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Persistence of the Fluoroquinolone Antibiotic Difloxacin in Soil and Lacking Effects on Nitrogen Turnover

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The environmental risks caused by the use of fluoroquinolone antibiotics in human therapeutics and animal husbandry are associated with their persistence and (bio)accessibility in soil. To assess these aspects, we administered difloxacin to pigs and applied the contaminated manure to soil. We then evaluated the dissipation and sequestration of difloxacin in soil in the absence and presence of plants within a laboratory trial, a mesocosm trial, and a field trial. A sequential extraction yielded antibiotic fractions of differing binding strength. We also assessed the antibiotic's effects on nitrogen turnover in soil (potential nitrification and denitrification). Difloxacin was hardly (bio)accessible and was very persistent under all conditions studied (dissipation half-life in bulk soil, >217 d), rapidly forming nonextractable residues. Although varying environmental conditions did not affect persistence, dissipation was accelerated in soil surrounding plant roots. Effects on nitrogen turnover were limited due to the compound's strong binding and small (bio)accessibility despite its persistence.

The introduction of veterinary antibiotics into the soil environment generates the risk of spreading antibiotic resistance, perturbing soil microbial communities and their functions, and allowing the entry of antibiotics into the food chain (Sarmah et al., 2006). Extensive research has been done into the environmental fate and effects of antibiotics (mostly macrolides, sulfonamides, and tetracyclines, as reviewed by Sarmah et al. [2006] and Chee-Sanford et al. [2009]) to assess these risks, but the research has largely disregarded fluoroquinolone antibiotics up to now. Fluoroquinolones are of growing importance in veterinary and even more in human medicine (Sukul and Spiteller, 2007), with a usage of 43 Mg in 1997 in the European Union for veterinary purposes only (European Medicines Agency, 1999). Despite the European Union's ban on the use of antibiotics as growth promoters in 2006, there is little evidence that the use of fluoroquinolones in animal husbandry is declining substantially (e.g., Veterinary Medicines Directorate, 2004, 2007). Nevertheless, we are not aware of a study that comprehensively covers the fluoroquinolones' fate and effects in soils.

Fluoroquinolones are metabolized to a varying degree after administration, and most primary metabolites still exhibit antibacterial activity (Sarkozy, 2001). Due to a strong adsorption to the solid phase during wastewater treatment (log K_d, 3.5–4.3) (Golet et al., 2003) and a pronounced stability in sewage sludge (i.e., no decrease of ciprofloxacin concentrations over 77 d) (Chenxi et al., 2008) and manure (dissipation half-life [DT_{50}] 100 d) (UK Environmental Agency, 2002), fluoroquinolones mainly reach the soil environment with the application of manure and sewage sludge to agricultural lands. Here, fluoroquinolones have been detected in concentrations of 0.1 to 370 μg kg^{-1} in soils

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Abbreviations: ASE, accelerated solvent extraction; DIF, difloxacin; DT_{50}, dissipation half-life; EAS, easily extractable; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NER, nonextractable residue; RLOQ, routine limit of quantitation; SAR, sarafloxacin; SPE, solid phase extraction; WHC_{max}, maximum water holding capacity.

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from various geographic origins (e.g., Hu et al., 2010; Martinez-Carballo et al., 2007).

Fluoroquinolones have shown low biodegradation by wastewater (i.e., no degradation of ciprofloxacin could be observed over 28 d in a closed-bottle test that was inoculated with an aliquot of the effluent from a wastewater treatment plant) (Alexy et al., 2004) and soil microorganisms (i.e., >80% of screened soil microorganisms were not capable of degrading danofloxacin) (Chen et al., 1997). This poor degradability and the strong binding to soil (Tolls, 2001) are the primary causes for the persistence of fluoroquinolones in soils and sediments. The DT_{50} values of enrofloxacin in soil exceeded 152 d in a pot experiment (Boxall et al., 2006). Half-lives of flumequine and oxolinic acid in sediment ranged from 99 to >180 d (Lai and Lin, 2009; Samuelsen et al., 1994) when incubated under laboratory conditions in the dark. The photolytic sensitivity of fluoroquinolones (Sukul and Spiteller, 2007) resulted in considerably reduced half-lives when incubated as a sediment slurry under light (DT_{50}, 4–15 d) (Lai and Lin, 2009).

Generally, the environmental effects of pollutants depend mainly on the (bio)accessible contaminant fraction rather than on the total concentrations in soil (Alexander, 2000). An estimate of this freely dissolved or weakly bound (bio)accessible fraction is the amount that can be extracted using "mild" reagents, such as 0.01 mol L^{-1} $CaCl_2$ solution (Barriuso et al., 2004). The contaminant fraction that is more strongly bound or sequestered in soil may then be extracted using harsher extraction methods, thus separating it from a further nonextractable fraction (Stokes et al., 2006). In the course of sequestration, a redistribution from weaker to stronger binding sites and a diffusion into nonaccessible microsites within the soil matrix is reflected by increasing concentrations of sequestered yet extractable and nonextractable contaminant fractions with time at the expense of the (bio)accessible fraction (Förster et al., 2009; Reid et al., 2000). At the same time, both strongly bound fractions may constitute a reservoir from which (bio)accessible concentrations can be remobilized (Alexander, 2000; Gevao et al., 2000) and hence maintained for extended periods of time. Sorption coefficients ($\log K_d$) in the range of 2 to 4 (Figueroa-Diva et al., 2010) indicate a limited (bio)accessibility of fluoroquinolones in soil. This presumably causes fluoroquinolone effects on selected microbial parameters to be small and short lived or even lacking, as observed for the fluoroquinolone difloxacin (DIF) at concentrations in the mg kg^{-1} range in laboratory experiments with homogenized soil without plants under constant temperature and moisture conditions (Kotzerke et al., 2011). However, neither the fluoroquinolones' (bio)accessibility and their effects at more realistic concentrations nor sequestration and formation of nonextractable residues (NERs) have been investigated in previous studies. As well, little is known about the long-term toxicity of persistent fluoroquinolone residues.

