



Research article

Long-term monitoring of sulfonamides and tetracyclines in manure amended soils and leachate samples - A follow-up study

Astrid Spielmeier^a, Madeleine S. Petri^a, Heinrich Höper^b, Gerd Hamscher^{a,*}^a Justus Liebig University, Institute for Food Chemistry and Food Biotechnology, Heinrich-Buff-Ring 17, D-35392, Giessen, Germany^b State Authority for Mining, Energy and Geology (LBEG), Stilleweg 2, D-30655, Hannover, Germany

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ABSTRACT

Antibiotics can be detected in manure and digestate samples worldwide. As manure is a frequently used fertilizer, antibiotics are found in soil and leachate samples. Only little is known about the long-term fate of antibiotics in the soil environment. One shortcut is the lack of appropriate monitoring studies. Here we present the results of an unequalled soil monitoring study over 18 years from an agricultural field site in Lower Saxony (Germany). Sulfonamides and tetracycline are mainly fixed in the upper soil layer. Contents showed a sharp decrease below sampling depth of 30 cm (plough depth). Sulfaguanidine and sulfamethazine (SMZ) were detected down to 90 cm. Water samples taken below the field site revealed the transfer of sulfonamides into leachate. High variances were observed between sampling points emphasizing the need for sampling strategies for environmental studies. In addition, field lysimeters with defined input of sulfonamides enabled a long-term monitoring and mass balance of antibiotic transfer into leachate over 10 years. SMZ showed the highest mobility with concentrations up to 65 ng L⁻¹. Less than 0.5% of the applied SMZ was transferred into the leachate. Data of lysimeter and field water samples support the theory of a steady state process with a continuous input of sulfonamides such as SMZ into leachate. Soils contaminated with antibiotics can be a long-term source for the input of antibiotic active compounds into deeper soil layers and groundwater.

1. Introduction

Due to its high nutrient content, easy applicability and availability, manure is frequently used as soil fertilizer (Weiland, 2010). According to the Federal Statistical Office, more than 200 Mio. m³ (200 billion liter) liquid manure and 20 Mio. tons (200 Mio. Mg) dung were spread on agricultural land in Germany in 2015 (Statistisches Bundesamt). However, beside nutrients, manure can contain (micro) pollutants such as synthetic hormones, heavy metals or pharmaceuticals (Hu et al., 2017). Pharmaceuticals such as antibiotics are used worldwide in animal husbandry with upward trend (Van Boeckel et al., 2015). Due to incomplete absorption and poor metabolism, antibiotics are partially excreted by the treated animal. Excretion rates can vary remarkably ranging from 10 to 90% depending on the compound (Sarmah et al., 2006; Spielmeier, 2018). In 2018, 722 tons antibiotics were sold to German veterinarians (Wallmann et al., 2019). The main part of the active ingredients (37.5%, 271 tons) belonged to the beta-lactams. These compounds are known to possess a reduced stability in manure matrices due to the alkaline pH (Tsuji et al., 1978; Berendsen et al., 2018). Assuming a mean excretion

rate of 50% for the other ingredient classes, this would make up 225 tons of antibiotics that were transferred into manure in 2018. Taken into account the mass of manure produced in Germany per year (see above), this would correspond to an average antibiotic content in manure of 1 mg kg⁻¹. As there are hotspots for the distribution of antibiotics (Wallmann et al., 2019), remarkable higher contents can be found in manure and dung samples in Germany (Wohde et al., 2016; Spielmeier, 2018). Zhou et al. (2013) calculated that 3244 tons of antibiotics might be transferred into manure in Chinese cattle and swine breeding operation systems. Although being estimations, these numbers illustrate the potential dimension of the excretion of antibiotic active compounds by treated animals.

As manure is frequently utilized as soil fertilizer, antibiotics enter the environment via this pathway. Especially in regions with a high density of animal breeding, soils are vulnerable towards contamination with antibiotics (de la Torre et al., 2012). The question whether the contents found in manure or soil can enhance the formation of antibiotic resistances is not finally clarified.

* Corresponding author.

E-mail address: gerd.hamscher@uni-giessen.de (G. Hamscher).

Antibiotics are detected in soil and leachate samples worldwide. There are several monitoring studies dealing with the occurrence of antibiotics in those matrices; however, long-term studies are rare (e. g. Kivits et al., 2018). A long-term approach is essential to gain insight into the fate of antibiotics in the soil environment concerning potential elimination pathways, compound stability and mobility within soil matrices. In 2017, we demonstrated the transfer of sulfonamides into leachate samples in field lysimeters over six years (Spielmeier et al., 2017). Here, the results of the subsequent sampling years (2016–2019) are presented.

This recent work also contained results for leachate samples from an agricultural field site in Lower Saxony, Germany. From the same site, data regarding the antibiotic content in soil samples are available (Hamscher et al., 2002, 2005). Both data sets were extended with data until 2019 enabling statements about the behavior of sulfonamides and tetracyclines in soil and leachate samples. Compared to the initial publications, the range of compounds being analyzed was enhanced by sulfaguanidine (SGU), 4-hydroxy sulfadiazine (4-OH-SDZ, metabolite of sulfadiazine) and iso-chlortetracycline (isoCTC, abiotic transformation product of chlortetracycline). IsoCTC was not investigated by Hamscher et al. (2002, 2005) as their study focused on the initial compounds, not the transformation products. SGU and 4-OH-SDZ were not included in the previous publication by Spielmeier et al. (2017) due to an uncertain or the lack of an unequivocal compound identification. In this study, identification was enabled by high resolution mass spectrometric analyses (HR-MS). Additionally, we were able to obtain an isolated standard of 4-OH-SDZ for comparison of MS spectra and analytical retention time. Thus, our studies performed over a period of almost two decades provide clear evidence of a permanent contamination of agricultural fields and corresponding leachate with veterinary antibiotics and some of their metabolites. Several of the compounds under investigation are among the best-selling veterinary drugs worldwide and therefore, the time has come, to rethink their current applications.

