Metabolism and Persistence of Atrazine in Several Field Soils with Different Atrazine Application Histories

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Abstract

To assess the potential occurrence of accelerated herbicide degradation in soils, the mineralization and persistence of ^{14}C-labeled and non-labeled atrazine was evaluated over three months in two soils from Belgium (BS: atrazine treated 1973-2008; BC: non-treated) and two soils from Germany (CK: atrazine treated 1986-1989; CM: non-treated). Prior to the experiment, accelerated solvent extraction of bulk field soils revealed atrazine (8.3 and 15.2 μg kg^{-1} in BS and CK soil), and a number of metabolites directly after field sampling, even in BC and CM soils without previous atrazine treatment, by means of LC-MS/MS analyses. For atrazine degradation studies, all soils were incubated under different moisture conditions (50% maximum soil water holding capacity -WHC$_{max}$-/slurried conditions). At the end of the incubation, the ^{14}C-atrazine mineralization was high in BS soil (81% and 83%), and also unexpectedly high in BC soil (40% and 81%), at 50% WHC$_{max}$ and slurried conditions, respectively. In CK soil, the ^{14}C-atrazine mineralization was higher (10% and 6%) than in CM soil (4.7% and 2.7%), but was not stimulated by slurried conditions. The results revealed that atrazine application history dramatically influences its degradation and mineralization. For the incubation period, the amount of extractable atrazine, composed of residues from freshly applied atrazine and residues from former field applications, remained significantly greater (statistical significance = 99.5% and 99.95%) for BS and CK soils, respectively, than the amount of extractable atrazine in the bulk field soils. This suggests that i) mostly freshly applied atrazine is accessible for a complex microbial community, ii) the applied atrazine is not completely mineralized and remains extractable even in adapted soils, and iii) the microbial atrazine-mineralizing capacity strongly depends on atrazine application history and appears to be conserved on long time scales after the last application.

KEYWORDS: metabolites, microbial adaptation, enhanced biodegradation, mineralization, persistence.

INTRODUCTION

An increasing demand for food and feed biomass for energy, and a simultaneous decline in available arable land caused by soil degradation and land-use changes (*1*, *2*) may require an increased use of pesticides to meet social and economic demands (*3*). Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] is one of the most widely used herbicides in the world, particularly for high-energy crops such as maize and sugar cane. The use of atrazine was banned in all EU Member States in September 2004, with permission to consume existing stocks until October 2005 (*4*). Even though its use is banned in the EU, it is still widely used throughout America and the Asia-Pacific region (*5*). In the USA, 35,000 tons have been applied annually to maize plantations during the last 20 years. The pesticide is of environmental concern due to its widespread use, persistence in soil, and frequent detection in ground and surface waters (*6*, *7*). In past years, an accelerated degradation of pesticides resulting in reduced efficiency has been reported for a number of chemicals, such as carbofuran, 2,4-D and isoproturon among others (*8*, *9*). More recently, several reports described similar enhanced atrazine degradation throughout the world (*10*). Due to these findings, soils exhibiting the phenomena of an accelerated atrazine mineralization related to previous atrazine applications have been defined as “atrazine-adapted” soils.

Due to its degradability, the transformation of atrazine reduces its intended herbicidal effects (*11*). However, the bioavailability and transformation of atrazine is influenced by the sorption and binding processes of the chemical on the soil matrix (*12*). The microbial accessibility and availability of atrazine depends strongly on a variety of abiotic factors, such as soil properties and moisture conditions (*13*), temperature, pH value (*14*), atrazine application history (*15*), the presence of other pesticides or available nutrients (*16*), organic carbon content (*17*), agricultural management, and tillage practices (*18*, *19*). In general, the degradation of pesticides in soils

is a complex interaction between different dynamic processes which do not allow a precise prediction of the long-term behavior in soils (*20*). Residue accessibility and accumulation are focal points for environmental risk assessment strategies.

The understanding and monitoring of field soils microbiologically adapted to regular and repeated pesticide applications provide important information for a sustainable use of agricultural land, stimulating the search for novel cultivation techniques. Several investigations in the past have described an enhanced atrazine degradation in soils from Belgium (*21, 22*). Microbial communities capable of rapidly degrading and even mineralizing atrazine from these soils have been described, revealing a wide diversity of substrate utilization abilities (*23*). However, the amount of residual atrazine originating from earlier field applications as well as the potential atrazine accumulation in atrazine-adapted soils has not yet been considered. Further studies are required on the simultaneous formation and determination of atrazine fractions, extractable by means of water and organic solvents, and non-extractable atrazine fractions, preventing the microbial degradation of this molecule.

In the present study, accelerated atrazine degradation and atrazine persistence in an atrazine-adapted, agricultural soil from Belgium and in three additional control soils from Belgium and Germany were examined under laboratory conditions. The aim of this study was to evaluate i) the kinetics of an accelerated atrazine mineralization in the various field soils using ^{14}C-labeled atrazine, ii) the kinetics of simultaneous metabolite formation, iii) the respective extractability of atrazine and its metabolites and the formation of non-extractable atrazine residues as a function of time, and iv) the presence and extractable quantities of residual atrazine from former atrazine field applications in the different soils and changes in these quantities during the incubation study.

MATERIALS AND METHODS

Soil characteristics, history and sampling

The Belgian field soil (BS; 0-10 cm depth) was a Gleyic Luvisol soil, pH value 5.94 (10 g / 25 mL 0.01 M $CaCl_2$), containing 1.26% C_{org}. Detailed data on soil texture analyses and soil properties are given in **Table 1**. The sampling field was located in Beverst, Belgium. It was used for continuous maize cultivation, and atrazine was applied annually at the recommended rates (0.5 - 2.0 kg active ingredient - a.i. ha^{-1}) from 1973 to 2004. Until 2008, atrazine was manually applied to a specified field plot of 324 m^2 located on the same field by scientists from the Department of Earth and Environmental Sciences, Katholieke Universiteit Leuven (K.U. Leuven), Belgium. The commercial atrazine product Gesaprim 500 (480 g a.i. L^{-1}) was applied at an application rate of 1 L ha^{-1}. Prior to soil sampling, the last reported atrazine application was conducted in autumn 2008.