It remains unclear to which extent the limited knowledge on fluoroquinolones gained up to now from laboratory studies can be transferred to the field situation. The presence of plant roots, for example, is rarely simulated in laboratory experiments, although it is known that organic pollutants are frequently less persistent in the rhizosphere, where microbial activity and the resulting degradative potential are higher than in bulk soil (Anderson et al., 1993). The rhizosphere effect on pollutant dissipation may also result from plant uptake or an increased sequestration of the respective compound in rhizosphere soil (Ma et al., 2010). There is also evidence that bacterial antibiotic tolerance and eventually resistance may be more pronounced and develop faster in the rhizosphere (Brandt et al., 2009). Further modifications to the fate of organic pollutants under field conditions comprise the presence of a structured soil, the transient flow of water, and variable soil temperature and moisture. Although an estimation of field dissipation rates from laboratory data may be feasible using temperature-adjusted dissipation rates (e.g., Rosendahl et al., 2011), a transfer of laboratory results to the field situation is not necessarily valid and often leads to an overestimation of persistence in the field (Beulke et al., 2000). There is evidence that the impact of temperature on the degradation of fluoroquinolones is limited (Lai and Lin, 2009), yet very little is known about the fate of fluoroquinolones in soil under varying environmental conditions.

To study the fate and effects of fluoroquinolones in soil comprehensively, we conducted (i) a controlled laboratory trial without plants, (ii) a controlled laboratory trial with plants (mesocosm trial), and (iii) a field trial using DIF and its metabolite sarafloxacin (SAR) as fluoroquinolone model compounds (see Table 1 for structure and physicochemical properties). To simulate realistic exposure conditions, DIF was administered to pigs, and the contaminated manure was applied to soil. Soil concentrations of DIF and SAR were analyzed in fractions of different binding strength using extractions of increasing harshness and efficiency. The influence of plant roots was investigated by selective sampling of rhizosphere soil. Due to their environmental relevance, parameters of the N-cycle (potential nitrification and denitrification) were chosen to test the effects of DIF and SAR on the soil microbial community. Our hypotheses were that (i) DIF and SAR are persistent in soil and show limited (bio)accessibility, (ii) persistence is reduced under elevated temperatures and/or field conditions, (iii) dissipation is enhanced in rhizosphere soil, and (iv) the effects on microbial processes are weak as a result of poor (bio)accessibility.

Materials and Methods

Manure and Soil

Contaminated manure was produced for each trial by oral administration of the prescribed dose of DIF (5 mg kg^{-1} body weight) in capsules on five consecutive days and collection of manure for 10 d (laboratory trial) or by intramuscular administration (Dicural injectable solution) of the prescribed dose of DIF on four consecutive days and collection of manure for 10 d (mesocosm trial and field trial). Manure for the laboratory trial was produced as a nonlabeled and ^{14}C-labeled variant (details available from Sukul et al., 2009). Antibiotic concentrations of the used manures and resulting concentrations applied to soil are shown in Supplemental Table S1. Manure free from antibiotics was produced as a control for the microbiological test for the mesocosm trial and the field trial by collecting manure from nontreated pigs. The soil used for all trials was the topsoil (silt loam) of a typic Hapludalf (Soil Survey Staff, 1999) with 12 g organic C kg^{-1} soil, a pH (0.01 mol L^{-1} $CaCl_2$) of 6.3, a cation exchange capacity of 11.4 $cmol_c$ kg^{-1} (measured at pH 8.1), a maximum water holding capacity (WHC_{max}) of 45.8%, and a

texture of 160 g clay kg^{-1} soil, 780 g silt kg^{-1} soil, and 60 g sand kg^{-1} soil.

Experiments

We conducted the following three experiments: (i) a laboratory trial (10°C, 14% soil moisture, no plants, radiolabeled and nonlabeled DIF), (ii) a mesocosm trial under controlled greenhouse conditions (21°C, 14% soil moisture, corn grown), and (iii) a field trial (variable soil temperature and moisture, corn grown). The mesocosm trial and the field trial were run in two treatments, one with manure from DIF-treated pigs and one with manure from nontreated pigs that served as a control for the testing of effects.

Laboratory Trial

This experiment was run in two parallels with soil samples being treated with ^{12}C-manure or with ^{14}C-manure. For both parallels, manure and soil were mixed at a ratio of 1:25 (w/dw, corresponding to an application of 30 m^3 manure ha^{-1} and subsequent incorporation to 5 cm depth), and the soil was adjusted to 30% WHC_{max}. The soil was incubated in aerated 2-L plastic vessels ($n = 4$) at 10°C in the dark, and samples were taken on 0, 1, 4, 8, 15, 29, 57, 120, and 218 d after manure application. For this trial and the following trials, samples for antibiotic analysis were stored on ice and immediately transported to the laboratory, where they were kept at −25°C until further processing.