2. Material and methods

2.1. Reagents and materials

Eleven sulfonamides and five tetracyclines were investigated, including sulfachlorpyridazine (SCY), sulfadiazine (SDZ), 4-hydroxy sulfadiazine (4-OH-SDZ), sulfadimethoxine (SDM), sulfaguanidine (SGU), sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxazole (SMX), sulfamethoxypyridazine (SMPD), sulfapyridine (SPY), sulfathiazole (STZ) as well as chlortetracycline (CTC), doxycycline (DC), iso-chlortetracycline (isoCTC), oxytetracycline (OTC), tetracycline (TC). An overview regarding structures, molecular weights, utilized precursor and product ions for analysis is provided in the Supplemental Material (Table A1). Standards for analysis and manure fortification were purchased in VETANAL™ grade from Sigma-Aldrich (Taufkirchen, Germany). IsoCTC, the main transformation product of CTC, was obtained from TRC (Toronto Research Chemicals, Canada). In case of 4-OH-SDZ, no analytical standard is commercially available. For qualitative compound identification, the substance was isolated from manure of pigs treated with SDZ. Details about the isolation process as well as NMR and HR-MS data are provided in the Supplemental Material (Section A1, Figure A1; Table A2). For quantification, signals of 4-OH-SDZ were referred to the standard of SDZ assuming comparable ionization. Solid phase extraction (SPE) was conducted with SPE cartridges Chromabond® HR-X from Macherey-Nagel (Düren, Germany). Details concerning the reagents and materials utilized for the lysimeter studies as well as the manure treatment before application to the field lysimeters are given in Spielmeier et al. (2017).

2.2. Samples from field site close to intensive livestock farming

Soil and water samples were obtained from a soil monitoring area in Lower Saxony (Germany). Detailed descriptions of the soil parameters

and the sampling procedures are provided by Hamscher et al. (2002). Briefly, soil samples were taken from the middle of the field site which was divided into four sampling areas (16 m × 16 m each). Soil samples were taken in layers of 10 cm down to a maximum depth of 90 cm. One sample per soil layer was generated from each sampling area by thoroughly mixing soil material from 16 randomly placed insertions with a soil sampling auger. Approximately 1 kg soil was taken per sampling area and day. Samples of the four sampling areas were analyzed separately (n = 4 for each soil layer). Water samples were fortnightly taken in 1.4 m depth during the winter season, when water seepage was occurring. Soil water was sampled using four borosilicate suction cups (S1 to S4) and applying a slight vacuum of about -150 hPa (at the tip of the suction cup), generating 4 water samples per sampling date. Water samples were frozen (-18 °C), soil samples were stored in the dark at room temperature (15–20 °C) until sample preparation.

In addition, a manure sample from an associated pig fattening farm was kindly provided by the respective farmer. The sample was taken in March 2018 and analyzed for the presence of sulfonamides and tetracyclines according to Spielmeier et al. (2014). Contents in manure are given in mg per kg fresh weight (fw).

2.3. Lysimeter studies

Pig manure fortified with ten sulfonamides (SCY, SDZ, SDM, SGU, SMPD, SMR, SMX, SMZ, SPY, STZ) was applied to two field lysimeters in Lower Saxony (Germany). A detailed description of the soil parameters, manure application and sampling procedure is provided by Spielmeier et al. (2017). Leachate samples at field site A (bedrock derived of limestone) were taken in 1 m depth, at field site B (sandy soil) in 2 m depth. Samples were stored at -18 °C before analysis.

For site B, data about the lysimeter seepage rate ($L m^{-2} d^{-1}$) were utilized to set up a mass balance. Based on concentrations determined for SMZ, an estimation of the remaining sulfonamide proportion within the soil column was performed. In October 2018, soil samples were taken from the same lysimeter to investigate the remaining antibiotic content.

2.4. Sample preparation and analysis - water samples

The procedure is described in detail in Spielmeier et al. (2017) and Bailey et al. (2016). Briefly, an internal standard consisting of $^{13}C_6$ SDZ, SMX and SMZ was added to 30 mL water sample to reach a final concentration of 100 ng L^{-1} . Samples were automatically applied to SPE using the system GX-271 ASPEC™ (Gilson, Limburg-Offenheim, Germany). Blank samples and standards (50 ng L^{-1}) were prepared using 30 mL tap water.

For LC-MS/MS analysis, a Dionex Ultimate RS3000 UHPLC (Dionex Thermo Fisher Scientific, Germering, Germany) connected to an ABSciex QTrap 3200 triple quadrupole mass spectrometer (ABSciex, Darmstadt, Germany) was utilized. Column and gradient details are provided in the Supplemental Material (Section A2). MS data acquisition was conducted in multiple reaction monitoring mode (MRM) recording two characteristic fragment ions. Details are provided in the Supplemental Material (Table A1).

2.5. Sample preparation and analysis - soil samples

One g soil was weighed in a 15 mL polypropylene centrifuge tube. The repeatability of selecting 1 g portion of 1 kg sample was not tested. Each sample was fortified with the internal standard consisting of $^{13}C_6$ SDZ, SMX and SMZ to reach a final content of 70 $\mu g kg^{-1}$. Samples were incubated at room temperature in the dark for seven days to allow an interaction of the internal standard with the soil matrix. This interaction respective the incubation time was found to influence the recovery rates of the investigated antibiotics (see Table A4 in the Supplemental Material). The chosen procedure (7 d incubation of internal standard before extraction) was considered as suitable compromise between the

simulation of long-term storage of soil samples and time spent for sample preparation.

The first extraction step was conducted with 3 mL 1 M citrate buffer (pH 4.7) which contained 0.05 M EDTA. After addition, the samples were vigorously shaken for 10 min and phases were separated by centrifugation (3 min, 3000 g). The supernatant was removed and stored in a 50 mL polypropylene centrifuge tube at 10 °C before further treatment. The solid residue was resuspended in 2 mL citrate buffer and 4 mL methanol and the mixture was shaken on a horizontal shaker at room temperature in the dark for 24 h. Phases were separated by centrifugation (3 min, 3000 g) and the supernatant was combined with the solution of the first extraction step. For reducing the organic fraction, the extract was incubated in a stream of nitrogen at 40 °C for 30 min. Afterwards, samples were diluted to 27.5 ml with deionized water and applied to SPE. SPE was performed via an automatic SPE system (GX-271 ASPEC™, Gilson, Limburg-Offheim, Germany). Cartridges were preconditioned with each 3 mL of methanol + 0.1% formic acid, methanol and citrate buffer. After application of 25 mL sample volume, elution was performed with two times 1 mL methanol + 0.1% formic acid and two times 1 mL methanol. Eluates were reduced to dryness (rotary evaporator) and the residue was reconstituted in two times 100 µL methanol/water (1:1, v/v). Samples were stored at -20 °C before analysis.