The corresponding control soil (BC; 0-10 cm depth) was collected in the same area as the atrazine-treated field soil (BS) but from an adjacent field plot used as a pear orchard, approximately 20 m from the herbicide-treated site. In addition, two further control soils were collected: an Orthic Luvisol from Merzenhausen, Germany (CM; 0-20 cm depth; pH: 7.0), and a Gleyic Cambisol from Kaldenkirchen, Germany (CK; 0-30 cm depth; pH: 5.35). Details on BC, CM, and CK are presented in **Table 1**. For BC and CM control soils, no atrazine application was documented. Due to maize cultivation during 1986-1989 on CK soil, an application of 1.5 L ha^{-1} (approx. 0.72 kg a.i. ha^{-1}) per annum of the commercial atrazine formulation Gesaprim 500 was reported for that four-year period (*24*). The soil types CM and CK were included as controls to demonstrate that accelerated atrazine degradation is strongly correlated with regular field applications of this pesticide. All control soils were treated in the same way as BS soil. All soils were collected randomly on the respective field plots from the 0-30 cm soil layer, as indicated in parenthesis above. A total of approximately 50 samples, corresponding to approx-

imately 5 kg, were collected using a Humax soil sampler 3 cm in diameter in May 2009. The soils were freshly sieved $\leq$2 mm and stored in the dark at 2-5 °C until further analysis. All degradation experiments were initiated one week after soil sampling.

Soil microcosms

Sample preparation and evaluation of atrazine degradation capacity

The experiment was performed using uniformly ^{14}C ring-labeled and unlabeled atrazine in accordance with a field application dose of 1.31 mg a.i. kg^{-1} soil. The amount used was equivalent to approximately 1.23 kg a.i. ha^{-1} (with respect to a sampling depth of 10 cm and assuming a bulk density of 1 g cm^{-3}, in accordance with OECD guideline 307 (*25*)). An ethanol stock solution of 1.5 mL was prepared containing 5.9 mg ^{14}C-atrazine at a specific ^{14}C-activity of 351 MBq mmol^{-1} (chemical purity 99%, American Radiolabeled Chemicals, Inc.), resulting in 6.4 kBq μL^{-1}. For the field (BS) and the control soils (BC, CM, CK), an atrazine application solution was prepared using 2500 μg atrazine (chemical purity 98%, Riedel-de-Haen, Seelze, Germany) dissolved in 60 mL ethanol. For each soil, 10 mL unlabeled atrazine stock solution was individually mixed with 35.1 μL of ^{14}C-atrazine stock solution. For each soil, 555 μg atrazine in total, associated with a total ^{14}C-activity of 225 kBq, was added to a homogenized subsample of 5% dry soil equivalents of each bulk soil (450 g dry soil equivalents). The ethanol was evaporated, and the sample was homogeneously mixed with the bulk soil using a tumble mixer (JEL, RRmini) for 30 min. Before spiking, the bulk soils were adjusted to the incubation temperature (20 $\pm$2 °C) for 8 hours. The homogeneous ^{14}C-atrazine distribution was confirmed by oxidation of 10 soil aliquots of 0.5 g using a Biological Oxidizer OX500 (R.J. Harvey Instrument Corporation). The emerging $^{14}CO_2$ was trapped in Oxysolve C-400 scintillation cocktail (Zinser Analytik). A 2500 TR, Tri-Carb, Packard Liquid Scintillation Analyzer (LSC) was used to detect ^{14}C-radioactivity using an internal standard. The values for ^{14}C-activity distribution in the bulk soils were 468 ($\pm$15) Bq g^{-1} for BS, 477 ($\pm$33) Bq g^{-1} for BC, 552 ($\pm$20) Bq g^{-1} for

CM, and 478 ($\pm$37) Bq g^{-1} for CK, respectively. Triplicates (20 g dry soil equivalents) of ^{14}C-atrazine-spiked soils were incubated at 50% WHC_{max} in accordance with OECD guideline 307. During the entire incubation period, the moisture content was controlled weekly by weighing the incubation flasks, and water losses were compensated by adding distilled water.

In a second approach, triplicates of 10 g soil samples were incubated under slurry conditions (1:4 soil:distilled water, w:w). Slurried conditions were applied to favor the diffusion of the herbicide and to increase the mobility of the soil microorganisms in order to stimulate atrazine mineralization (*26*).

For the slurries, 10 g subsamples were chosen to ensure sufficient turbation of the samples and to avoid a separation of the soil from the liquid phase. The pH value for all soils was measured at the beginning and at the end of the incubation period in the slurry reactors, and remained in the same range (BS: 6.2; BC: 6.0; CM: 6.8; CK: 5.5, compared to data in **Table 1**). Slurries were shaken continuously on a rotary shaker at 125 rpm to promote aeration of the samples.

All samples were placed in the dark at 20 $\pm$2 °C. Evolving $^{14}CO_2$ was trapped in 1.5 mL 2 M NaOH solution, which was placed in a glass vial inside the hermetically closed bottles. Traps were periodically replaced and washed with 4 mL dionized water, and the combined liquids were analyzed via LSC using 10 mL Instant Scint-Gel Plus^{TM} as scintillation cocktail (Perkin-Elmer).

Analysis

Soil sample extraction

Bulk field soils In a first step, an accelerated solvent extraction (ASE; ASE 200, Dionex; in accordance with (*7*)) was applied to all bulk field soils directly after field sampling. A water-methanol solution (1:4 v:v) was used to determine potential atrazine background levels in the soils as a result of former field applications or shift contamination. Freeze-dried (Lyovac GT2,

Steris) soil samples were weighed in 11 mL stainless steel ASE cells. The remaining space above the samples was filled with fine, annealed sea sand (Merck) to reduce the extract volume and to avoid clogging of the ASE steel filter lid. The extraction temperature was adjusted to 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 cell purging was set to 100 sec using nitrogen gas.

Experimental soils To distinguish between a potentially mobile and a soil-bound atrazine fraction, soil samples were initially water-extracted prior to ASE. At days 0, 4, 8, 15, 30, after incubation, and 92 for BS/BC and 98 for CK/CM, respectively, the triplicates of 20 g dry soil equivalents of ^{14}C-atrazine spiked soil samples were placed in 250 mL poly-ethylene centrifuge vials (Beckman) and deionized water was added (soil+water 1+8 w:w(*27*)). The soil-water mixture was shaken for 6 h at 150 rpm (Horizontal Shaker SM 25, Edmund Büler), and samples were centrifuged at 10,000 g for 90 min (Beckman J2-21, Rotor JA 14). An aliquot of 100 mL of the clear supernatant was filtered (0.45 μm, cellulose-acetate filter, Type 111, Sartorius). The filtered samples were used for pH, extracted ^{14}C-activity (LSC) and extracted atrazine and atrazine metabolite analyses (LC-MS/MS). The residual ^{14}C-activity in the aqueous phase remaining in the soil sample was considered for the calculations. The amount of remaining water-extracted ^{14}C-activity was discounted from the subsequent first ASE extract and added to the calculated amount of total water-extractable ^{14}C-activity. Prior to ASE, the water-extracted soil samples were freeze-dried.