Mesocosm Trial

Manure and soil were mixed at a ratio of 1: 25 (w/dw), and the soil was filled into polystyrene tubs (140 cm × 80 cm × 40 cm, $n = 4$) that were planted with corn (*Zea mays* L., var. PR39K13 Pioneer Hi-Bred) the next day. The temperature was kept constant during incubation (21°C), and each tub was illuminated by two halogen lamps (400 W) with a day/night cycle of 16:8 h. Soil moisture varied between 8.5 and 15.2% (w/w), and water loss was replenished regularly. Soil was sampled on Days 0, 1, 7, 14, 28, 42, and 63 after manure application. Bulk soil (≥5 subsamples) was taken from in between corn rows using a soil corer (9.2 cm height × 1.2 cm i.d.). Rhizosphere soil was defined as soil adhering to the roots after gentle shaking (modified from Costa et al., 2006). Soil was sampled by digging out at least four corn plants, shaking, cutting roots plus remaining soil into small pieces, and thorough mixing. For this trial and for the following trial, samples for microbiological analyses were immediately transported to the laboratory and processed on arrival.

Field Trial

The field trial was conducted on a typic Hapludalf (see above) near Jülich (Western Germany; 50°55′48,77′′ N, 6°17′20,02′′ E) from May 2010 until February 2011. The site has a mean annual precipitation of 700 mm and a mean annual temperature of 9.9°C. A meteorological station recorded weather conditions during the trial period (Supplemental Fig. S1). Experimental corn plots (3 × 6 m; $n = 4$) were established in May 2010. The collected manure was applied at 30 m^3 ha^{-1} on 31 May 2010 (Day 0). It was incorporated to a depth of 12 cm, and corn was sown. Soil samples were collected on 0, 7, 14, 28, 71, 105, 140, 178, and 252 d after manure application. Corn was harvested after sampling on Day 140. Bulk soil was sampled between rows using a soil corer (5.6 cm i.d., 12 cm height) by mixing three subsamples. Rhizosphere soil was sampled from Day 28 until Day 140 only by digging out ≥3 plants, shaking, cutting roots plus remaining soil into small pieces, and thorough mixing. Corn plants were sampled from Day 28 until Day 140 and were separated into roots and plants (Day 28) and into roots, stem, leaves, and corn (Days 71, 105, and 140).

Laboratory Analyses

Chemicals and Reagents

All solvents used were of HPLC grade. Sand for accelerated solvent extraction (ASE), salts, and acids were proanalysis grade. Water was purified using a Millipore Synergy water treatment system. The analytical standards difloxacin

Table 1. Chemical structures and physicochemical properties of difloxacin and sarafloxacin.

Substance	Chemical Abstracts Service no.	Structure	Formula	pK_a†	K_{ow}‡
Difloxacin	98106–17–3		$C_{21}H_{19}F_2N_3O_3$	pK_a1: 5.7–6.1§ pK_a2: 7.2–7.6§	0.89¶
Sarafloxacin	98105–99–8		$C_{20}H_{17}F_2N_3O_3$	pK_a1: 5.6# pK_a2: 8.2#	NA††

† Acid dissociation constant.

‡ Octanol-water partition coefficient.

§ Taken from Ross and Riley (1990) and Barron et al. (2000).

¶ Taken from Ross et al. (1992).

Taken from Barron et al. (2000).

†† Data not available.

hydrochloride (6-Fluoro-1-(4-fluorophenyl)-1,4-dihydro-7-(4-methylpiperazino)-4-oxo-3-quinolinecarboxylic acid hydrochloride; purity >99%), sarafloxacin hydrochloride trihydrate (6-Fluoro-1-(4-fluorophenyl)-1,4-dihydro-4-oxo-7-(1-piperzinyl)-3-quinolinecarboxylic acid hydrochloride trihydrate; purity >97%), and sarafloxacin-D_8–hydrochloride trihydrate (purity >99%) were purchased from Sigma-Aldrich. ^{14}C-Difloxacin hydrochloride (99.1%) with a specific radioactivity of 2.15 GBq mmol L^{-1} and a labeling position at 2-pyridine was obtained from GE Healthcare. Enrofloxacin-D_5 hydrogen iodide (1-cyclopropyl-7-(4-ethyl-d_5–1-piperazinyl)-6-fluoro-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid hydrogen iodide; purity >99%) was purchased from C/D/N Isotopes, and $^{13}C^{15}N$-difloxacin (purity >99%) was provided by the Institute of Environmental Biology and Chemodynamics of the RWTH Aachen University.

Extraction of Antibiotics from Soil

To obtain fractions of differing binding strength and (bio)-accessibility, antibiotics and their metabolites were extracted from soil using extractants of increasing harshness. An easily extractable fraction (EAS), serving as a proxy of (bio)-accessibility, was obtained using 0.01 mol L^{-1} $CaCl_2$ followed by methanol (MeOH) (laboratory trial, adapted from Barriuso et al. [2004] and Förster et al. [2009]) or 0.01 mol L^{-1} $CaCl_2$ alone (mesocosm and field trial). A more strongly bound fraction was gained by an exhaustive extraction using ASE.

In detail, 10 g of soil were shaken end-over-end with 25 mL of 0.01 mol L^{-1} $CaCl_2$ for 24 h. The sample was centrifuged, and an aliquot was transferred to an autosampler vial and spiked with the internal standards ($^{13}C^{15}N$-DIF and D_8–SAR). The supernatant was decanted, 25 mL of MeOH was added, and the sample was shaken end-over-end for 4 h. After centrifugation, an aliquot was transferred to an autosampler vial and spiked with the internal standards. Results from the laboratory trial showed that MeOH extracted an antibiotic fraction of similar (bio)accessibility and dissipation behavior as 0.01 mol L^{-1} $CaCl_2$; the MeOH step was thus omitted during analysis of the mesocosm and the field trial. Because the MeOH-extractable fraction constituted on average 30% of the EAS fraction, omitting the MeOH step slightly underestimated the total concentrations of fluoroquinolones in easily extractable forms but had little effect on the concentrations in the ASE fraction, which were larger by a factor of 50 to 100. For analysis of the field trial, the $CaCl_2$-extractable fraction was subjected to a solid-phase extraction (SPE), which was modified from Herrera-Herrera et al. (2008) (dilution of samples with water, 1:1; no addition of EDTA disodium salt). Recoveries of the SPE method are given in Supplemental Table S2.