For quantitative analysis, a system consisting of a Dionex Ultimate RS3000 UHPLC (Dionex Thermo Fisher Scientific, Germering, Germany) was connected to an ABSciex QTrap 3200 triple quadrupole mass spectrometer (ABSciex, Darmstadt, Germany). UHPLC conditions are given in the Supporting Information (Section A2). MS analyses were performed using electrospray ionization (ESI) in positive mode. Capillary voltage and temperature were set to 5000 V and 500 °C, respectively. Data acquisition was conducted via multiple reaction monitoring mode (MRM) recording two fragment ions (except iso-chlortetracycline, only one intense fragment is formed) (Table A1). Scan time for each transition was set to 100 ms.

Details concerning method validation are provided in the Supplemental Material (Section A3, Table A3–A5). For quantification, a solvent standard was utilized with a concentration of 500 µg L⁻¹ (internal standard 350 µg L⁻¹) which corresponds to 100 µg kg⁻¹ (internal standard 70 µg kg⁻¹) in case of 100% recovery. Results are presented as µg antibiotic per kg soil dry matter. Results were corrected by recovery rates obtained after 14 d analyte and 7 d internal standard incubation on soil matrix (Table A4). Data below MLOQ are often presented as 0.5 MLOQ. However, this was not applicable in our case. For several compounds, MLOD and MLOQ differed by less than factor 2 (Table A3) which would lead to (theoretical) contents below the limit of detection. Therefore, contents below MLOQ were set to median value of MLOD and MLOQ (Table A3).

2.6. HR-MS measurements

To verify the presence of SGU and 4-OH-SDZ in water and soil samples, extracts were additionally analyzed using a Shimadzu Nexera X2 UHPLC (Shimadzu, Darmstadt, Germany) connected to an ABSciex TripleToF 5600 + quadrupole - time of flight mass spectrometer. Both full scan as well as product ion data acquisition were conducted. Detailed method parameters are provided in the Supplemental Material (Section A2). Calibration of the MS system was performed before measurements with sodium formate (Juo et al., 2014).

3. Results and discussion

3.1. Soil and leachate samples from livestock farming area

The retrospective soil study includes samples from 2001, 2005, 2009, 2015 and 2018 and, thus, covers a period of 18 years. Depth profiles down to 90 cm were available for the years 2001 and 2015.

3.1.1. Sulfonamides

Of all sulfonamides investigated, four compounds were detected in the soil samples, namely SDZ, 4-OH-SDZ, SGU, SMZ. They were found in all samples taken between 0 and 30 cm (Figure 1 A-D). Presence of SGU and 4-OH-SDZ were verified by HR-MS measurements (data provided in Table A6). In Germany, SDZ and SMZ are the most applied sulfonamides in veterinary medicine making up 80% of the distributed sulfonamides in 2015 (Wallmann et al., 2016). Thus, their detection in soil samples in areas of intense livestock farming can be expected whereas the detection of other sulfonamides is less likely.

4-OH-SDZ is a metabolite of SDZ formed e.g. by pigs treated with the sulfonamide. As no complete conversion takes place, both SDZ and 4-OH-SDZ are excreted in pig manure with a ratio of approximately 2:1 (Lamshöft et al., 2007). The investigated field site is frequently fertilized with manure from a pig breeding farm. In one sample from 2018, 4-OH-SDZ was present with a content of 2.3 mg kg⁻¹ fw whereas SDZ was surprisingly not found. Thus, the metabolite was (and might be still) introduced in the soil via manure application. Another possibility might be the formation of 4-OH-SDZ in the soil itself (see Section 3.2).

SDZ and 4-OH-SDZ showed comparable contents within the upper soil (0–30 cm, maximum contents of 5.8 and 8.0 µg kg⁻¹ dry matter for SDZ and 4-OH-SDZ, respectively). It should be taken into account that for all matrices investigated the quantification of 4-OH-SDZ was performed using a SDZ standard assuming similar ionization of both compounds. Comparison of the isolated 4-OH-SDZ with an analytical standard of SDZ implies that the metabolite gives an up to factor 5 less intense MS signal than the parent compound. Thus, contents of 4-OH-SDZ presented here might be actually higher. Due to uncertainties concerning compound purity and matrix effects for 4-OH-SDZ in soil and leachate extracts (isolated compound was not available during method validation), no correction of the analytical results was performed.

In case of samples taken below 30 cm, samples showing detectable amounts of SDZ did not show the presence of 4-OH-SDZ (Figure 1 A and B). On the one hand, this point is attributed to the low analyte content (contents close to MLOD). Another explanation might be different sorption processes of the compounds. 4-OH-SDZ was frequently detected in water samples taken below the field site (88 out 116 samples), while positive results for SDZ were found only sporadically (less than ten samples). 4-OH-SDZ might be transferred more easily to deeper soil layers than SDZ leading to non-detectable amounts of the metabolite in the soil column. This example underlines that analyses of soil or leachate samples only would provide an incomplete picture of the fate of antibiotics in environmental matrices. Based on soil sample results, one might consider 4-OH-SDZ to be retained in the upper soil layer. However, leachate samples prove the transfer of this compound within the soil column. Whenever possible, both sample types should be investigated to obtain a deeper insight in the mobility of compounds in the soil environment.

A sharp decrease of the antibiotic content and detection frequency was observed below 30 cm for all analytes (Figure 1). For all years, there was a clear separation between samples taken in the upper soil layer (A-horizon, 0–30 cm) and the subsoil (B-horizon, 30–90 cm). The A-horizon corresponds to the plough layer and is visually distinguishable from the other layers due to its high content of organic matter (2.01% organic carbon vs. 0.64% organic carbon in the Bhs- and 0.2% organic carbon in the Bhs-Go-horizons, see Figure A2 for details) (LBEG, 2005). The interaction of sulfonamides with these components leads to their retardation within the upper soil layer (Bialk et al., 2005; Bialk and Pedersen, 2008; Förster et al., 2009; Gulkowska et al., 2013). The subsoil layer lacks this high proportion of organic matter. Thus, compounds entering the B-horizon can be more easily washed-out into deeper soil layers. This is reflected by the results of SMZ and SGU. These compounds could be detected in all segments down to 90 cm and in the leachate (Figure 1 C, D, Table 1).

