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Effect of Swine Manure on Sulfamethazine Degradation in Aerobic and Anaerobic Soils

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Abstract Degradation and fate of sulfamethazine (SMZ) were determined under aerobic and anaerobic conditions in soil with and without swine manure amendment. For both aerobic and anaerobic conditions, SMZ disappeared rapidly during the first 7 days followed by slow disappearance which may indicate that SMZ had become more persistent and less available. For soils receiving 100 mg/kg of SMZ, the percent of SMZ remaining in the soil after 63 days were between 25 and 60 %. Depending on the initial SMZ concentration, estimated half-lives for aerobic and anaerobic incubations ranged from 1.2 to 6.6 and 2.3 days to more than 63 days, respectively. Addition of manure (0.054 g/g soil) did not significantly affect the half-lives of SMZ. Inhibitory effects of SMZ on anaerobic microbial respiration were observed in unamended soil at concentrations of 50 mg/kg or higher, but only transient inhibitory effects were found in aerobic soil. Five to 22 % of the ^{14}C[phenyl]-SMZ added were extracted at the end of the incubations while 70 to 91 % of the ^{14}C were converted to bound (non-extractable) forms in both manure amended and unamended soil. Only 0.1 to 1.5 % of ^{14}C-SMZ was mineralized to $^{14}CO_2$. Disappearance of SMZ in sterilized soil was not completely halted indicating possible contribution of abiotic processes to the disappearance of SMZ in soil.

Keywords Antibiotic · Sulfonamide · Sulfamethazine · Inhibition · Metabolites · Degradation

1 Introduction

Veterinary antibiotics from animal feedlots are of concern as they may result in an increase in antimicrobial resistant bacteria (Sengeløv et al. 2003; Sapkota et al. 2007) and may have ecotoxicological impacts on aquatic organisms and humans (Sarmah et al. 2006; Schauss et al. 2009). Sulfamethazine (SMZ), a sulfonamide compound, is a common antibiotic used in the swine industry (Sarmah et al. 2006). Sulfonamide antibiotics and their metabolites excreted by animals enter the environment via land application of manure (Haller et al. 2002; Hamscher et al. 2005). Sulfonamide concentrations in manure were found to be 20 mg/kg (wet manure) (Haller et al. 2002) and SMZ concentrations were found to be 7.2 mg/kg on a dry matter basis (Hamscher et al. 2005; Aust et al. 2008). Aqueous concentrations of sulfonamides greater than 20 μg/L were found in the manure lagoons in Iowa and Ohio (Campagnolo et al. 2002). In runoff water from fields treated with beef cattle manure, SMZ concentrations ranged from 2.6 to 3.9 μg/L (Amarakoon et al. 2014). At a swine

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manure composting facility in Korea, the maximum concentration found in surface waters was 9.60 μg/L while the concentrations of SMZ in soils were found to be 1.1 μg/kg (Awad et al. 2014). In a livestock farm in northern Germany, SMZ concentration in groundwater was found to be as high as 0.24 μg/L and a soil concentration of 2 μg/kg (Hamscher et al. 2005).

Data on the sorption and degradation of sulfonamides are important in understanding the fate and impact of sulfonamides in the environment. Sorption of SMZ and other sulfonamides to soils are a function of soil organic matter and soil pH (Tolls 2001; Sarmah et al. 2006; ter Laak et al. 2006; Lertpaitoonpan et al. 2009). Half-lives of sulfonamides in aerobic soils ranged from <1 to 30 days (Boxall et al. 2004; Blackwell et al. 2005, 2007; Wang et al. 2006b; Accinelli et al. 2007; Yang et al. 2009; Topp et al. 2012). N^4-acetyl-sulfamethazine (N^4-acetyl-SMZ), des-amino-sulfamethazine (des-amino-SMZ), and N^1-methyl sulfamethazine (N^1-methyl-SMZ) were the main metabolic forms of SMZ found in swine manure (Paulson et al. 1981) and other animal excreta (Garcia-Galán et al. 2008). It is not clear whether these metabolites were formed solely by chemical reactions or by microbial degradation in the animal digestive systems. Several researchers have found that degradation of sulfonamides in manure and in soils were affected by the initial antibiotic concentration, moisture, temperature (Wang et al. 2006a), and soil texture (Accinelli et al. 2007). Addition of manure slurry to soil increased the degradation rates of SMZ and sulfadimethoxine, which may be due to increased microbial population or activity (Wang et al. 2006a; Accinelli et al. 2007). Doses of sulfapyridine ranging from 0.003 to 1.14 mg/kg, and from 6.45 to 86.5 mg/kg caused 10 and 50 % inhibition of microbial activity, respectively, in a Fe(III) reduction test (Thiele-Bruhn and Beck 2005). Inhibition of microbial degradation is probably due to the impact of sulfonamides on the folic acid synthesis process (Skold 2000).

This study investigated the degradation of SMZ at different initial concentrations under aerobic and anaerobic conditions in soil, and with and without swine manure amendment. ^{14}C-SMZ was used to determine the extent of SMZ mineralization and the distribution of SMZ and its metabolites in soils. In addition, the impact of initial SMZ concentrations on soil microbial respiration was investigated.