Another 10-g aliquot of soil was extracted by ASE with ethylacetate:methanol:25% ammonia solution (63:25:12, v/v). The ASE (ASE 350, Dionex) was operating at 100°C and 100 bar with a preheat time of 5 min, a flush volume of 50%, and two cycles with a static time of 30 min each. An aliquot of the extract was transferred to an autosampler vial and spiked with the internal standards ($^{13}C^{15}N$-DIF and D_8–SAR). Despite using very harsh extraction conditions (elevated temperature, pressure, and pH), the recoveries of the ASE method were rather low (20–50%; see Supplemental Table S2). Low recoveries of fluoroquinolones have been reported elsewhere (Marengo et al., 1997), and we ascribe this phenomenon to the strong binding of fluoroquinolones to soil (Figueroa-Diva et al., 2010). All samples were stored frozen at −25°C until analysis via liquid chromatography–tandem mass spectrometry (LC-MS/MS) (see below).

Extraction of Antibiotics from Plants

After freeze-drying and grinding, plants were extracted with ASE (ASE 200, Dionex) with a methanol/water solvent mixture (1:1, v/v) with 1% formic acid. Because we expected to find the largest antibiotic amounts in the roots, the method was optimized with respect to roots. Parameters of the applied ASE method were: 75°C, 100 bar, 5 min preheat, two cycles, 10 min static time, 50% flush volume, and 5 min purge time. Plant extracts were concentrated, and the remaining residue was dissolved in water (200 μL) containing 1% formic acid and 200 ng internal standard (enrofloxacin-D_5). Method recoveries are shown in Supplemental Table S2. All samples were stored frozen (−25°C) until analysis by high-resolution mass spectrometry (see below).

Radioactivity Measurement

In the labeled samples from the laboratory trial, total radioactivity was determined in five replicates via combustion using a biological oxidizer (Robox 192, Zinsser Analytic) and determination of evolving $^{14}CO_2$ in Oxysolve C-400 (Zinsser Analytic) using liquid scintillation counting.

Liquid Chromatography–Tandem Mass Spectrometry Analysis

For LC-MS/MS analysis of the soil extracts, a Thermo Finnigan system was used, which was composed of a Surveyor autosampler plus, a Surveyor MS pump plus, and a TSQ Quantum Ultra equipped with a (heated) electrospray ionization (H-ESI) ion source operating in positive mode (Thermo Finnigan). We used a Nucleodur Sphinx RP (150 × 3 mm, 3-μm particle size) column (Macherey-Nagel). Eluents were 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The gradient was controlled as follows: 95% eluent A for 1 min, linear gradient to 70% eluent A over 6 min, hold for 3 min, linear gradient to 30% eluent A over 8 min, hold for 2 min, then 100% eluent B for 3 min, linear gradient to 5% eluent A over 1 min, and hold for 6 min. A 10-μL sample was injected using partial loop injection. Ionization parameters were spray voltage 3000 V, vaporizer temperature 400°C, and capillary temperature 270°C. Nitrogen served as sheath and auxiliary gas and helium was used as collision gas at a pressure of 1.5 mm Hg. The MS was operated in selected reaction monitoring mode with three transitions being measured for each compound. Routine limits of quantitation (RLOQ) obtained for both compounds were 0.25 μg kg^{-1} dry soil in the $CaCl_2$- and MeOH-extractable fractions, 0.7 μg kg^{-1} dry soil in the ASE-extractable fraction, and 0.01 μg kg^{-1} after SPE.

Analysis of the plant samples was performed with an LTQ-Orbitrap Mass Spectrometer (Thermo Finnigan) equipped with an H-ESI II source. The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range, 200–600 with nominal mass resolving power of 60,000 at m/z 400 and a scan rate of 1 Hz) with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal

calibration standard (bis(2-ethylhexyl) phthalate: m/z = 391.284286). The spectrometer was coupled to an Agilent 1200 HPLC system consisting of a pump, a diode array detector, a column oven, and an auto-sampler (injection volume, 10 μL). Nitrogen was used as sheath gas (50 arbitrary units) and auxiliary gas (10 arbitrary units), and helium served as the collision gas. The separations were performed using a Macherey-Nagel Nucleodur Gravity C18 column (1.8 μm, 2 × 50 mm; column temperature, 30°C) with water containing 0.5% formic acid (eluent A) and methanol containing 0.1% formic acid (eluent B) as the mobile phase at a flow rate of 300 μL min^{-1}. The gradient was programmed as follows: 98% eluent A isocratic for 2 min, linear gradient to 50% eluent B over 10 min, then to 100% eluent B, hold 100% eluent B for 4.5 min before returning to the initial condition (98% eluent A) within 1 min, and hold 98% eluent A for 5 min. The limit of quantification based on these data was 50 ng DIF g^{-1} for dry plant material and 3 ng DIF g^{-1} for dry root material. The limit of detection was 17 ng DIF g^{-1} for dry plant material and 1 ng DIF g^{-1} for dry root material.