In contrast to SDZ, detectable and quantifiable amounts of SMZ were observed in the subsoil although both compounds were present in

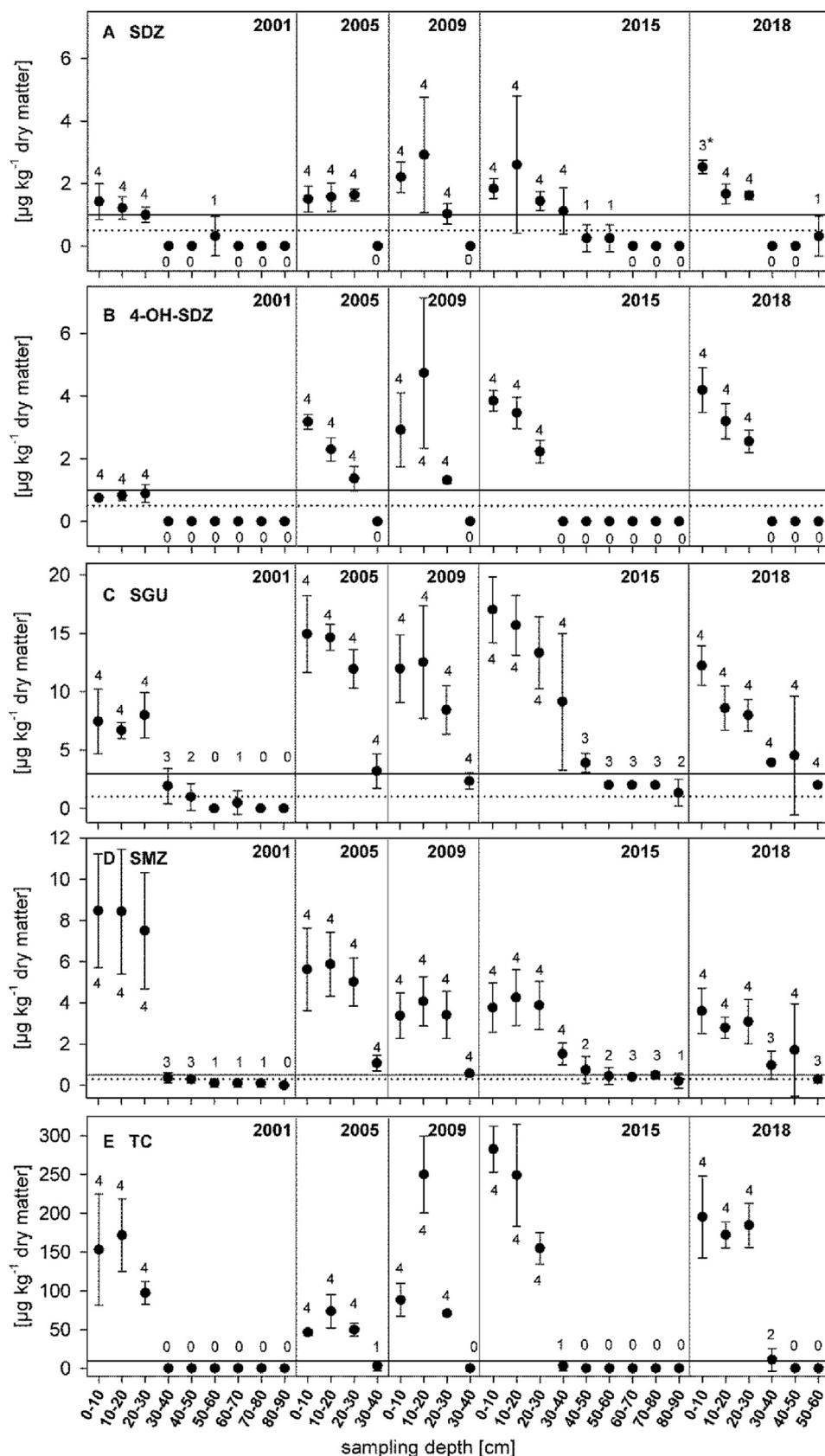


Figure 1. Contents of (A) SDZ, (B) 4-OH-SDZ, (C) SGU, (D) SMZ, (E) TC in soil samples taken on a monitoring field site in Lower Saxony (Germany) over the course of 18 years; contents are given in μg antibiotic per kg dry matter, contents were corrected by recovery rate (see Table A4); values are given as arithmetic mean \pm standard deviation ($n = 4$; except 2015, only 3 samples were available for depths below 40 cm); numbers indicate the number of positive samples with content above MLOD, negative samples were set to 0 for mean calculation; dotted and solid line represent the MLOD and MLOQ, respectively (due to scaling dotted line not visible in case of TC); * - one value eliminated as outlier.

comparable contents in the upper soil layer. One explanation can be the lower MLOD and MLOQ for SMZ (Figure 1, Table A3). SDZ contents were already close to the MLOQ in the A-horizon, making a detection of the compound less likely in the layers below. Another reason can be a different mobility of SDZ and SMZ within the soil matrix. In several studies investigating the fate of sulfonamides in soil columns, SMZ was found to possess a higher mobility than other investigated compounds (Kurwadkar et al., 2011; Vithanage et al., 2014; Kivits et al., 2018). This assumption is supported by the results of the leachate samples. Although SMZ, SDZ and 4-OH-SDZ possessed similar contents in the upper soil layer (Figure 1), only SMZ and 4-OH-SDZ were detected in the leachate with concentrations up to 97 and 37 ng L⁻¹, respectively (Table 1).

Leachate values of SMZ for the different sampling points confirm the results of previous studies both in tendency and order of magnitude (Hamscher et al., 2005; Spielmeyer et al., 2017). Although being collected under the same field, leachate samples of the four suction cups showed distinct differences concerning the SMZ concentration. Similar tendencies were found for SGU and 4-OH-SDZ (Table A7 and A8). This can be attributed to an inhomogeneous distribution of SMZ in the soil matrix. Within the upper soil layer samples, relative standard deviations up to 40% were obtained per sampling day (Figure 1 D). Another explanation can be an inhomogeneous soil matrix itself, for instance slightly varying contents of humic matter or different proportions of soil

constituents such as sand or loam. These factors are known to influence the mobility of sulfonamides in soil matrices (Unold et al., 2009; Bailey et al., 2016; Pan and Chu, 2017). Leachate samples at S3 and S4 showed a more intense color (yellow to yellow-brown) than samples at S1 and S2 (slightly yellow), which is probably due to an enhanced transfer of soil ingredients such as humic matter. This could favor the wash out of other compounds present in the soil such as sulfonamides consequently leading to maximum values of SMZ in the leachate at S3 and S4 (Table 1).