2 Materials and Methods

2.1 Soil and Swine Manure

Clarion soil was collected at a depth of 0 to 15 cm from a cornfield near Ames, Iowa. The soil was thoroughly homogenized, partially dried at room temperature, sieved using a 2-mm opening sieve, and stored moist in a refrigerator. Soil moisture content was determined by weight loss after drying in an oven at 105 °C for at least 24 h. The soil was a loam with 44 % sand, 36 % silt, 20 % clay, and an organic carbon content of 2.6 %. The soil pH was 6.4, and the cation exchange capacity was 13.9 meq/100 g (analyzed by Midwest Laboratories, Omaha, NE). Swine manure slurry was collected from the deep pit of a swine farm near Boone, Iowa and stored in a refrigerator until use. The pH of liquid manure was 8.9. The liquid manure was analyzed for its carbon content using a CNS analyzer (Elementar Vario MAX CNS Analyzer, Hanau, Germany). The manure had 8.1 % dry matter, 36.8 % carbon, and 3.9 % nitrogen content on a dry matter basis.

2.2 Chemicals

Sulfamethazine (4-amino-N-[4, 6-dimethyl-2-pyrimidinyl]-benzenesulfonamide; CAS number 57-68-1) with a purity of 99 % was purchased from Sigma-Aldrich (St. Louis, MO). Physical-chemical properties of SMZ include the following: molecular weight of 278.34 ($C_{12}H_{14}N_4O_2S$), log K_{ow} of 0.89, solubility of 1.5 g/L, and pK_1 and pK_2 of 2.65±0.2 and 7.4±0.2, respectively. A stock solution of 375 mg/L of SMZ in 10 % methanol and 90 % deionized water was prepared. Concentrations of 37.5 and 3.75 mg/L of SMZ solutions were prepared by diluting the 375 mg/L solution with deionized water. ^{14}C[phenyl]-SMZ was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Acetonitrile, HPLC and chromatography grade water for HPLC analysis, and methanol were purchased from Burdick & Jackson (Muskegon, MI).

2.3 Aerobic and Anaerobic Degradation Experiments

Aerobic degradation of SMZ was determined in laboratory incubation experiments. Moist soil was weighed (equivalent to 15 g dry weight) and placed in 40-mL screw-top amber-glass tubes. SMZ stock solutions were added to the soil to give initial SMZ concentrations in

the soils of 0.5, 5, 50, and 100 mg/kg. Deionized water was added to achieve a soil moisture content of 25 %. For manure-amended soil, 1 g of liquid swine manure was added to each tube. The tubes were capped, weighed, and incubated at 22±1 °C. Each tube was weighed every 3 days interval to determine the soil moisture, and water was added, if needed. Triplicate samples for all treatments were prepared. To determine the disappearance of SMZ, tubes were sacrificed, and samples extracted and analyzed with high pressure liquid chromatography (HPLC) for SMZ at 4, 7, 14, 21, and 28 days after the start of the experiment.

Sterile soil was used as a control to determine the contribution of abiotic processes in the degradation experiments. To prepare sterilized samples, unamended and manure-amended soil samples were weighed and placed in tubes in the same way as mentioned previously. The tubes were autoclaved for 30 min. To further ensure inhibition of microbial activities, 700 mg of sodium azide was added to each tube. Samples were treated with SMZ to obtain a 5 mg/kg initial concentration and incubated as described previously. At each sampling time, soil in the tubes were extracted and analyzed for the SMZ using HPLC. All the sterilized samples were prepared in a sterile hood.

Anaerobic degradation experiments were conducted in a similar manner as the aerobic degradation experiments. Soil samples were prepared in tubes and treated with SMZ. Tubes were then capped tightly with plastic screw caps and Teflon-faced rubber septa. The septa of the tubes were then pierced with a syringe needle connected to a vacuum pump, and the tubes were evacuated. Helium gas was then injected to purge the tubes, resulting in a helium gas headspace at atmospheric pressure. Soil samples were extracted at 7, 14, 21, 35, and 63 days after the start of the experiment.

2.4 Extraction of SMZ

The extractant used was a mixture of 80 % methanol and 20 % of a 0.1 M KOH solution. The KOH was added to adjust the pH of the soil-solution system above the pK_2 of SMZ to increase extractability. To extract the SMZ in soil, 10 mL of the methanol/KOH mixture was added to each tube. Samples were agitated for 3 h at 22±1 °C, and centrifuged at 2700×*g* for 15 min. The supernatant was then transferred to 15-mL glass volumetric tubes. The extraction was conducted three times for a given soil sample and the supernatants combined. The combined supernatants were evaporated using nitrogen gas in a N-EVAP analytical evaporator (Organomation Associates, Berlin, MA) at 41 °C, and the remaining residuals redissolved with 80 % phase A and 20 % phase B of HPLC mobile phase (details presented later). The liquid was filtered with 0.2 μm nylon membrane filter (13 mm polypropylene-encased) (Alltech, Deerfield, IL) and 2 mL of the filtrate transferred to HPLC vials for analysis.

Preliminary tests were conducted before the experiments to investigate the recoveries of SMZ. The extraction recoveries for a spiked concentration of 5 mg/kg in unamended soil, manure-amended soil, and sterilized manure-amended soil were 90, 88, and 92 %, respectively.

2.5 HPLC Analysis

SMZ was analyzed using an Agilent HPLC Series 1100 (Eagan, MN) with a photodiode array detector. The detection wavelength was 254 nm. Triplicate 50 μL injections were made for each sample. Mobile phase A contained water with 1 mM ammonium acetate and 0.1 % (*v*/*v*) glacial acetic acid and phase B consisted of acetonitrile and 0.1 % (*v*/*v*) glacial acetic acid. The initial flow rate was 0.5 mL/min and consisted of 10 % phase B. Phase B in the eluent was increased from 10 to 100 % at various times through the analysis (details provided in Lertpaitoonpan et al. 2009). Selected samples were analyzed by HPLC coupled with mass spectrometry to evaluate the identity of N^4-acetyl-SMZ, N^1-methyl SMZ, and des-amino-SMZ. Standards were obtained from USDA-ARS, Fargo, ND.