Determination of Potential Nitrification and Denitrification Activities

The effects on potential nitrification and potential denitrification were tested on selected samples of the mesocosm trial (Day 63 only) and for the entire field trial. Potential nitrification was determined according to Hoffmann et al. (2007). Briefly, the nitrite production was measured photometrically at a wavelength of 530 nm in a microtiter plate reader after 2- and 6-h incubation of 2.5 g soil with 10 mL substrate solution (1 mol L^{-1} potassium phosphate buffer [pH 7.2] with 5.63 mol L^{-1} sodium chlorate and 1.5 mol L^{-1} diammonium sulfate). The photometric detection was enabled after a reaction of nitrate with the color reagent (0.06 mol L^{-1} sulfanilamide acidified with phosphoric acid and 1.72 mmol L^{-1} N-(1-naphthyl)-ethyleneaminedihydrochloride).

The potential denitrification activity was measured with a method based on the acetylene inhibition method (Ryden et al., 1979). Three grams of soil (water content adjusted to 45% WHC_{max}) was placed into 10-mL gas-tight vials that were sealed with septum caps. The addition of nitrate was not necessary due to the high nitrate concentration in soil. After exchanging the vial atmosphere to nitrogen and replacing 10% of the headspace volume with acetylene, the samples were incubated at 20°C for 24 h. The evolved nitrous oxide was quantified with a gas chromatograph (GC-2014AF gas chromatograph with an AOC-5000 autosampler, Shimadzu) equipped with an electron capture detector (250°C) and a 1 m × 1/8” HayeSep Q 80/100-mesh column (Vici Valco Instruments). The column temperature was set to 40°C. Dinitrogen with a flow rate of 25 mL min^{-1} was used as carrier gas.

Data Evaluation

Concentrations below RLOQ were set to zero for mathematical operations and statistical tests. For the laboratory trial, concentrations in the $CaCl_2$ and MeOH extracts were summed to give the EAS fraction. Easily extractable concentrations were subtracted from ASE-extractable concentrations for all trials. Total radioactivity determined in radiolabeled samples from the laboratory incubation was corrected for differences in antibiotic concentrations between the ^{12}C-manure and the ^{14}C-manure by dividing with the factor by which concentrations in ^{14}C-manure were higher. Nonextractable residues were then calculated by subtracting total extractable amounts (^{12}C parallel). The fitting of first-order dissipation models (Eq. [1]) was performed using nonlinear regression (Systat Software GmbH, 2008; Sigma Plot 11.0S):

$$C(t) = C_0 e^{(-kt)} \qquad [1]$$

where $C(t)$ is the concentration (μg kg^{-1}) still present in soil at time t (d), C_0 is the initial concentration (μg kg^{-1}), and k (d^{-1}) is the dissipation rate constant. Dissipation half-lives (DT_{50}) were calculated from Eq. [1] with $DT_{50} = \ln(2)\ k^{-1}$. Adding a constant to Eq. [1] sometimes yielded a better fit, and half-lives were then calculated stepwise from the model equation.

Differences between antibiotic concentrations in bulk soil and rhizosphere soil and between potential (de-)nitrification rates in control samples and DIF samples were tested for statistical significance for each sampling date using a Mann-Whitney U test ($p < 0.05$) (Statistica 8.0.361.0, Statsoft).

Results and Discussion

The results of different trials will be presented and discussed jointly in this section when covering the same aspects of the environmental fate of DIF and SAR.

In the laboratory trial, easily extractable concentrations of DIF varied between 7 and 12 μg kg^{-1} dry soil. The EAS fraction of SAR was below RLOQ (Fig. 1), reflecting the low metabolization of DIF to SAR in the pig (Sukul et al., 2009) (i.e., the low concentrations of SAR in manure) (see Supplemental Table S1) and hence a smaller amount of SAR applied to soil. Similar results were obtained from the subsequent trials, where easily extractable concentrations of DIF and SAR were below RLOQ (mesocosm, data not shown) or below 0.15 μg kg^{-1} (field trial; Supplemental Fig. S2). Fitting of dissipation models to easily extractable concentrations of DIF and SAR and calculation of DT_{50} was not feasible (Table 2) due to the recurring lack of a distinct temporal trend in the EAS fraction. In accordance with our first hypothesis, the (bio)accessible concentrations of DIF and SAR were thus on a very low (below 2% of the applied amount) but constant level, which was maintained over extended periods of time. This finding suggests that dissipation of easily extractable DIF and SAR, if occurring at all, was presumably balanced by equilibrium exchange of both compounds with the more strongly bound fraction.

The ASE-extractable concentrations of DIF and SAR decreased during the laboratory incubation with a DT_{50} of 290 and 2.7 d, respectively (Table 2). The field trial yielded a DT_{50} of similar magnitude (220 d for DIF) (Table 2), whereas dissipation models could not be fitted to the mesocosm data for bulk soil (Supplemental Fig. S3). The 63-d experimental period was too short to observe substantial dissipation. Because DT_{50} exceeded 180 d in the laboratory trial and the field trial, DIF has to be classified as persistent according to the Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention, 2009) and the European Union