Interestingly, soil and leachate samples showed different patterns concerning the distribution of minimum and maximum antibiotic contents. In most cases, soil samples taken at S4 delivered the lowest SMZ content while samples taken at S2 showed the maximum value (Table A9). The opposite was found for the leachate samples with lowest concentrations at S2 (<MLOQ up to 18 ng L⁻¹) and highest values at S4 (35–97 ng L⁻¹) (Table 1). As stated before, inhomogeneities within the soil matrix can influence the mobility of compounds such as sulfonamides within the soil column. These results underline the need for selecting several sampling points within one field site to gain a comprehensive insight in the fate and transfer of contaminants in natural matrices. Taking water samples at S1 or S2 only would have implicated a low potential of sulfonamides being transferred into leachate whereas results for sampling point S4 only might have overestimated the mobility (Table 1, Table A7 and A8).

Table 1. SMZ, SGU and 4-OH-SDZ in leachate samples below an agriculture field within an area of intensive livestock farming.

sampling date	SMZ	SMZ	SMZ	SMZ	SGU	4-OH-SDZ*
	S1	S2	S3	S4	S4	S4
October 27, 2016	15	<MLOQ	29	92	106	12
November 10, 2016	13	<MLOQ	41	79	91	11
November 24, 2016	15	12	40	81	234	18
December 10, 2016	15	10	40	82	150	16
December 19, 2016	<MLOQ	<MLOQ	27	78	186	13
January 02, 2017	<MLOQ	<MLOQ	41	78	180	17
January 16, 2017	<MLOQ	<MLOQ	23	73	200	15
February 07, 2017	14	<MLOQ	36	44	111	<MLOQ
February 25, 2017	12	<MLOQ	37	43	111	10
March 05, 2017	<MLOQ	<MLOQ	27	40	95	<MLOQ
March 26, 2017	<MLOQ	<MLOQ	41	35	92	<MLOQ
April 01, 2017	<MLOQ	<MLOQ	49	36	124	10
December 19, 2017	27	<MLOQ	51	61	99	16
January 01, 2018	19	<MLOQ	46	50	104	13
January 15, 2018	16	<MLOQ	60	47	80	11
February 13, 2018	10	<MLOQ	34	40	72	<MLOQ
February 25, 2018	11	<MLOQ	49	48	86	10
March 17, 2018	12	<MLOQ	53	48	100	11
April 02, 2018	17	<MLOQ	56	52	126	12
April 17, 2018	16	<MLOQ	54	57	143	14
May 02, 2018	19	<MLOQ	59	65	145	16
December 18, 2018	11	14	16	86	107	33
December 31, 2018	33	11	35	97	105	28
January 10, 2019	24	<MLOQ	27	89	132	31
January 20, 2019	14	18	15	82	168	37
February 12, 2019	<MLOQ	10	<MLOQ	45	139	30
February 27, 2019	18	15	22	42	85	18
March 13, 2019	16	12	28	43	93	20
March 28, 2019	<MLOQ	<MLOQ	24	44	95	17

Concentrations given in ng L⁻¹.

<MLOQ - concentrations below 10 ng L⁻¹.

MLOQ - method limit of quantification, 4-OH-SDZ - 4-hydroxy-sulfadiazine, SGU - sulfaguandine, SMZ - sulfamethazine; for description of sampling sites S1 to S4 see Hamscher et al. (2002).

Data for S1 to S3 for SGU and 4-OH-SDZ are provided in Table A7 and Table A8, respectively.

* Values for MLOQ were taken from sulfadiazine (Table 2).

SGU showed the highest soil contents of all sulfonamides investigated (Figure 1 C). Like SMZ, it revealed a high mobility within the soil column and samples down to 90 cm were found to contain SGU. Consequently, this sulfonamide was also detected in leachate possessing the highest concentration of the compounds analyzed (Table 1). SGU is the most polar sulfonamide investigated in this study and comparable low K_d values were previously determined (Bialk-Bielinska, 2012; Maszkowska et al., 2013). Both factors might favor the transfer of the compound in the soil column.

In contrast to SDZ and SMZ, SGU is not authorized as active ingredient in human and veterinary medicine in Germany (VETIDATA). According to data from the German Federal Office of Consumer Protection and Food Safety, SGU was not distributed to German veterinarians from 2013-2015 (afterwards, no detailed data available) (Wallmann et al., 2014, 2015; 2016). Analysis of soil and manure samples in Germany, Spain and in the USA did not reveal SGU in any sample (Haller et al., 2002; Shelver et al., 2010; Biel-Maeso et al., 2017). Also manure and digestate samples from the respective field site did not contain SGU in amounts above the MLOD. This raises the question about the source of this antibiotic in the soil.

As a permanent input via manure can be excluded, the formation of SGU within the soil might be an explanation. SDZ, SGU and SMZ possess the same substructure units. A cleavage of the diazine respective methazine ring would lead to the guanidine subunit present in SGU. Sur and Stork (2003) found a similar transformation pathway for imidacloprid in plants, leading to imidacloprid guanidine. It must be taken into account that in case of imidacloprid no aromatic ring, but a hydrogenated N-nitro-imidazolamine moiety is present. The transformation reaction appears less likely in case of an aromatic ring cleavage. Furthermore, SGU and SMZ occur in the same order of magnitude within the soil samples (Figure 1 C and D). A formation of SGU starting from SMZ should lead to SGU contents being lower than contents of SMZ. This was not observed.

One explanation might be the high persistence of sulfonamides in soil due to sequestration processes (Förster et al., 2009). SGU might derive from an application which occurred decades ago. At first sight, the depth profile analysis and results of the leachate sample (Figure 1C, Table 1) contradict this theory. If positive SGU findings were the result of a former application, one should expect a decrease of the soil SGU content over a period of 18 years. This was not observed for the values within the upper soil layer (0–30 cm, Figure 1 C). As discussed in detail for the lysimeter study (Section 3.2), even comparable high leachate concentrations of 200 ng L⁻¹ over several years correspond to less than 1% of the initial soil content being washed out. Thus, the positive findings of SGU might be the result of a former application of the antibiotic even though soil contents remain on a comparable level over years. This would underline the high stability of sulfonamides in soil matrices and their potential for a long-term transfer into leachate samples.