To quantify the more strongly soil-associated atrazine fraction, an accelerated solvent extraction was applied subsequent to the water extraction, as described above. From each sample, a 10 g freeze-dried and homogenized soil subsample was used for ASE. Each sample was extracted four consecutive times under the same ASE conditions as mentioned above to recover the extractable fraction.

The possible influence of the applied ASE settings on the atrazine molecule was previously tested as follows: 3 mL of standard ethanol solution containing a total of 300 μg atrazine

was added to annealed sea sand; after the ethanol was evaporated, the sample was extracted as described. Periodic high-performance liquid chromatography (HPLC; Dionex, pump M480, sampler Gina 50, UV-detector UVD 3405) analyses of the extract detected only atrazine. No metabolites were found even over a lengthy extract storage period of 6 weeks.

Analyses of extracted ^{14}C-activity

To determine the water- and ASE-extracted residual ^{14}C-activity, a triplicate sample of 1.0 mL of each extract was mixed with 4.5 mL scintillation cocktail (Instant Scint-Gel Plus^{TM}, Perkin-Elmer), and radioactivity detection was performed by LSC. An external standard was used for quenching correction. Since most ^{14}C-activity was obtained in the first ASE step, the first extract was used for further LC-MS/MS analyses. The remaining ^{14}C-activity in the soil samples after four consecutive ASE extractions was determined by combustion and LSC analysis as described above and is referred to as non-extractable residues (NER).

LC-MS/MS analyses of soil extracts

Atrazine (chemical purity: 97.4%) and its metabolite 2-hydroxy-atrazine (96.0%) were purchased from Riedel-de Haën. All further atrazine metabolites such as desethyl-desisopropyl-2-hydroxy-atrazine (98.0%), desisopropyl-2-hydroxy-atrazine (98.5%), desethyl-desisopropyl-atrazine (98.5%), desethyl-2-hydroxy-atrazine (99.5%), desethyl-atrazine (96.0%), and desisopropyl-atrazine (98.7%) were purchased from Dr. Ehrenstorfer GmbH, Germany. For the quantification of atrazine and its metabolites, deuterated (D_5)-atrazine (99.0%), (D_5)-2-hydroxy-atrazine (97.0%), and (D_5)-desethyl-atrazine (99.0%, all from Dr. Ehrenstorfer GmbH, Germany) were used as internal standards with a sample concentration of 0.1 μg mL^{-1} for (D_5)-atrazine and (D_5)-2-hydroxy-atrazine, respectively, and 0.5 μg mL^{-1} for (D_5)-desethyl-atrazine, due to its smaller ionization efficiency.

Liquid extracts were analyzed by HPLC-MS/MS in triplicates for atrazine and its metabo-

lites as described elsewhere (*28*). An HPLC (Agilent Series 1100) with binary pump and temperature-controlled column compartment (22°C), equipped with CTC-HTC-PAL sampler was used. MZ Perfect Sil Target ODS-3 was used as the stationary phase (2.1 mm × 125 mm × 3 μm), and an additional HPLC pre-column was applied (2.1 mm × 10 mm × 3 μm, both MZ Analysentechnik Mainz, Germany). A mixture of acetonitrile (solvent A; Biosolve LCMS) and 2 mmol ammonium acetate solution (solvent B; Merck) was used as the HPLC eluent (gradient program: 0-20 min 5% solvent A + 95% solvent B; 20-30 min 100% solvent A; 30-40 min 5% solvent A + 95% solvent B). The flow rate was 0.15 mL min^{-1}. The total injection volume of each sample was 10 μL.

The MS analyses were performed with a Thermo Electron Model TSQ-Quantum 2002 in positive electrospray ionization (ESI+) and in multiple reaction monitoring (MRM) mode.

To consider potential quenching effects of the additionally extracted soil matrix, 30 μL of D_5-standard solution containing the individual deuterated standard were dissolved in 100 μL aliquots of water or ASE soil extracts ("blanks"), respectively.

The linear operating ranges including the limits of quantification were evaluated for each compound in its respective liquid matrix (water- or ASE-extracted soil matrix). The differing linear operating ranges are presented in **Table 2**. The results of the analyses were calculated in accordance with the standardized signal-to-noise ratio of 3:1 for the detection limit, and 10:1 for the limit of quantification, respectively.

Elementary analysis of soil samples

Analysis of C_{org} and the soil elements was performed as described elsewhere (*7*). Carbon quantification was conducted using a Leco RC-412 multiphase carbon determinator.

Statistical analysis

The significance of differences between the mean values of ^{14}C-atrazine mineralization at 50% WHC_{max} and under slurried conditions was calculated using the independent two-sample *t*-test. Statistical significance values are given as *Si* in %.

RESULTS AND DISCUSSION

Mineralization of ^{14}C-atrazine

The amount of mineralized ^{14}C-atrazine in BS soil reached a plateau after 20 days of incubation and accounted for 81.1% at 50% WHC_{max} and 83.5% under slurried conditions after 92 days (**Figure 1(a)**). The pattern and amount of observed ^{14}C-atrazine mineralization is in accordance with other investigations using atrazine-treated farmland soil from Brazil, France, Canada and the US (*14*, *16*, *29*). In these studies, an atrazine mineralization of 60-85% after 20 days indicated an atrazine-degrading soil microbial community. The mineralization of ^{14}C-atrazine reached a maximum of 14% d^{-1} after 7 days of incubation for both 50% WHC_{max} and slurried conditions. The mineralization decreased rapidly after 13 and 20 days, respectively, accounting for <1% d^{-1} until the end of the experiment (**Figure 1(b)**).

