Rüdiger Reichel

Soil microbial community responses to antibiotic pharmaceuticals: influence of different soil habitats and moisture regimes

Dem Fachbereich VI
(Raum- und Umweltwissenschaften)
der Universität Trier
zur Verleihung des akademischen Grades
Doktor der Naturwissenschaften (Dr. rer. nat.)
genehmigte Dissertation

Betreuender:
Prof. Dr. agr. habil. Sören Thiele-Bruhn, Bodenkunde, Universität Trier

Berichtende:
Prof. Dr. agr. habil. Sören Thiele-Bruhn, Bodenkunde, Universität Trier
Prof. Dr. rer. nat. habil. Michael Veith, Biogeographie, Universität Trier
Prof. Dr. rer. nat. habil. Michael Schloter, TU & Helmholtz Zentrum München

Datum der wissenschaftlichen Aussprache: 02. Juli 2014

Trier 2014

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Abstract

Veterinary antibiotics are released to arable agricultural soil together with manure, including nutrients, organic matter, and microorganisms. Previously, the effects of antibiotic-contaminated manure on soil microbial community activity, function, structure, and resistance have been reported under controlled experimental conditions. This thesis further evaluated the antimicrobial effects as influenced by different manure compositions, soil microhabitats and moisture regimes, plants, and different distances to roots. Microbial community responses were determined by phenotypic phospholipid fatty acid (PLFA) and genotypic 16S rRNA gene fragment analyses. (Chapter 3) demonstrates that medication of pigs with difloxacin (DIF) and sulfadiazine (SDZ) alters the molecular-chemical pattern of slurries, confounding the detection of a consistent antibiotic effect in bulk and respective rhizosphere soil. This was evaluated in a 63-day mesocosm experiment considering typical agricultural manure applications to maize planted soil. Fecal bacteria were detected even 14 days after manure amendment. Manure of DIF- and SDZ-medicated pigs clearly affected the microbial community in mesocosm bulk and rhizosphere soil, temporarily matching antibiotic effects reported in previous studies. (Chapter 4) discusses the influences of different soil microhabitats on antibiotic fate and the effects on soil microflora. Total extractable SDZ was more than two-fold larger in earthworm burrows and soil macroaggregate surfaces compared to bulk soil or the interior fraction of aggregates. Furthermore, soil microbial communities were affected by a combination of soil microhabitat and treatment, which was reflected by different structural and functional community responses to SDZ in laboratory and under field conditions. (Chapter 5) evaluates if SDZ effects on microbial communities are more pronounced in soils which undergo periodic changes in soil moisture by drying-rewetting dynamics compared to soils without such moisture fluctuations. This was tested in a 49-day climate chamber soil pot experiment grown with grass. Manure-amended pots without or with SDZ contamination were incubated under a dynamic moisture regime with repeated drying and rewetting changes of more than twenty percent maximum water holding capacity compared to the control moisture regime. The microbial biomass, but less pronouncedly the community structure, showed an increased responsiveness to the combined stress of SDZ and dynamic moisture changes in the laboratory. Similar responses were documented under field conditions. (Chapter 6) indicated adverse effects of SDZ on root geotropism, number of lateral roots, and water uptake by plants in a 40-day greenhouse experiment with willow and maize grown in soil with environmentally relevant and worst-case antibiotic contamination. (Chapter 7) showed that the associated microbial community responded to a combination of plant species, distance to the root, and antibiotic spiking concentration. In highly antibiotic-contaminated soils, the structural and functional responses of the microbial community were dominated by indirect antibiotic effects on plants and roots.