Thus, the origin of SGU cannot be clarified so far. Studies investigating the presence and fate of sulfonamides in soil and leachate samples should include the analysis of SGU to check whether this specific sulfonamide can only be found in places where SDZ and SMZ are present. This might help to clarify whether SGU could be potentially formed in soil matrices. However, studies performed with ¹⁴C-labelled SDZ applied to soil did not report a formation of SGU so far (Unold et al., 2009; Kasteel et al., 2010; Sittig et al., 2014). A detection of SGU only or in higher amounts than SDZ and SMZ would give a hint about a potential SGU usage in animal husbandry before it was replaced by sulfonamides such as SDZ and SMZ. This would deliver useful information about the stability of sulfonamides in soil matrices and a potential long-term transfer of the antibiotics into leachate.

3.1.2. Tetracyclines

TC was detected in all samples taken between 0 and 30 cm (Figure 1E). CTC, DC and OTC were not found. Hamscher et al. (2002, 2005) reported traces of CTC for the same field site with contents below 10 µg

kg⁻¹. As the MLOD of the method presented here was 25 µg kg⁻¹ (Table A3), negative results are probably due to analytical shortcomings. In contrast to this study (triple-quadrupole mass spectrometer), Hamscher et al. (2002, 2005) utilized an ion trap. Ion traps enable the accumulation of ions potentially leading to lower MLOD. CTC was detected in a manure sample from an associated pig fattening farm (2.3 mg kg⁻¹ fw). Thus, an input of this antibiotic in the soil occurs. In manure, CTC undergoes an isomerization to its main transformation product isoCTC. This abiotic reaction occurs at pH values above 7.5 (Halling-Sørensen et al., 2002). The presence of isoCTC could be proven in both manure (5.1 mg kg⁻¹) and soil samples (contents up to 23.5 µg kg⁻¹, Table A10). Thus, the detection of isoCTC might serve as indirect proof for CTC.

Tetracyclines are known to show strong sorption to soil matter due to physisorption processes like chelate complex formation with multivalent cations (Eichhorn and Aga, 2004). A transfer to groundwater samples below the investigated field site was not observed within previous studies (Hamscher et al., 2002, 2005). Laboratory studies did not find a transfer of TC along an artificial, irrigated soil column (Pan and Chu, 2017). Within this study, both TC and isoCTC were detected in sampling depth of 0–30 cm which corresponds to the plough depth. In few cases, TC was detected in samples taken between 30 and 40 cm (Figure 1E). This would suggest a mobility of the antibiotic within the soil matrix. However, the positive findings are mainly ascribed to sampling issues. During separation of the soil column into 10 cm segments, a partial mixture of sample material above and below 30 cm probably occurred. Moreover, the plough depth is not exactly 30 cm everywhere in the field and may be up to 35 cm, thus, topsoil material will be included in the layer 30–40 cm. A transfer due to leaching processes should have led to positive findings in samples below 40 cm, as it was observed for sulfonamides (see discussion in Section 3.1.1), but this was not the case.

Over the course of the years, TC contents did not show a clear trend which would imply an accumulation or an elimination of the compound (Figure 1E). A pig manure sample from 2018 possessed a TC content of 0.81 mg kg⁻¹ fw. In previous manure samples, TC was detected as well ranging from 0.5 to 9.7 mg kg⁻¹ fw (Hamscher et al., 2002, 2005; Spielmeyer et al., 2017). This indicates a permanent input of the antibiotic into the soil and an increase of the TC soil content would be expected. Based on a plough depth of 30 cm, a soil density of 1,500 kg per m³ (taken from "Guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 and GL38") and a manure application of 45 m³ fresh manure per hectare and year (Hamscher et al., 2002), a TC content of 1 mg kg⁻¹ in manure would correspond to an annual increase of the soil content by 10 µg kg⁻¹ in soil after ploughing. Based on the variability of the four samples per sampling point (Figure 1E), this increase could not be represented by the analytical method. Within the method validation, an interday precision of 3.2% for TC was determined (Table A3). Depending on the initial soil content, an addition of 10 µg kg⁻¹ might not be distinguishable from the analytical variation (3.2% of 300 µg kg⁻¹ corresponds to 9.6 µg kg⁻¹). Furthermore, the four sampling points themselves provide a variation as already discussed for sulfonamides (see Section 3.1.1) (Figure 1E). Consequently, a remarkable increase of the TC soil content over the study period can only be observed when the TC content of the utilized manure is continuously higher than 1 mg kg⁻¹ or when more than 45 m³ are applied. Both factors cannot be excluded. However, the observed fluctuations between sampling years are probably the result of an inhomogeneous distribution of the analyte within the matrix. In addition, 1 g sample material was utilized for sample preparation and this amount is supposed to represent an area of 256 m². Samples were homogenized before usage, but the risk of hot spots within the material cannot be completely eliminated. This is especially emphasized by the results for 2009. Ploughing should lead to a homogenization of the soil within the first 30 cm. However, samples from 10-20 cm showed a more than two times higher TC content than the surrounding segments (Figure 1E). Similar inhomogeneities were reported in previous studies from the same field site (Hamscher et al., 2002,

2005). This underlines the need for developing sampling strategies before taking field samples to reduce errors to a minimum.

The constant TC content could also represent a pseudo-persistence, a term first utilized for contaminants in sewage effluents (Daughton, 2002; Mackay et al., 2014). On the one hand, sequestration processes would lead to a reduction of the TC content. On the other hand, the application of contaminated manure leads to a permanent input of TC into the soil. If both processes occurred in the same order of magnitude, an (apparent) constant TC content would be the result. For sulfonamides, microwave extraction with elevated temperatures (e.g. 150 °C for 15 min) can be employed to enhance the recovery of the analytes from (aged) soil samples (Förster et al., 2008). However, tetracyclines possess a reduced temperature stability (Loftin et al., 2008), so these enhanced extraction methods are not applicable in their case.

3.2. Lysimeter studies

The results presented here are a follow-up survey of a previously published study (Spielmeier et al., 2017). To avoid redundancy, the discussion presented here is limited to new aspects based on the results obtained for the years 2016–2019.