2.6 Sulfamethazine Effects on Microbial Respiration

Aerobic and anaerobic respiration experiments in unamended soil and manure-amended soil treated with SMZ at concentrations of 0, 0.5, 5, 10, 50, 100, and 150 mg/kg were prepared by placing moist soil (equivalent to 15 g dry weight) in 40-mL screw-top amber-glass tubes. For manure-amended soil, 1 mL of liquid manure was added into each tube. SMZ stock solutions were added to the soil to obtain the initial concentrations listed above and deionized water added to achieve a soil moisture of 25 %. For the aerobic experiments, tubes were capped loosely (to allow for exchange of air) and weighed. Tubes were uncapped once every 3 days and weighed to check if water was needed to maintain the 25 % moisture

content. The tubes were incubated at 22±1 °C. Soil without addition of SMZ was used as a control. Anaerobic conditions were obtained by capping the tubes tightly with screw caps and rubber septa. The tubes were evacuated using a syringe needle connected to a vacuum pump and the tubes purged with helium gas to create a helium gas headspace in the tubes.

Gas produced from samples were analyzed for carbon dioxide on days 2, 4, 6, 10, 14, 18, 24, 32, and 40 for aerobic incubations and on days 6, 14, 24, 32, 40, 52, 66, and 80 for the anaerobic incubations. During each gas sampling event for the aerobic experiment, the tubes were uncapped to release all gasses accumulated previously, and then capped tightly and incubated for 1 h. A 10-mL gas sample was obtained and transferred to a previously evacuated vial. After sampling the headspace for aerobic respiration, tubes were uncapped, weighed, and water added (if needed) to replenish moisture loss. All tubes were then incubated as described for aerobic respiration until the next sampling time.

For anaerobic incubations, the tubes were evacuated and purged with helium gas three times to flush out the gas produced earlier, and the tubes were incubated for 1 h before gas samples were collected. The procedures for collecting headspace gas were similar to that of aerobic incubations. After sampling, the tubes were evacuated and purged with helium gas and incubated anaerobically until the next sampling time.

Gas samples were analyzed for carbon dioxide using a SRI 8610C gas chromatograph (SRI Instruments, Torrance, CA) equipped with a flame ionization detector (FID), a HaySep D column (Alltech, Deerfield, IL) and an autosampler. The oven temperature was set at 50 °C. Standard curves were established using carbon dioxide concentrations of 503 to 100, 400 ppmv (SCOTTY®II standard gases, Scott Specialty Gas, Plumsteadville, PA). The amounts of carbon dioxide produced represented the hourly rate of gas generation at the particular time of sampling.

2.7 Fate of ^{14}C-SMZ

For this experiment, soils were prepared and treated in the same manner as the aerobic and anaerobic degradation experiments. Tubes were individually prepared with a total SMZ soil concentration of 0.5, 5, or 50 mg/kg. The SMZ consisted of unlabeled SMZ and ^{14}C-SMZ at a concentration of 1739 Bq of ^{14}C[phenyl]-SMZ per tube. A 2-mL glass vial containing 1 mL of 1 M NaOH was placed in each tube to trap $^{14}CO_2$ evolved. Tubes were capped and incubated. At each sampling event, NaOH solution was transferred into a scintillation vial and 6 mL of Ultima Gold™ XR cocktail (Perkin Elmer, Waltham, MA) was added. The ^{14}C in the scintillation vials were counted for 5 min using a Packard 1900TR liquid scintillation analyzer (Perkin Elmer, Waltham, MA). New vials filled with fresh NaOH were then placed back into the tubes.

At the last sampling event (28 days for aerobic treatment and 77 days for anaerobic treatment), SMZ was extracted from the soil in the same manner as described previously and the extracts evaporated to 5 mL. One mL of the extract was transferred into a scintillation vial with 6-mL cocktail and counted for 5 min to determine the total extractable ^{14}C. Two hundred microliters of the extracts were analyzed for extractable ^{14}C-SMZ and metabolites using a HPLC (Hewlett-Packard series 1100, Palo Alto, CA) with a mobile phase of 30 % methanol and a flow rate of 1 mL/min. The detection wavelength used was 254 nm. The HPLC was connected to a Beta-RAM radioactive detector (IN/US Systems, Tampa, FL) with a 30-s residence time and IN-FLOW® cocktail of 1:1 ratio.

The extracted soils remaining were air-dried and ground, and a 0.5-g subsample combusted at 900 °C using an OX500 Biological Oxidizer (R.J. Harvey Instrument Corporation, Tappan, NY). The $^{14}CO_2$ generated from the combustion was trapped in NaOH solution and measured using liquid scintillation counting to determine the ^{14}C-SMZ bound to soil. Mass balances were conducted by summing the $^{14}CO_2$ evolved, extractable ^{14}C, and the ^{14}C bound to the soil.