Kurzzusammenfassung

Veterinärantibiotika gelangen zusammen mit Gülle in landwirtschaftlich genutzte Böden. Es wurde gezeigt, dass Antibiotika Aktivität, Funktion, Struktur und Resistenzbildung der mikrobiellen Gemeinschaften in Böden beeinflussen. Erstmals wurden hier die Auswirkungen variierender Güllequalität, Mikrohabitate, Bodenfeuchte und Pflanzen auf die Effekte synthetischer Antibiotika durch phänotypische und genotypische Charakterisierung der mikrobiellen Gemeinschaft untersucht. (Kapitel 3) untersucht wie sich eine nach Antibiotikabehandlung von Schweinen geänderte Güllezusammensetzung, in Kombination mit dem Antibiotikum, auf die mikrobielle Gemeinschaft im wurzelfernen und Rhizosphäre-Boden auswirkt. Dazu wurden die Effekte einer typischen Applikation von Schweinegülle ohne und mit Sulfadiazin (SDZ) und Difloxacin über einen Zeitraum von 63 Tagen untersucht. Fäkalbakterien waren auch zwei Wochen nach Applikation der Gülle im Boden nachweisbar. Die Güllequalität veränderte sich nach Antibiotikabehandlung, mit indirekten Auswirkungen auf die mikrobielle Gemeinschaft im wurzelfernen und Rhizosphären-Boden, was den Nachweis direkter Antibiotika-Effekte erschwerte. (Kapitel 4) umfasst Labor- und Feldversuche, die den Einfluss verschiedener Mikrohabitate, z.B. Rhizosphäre, Regenwurmgang, und Makroaggregate, auf die Verteilung und die Effekte von SDZ untersuchten. Im Regenwurmgang und Makroaggregat-Schale waren die extrahierbaren SDZ-Gehalte durchschnittlich doppelt so hoch als im angrenzenden Boden. Die mikrobielle Gemeinschaft im Labor- und Feldversuch war durch das jeweilige Mikrohabitat geprägt und wies in Kombination mit SDZ charakteristische, meist strukturelle Veränderungen auf. (Kapitel 5) hinterfragt den Zusammenhang von SDZ-Effekten und dynamischer Bodenfeuchteänderung. Der mit Gras bepflanzte Boden wurde mit unbehandelter und SDZ-angereicherter Gülle behandelt. In Klimakammern wurden die Töpfe ohne und mit dynamisch wechselnder Bodenfeuchte über 49 Tage inkubiert. Chronologische Analysen der SDZ-Konzentration und Veränderungen der mikrobiellen Gemeinschaft zeigten, dass dynamisch wechselnde Bodenfeuchte die Sequestrierung von SDZ fördert, zeitweise aber auch den extrahierbaren Anteil erhöhen kann. Insbesondere die mikrobielle Biomasse wurde durch eine kombinierte Wirkung von dynamischer Bodenfeuchte und SDZ beeinflusst. Diese wurde im Feldversuch bestätigt. (Kapitel 6) zeigt in einem 40-tägigen Gewächshausversuch mit Mais- und Weidenpflanzen, dass SDZ von Wurzeln absorbiert werden kann und sich in kontaminierten Böden die Wurzelfunktion und -struktur nachteilig verändern kann. (Kapitel 7) ergänzt, dass SDZ-Effekte auf Mikroorganismen durch die Pflanze, den Abstand zur Wurzel und die SDZ-Konzentration bestimmt werden, wobei in stark belasteten Böden indirekte Effekte auf Mikroorganismen durch nachteilige Veränderungen der Wurzel dominieren.

List of Abbreviations (Chapters 1-2 and 8-9)

AHI. ..... Animal Health Institute, USA
ANSES. ..... Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail, France
BfT. ..... Bundesverband für Tiergesundheit e.V., Germany
BVET. ..... Bundesamt für Veterinärwesen, Switzerland
BVL. ..... Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, Germany
Cf.. ..... Confer (lat.), consult
DANMAP. ..... Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
DGGE. ..... Denaturing Gradient Gel Elecrophoresis
DIF. ..... Difloxacin
DNA. ..... Deoxyribonucleic acid
DRIFT. ..... Diffuse Reflectance Infrared Fourier Transformed
EMA. ..... European Medicines Agency
EU. ..... European Union
FAO. ..... Food and Agriculture Organization of the United Nations
FDA. ..... Food and Drug Administration, USA
Fe. ..... Ferrum (lat.), iron
FQ. ..... Fluoroquinolone
$Gram^-$. ..... Gram-negative
$Gram^+$. ..... Gram-positive
LEI. ..... Agricultural Economics Research Institute of Wageningen University
NA. ..... Data not available
PLFA. ..... Phospholipid fatty acid
rRNA. ..... Ribosomal ribonucleic acid
SA. ..... Sulfonamide
SDZ. ..... Sulfadiazine
tRNA. ..... Transfer ribonucleic acid
USA. ..... United States of America
VA. ..... Veterinary antibiotics
VMD. ..... Veterinary Medicines Directorate, United Kingdom
W/UR. ..... Wageningen Universiteit en Researchcentrum, the Netherlands

List of Publications

My cumulative PhD thesis comprises four manuscripts in peer-reviewed journals (Chapters 3-6) and one draft manuscript (Chapter 7), which were additionally communicated to a broad scientific community at national and international congresses.