Ten sulfonamides were applied to the lysimeters at site A and B from autumn 2009 until autumn 2011. Within the years of manure application, all sulfonamides except STZ were detected in the leachate at site A (bedrock soil) (see Table 2 in Spielmeier et al. (2017)). From 2015–2019, detections were limited to SDZ, SGU, SMX, and SMZ and most results were below the MLOQ (Table 2, Figure 2A). In other studies, these compounds were found to possess a high mobility in the soil matrix compared to other sulfonamides (Kurwadkar et al., 2011;

Bialk-Bielinska, 2012; Vithanage et al., 2014). Compared to site B, both detection frequency and concentration in the leachate were lower at site A. Pores within the soil structure allow water to bypass the soil matrix during rain events or irrigation which limits the interaction between liquid and solid phase. This hampers the transfer of soil components into deeper soil layers in general and of antibiotics in the case of the lysimeter at site A. In addition, 2018 and the first half of 2019 were characterized by long drought periods. Both factors led to low proportions of the sulfonamides being desorbed and transferred into the leachate at site A.

A different trend was observed at site B (sandy soil). Here, all applied sulfonamides except SDM were detected in the leachate (Table 2). SDM is the least polar of the investigated substances and showed a lower mobility than other sulfonamides in several studies (Figuroa-Diva et al., 2010; Kurwadkar et al., 2011; Bialk-Bielinska, 2012; Maszkowska et al., 2013). Doretto et al. (2014) found higher sorption coefficients for the unipolar SDM than for the more polar SMZ on sand, clay and sandy clay. However, different mobilities cannot be attributed to polarity only. SMX is less polar than SDZ and showed a higher detection frequency (Table 2). As discussed before (Spielmeier et al., 2017), other parameters such as pKs values or interaction with organic soil constituents influence the transfer of sulfonamides in the soil column.

SMZ was the first sulfonamide to be detected at site B which confirms the high mobility of this compound within the soil matrix. From 2012 onwards, SMZ was present in all samples except one (Table 2 here and in Spielmeier et al. (2017)). After restarting sampling in 2015, most results were above the MLOQ (86/89, Table 2, Figure 2B) which shows a constant transfer of the antibiotic into the leachate. In this context it is interesting to note that concentrations were between 30 to 65 ng L⁻¹ for the majority of the samples (Figure 2B). No permanent increase of the

Table 2. Detection frequency of sulfonamides in lysimeter leachate.

site A	2015 (6)				2016 (8)		2017 (16)		2018 (10)		2019 (10)	
	MLOD	MLOQ	>MLOD	>MLOQ	>MLOD	>MLOQ	>MLOD	>MLOQ	>MLOD	>MLOQ	>MLOD	>MLOQ
SCY	7.5	20	0	0	0	0	0	0	0	0	0	0
SDZ	2	10	0	0	0	0	2	0	2	0	0	0
4-OH-SDZ	2*	10*	n.a.	n.a.	n.a.	n.a.	0	0	0	0	0	0
SDM	5	10	0	0	0	0	0	0	0	0	0	0
SGU	10	60	2	1	4	1	16	0	10	1	10	0
SMR	5	10	0	0	0	0	0	0	0	0	0	0
SMZ	2	10	6	1	8	0	15	2	10	1	10	0
SMX	2	7.5	6	0	4	0	5	0	0	0	4	0
SMPD	2	10	0	0	0	0	0	0	0	0	0	0
SPY	5	10	0	0	0	0	0	0	0	0	0	0
STZ	7.5	20	0	0	0	0	0	0	0	0	0	0
site B	2015 (8)				2016 (12)		2017 (31)		2018 (18)		2019 (20)	
	MLOD	MLOQ	>MLOD	>MLOQ	>MLOD	>MLOQ	>MLOD	>MLOQ	>MLOD	>MLOQ	>MLOD	>MLOQ
SCY	7.5	20	0	0	0	0	0	0	0	0	1	1
SDZ	2	10	0	0	0	0	4	0	0	0	1	1
4-OH-SDZ	2*	10*	n.a.	n.a.	n.a.	n.a.	20	0	8	0	16	0
SDM	5	10	0	0	1	0	0	0	0	0	0	0
SGU	10	60	2	1	8	2	30	19	18	0	20	1
SMR	5	10	0	0	0	0	0	0	0	0	1	1
SMZ	2	10	8	8	12	12	30	28	18	18	20	20
SMX	2	7.5	8	0	11	0	21	0	18	0	15	0
SMPD	2	10	8	0	9	0	18	0	9	0	12	0
SPY	5	10	7	0	8	0	24	6	17	1	18	1
STZ	7.5	20	0	0	0	0	0	0	0	0	5	3

MLOD/Q - method limit of detection/quantification, n.a. - not analyzed, SCY - sulfachloropyridazine, SDZ - sulfadiazine, 4-OH-SDZ - 4-hydroxy sulfadiazine, SDM - sulfadimethoxine, SGU - sulfaguandine, SMR - sulfamerazine, SMZ - sulfamethazine, SMX - sulfamethoxazole, SMPD - sulfamethoxypyridazine, SPY - sulfapyridine, STZ - sulfathiazole; 2015 (6) - sampling year and number of investigated samples.

Data from 2015 taken from Spielmeier et al. (2017) (except data for SGU); data from 2016 from Spielmeier et al. (2017) were enhanced by data from second half of 2016 investigated within the study presented here.