In the control BC soil, 39.7% and 81% of the total applied ^{14}C-atrazine was mineralized at the end of the incubation period (92 days) at 50% WHC_{max} and under sluried conditions. In BC soil, mineralization increased after an extended lag-phase of 25 and 13 days, respectively (**Figure 1(a)**). Since the BC soil was collected from a pear orchard adjacent to the atrazine-adapted field, it can be assumed that atrazine contamination occurred due to drifting during field application or surface runoff. This would have stimulated microbial communities in this soil capable of mineralizing atrazine. Such a shift by surface runoff or airborne transport of atrazine-degrading organisms is undocumented, but should be considered since even interhemispheric soil bacteria deposition has been described in earlier studies (*30*, *31*). It should be taken

into account that certain microbes in this soil have native atrazine-degrading ability. Similar degradation patterns for soils from plots to which atrazine was not directly applied have been reported previously, and in these studies atrazine degradation in the control soils was attributed to the transport of degrading organisms from implements used for bed formation, tractor tires, harvesting equipment, and irrigation or runoff (*32*). Mineralization was significantly higher under slurried conditions in BS (*Si* = 95-99.95%) and BC (*Si* = 75-99.95%), which can be explained by a disaggregation of soil particles and homogeneous distribution of degrading strains and atrazine molecules in solution resulting in an increased microbial accessibility. An earlier study on atrazine mineralization using an atrazine-degrading *Pseudomonas* strain demonstrated that the rate of atrazine removal was proportional to the soil water content and the amount of degrading bacteria in the soil and that enhanced atrazine mineralization was promoted by slurry conditions (*26*). The mineralization rate in BC slurry was delayed compared to BS, accounting for 6% d^{-1} after 20 days of incubation (**Figure 1(b)**). The relatively high standard deviation in the BC slurry was attributed to initial differences in the extent of ^{14}C-atrazine mineralization, which equalized as a function of time.

After an extended lag-phase of 30 d, 10.3% and 6% of applied ^{14}C-atrazine was mineralized in CK soil after 98 days of incubation, at 50% WHC_{max} and under slurried conditions, respectively (**Figure 1(c)**). Even though CK soil was subjected to repeated atrazine application from 1986 to 1989, it remains rather speculative as to whether these limited applications induced an enhanced microbial atrazine degradation ability at that time. However, the experimental reapplication of atrazine after two decades without any additional atrazine treatment resulted in a slightly enhanced degradation of the chemical within the time frame of the investigation (**Figure 1(d)**). This may support previous observations of an enhanced atrazine mineralization after a single annual atrazine application (*32*).

For the CM soil, the mineralization of ^{14}C-atrazine remained low for the entire incubation period, totaling 4.7% at 50% WHC_{max} and 2.7% under slurried conditions, respectively (**Figure 1(c)**). This observation was expected since no atrazine application has ever been re-

ported for CM soil and the mineralization rate remained lowest for all soils tested (**Figure 1(d)**). In the CK and CM soils, slurried conditions did not favor an accelerated atrazine mineralization, and mineralization was statistically significantly higher at 50% WHC_{max} conditions (both *Si* = 75-99.95%). However, since the atrazine mineralization remained comparably low in both CK and CM soils under both moisture regimes within the experimental period, an adapted soil phenomena could not be adressed at this point.

For the atrazine-adapted BS soil, the graphically determined half-life for atrazine amounted to 9 days for both 50% WHC_{max} and slurried conditions (**Figure 1(a)**) and was in accordance with previous studies (*11*). In accordance with Krutz et al. (*10*), who applied a multiple linear regression model to generate atrazine persistence estimates, the BS soil can be categorized as an adapted soil with a half-life of $\leq$15 days. The BC slurry reactors exhibited a mean half-life of 21 days and can therefore be classified as an intermediately adapted soil with a half-life between 15 and 30 days (*10*). A half-life was not determined for BC 50% WHC_{max}, CK or CM because the mineralization remained less than 50% after the entire incubation time (**Figure 1(b)**). It is important to note that half-life determinations based on short-term laboratory experiments can be misleading. Firstly, stringent soil sorption/entrapment of small atrazine fractions may exclude the molecules from microbial attack, resulting in an unexpected persistence of these atrazine fractions. Secondly, results of a rapid dissipation in the initial phase of an experiment due to enhanced microbial degradation processes may not consider the microbially inaccessible and stable atrazine fraction.

Extractable atrazine residues and metabolites

ASE extractions of bulk field soils

Atrazine and a number of its metabolites were extracted and quantified in all bulk soils directly after field sampling by means of ASE and LC-MS/MS analyses (**Table 3**, **Figure 2(b)/(d)**, and **Figure 3(b)/(d)**). The lower amount of extractable atrazine in BS compared to CK soil (8.3 $\pm$0.9 versus 15.2 $\pm$0.2 μg kg^{-1}; **Table 3**) must be attributed to the high atrazine miner-

alization capacity in BS soil due to regular atrazine applications over the last 30 years. The fact that atrazine is still detectable in CK soil two decades after the last atrazine application demonstrates the long-term persistence of atrazine in soil. These results may provide evidence of a wide-scale presence and persistence of this pesticide compound in soils with and without recent atrazine application history. These findings verify previously published results highlighting the recalcitrant behavior of atrazine and its metabolites in field soils on long time scales (*7*). Even though no atrazine or other *s*-triazine pesticide applications were reported for the BC and CM soils, desisopropyl-2-hydroxy-atrazine and desethyl-desisopropyl-2-hydroxy-atrazine were extracted and quantified, yielding 55.6 $\pm$13.3 (BC) / 25.9 $\pm$8.3 (CM) and 100.9 $\pm$2.3 μg kg^{-1}, respectively. The background level of atrazine and its metabolites thus obtained must be attributed directly to previous atrazine applications (BS and CK soils) or indirectly to drift contamination or undocumented atrazine or triazine pesticide application (BC and CM soils). Blank samples were analyzed regularly within the analytical process to exclude potential contamination of samples and laboratory/analytical devices. For atrazine, the overall background level was <1.5% of freshly applied atrazine for the incubation experiments, and was therefore not considered in the calculation. However, the occurrence of atrazine and its metabolites in all soils used, particularly in the various soils used as controls (BC, CM, CK) must be considered carefully.

Water extraction of atrazine-spiked and incubated soils

Prior to ASE extraction, water extraction was used to obtain a readily available fraction of atrazine and/or its metabolites at different incubation times from the incubated soil samples. Subsequent to the experimental atrazine application, the major metabolite 2-hydroxy-atrazine was desorbed and detected as well as the parent compound atrazine at T0-T8 for BS, T0-T92 for BC, T0-T30 for CK, and T0-T98 for CM soils, respectively (**Figure 2(a)/(c)**; **Figure 3(a)/(c)**). The water extractability of 2-hydroxy-atrazine from soil, even after long-term environmental aging, has been reported previously (*28*), and the rapid occurrence of 2-hydroxy-atrazine is

strongly related to microbial degradation activity (*33*). However, minor differences in pH and C_{org} content in the soils and within the individual reactors may have influenced sorption and desorption behavior, which could explain the relatively high standard deviations in the water extracts of BS and BC soils.