Publications

Ollivier, J., Kleineidam, K., **Reichel, R.,** Thiele-Bruhn, S., Kotzerke, A., Kindler, R., Wilke, B.-M., Schloter, M. (**2010**): Effect of Sulfadiazine-Contaminated Pig Manure on the Abundances of Genes and Transcripts Involved in Nitrogen Transformation in the Root-Rhizosphere Complexes of Maize and Clover. *Applied and Environmental Microbiology* 76, 7903–7909. DOI: 10.1128/AEM.01252-10.

Michelini L., **Reichel R.**, Werner W., Ghisi R., Thiele-Bruhn S. (**2012**) Sulfadiazine uptake and effects on *Salix fragilis* L. and *Zea maize* L. plants. *Water, Air and Soil Pollution* 223, 5243-5257. DOI: 10.1007/s11270-012-1275-5. (Chapter 6) and also part of the PhD-thesis of Michelini (2013).

Reichel R., Rosendahl I., Peeters E.T.H.M., Focks A., Groeneweg J., Bierl R., Schlichting A., Amelung W., Thiele-Bruhn S. (**2013**) Effects of slurry from sulfadiazine- (SDZ) and difloxacin- (DIF) medicated pigs on the structural diversity of microorganisms in rhizosphere soil. Soil *Biology and Biochemistry* 62, 82–91. DOI: 10.1016/j.soilbio.2013.03.007. (Chapter 3).

Reichel, R., Patzelt, D., Barleben, C., Rosendahl, I., Ellerbrock, R. H., Thiele-Bruhn, S. (**in press**): Soil microbial community responses to sulfadiazine-contaminated manure in different soil microhabitats. *Applied Soil Ecology*. DOI: 10.1016/j.apsoil.2014.03.010 (Chapter 4).

Reichel, R., Viviane Radl, Ingrid Rosendahl, Andreas Albert, Wulf Amelung, Michael Schloter, Sören Thiele-Bruhn (**in press**): Soil microbial community responses to antibiotic-contaminated manure under different soil moisture regimes. *Applied Microbiology and Biotechnology*. DOI: 10.1007/s00253-014-5717-4 (Chapter 5).

Reichel R., Michelini L., Ghisi R., Thiele-Bruhn S. (draft for submission to the *Journal of Plant Nutrition and Soil Science*): Soil microbial community responses to sulfadiazine at different distances to plant roots (Chapter 7).

Oral presentations

Reichel R., **2009**. Wirkung von Veterinärmedikamenten auf die strukturelle Diversität der Prokaryoten in Boden-Mikrokompartimenten. Oral presentation, Jahrestagung der Deutschen Bodenkundlichen Gesellschaft, 07.09.2009, Bonn, Germany

Reichel R., Peeters E., Focks A., Thiele-Bruhn S., **2010**. Effekte von Veterinärantibiotika auf die strukturelle Diversität der mikrobiellen Gemeinschaft im Rhizosphären-Boden. Oral presentation, Jahrestagung der Deutschen Bodenkundlichen Gesellschaft, 07.09.2011, Berlin, Germany

Reichel R., Rosendahl I., Amelung W., Peeters E., Focks A., Thiele-Bruhn S., **2011**. Effects of slurry from sulfadiazine (SDZ) treated pigs on the structural diversity of microorganisms in rhizosphere soil. Oral presentation, 21st Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC Europe), 16.05.2011, Milan, Italy

Reichel R., Patzelt D., Ellerbrock R.H., Thiele-Bruhn S., **2012**. Effects of pig slurry co-applied sulfadiazine (SDZ) on the microbial diversity in soil microcompartments such as earthworm channels and rhizosphere soil. Oral presentation, 6th SETAC World Congress / SETAC Europe 22nd Meeting of the Society of Environmental Toxicology and Chemistry (SETAC), 21.05.2011, Berlin, Germany

