herbicide atrazine. It is noteworthy that atrazine as the parent compound is still detectable in soil and deeper layers even after 22 years of environmental exposure. This finding indicates a potential long-term risk for soil and groundwater contamination by atrazine. These findings can be useful for environmental and agricultural assessments and environmental policy decisions concerning pest management.

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