2.8 Degradation Kinetics

The kinetics of SMZ degradation were evaluated using the availability-adjusted first-order model which has been applied to the degradation of pesticides and organic contaminants in soil (Wang et al. 2006b; Wang and Yates 2008; Krogh et al. 2009):

$$\frac{dC}{dt} = -k\} C e^{-(at)} \quad (1)$$

$$C_t = C_0 e^{-\frac{k\}\left(1-e^{-(at)}\right)}{a}} \quad (2)$$

where C_t is the concentration of the SMZ (mg/kg) at time t (d); C_0 is the initial concentration of SMZ (mg/

kg); k'' is the availability adjusted rate constant; and a is a first-order coefficient describing change in the available fraction. The availability-adjusted first-order model is pseudo first-order where the availability of the SMZ is regulated by a as discussed in Wang et al. (2006b). The parameters for Eq. 2 were determined by fitting the model to the experimental data using non-linear regression analysis.

Half-lives ($t_{1/2}$) for SMZ were estimated as follows:

$$t_{1/2} = -\frac{1}{a} ln\left(1 - \frac{0.693a}{k''}\right) \quad (3)$$

This half-life is equivalent to a DT_{50}, the time required for 50 % dissipation.

3 Results and Discussion

3.1 Aerobic Degradation of SMZ

The SMZ concentrations over time in the soil samples (unamended and manure-amended) under aerobic conditions are presented in Fig. 1. The concentrations of SMZ remaining in the soil varied according to the initial concentration applied, with an initial rapid decrease in SMZ concentrations (within 7 days) to concentrations as low as 2 % of the initial concentrations for 0.5, 5, and 50 mg/kg treatments over 21 days of incubation. For manure-amended soil, the degradation of SMZ was more rapid than that of unamended soils at the 5 mg/kg initial concentration. One possible reason for the more rapid degradation in the manure-amended soil was the higher microbial concentration due to the addition of manure. The slower degradation in the later phase of incubation is probably due to less available SMZ remaining in the soil. For the 100 mg/kg initial concentration, the SMZ concentration in both unamended and manure-amended soils declined in the first 7 days of incubation and then slowly reaching an asymptotic concentration of about 30 % of the initial concentration. A reason for the higher concentration remaining may be due to the inhibition effects of SMZ. For the control experiments using sterilized soil (unamended and manure-amended) and 5 mg/kg of SMZ, the SMZ concentration decreased to about 50 % of the initial concentration. Similar losses of sulfadiazine were observed in autoclaved soils by Yang et al. (2009), and they attributed these losses to abiotic binding.

3.2 Anaerobic Degradation of SMZ

Under anaerobic conditions, the changes in SMZ concentrations were similar to that of aerobic experiments, with a rapid initial decrease in the concentration and followed by a slow decrease (Fig. 2). The impact of initial concentration on anaerobic degradation was also similar to that of aerobic degradation experiments, with lower percentages of SMZ degraded for high initial concentrations of SMZ. The remaining SMZ concentrations (>21 days incubation) under anaerobic conditions were found to be 10 to 30 % higher than under aerobic conditions. The degradation of SMZ in manure-amended soil was generally similar to that in unamended soils, except for the 50 mg/kg concentration in the unamended soil where the final SMZ concentration was similar to that for the 100 mg/kg concentration. Another observation is that the asymptotic concentration reached for the 100 mg/kg initial SMZ concentration treatment was similar to that of the sterilized manure-amended soil. As in the aerobic incubations, the loss of SMZ in the control incubations may be due to abiotic processes.

Extracts of unamended soil and manure-amended soil from anaerobic degradation experiments at day 63 were analyzed for potential SMZ metabolites, using LC-MS and MRM MS/MS. These analyses found N^4-acetyl-SMZ and des-amino SMZ in all samples, except for the sterilized samples, but there was no evidence of N^1-methyl-SMZ in either degradation experiment (data not shown). Identification of the compounds was made by comparing m/z+1 and retention times to standards prepared using the pure compounds.

3.3 Degradation Kinetics

The biphasic degradation curves as presented in Figs. 1 and 2 showed a deviation from first-order kinetics. This is due to the lack of availability of the SMZ as seen for a number of other xenobiotics (Wang et al. 1995; Anhalt et al. 2008). The biphasic degradation of SMZ for both aerobic and anaerobic treatments may be modeled using an availability-adjusted first-order model (Eq. 2). The adjusted degradation rate constants, k'', and a values are presented in Table 1. R^2 values for all the regressions were >0.88 for aerobic treatments and >0.90 for anaerobic treatments. Similar modeling efforts were conducted using a standard first-order model for both aerobic and anaerobic treatments, but the first-order model did not fit the data well (data not shown) with the first-order