Reichel R., Thiele-Bruhn S., **2013**. Multiple factors govern the effects of pharmaceutical antibiotics in structured field soil. Oral presentation, 23rd Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC Europe), 13.05.2013, Glasgow, Scotland

Reichel R., Rosendahl I., Radl V., Schloter M., Thiele-Bruhn S., **2013**. Microbial response to SDZ-contaminated manure as influenced by soil moisture changes of different intensity. Oral presentation, Jahrestagung der Deutschen Bodenkundlichen Gesellschaft, 11.09.2013, Rostock, Germany

Reichel R., Patzelt D., Barleben C., Rosendahl I., Ellerbrock R.H., Thiele-Bruhn S., **2014**. Are soil microhabitats with characteristic microbial communities influencing the fate and effect of antibiotics in soil? Oral presentation, DBG-Workshop "Soil processes – is the whole system regulated at hot spots? From micro-scales to the pedon", 04.05.2014, Freising, Germany

Poster presentations

Reichel R., Patzelt D., Ellerbrock R.H., Thiele-Bruhn S., **2012**. Effects of sulfadiazine (SDZ) co-applied with pig slurry on structural and functional diversity of microorganisms in soil microcompartments. Poster presentation, 4th International Congress EUROSOIL 2012, 02.07.2012, Bari, Italy

Reichel R., Patzelt D., Ellerbrock R.H., Thiele-Bruhn S., **2013**. Microbial community structure at habitat level controls the response to antibiotics. Poster presentation, 2nd Thünen Symposium on Soil Metagenomics, Mining and learning from metagenomes, 11.12.2013, Braunschweig, Germany

Thiele-Bruhn S., Hammesfahr U., **Reichel** R., **2014**. Ecotoxic effects of antibiotics depend on micro-scale variability of soil microbial diversity. Poster presentation, 24th Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC Europe), 11.05.2014, Basel, Switzerland

1. Introduction

1.1 Relevance of anthropogenic antibiotic contamination

The anthropogenic era of antibiotics was founded by Alexander Fleming, who discovered penicillin in 1928 (Diggins, 1999). Biosynthesis of antimicrobial secondary metabolites by autochthonous soil microorganisms is widespread in natural soils (Thomashow and Weller, 1995; Davies, 2006). Nevertheless, since the first introduction of antibiotics, new chemical entities of antimicrobials have been discovered and were synthesized on an industrial scale (Kumar et al., 2012a). Roughly estimated, more than 250 antibiotic compounds are licensed in Germany today (Kümmerer and Henninger, 2003). About half of the antibiotics produced are consumed in agriculture (Teuber et al., 2001). Globally, agricultural antibiotic use is estimated at 50,000 to 200,000 tons/year (Ok et al., 2011; Du and Liu, 2012; Kumar et al., 2012a). This can no doubt be revised upwards because Chinese livestock production alone consumes approximately 97,000 tons veterinary antibiotics (VA) per year (Liu and Wong, 2013). The rising consumption of VAs correlates with the increasing demand for meat products in European countries, the USA and worldwide (see below, Table 1-1). The use of antibiotics is common practice in livestock farming for prophylaxis or to cure infectious diseases. Additionally, as in USA and Canada, antimicrobial feed additives are still permitted to enhance the feed-to-gain ratio (food-use efficiency) in pig and calve fattening by up to 10% (Kumar et al., 2005; Aust et al., 2008; Kumar et al., 2012a). In the USA, 13% of VAs served non-therapeutic, growth-promoting purposes in 2007 (AHI, 2012). Unsurprisingly, since the 1970s the intestinal flora of workers and animals on farms with in-feed antimicrobials are populated with resistant bacteria (Marshall and Levy, 2011). Sub-therapeutic conditions can promote the evolution of highly antibiotic unsusceptible mutants that are able to maintain their resistance with low fitness costs (Andersson and Hughes, 2012). This was one reason why the European Union (EU) started to phase out antibiotics as growth promoters in 1998 until the ultimate ban in 2006 (Kim et al., 2011), but without limiting the preventive and therapeutic use in livestock farming. Infections with multi-resistant “superbugs” after contact with medicated animal and contaminated foods are increasingly being reported (Marshall and Levy, 2011; Du and Liu, 2012). This also raises the probability that reserve antibiotics of human medicine lose their efficiency. The VA consumption continues to increase, including in countries such as the USA and Germany, leading to an estimated antibiotic consumption of 319 and 207 milligrams per kilogram meat product in 2011 (Table 1-1). In other countries such as the Netherlands, tightened administrative and hygiene regulations lowered the VA consumption without limiting meat production (Table 1-1). Excluding Germany, the total consumption of VAs in 19 EU countries was estimated at 4,802 tons in 2010, with 39% tetracyclines, 23% penicillins, 11% sulfonamides, 6% macrolides and 5% others such as