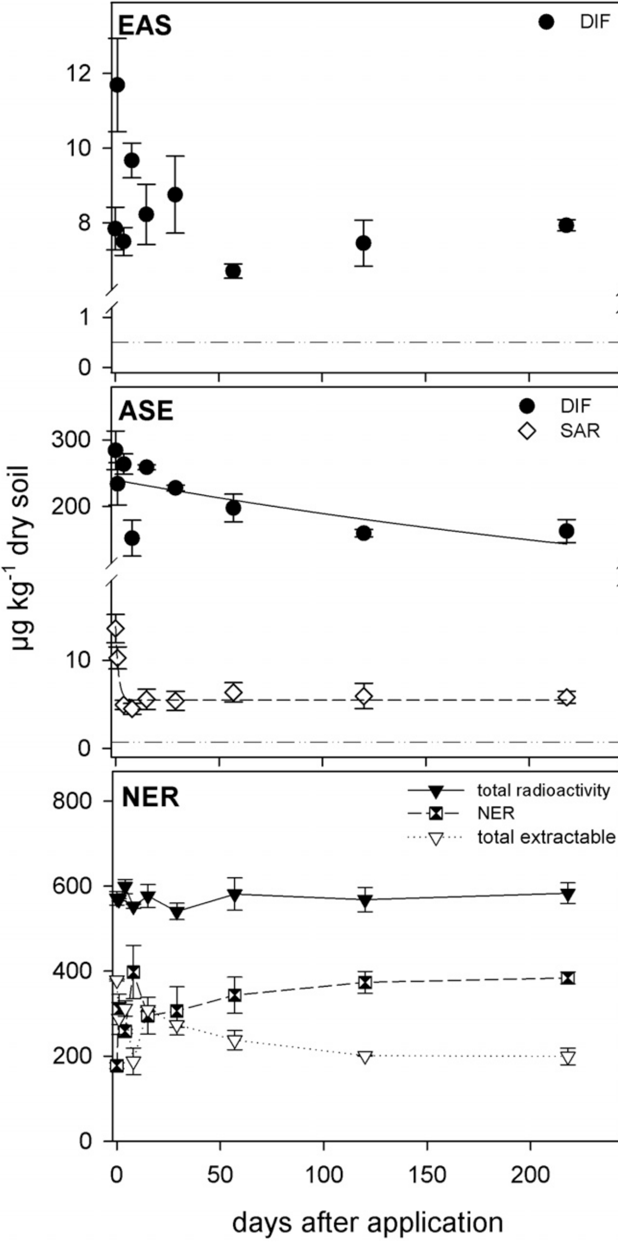

Fig. 1. Easily extractable (EAS, upper panel) and accelerated solvent-extractable (ASE, middle panel) concentrations as well as nonextractable residues (NERs) (lower panel) of difloxacin (DIF) and sarafloxacin (SAR) in the laboratory trial. Data points and error bars represent the mean of four replicates ± SE. Dashed-dotted horizontal lines in the upper and middle panel represent the routine limit of quantification; solid and dashed curves in the middle panel represent fitted dissipation curves.

pesticide legislation (Regulation [EC] no. 1107/2009; http://eur-ex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0001:0050:en:PDF). Persistence of DIF and SAR in soil was additionally reflected in (i) a leveling off of ASE-extractable concentrations at approximately 30% (DIF) and 16% (SAR) of the applied amount over the last 150 d of the experimental period (laboratory and field trial) (Fig. 2) and in (ii) a maintenance of initial values of total radioactivity over the entire trial period (Fig. 1). The latter implies the occurrence of NERs, which made up 30% of the applied amount on Day 0 and plateaued at 60 to 65% of the applied amount over the last 150 d of the experimental period (Fig. 1). The rapid formation of NERs suggests that it was not caused by the slow processes of intraparticle or intraorganic matter diffusion. It seems that strong sorptive interactions between fluoroquinolones and soil (Figueroa-Diva et al., 2010) prevented extraction as well as dissipation of fluoroquinolones in general. The rapid and strong sorption of fluoroquinolones to soil thus dominates their environmental fate, limits their (bio)accessibility, and causes NER formation and the pronounced persistence observed.

Dissipation and sequestration seemed virtually independent of experimental conditions (i.e., the ASE-extractable portion approached the same constant level in all trials) (Fig. 2), whereas soil moisture and temperature varied widely between experiments (laboratory: 10°C and 14% soil moisture; mesocosm: 21°C and 14% soil moisture; field: −8 to 28°C, 11–24% soil moisture) (Supplemental Fig. S1). Especially temperature typically determines dissipation and sequestration of organic pollutants in soil, as we could show for the sulfonamide antibiotic sulfadiazine (Rosendahl et al., 2011). The small temperature dependence of the dissipation and sequestration of DIF and SAR suggests that these processes are not governed by biological processes or by the exothermic formation of chemical bonds. Sorption of fluoroquinolones, however, is only weekly temperature dependent. Sorption coefficients (K_f or log K_d) for the interaction of enrofloxacin with zeolite (Ötker and Akmehmet-Balcıoğlu, 2005) and of norfloxacin with surface-modified carbon nanotubes (Wang et al., 2010) varied by less than 5% in the temperature range relevant to our trials (5–37°C). Although obtained for enrofloxacin and norfloxacin, these results can be expected to reflect the behavior of DIF quite well because the fluoroquinolone base structure governs their sorption, the various substituents having no great impact (Figueroa-Diva et al., 2010). Also, the processes governing sorption to zeolites, carbon nanotubes, and soil should be similar (Ötker and Akmehmet-Balcıoğlu, 2005; Vasudevan et al., 2009; Wang et al., 2010). Overall, the compiled results from all experiments and especially the uncertainty of DT_{50} (Table 2) give no indications to support our second hypothesis (i.e., persistence of DIF and SAR is neither reduced under elevated temperatures nor under field conditions as compared with laboratory conditions).