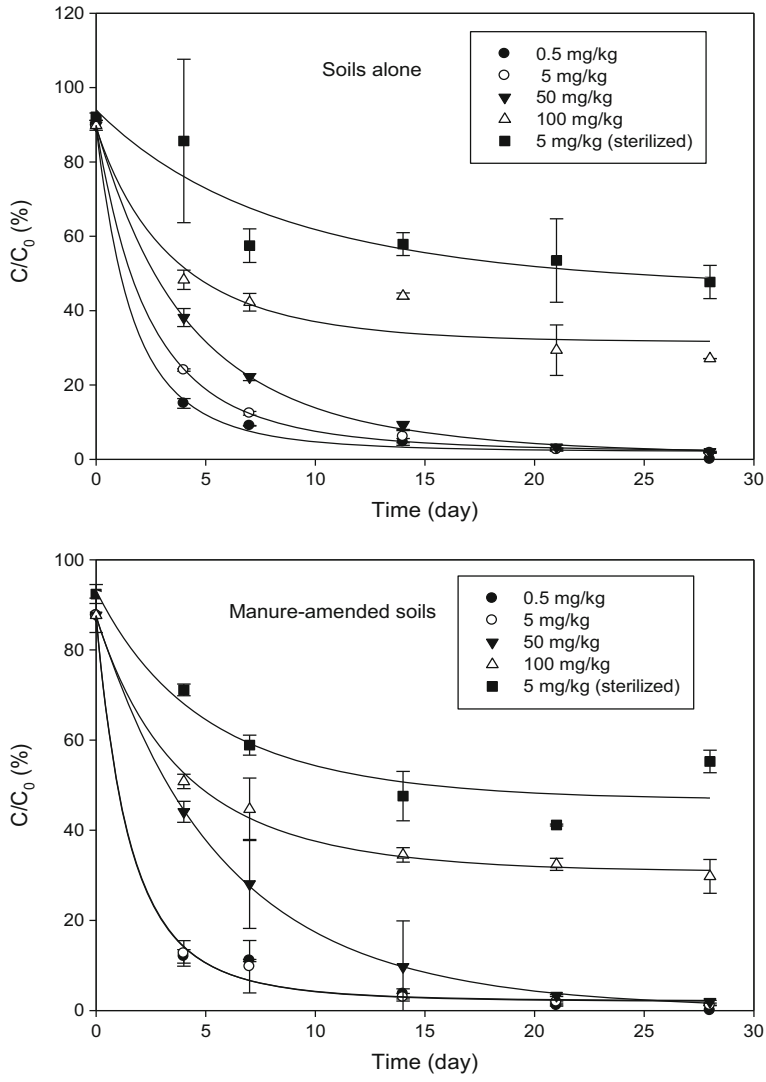

Fig. 1 Sulfamethazine remaining in soil or in manure-amended soil under aerobic conditions for various initial concentrations (C_0) (*solid lines* fit of availability adjusted first-order kinetic model)

model consistently underestimating the concentrations of SMZ for longer incubation times.

Figure 3 shows that the adjusted degradation rate constants (k'') for aerobic and anaerobic treatment (soil only) declined with increasing initial concentrations of SMZ. Addition of manure to the soil helped to maintain the degradation of SMZ, but for higher SMZ concentrations, the adjusted degradation rate constants for both unamended and manure-amended for both anaerobic and aerobic treatments were similar. The values of a were found to be statistically similar ($P \geq 0.05$) for all initial SMZ concentrations and for both aerobic and anaerobic incubations tested except for the 50 mg/kg concentrations for the aerobic incubations (see Table 1). Parameter a is an empirical indicator of the availability of the compound where a high value of a shows that the compound is less available resulting in greater residual concentrations in the soil. The fairly constant value of a may indicate that the initially available fraction may be similar for all treatments which in turn would imply that the variation of k'' in Fig. 3 may be due to the saturation of SMZ-degrading microbial enzymes at high concentrations of SMZ or the inhibition effects of SMZ. The results for this study were different from that of Wang et al. (2006a) who reported a decrease in both k'' and a values as the initial sulfadimethoxine concentrations were increased. Wang et al. (2006b) also suggested that sulfonamide toxicity at higher concentrations in soil may explain the decline of estimated k'' at higher concentrations.