ASE extraction of water-extracted soils

Since most ^{14}C-activity was obtained in the first ASE extraction step (BS: 86.3-99.6%; BC: 70.9-98.0%; CK: 88.4-98.9%; CM: 84.6-99.3%; calculated for total ASE-extractable ^{14}C-activity in triplicate samples on the individual sampling days), the first extract was used to detect and quantify residual atrazine and its metabolites by means of LC-MS/MS analyses. A number of hydroxy-atrazine analogues were detected in all soils after experimental atrazine application throughout the entire incubation period (**Figure 2(b)/(d)**, **Figure 3(b)/(d)**). These results indicate that a dechlorination of the atrazine molecule occurs rapidly, and that the hydroxy analogues, such as 2-hydroxy-atrazine, desethyl-2-hydroxy-atrazine, and desisopropyl-2-hydroxy-atrazine, remain fairly stable in the soil (*33*). At the end of the incubation period (T92/T98), the amount of extractable atrazine and most of its metabolites remained significantly higher (*Si* = 95-99.5%) at 50% WHC_{max} as well as under slurried conditions than the amount of extractable atrazine and its metabolites in all respective bulk soils directly after field sampling (**Figure 2(b)**, **Figure 3(b)**). To our knowledge, background atrazine levels in atrazine-adapted soils and the bioaccessibility of these aged atrazine residues have not been considered or monitored in previous investigations. The results suggest that only freshly applied atrazine is mineralized to a large extent. However, it can be assumed that even in adapted soils, atrazine is not completely degraded over extended time periods. It remains more or less stabilized in the soil matrix, and may slowly accumulate. Even though earlier studies reported reduced bioavailability/biodegradation due to soil sorption (*34*, *35*), nothing is known about the potential leaching of these persistent atrazine pools into deeper soil layers, where microbial activity is reduced, causing long-term environmental persistence. The influence of organic carbon on atrazine sorption has already

been described elsewhere (*36*). Binding or intrusion of the chemical to soil components, such as organic carbon, could result in a decrease in its accessibility for soil microbes, which does not necessarily exclude the molecule from environmental interactions.

^{14}C-activity distribution and total recovery

Depending on the rate of mineralization the water-extractable ^{14}C-activity decreased for all soils under 50% WHC_{max} conditions, except for CK. Simultaneously, the ASE-extractable ^{14}C-fraction increased, indicating a redistribution of the ^{14}C-labeled compound from weaker to stronger adsorption sites (*29*, *37*).

In BS, the amount of water-extractable ^{14}C-activity accounted for 78.5% at day 0 (T0), decreasing to 1.6% at the end of the incubation period (T92). The simultaneously high increase in mineralized ^{14}C-labeled atrazine at 50% WHC_{max} conditions (4.5% at T4 to 81.2% at T92) provides evidence that a water-extractable atrazine fraction favors enhanced bioaccessibility and bioavailability (**Figure 4(a)**). Even though the total amount of the water- and ASE-extractable fractions decreases over the entire incubation period, the amount of NER remained constant at 4.1% from T30 to the end of the incubation period (T92) for the atrazine-adapted BS soil (**Figure 4(a)**). Since we were able to extract atrazine from BS soil directly after field sampling, this result provides evidence that a certain amount of atrazine remains stable, even in this soil exhibiting accelerated atrazine degradation. Since no perceptible mineralization is observed between T30 and T92, the ASE-extractable fraction, accounting for 8.4% and 6.5%, respectively, can be considered to be less accessible for biodegradation.

Decreased water-extractable ^{14}C-activity in BC soil from 79.8% (T0) to 54% (T15) with no conspicuous mineralization until day 30 (5.4% at T30 to 39.7% at T92) implies a rapid increase in the adsorption of the pesticide to the soil matrix and the formation of NER (2.5% at T0 to 15.6% at T92; **Figure 4(b)**). This observation is in accordance with the general opinion that pesticide sorption increases with soil-contact time, mostly in relation to the organic carbon content in the soil (*38*). Similar observations on atrazine-NER formation were presented previously

for a non-atrazine-mineralizing soil, suggesting that the slow decrease in the aqueous fraction may explain the common occurrence of atrazine and its metabolites in ground waters through successive leaching from the persistent pesticide pool (*39*).

A fairly constant amount of water-extractable and ASE-extractable ^{14}C-activity in CK soil over the entire incubation period (58.5% at T0 to 38.3% at T98 and 41.7% to 44.7%, respectively; **Figure 4(c)**) might be related to the different soil properties and soil texture in CK soil compared to BS, BC and CM soils. A high sand content and only small amounts of organic C, silt and clay (**Table 1**), as influential parameters for atrazine sorption and molecule entrapment, can be held responsible for the steady water-extractable residual ^{14}C-activity in CK soil. This finding may provide evidence of successive atrazine leaching in sandy field soils, where only minor atrazine degradation occurs.

The pattern of ^{14}C-activity distribution in the different fractions in CM soil describe a constant decline in water-extractable ^{14}C-activity and an increase in ASE-extractable and non-extractable ^{14}C-activity residues (**Figure 4(d)**). Since no remarkable ^{14}C-atrazine mineralization can be observed in CM soil, the behavior of the applied ^{14}C-atrazine in CM soil may be representative for a non-atrazine-adapted Orthic Luvisol under the applied experimental conditions.

Since most ASE-extractable residues were identified at the end of the study as atrazine or its metabolites under both 50% WHC_{max} and slurried conditions (**Figure 2(b)/(d)** and **Figure 3(b)/(d)**), it can be assumed that the non-extractable ^{14}C-fraction may partly consist of these molecules or e.g. biogenic ^{14}C-residues, which are strongly absorbed or entrapped in the soil matrix. Further research is needed to evaluate the nature of the non-extractable pesticide residues and their remobilization potential as a possible consequence of changing environmental conditions in order to predict the long-term fate of these compounds. The influence of changing dry-wet cycles on the enhanced water extractability of several environmentally long-term aged ^{14}C-labeled pesticide residues is currently being investigated.

Abbreviations used

1. BS: Belgian Gleyic Luvisol atrazine-treated field soil
2. BC: Belgian Gleyic Luvisol control soil from an adjacent field plot used as a pear orchard
3. CM: control soil, Orthic Luvisol from Merzenhausen, Germany
4. CK: control soil, Gleyic Cambisol from Kaldenkirchen, Germany
5. WHC_{max}: maximum soil water holding capacity
6. ASE: accelerated solvent extraction
7. LC-MS/MS: liquid chromatography tandem mass spectrometry
8. MRM: multiple reaction monitoring
9. D_5 STD: deuterated standard
10. LSC: liquid scintillation counter
11. NER: non-extractable residues
12. HPLC: high-performance liquid chromatography

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FIGURE CAPTIONS

Figure 1: Cumulative $^{14}CO_2$ and rates of ^{14}C-atrazine mineralization in the (a)/(b) Belgian field (BS) and orchard control (BC) soils, and in the (c)/(d) German field soils CM and CK, at a moisture content of 50% WHC_{max} and under slurried conditions. Standard deviation of $n \geq 3$. Standard deviations are indicated only when larger than symbols.