As in bulk soil, easily extractable concentrations were small in rhizosphere soil (<RLOQ in the mesocosm trial, <0.15 μg kg^{-1} in the field trial; data not shown) and lacked a clear temporal trend. Because data close to RLOQ carry a high uncertainty, we refrain from a discussion of the differences between bulk soil and rhizosphere soil in the EAS fraction. ASE-extractable concentrations of DIF and SAR, however, tended to be smaller in rhizosphere soil than in bulk soil (Fig. 3). Differences between ASE-extractable concentrations in bulk soil and in rhizosphere soil were significant (Mann-Whitney U test, $p < 0.05$) on Days 42 (DIF) and 63 (DIF and SAR) in the mesocosm trial and on Day 105 (DIF) in the field trial (i.e., at later stages of the experiments). The DT_{50} values of the ASE-extractable fraction were also lower in rhizosphere soil (82 and 69 d for DIF in the mesocosm and field trial; Fig. 3 and Table 2) than in bulk soil. In line with our third hypothesis, dissipation was thus enhanced in the vicinity of plant roots. Several mechanisms can be responsible for this observation: (i) enhanced microbial degradation in rhizosphere soil (Anderson et al., 1993), (ii) plant uptake of

Table 2. Soil dissipation half-life of difloxacin and sarafloxacin in the easily extractable and the accelerated solvent-extractable fraction in the laboratory trial and in the mesocosm trial and the field trial.

Trial	Difloxacin		Sarafloxacin	
	Easily extractable fraction†	Solvent-extractable fraction†	Easily extractable fraction†	Solvent-extractable fraction†
	d			
Lab incubation	NS‡	290* (190–580)§	n.d.¶	2.7*,# (1.8–4.6)
Mesocosm trial				
Bulk soil	n.d.	NS	n.d.	NS
Rhizosphere soil	n.d.	82** (65–110)	n.d.	NS
Field trial				
Bulk soil	NS	220** (170–320)	n.d.	n.d.
Rhizosphere soil	NS	69* (51–110)	n.d.	n.d.

* Parameter estimation of the underlying dissipation model significant at $p < 0.1$.

** Parameter estimation of the underlying dissipation model significant at $p < 0.05$.

† Determined with the exponential dissipation model unless otherwise stated.

‡ Curve fitting not significant.

§ The uncertainty range of the dissipation half-life (determined from the fitted dissipation rate constant ± SE) is given in parentheses.

¶ Not determined because of few data points.

Exponential dissipation model plus constant.

antibiotics (Boxall et al., 2006), and (iii) enhanced sequestration in rhizosphere soil (Boucard et al., 2005).

Considering the poor biodegradability of fluoroquinolones (Alexy et al., 2004; Chen et al., 1997) and the small soil concentrations of easily extractable DIF and SAR, a rapid and efficient degradation of both compounds in rhizosphere soil seems rather unlikely as the sole explanation for a faster loss of these compounds from the ASE-extractable fraction. Plant uptake has been demonstrated for several fluoroquinolones (Boxall et al., 2006; Hu et al., 2010). Showing mass-spectrometric signals of DIF below the limit of quantitation (corn leaf and roots on Day 71, corn grains on Days 105 and 140; Supplemental Fig. S4), our data indicate an uptake of trace amounts of DIF into corn plants. A first estimation (see Supplemental Material) shows that plant uptake may quantitatively explain a part of the reduction of antibiotic concentrations in rhizosphere soil but most likely not all of it, so that other mechanisms must be involved in the rhizosphere effect.

Changes in biological, chemical, and physical soil properties induced by plant roots are manifold (Hinsinger et al., 2009), and their effects on sorption and sequestration are poorly understood. Potential mechanisms include the accumulation or depletion of cationic nutrients in rhizosphere soil (Hinsinger et

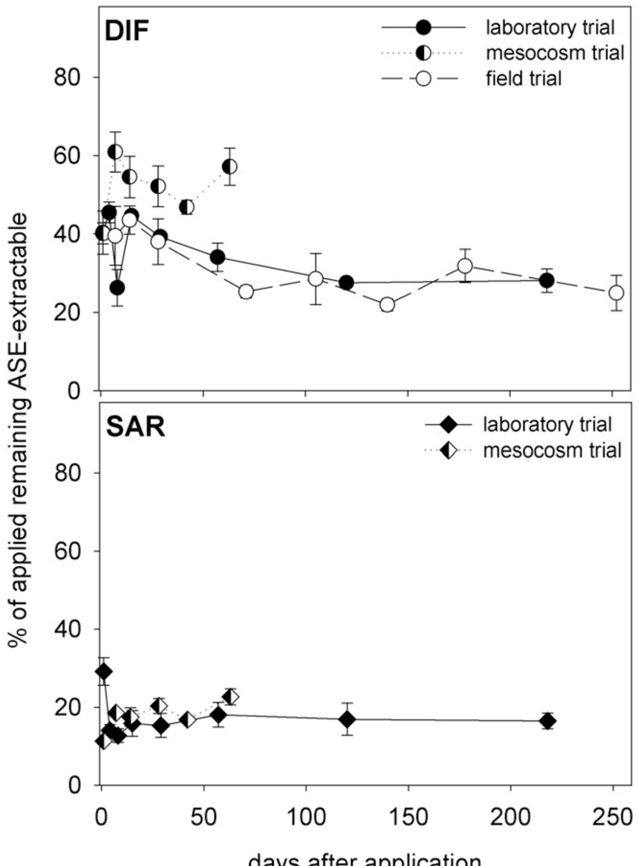

Fig. 2. Accelerated solvent-extractable (ASE) fraction of difloxacin (DIF) and sarafloxacin (SAR) as percent of the applied amount in the laboratory trial, mesocosm, and field trial. Data points and error bars represent the mean of four replicates ± SE.