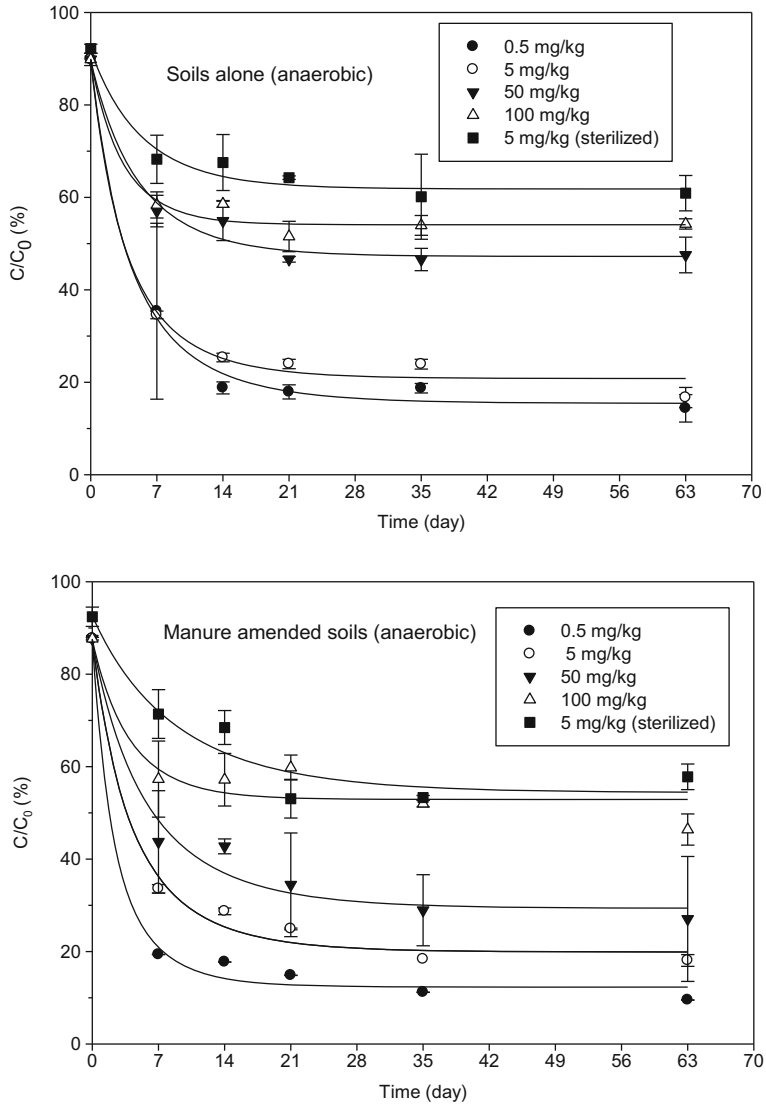

Fig. 2 Sulfamethazine remaining in soil alone or in manure-amended soil under anaerobic conditions for various initial concentrations (C_0) (*solid lines* fit of availability adjusted first-order kinetic model)

Half-lives of SMZ for both unamended and manure-amended soils ranged from 1 to 7 days, and 2 to 15 days, for aerobic and anaerobic conditions, respectively (Table 2). At a given concentration, half-lives of SMZ in both unamended soils and manure-amended soils under aerobic conditions were shorter than under anaerobic conditions. Other studies reported the half-lives of some sulfonamides ranging from 2 to 30 days in aerobic soils (Kay et al. 2004; Blackwell et al. 2005; Wang et al. 2006b; Accinelli et al. 2007; Topp et al. 2012). The half-life for an initial concentration of 100 mg/kg under anaerobic conditions was not estimated since the concentrations of SMZ did not decrease to 50 % of the initial applied SMZ concentration. Half-lives of sulfonamide antibiotics in anaerobic soils have not been previously reported.

3.4 Soil Respiration

For aerobic and anaerobic soil respiration tests, the net maximum cumulative CO_2 evolved (after subtracting from the control) are presented in Fig. 4. Cumulative amounts of CO_2 produced over time were estimated from the sampling procedure described in the methods and the net amounts of cumulative CO_2 production were obtained by subtraction of the cumulative CO_2 produced by the control (SMZ-free) soils. For the aerobic tests with unamended and manure-amended soils over the

Table 1 Degradation rate constants and half-lives for aerobic and anaerobic degradation of SMZ in soils alone and manure-amended soils (mean with 95 % confidence interval)

Treatment/initial conc.	Rate constant (k'')	Availability coefficient (a)	R^2	Half-life
(mg/kg soil)	(1/d)	(1/d)		(d)
Aerobic				
5 (sterilized)	0.06±0.08	0.09±0.14	0.88	36.2
0.5	0.58±0.14	0.16±0.08	>0.99	1.3
5	0.40±0.04	0.11±0.02	>0.99	1.9
50	0.23±0.02	0.05±0.02	>0.99	3.2
100	0.20±0.14	0.19±0.16	0.95	5.9
Aerobic + manure				
5 (sterilized)	0.11±0.16	0.15±0.14	0.92	17.1
0.5	0.62±0.25	0.17±0.14	>0.99	1.2
5	0.63±0.18	0.17±0.10	>0.99	1.2
50	0.17±0.00	0.02±0.00	>0.99	4.1
100	0.17±0.04	0.17±0.04	>0.99	6.6
Anaerobic				
5 (sterilized)	0.06±0.04	0.16±0.10	0.97	>63[a]
0.5	0.20±0.06	0.12±0.04	>0.99	4.3
5	0.21±0.08	0.15±0.04	0.99	4.4
50	0.10±0.04	0.15±0.08	0.98	15.1
100	0.12±0.08	0.24±0.16	0.98	>63[++]
Anaerobic + manure				
5 (sterilized)	0.05±0.04	0.09±0.08	0.93	>63[a]
0.5	0.37±0.18	0.19±0.10	0.99	2.3
5	0.19±0.06	0.13±0.06	0.99	4.9
50	0.12±0.06	0.11±0.06	0.97	9.1
100	0.11±0.14	0.21±0.27	0.90	>63[a]

[a]Half-lives were not estimated due to concentrations remaining above 50 % at 63 days

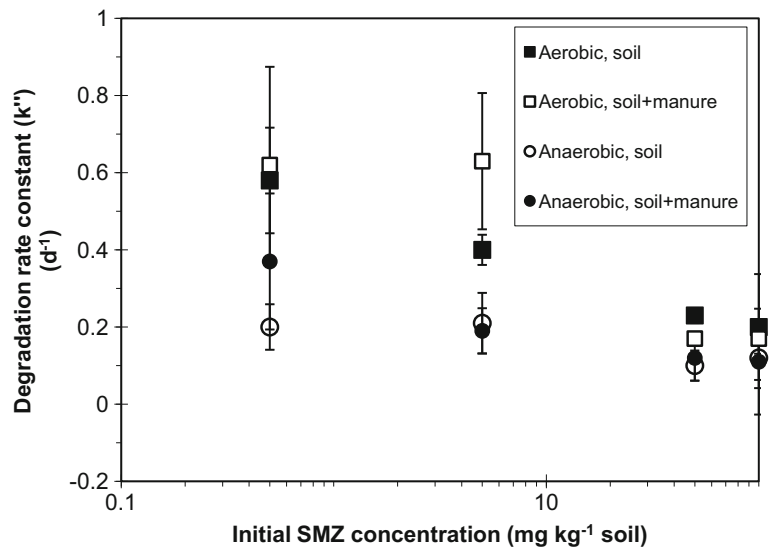

Fig. 3 Effect of initial sulfamethazine concentrations on the availability adjusted rate constants (k''±95 % confidence interval) for aerobic and anaerobic degradation experiments