Figure 2: Water-extractable atrazine and 2-hydroxy-atrazine in (a) BS and (c) BC soil, and atrazine and its metabolites detected in their corresponding ASE extracts in (b) BS and (d) BC soils. Standard deviation of $n \geq 3$. b.d.l. = below detection limit.

Figure 3: Water-extractable atrazine, 2-hydroxy-atrazine and desethyl-atrazine in (a) CK and (c) CM soils, and atrazine and its metabolites detected in their corresponding ASE extracts in (c) CK and (d) CM soils. Standard deviation of $n \geq 3$. b.d.l. = below detection limit.

Figure 4: Total recovery of all respective ^{14}C-fractions for (a) BS, (b) BC, (c) CK, and (d) CM soils. The ASE-fraction represents the total extracted ^{14}C-activity after four consecutive sample extractions. NER = non-extractable residues. Standard deviation of $n \geq 3$.

TABLES

Table 1: Soil Properties, pH and Organic Carbon Content. Data for the German Merzenhausen (CM) and Kaldenkirchen (CK) control soil have been presented elsewhere (*24*, *40*). BS: Belgian field soil; BC: Belgian control soil. ± standard deviation for C_{org} of $n = 3$. Values for sand, silt, clay, and C_{org} are given in weight-%.

soil	**(BS)**	**(BC)**	**(CM)**	**(CK)**
sand	30.12	30.12	3.00	73.30
silt	62.05	62.05	79.00	23.10
clay	7.83	7.83	18.00	3.60
pH	5.94	6.02	7.00	5.35
C_{org}	1.26 ±0.02	1.89 ±0.22	1.04	0.99
texture (US System)	silt loam	silt loam	silt loam	sandy loam

Table 2: LC-MS/MS Linear Operating Ranges for Atrazine and its Respective Metabolites in the Water- and ASE-Extract Soil Matrix. l.o.d.: limit of detection for each compound in the HPLC eluent consisting of 95% 2 mmol ammonium acetate solution + 5% acetonitrile.

	water matrix [μg mL^{-1}]	ASE matrix [μg mL^{-1}]	l.o.d. in HPLC eluent [ng mL^{-1}]
atrazine	0.0005 - 1.0	0.001 - 1.0	0.005
2-hydroxy-atrazine	0.001 - 1.0	0.005 - 1.0	0.01
desisopropyl-atrazine	0.005 - 1.0	0.02 - 1.0	0.06
desethyl-atrazine	0.015 - 1.0	0.03 - 1.0	0.04
desethyl-2-hydroxy-atrazine	0.0025 - 0.5	0.01 - 0.5	0.04
desethyl-desisopropyl-atrazine	0.03 - 0.5	0.05 - 1.0	0.25
desisopropyl-2-hydroxy-atrazine	0.0025 - 1.0	0.01 - 0.5	0.2
desethyl-desisopropyl-2-hydroxy-atrazine	0.01 - 0.5	0.05 - 1.0	0.4

Table 3: Background Levels of Atrazine and its Metabolites in the Field Bulk Soils as Determined by ASE and LC-MS/MS Analyses. ± standard deviation for n = 4. b.q.l. = below quantification limit. b.d.l. = below detection limit.

	soils used			
	BS	BC	CM	CK
compound background level	[μg kg^{-1}]	[μg kg^{-1}]	[μg kg^{-1}]	[μg kg^{-1}]
atrazine	8.3 ±0.9	b.d.l.	b.q.l.	15.2 ±0.2
2-hydroxy-atrazine	37.8 ±5.0	b.q.l.	b.q.l.	12.8 ±0.1
desisopropyl-atrazine	b.d.l.	b.d.l.	b.d.l.	b.d.l.
desethyl-atrazine	b.q.l.	b.d.l.	b.d.l.	b.d.l.
desethyl-2-hydroxy-atrazine	48.3 ±6.7	b.d.l.	b.d.l.	b.d.l.
desethyl-desisopropyl-atrazine	b.d.l.	b.d.l.	b.d.l.	b.d.l.
desisopropyl-2-hydroxy-atrazine	2.9 ±0.4	55.6 ±13.3	25.9 ±8.3	18.3 ±2.3
desethyl-desisopropyl-2-hydroxy-atrazine	b.q.l.	100.9 ±2.3	b.q.l.	b.q.l.