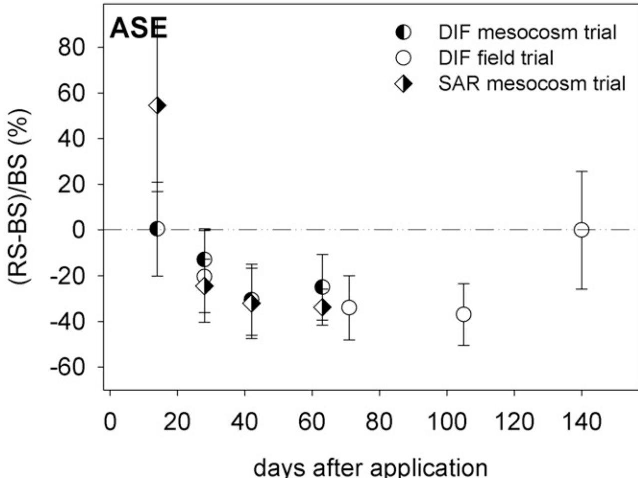

Fig. 3. Difference between antibiotic concentrations in rhizosphere soil (RS) and bulk soil (BS) as percent of bulk soil concentrations in the mesocosm and field trial. Data points and error bars represent the mean of four replicates ± SE.

al., 2009), which may affect fluoroquinolone complex formation with polyvalent metal cations (Sukul and Spiteller, 2007) with still unknown effects on sequestration. Changes of the pH in rhizosphere soil also affect sorption (Vasudevan et al., 2009) and thus sequestration, but we did not measure systematic differences between bulk soil pH and rhizosphere soil pH in any trial. However, if sorption and sequestration of DIF and SAR were enhanced in rhizosphere soil, the antibiotic fraction subjected to this enhancement must have been transferred directly into the NERs because no transient accumulation of both compounds in the ASE-extractable fractions was observed. Testing this possibility would require experiments with radiolabeled compounds.

There were no clear effects of DIF and SAR on the microbial activity, presumably as a consequence of the antibiotics' strong sequestration and low (bio)accessibility. Thus, the potential nitrification activity was not significantly different between samples treated with control manure and DIF manure during the mesocosm trial (Day 63; data not shown) and the entire field trial (Fig. 4). Also, the effects of DIF and SAR on the potential denitrification activity were not significant in both experiments (mesocosm: data not shown; field experiment: Fig. 4). Similarly, potential nitrification and denitrification were not affected by DIF and SAR in rhizosphere soil, which generally did not show elevated levels of both processes as compared with bulk soil (data not shown). This might be due to the applied sampling technique, which yielded adjacent soil in addition to soil particles adhering directly to the root surface.

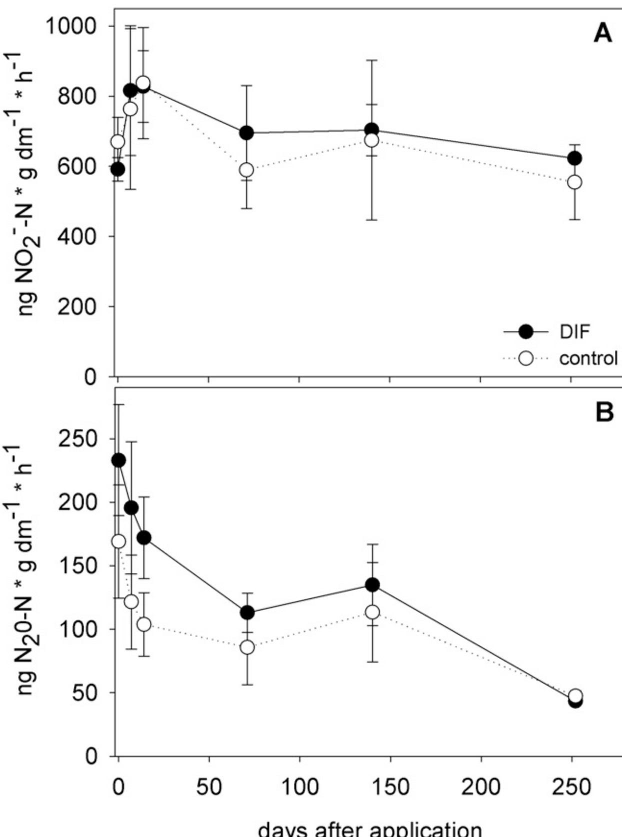

Fig. 4. Time course of potential nitrification activity (A) and potential denitrification activity (B) in bulk soil in the field trail. Data points and error bars represent the mean of four replicates ± SD.

In contrast to the results of our study, DIF was shown previously to slightly affect nitrogen transformation and the microbial community in soil but at concentrations exceeding those in the experiments described here for more than one order of magnitude (Kotzerke et al., 2011). Conversely, the more realistic soil concentrations of DIF used here did not affect the tested microbial parameters, although they were obtained for a worst-case scenario (i.e., the highest prescribed dosage, manure from treated animals only, and no storage of manure).

We conclude that fluoroquinolones are persistent in soil as a consequence of their rapid and strong sorption. This strong binding is hardly affected by soil moisture or temperature and reduces risks emerging from the presence of fluoroquinolones in soil. Plants promote the dissipation of fluoroquinolones in the direct vicinity of roots, but, given the small fraction of rhizosphere soil (assumed to account for <15% of soil volume), their impact on the overall fate of fluoroquinolones is also limited.

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