Table 2 Distribution of ^{14}C in soil after 28 days of aerobic incubation or 77 days anaerobic incubation (average with 95 % confidence interval)

Soil treatment	Initial conc. (mg/kg soil)	% of added ^{14}C		
		Bound ^{14}C	Extractable ^{14}C (^{14}C-SMZ)[a]	$^{14}CO_2$
Aerobic	0.5	88.6±0.5	5.4±0.6 (0.4±0.2)	1.5
	5	89.0±0.4	10.5±0.4 (4.5±2.4)	0.5
	50	84.5±0.2	15.8±1.6 (7.6±1.0)	0.6
Aerobic + manure	0.5	87.6±0.1	6.0±0.4 (0.00)	0.7
	5	81.2±0.2	12.1±6.9 (8.7±1.4)	0.2
	50	70.2±0.1	23.9±0.8 (18.3±2.4)	0.2
Anaerobic	0.5	91.2±0.1	11.3±0.4 (5.5±3.7)	0.2
	5	85.1±0.1	11.1±1.8 (4.2±1.4)	0.2
	50	79.9±0.2	13.8±3.9 (8.5±2.5)	0.1
Anaerobic + manure	0.5	86.4±0.1	10.0±2.0 (1.3±1.2)	0.3
	5	80.7±0.1	14.2±2.2 5.3±3.3)	0.2
	50	73.7±0.1	22.4±8.4 (15.9±4.7)	0.1

[a]Extractable ^{14}C-SMZ measured by HPLC

40 days of incubation, the net maximum cumulative CO_2 evolved were similar up to 10 mg/kg but gave higher net maximum cumulative CO_2 evolved for higher SMZ concentrations. In manure-amended soil, some inhibition of respiration in the first 6 days of incubation were observed (data not shown), but by the end of the 40-day incubation, the respiration from the 50, 100, and 150 mg/kg treatments exceeded the control. Sulfonamide-resistance genes increased in soils with respect to time after application and sulfadiazine concentration (Heuer et al. 2011). Under anaerobic conditions, the net maximum cumulative CO_2 evolved for unamended and manure-amended soils were lower than the control soil for the 50, 100, and 150 mg/kg concentration treatments over the entire 80-day period (Fig. 4), but for treatments with SMZ concentrations of 0.5, 5, and 10 mg/kg, more CO_2 was evolved than from the control. The greater inhibition of anaerobic respiration compared to aerobic respiration may reflect the different susceptibility of the microbial communities active under anaerobic conditions.

3.5 Fate of ^{14}C-SMZ

The distributions of ^{14}C at 28 days in aerobic soil and 77 days in anaerobic soil are presented in Table 2. Total ^{14}C (bound, CO_2, and extractable) recovered ranged from 93.8 to 101.7 % of the applied ^{14}C. In unamended soil under aerobic conditions, about 0.5 to 1.5 % of the SMZ was mineralized to CO_2 while only 0.1 to 0.2 % was mineralized under anaerobic conditions. For manure-amended soil under aerobic conditions, mineralization ranged from 0.2 to 0.7 % which was significantly lower than the unamended soil. The lower mineralization in manure-amended soil may be attributed to lower availability of SMZ to microorganisms due to possible abiotic interactions between SMZ and the manure. A similar effect of manure on SMZ mineralization

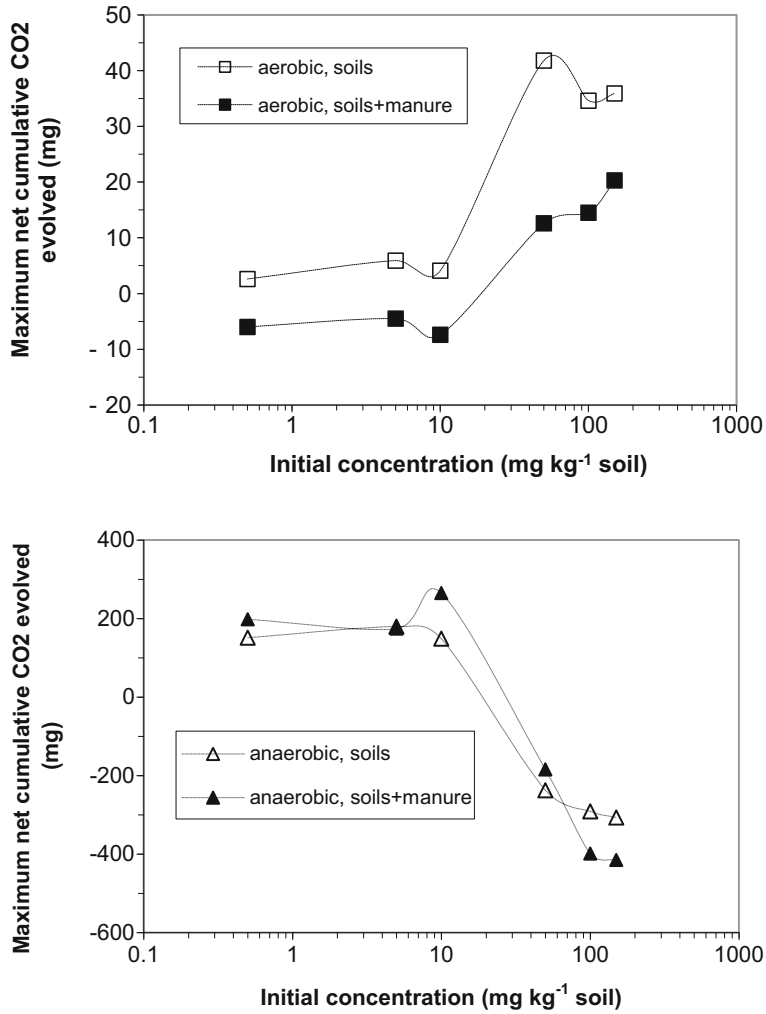

Fig. 4 Net maximum cumulative CO_2 evolved from unamended and manure-amended soils under aerobic and anaerobic incubations for various SMZ initial concentrations