FIGURES

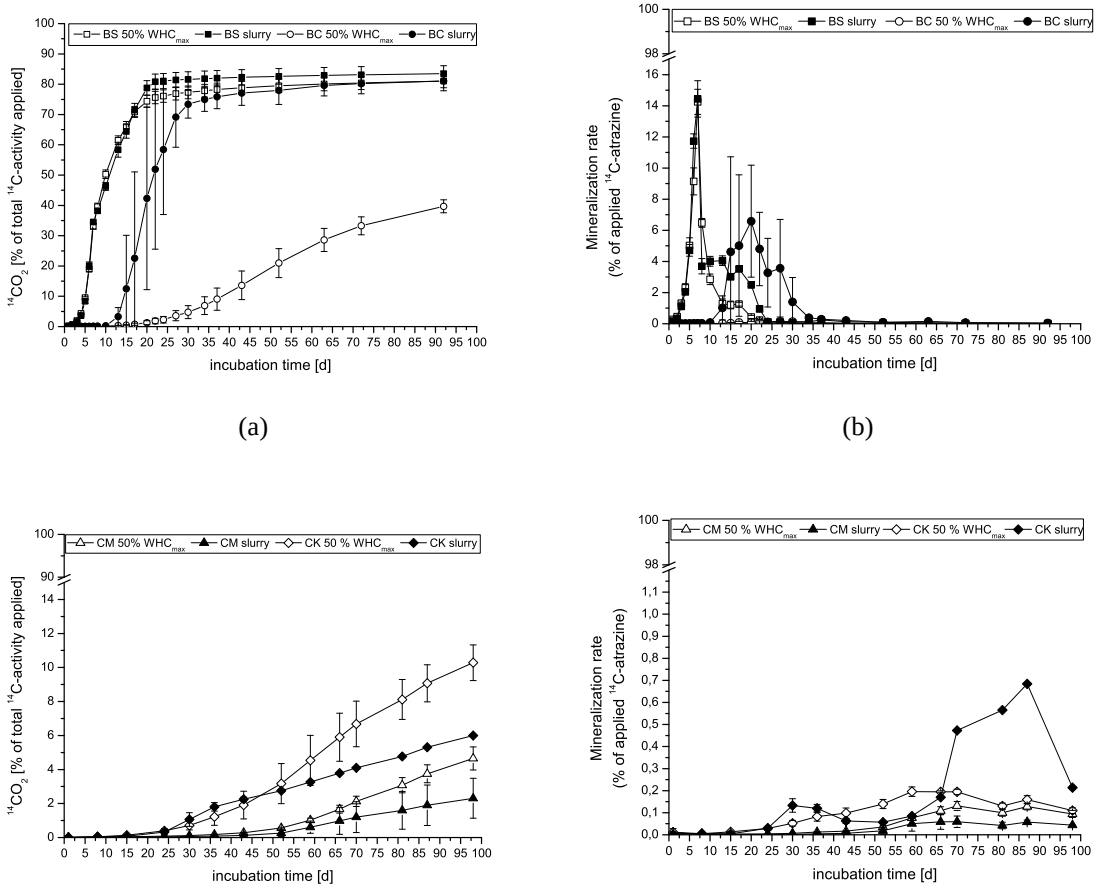

Figure 1

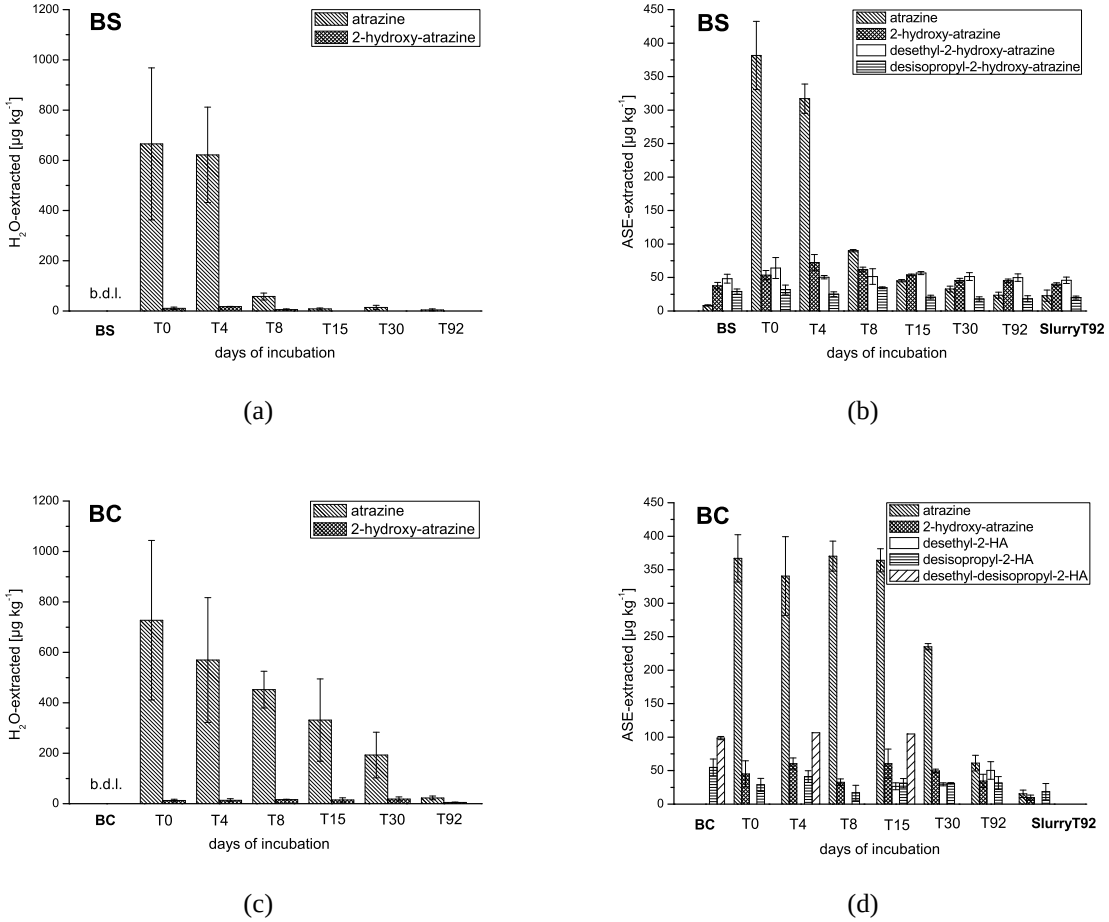
BS
atrazine
2-hydroxy-atrazine
H2O-extracted [µg kg-1]
b.d.l.
BS T0 T4 T8 T15 T30 T92
days of incubation
(a)
BS
atrazine
2-hydroxy-atrazine
desethyl-2-hydroxy-atrazine
desisopropyl-2-hydroxy-atrazine
ASE-extracted [µg kg-1]
BS T0 T4 T8 T15 T30 T92 SlurryT92
days of incubation
(b)
BC
atrazine
2-hydroxy-atrazine
H2O-extracted [µg kg-1]
b.d.l.
BC T0 T4 T8 T15 T30 T92
days of incubation
(c)
BC
atrazine
2-hydroxy-atrazine
desethyl-2-HA
desisopropyl-2-HA
desethyl-desisopropyl-2-HA
ASE-extracted [µg kg-1]
BC T0 T4 T8 T15 T30 T92 SlurryT92
days of incubation
(d)