* Due to the lack of a pure standard substance, values from SDZ were adapted

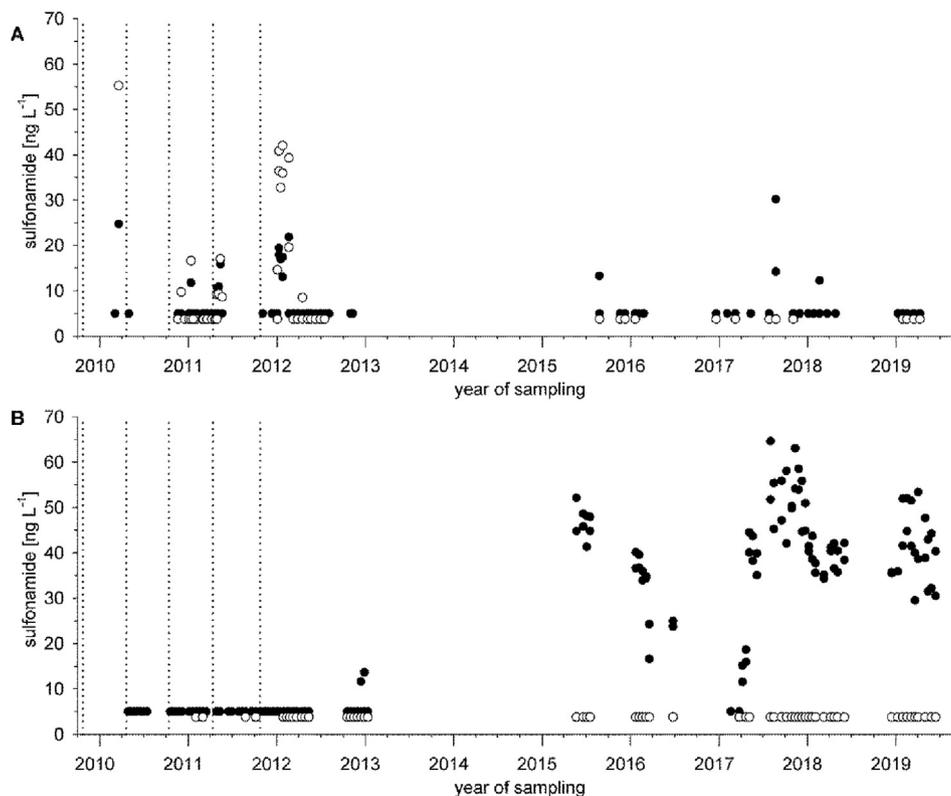


Figure 2. SMZ (black circle) and SMX (white circle) in leachate samples under (A) bedrock out of limestone decomposition (site A) and (B) sandy soil (site B); vertical lines represent the time points of application of fortified manure; samples below MLOQ are given as half MLOQ (see Table 2); year dates represent January of the respective year; data before spring 2016 were taken from Spielmeier et al. (2017).

concentration was observed during the monitoring period. This might indicate a saturation or a steady state of the transfer of the antibiotic in the soil column. An influence of the drought periods hampering the wash out of higher portions of the antibiotic can be excluded as the field at site B is artificially irrigated. The continuous input of water into the system might favor the formation of a steady state. This theory would be supported by the results obtained for the leachate below the monitoring field site (Table 1). There, comparable concentration ranges were obtained over a period of more than five years (30–100 ng L⁻¹, see also Table 1 in Spielmeier et al. (2017)). The consequence of a steady state would be the permanent input of the sulfonamide into the leachate even after stopping the application of the antibiotic into the soil.

Beside the intentionally applied sulfonamides, 4-OH-SDZ was detected in the leachate samples. This metabolite of SDZ was also detected in the manure utilized for sulfonamide application, but the 4-OH-SDZ content corresponded to less than 0.5% of the SDZ content. Thus, it appears highly unlikely that the positive findings in the leachate derive from the initial content in the applied manure only. In fact, the results support previous findings that 4-OH-SDZ cannot only be formed within the treated organism, but in soil samples as well. Several studies investigated the fate of SDZ in soil. They found a transformation of the antibiotic leading to different transformation products such as 4-OH-SDZ (Unold et al., 2009; Kasteel et al., 2010; Sittig et al., 2014). At field site B, one sample of the upper soil was taken from each lysimeter to check which portion of the sulfonamides can still be detected. Analyses revealed the presence of 4-OH-SDZ and contents were five times lower than those of SDZ (Table A11). This would support the assumption that 4-OH-SDZ derives from the transformation of the applied SDZ in the soil.

Based on the amount of fortified manure applied to the soil, a theoretical content of 133 µg kg⁻¹ would be expected for each of the applied sulfonamides. In fact, contents of all analytes were between 1.6 and 13.4 µg kg⁻¹ (Table A11). Due to the results of the leachate analyses, a wash-out and extended transfer into deeper soil layers can be excluded. A

degradation due to sunlight exposure can be neglected as well as the soil has been ploughed after manure application. The lysimeter setup does not enable a horizontal diffusion of the compounds. Consequently, most of the added sulfonamides still have to be present in the soil column, most likely as so-called non-extractable residues (NER) due to sequestration processes (Höltge and Kreuzig, 2007; Förster et al., 2009; Gulkowska et al., 2013; Müller et al., 2013). NER are formed within few hours after application of sulfonamides to soil matrix which is reflected by decreasing recovery rates with prolonged sample storage (Table A4) (Hamscher et al., 2005; Müller et al., 2013). They could be build up by covalent bond formation between sulfonamides and humic substances (Bialk and Pedersen, 2008; Gulkowska et al., 2013). Gulkowska et al. (2013) distinguished between desorbable, labile and stable SMZ within their incubation setup with synthetic humic acids. The constant release of a small amount of SMZ might be due to desorbable and labile SMZ still present in the soil matrix. Another explanation would be the permanent formation of desorbable SMZ within a steady state process. As discussed for the water sample analyses, the relative constant concentration of SMZ in the leachate of both the lysimeter and the agricultural field site suggests a steady state process leading to a continuous transfer of sulfonamides into deeper soil layers.

For site B, the seepage volume of the lysimeter was utilized for performing a mass balance. Since the first manure application in autumn 2009, 3300 L per m² passed through the soil column. If an average SMZ concentration of 40 ng L⁻¹ was assumed (low concentrations at the beginning, high concentrations towards the end of the study period), this would correspond to a transfer of 120 µg SMZ into the leachate over 10 years. Between 2009 and 2011, 50 mg per sulfonamide were applied to the study area. Within 10 years, less than 0.5% of the initially applied SMZ were transferred into the leachate. Assuming a steady state process, this would correspond to transfer of sulfonamides into the leachate for several decades even after stopping application of contaminated manure.

4. Conclusion

It was clearly demonstrated that especially long-term lysimeter and field studies accompanied by sophisticated analytical methods allow for a detailed investigation of the environmental fate of worldwide used veterinary drugs and their metabolites. Tetracyclines were shown to persist in higher concentrations ($>100 \mu\text{g kg}^{-1}$) in top soil, no leaching in deeper soil segments or groundwater occurred. Although certain sulfonamides have not been applied since several years to agricultural soils, low concentrations (ng L^{-1}) of the active drugs and one metabolite were transferred to leachate. We conclude that some veterinary drugs are even more persistent than expected and present in the soil environment even decades after their application. In regard of threshold values for the induction of antibiotic resistance, even low amounts may have an impact on the formation of antimicrobial resistance in the environment. This underlines the necessity of a precautionary limit for antimicrobial active compounds in manure and digestate.

Declarations

Author contribution statement

Astrid Spielmeier: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Madeleine S. Petri: Performed the experiments; Analyzed and interpreted the data.

Heinrich Höper, Gerd Hamscher: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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