in pond water microcosms was described by Henderson et al. (2009). Our result was also similar to the result of Schmidt et al. (2008) who reported about 2 % mineralization of ^{14}C-sulfadiazine in soil. Tappe et al. (2011) found that pyrimidine-ring labeled sulfadiazine disappeared completely in 10 days in soils enriched with swine manure but no $^{14}CO_2$ was released. Topp et al. (2012) found that soils that were previously exposed to sulfamethazine rapidly degraded ^{14}C-SMZ and mineralized to $^{14}CO_2$, but this degradation was not found in untreated soil. Table 2 also presents the total ^{14}C recovered from the soils by solvent extraction and the ^{14}C-SMZ found in those extracts. Transformation products of ^{14}C-SMZ, estimated from the difference between the total extractable ^{14}C recovered and the ^{14}C-SMZ, varied from 5 to 10 % of the applied ^{14}C.

The majority of the ^{14}C was bound to soil (non-extractable), ranging from 70 to 90 % of applied ^{14}C (Table 2). This high percentage of bound residual ^{14}C-SMZ suggested that binding to the soil was a major mechanism of SMZ removal. The form of ^{14}C bound to soil was not investigated, but likely consisted of ^{14}C-SMZ and/or SMZ metabolites. In addition, higher initial concentrations of SMZ resulted in lower percent of bound ^{14}C. The percent of ^{14}C bound residues were similar in magnitude, but statistically different for the aerobic and anaerobic treatments at the 50 mg/kg treatment. These results are in good agreement with other studies which reported that the non-extractable ^{14}C-sulfadiazine and ^{14}C-sulfamethoxazole remaining in soil exceeded 80 % (Heise et al. 2006; Schmidt et al. 2008). Using a variety of bioassay techniques, Kreuzig and

Höltage (2006) showed that non-extractable bound residues of ^{14}C-sulfonamide were generally not available for biological uptake.

The formation of bound residues has been attributed to several processes, including covalent bonding of SMZ to organic matter (Bialk et al. 2005) and irreversible sorption (Kahle and Stamm 2007; Wehrhan et al. 2010). Stoob et al. (2006) showed that extraction of aged sulfonamide antibiotic residues increased by changing the temperature from 100 to 200 °C during the extraction procedure. This may be indicative of strong hydrogen bonding by the sulfonamides to the soils.

For treatments with initial SMZ concentrations of 5 and 50 mg/kg, the amount of bound residue in the presence of manure was lower than the unamended soil (Table 2). Even though sorption of sulfonamides is expected to increase with higher soil organic matter contents, Thiele-Bruhn and Aust (2004) found that when a low concentration (2 % *w*/*w*) of pig manure slurry was added to soils, the sorption of sulfonamides decreased when compared to soils without manure. They suggested that this was due to competitive sorption between the antibiotic and dissolved organic compounds in manure, such as amino-N-containing soluble compounds and N-heterocyclic hydrocarbons (Liang et al. 1996). Alternatively, binding of SMZ to soluble manure-derived compounds might delay the formation of bound residues. The effect of manure on bound residue formation in this experiment was opposite to that reported by Kreuzig and Höltage (2005) where pre-incubation of the antibiotic with manure prior to addition to the soil resulted in nearly complete transformation of ^{14}C-sulfadiazine into bound residues within 3 days.

4 Conclusions

Change in SMZ concentrations in unamended and manure-amended soils for aerobic and anaerobic conditions showed a biphasic disappearance pattern. Disappearance of SMZ concentrations under aerobic conditions was faster than under anaerobic conditions. In addition, the percent of SMZ remaining in the soils were higher for treatments with higher initial SMZ concentrations. An availability-adjusted first-order model was able to model the disappearance of SMZ for all treatments. Half-lives of SMZ ranged from1.2 to 6.6 days and 2.3 and >63 days for aerobic and anaerobic conditions, respectively. Both unamended soil and manure-amended soils under anaerobic conditions with an initial concentrations of SMZ greater than 50 mg/kg, microbial respiration was reduced as compared to the SMZ-free control, indicating an inhibitory effect of SMZ. Less than 2 % of ^{14}C[phenyl]-SMZ was mineralized. The metabolites *N*-4-acetyl-SMZ and des-amino SMZ were found in soils, and 5 to 24 % of the applied ^{14}C were extractable at the end of the incubations. The primary mechanism accounting for the loss of SMZ in soils was the formation of bound residues which accounted for more than 70 % of the applied ^{14}C (depending on initial concentration). The addition of swine manure tended to reduce binding of SMZ to soil, particularly as the SMZ concentrations increased. The mechanisms of SMZ binding in soil are not fully known, but decreases in bioavailability and the potential transport and toxicological effects of SMZ.

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