Figure 2

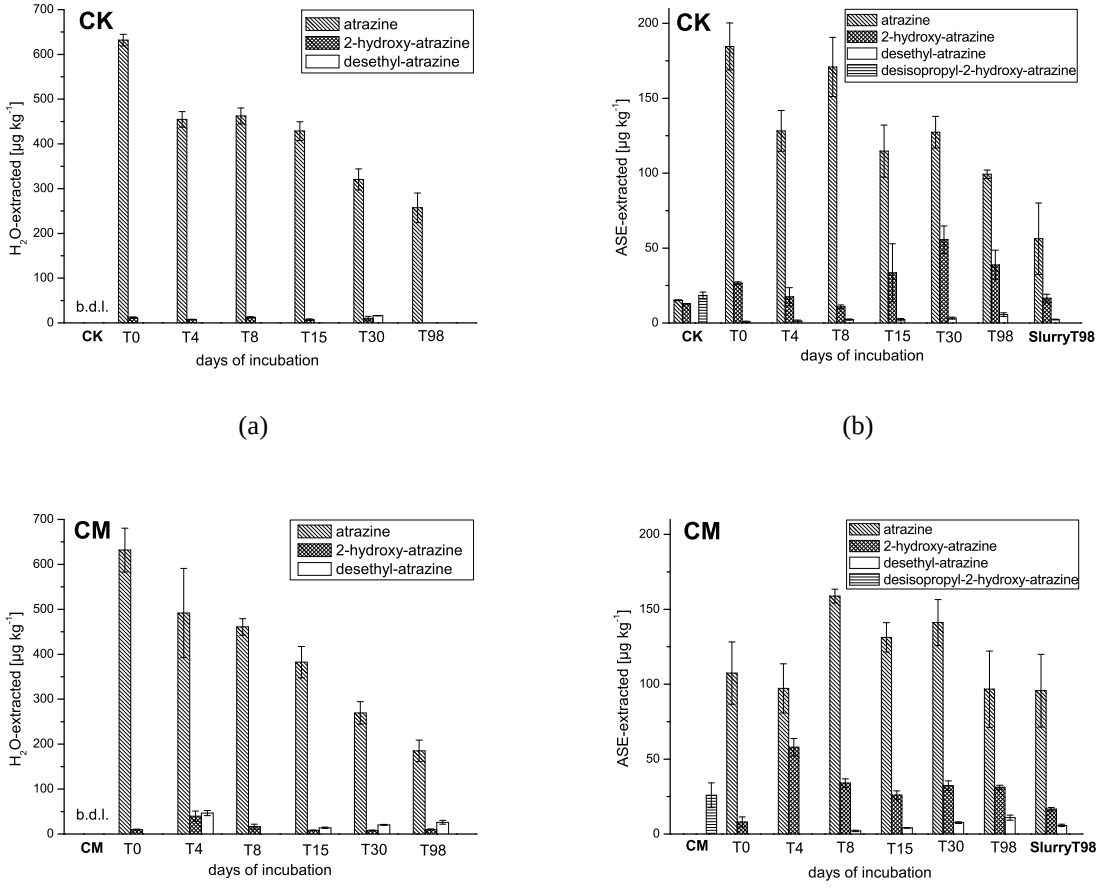
CK
atrazine
2-hydroxy-atrazine
desethyl-atrazine
desisopropyl-2-hydroxy-atrazine
H2O-extracted [µg kg-1]
ASE-extracted [µg kg-1]
b.d.l.
CK T0 T4 T8 T15 T30 T98
CK T0 T4 T8 T15 T30 T98 SlurryT98
days of incubation
(a)
(b)
CM
(c)
(d)

Figure 3

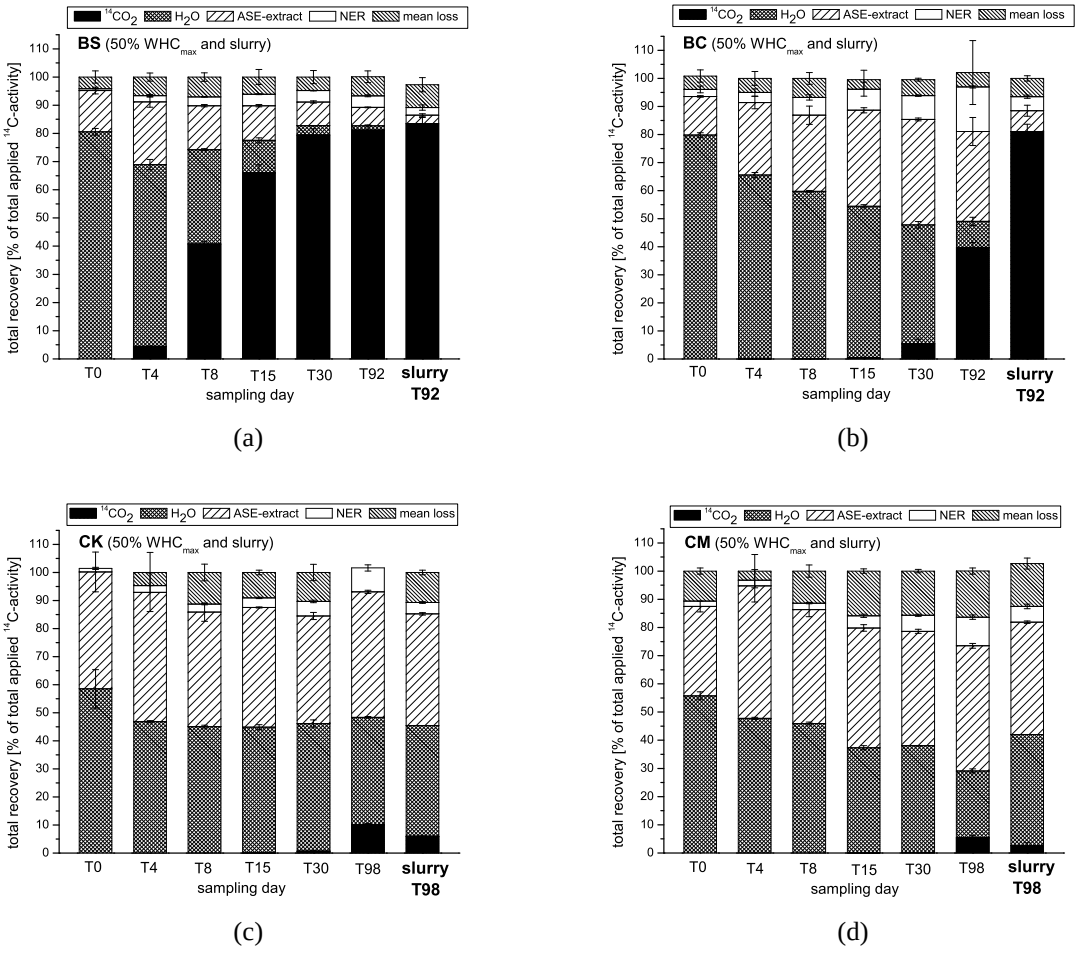
$^{14}CO_2$ H_2O ASE-extract NER mean loss
BS (50% WHC_{max} and slurry)
BC (50% WHC_{max} and slurry)
CK (50% WHC_{max} and slurry)
CM (50% WHC_{max} and slurry)
total recovery [% of total applied ^{14}C-activity]
sampling day
T0 T4 T8 T15 T30 T92 slurry T92
T0 T4 T8 T15 T30 T98 slurry T98
(a)
(b)
(c)
(d)

Figure 4