fluoroquinolones (EMA, 2012). In Germany, 1,706 tons of VAs were received by wholesalers and veterinarians in 2011 (Table 1-1), comprising 33% tetracyclines, 29% aminopenicillines, 11% sulfonamides, 10% macrolides, 7% polypeptide antibiotics, and others such as 0.5% fluoroquinolones (BVL, 2013).

Table 1-1. Consumption of veterinary antibiotics in eight selected countries over a six-year period

	Estimated use of veterinary antibiotic (tons/year)								Trends	
Selected countries	2005	2006	2007	2008	2009	2010	2011	VA mg/kg meat[15]	VA use [13]	Meat production [11, 13]
Czech Republic (EU)[1]	91	100	88	95	82	NA	NA	132	↓	↓**
Denmark (EU)[1, 4]	111	114	119	117	130	127	108	53	↓	↓
France (EU)[3]	1,293	1,234	1,327	1,172	1,058	1,014	914	160	↓**	↑
Germany (EU)[7,8]	784	839	917	926	903	921	1,706[14]	207	↑	↑**
Netherlands (EU)[2]	527	519	565	506	495	433	338	127	↓*	↑**
Switzerland (CH)[6]	NA	68	72	73	70	66	62	96	↓	↑**
United Kingdom (EU)[5]	445	405	387	384	402	447	346	131	↓	↑
United States of America (USA)[9,10]	11,087	11,999	12,631[12]	NA	13,067	13,241	13,542	319	↑**	↑*

NA, data not available

Marked citations: [1]EMA (2011), [2]LEI Wageningen UR (2012), [3]ANSES (2012), [4]DANMAP (2010, 2011), [5]VMD (2012), [6]BVET (2011), [7]BfT (2011), [8]BVL (2013), [9]AHI (2008), [10]FDA (2010, 2011, 2012), [11]FAO (2013)

[12] Non-therapeutically used antibiotics accounted for 13% in 2007 (AHI, 2012).

[13] Significant trends according to Pearson correlation test: $p < 0.05$ (*), $p < 0.01$ (**)

[14] Since 2011, quantities calculated based on veterinary antibiotics transferred to veterinarians

[15] Based on the veterinary antibiotic usage and meat production data from FAO (2013)

Most therapeutic antibiotics are administered in mg kg^{-1} body weight doses and exhibit short half-lives in animals, preventing undesired accumulation (Schadewinkel-Scherkl and Scherkl, 1995). From the VAs administered to livestock, 30-90% are excreted with urine and feces unchanged or as metabolites (Sarmah et al., 2006). Urine and feces from medicated animals are released into the environment, particularly to agricultural soil, either by grazing animals or application of contaminated manure as organic fertilizer (Sarmah et al., 2006). Typical pathways of antibiotics into the environment are (i) manure of medicated animals that received injection solutions, therapeutic feed/water additives, or ergotropics, (ii) sewage sludge with antibiotic residues of humane medicine, (iii) directly by application as preservatives against plant diseases, and (iv) unintended by releases during use and production (see below, Figure 1-1; adapted from Du and Liu, 2012). Consequently, VAs have been detected in groundwater of farms as well as in aquatic and soil environments (Sarmah

et al., 2006). In agricultural soil, antibiotic residues, typically ranging from µg kg^{-1} to mg kg^{-1}, reach field concentrations comparable to pesticides (Thiele-Bruhn, 2003a; Michelini et al., 2012). Consequently, for environmental risk assessment of pharmaceutical contaminants in soil, a trigger value of 100 µg kg^{-1} was adopted (European Medicines Agency, 1997). Nevertheless, the reliability of this value has to be evaluated by basic research on the fate and effect of pharmaceuticals in soil (Figure 1-1).

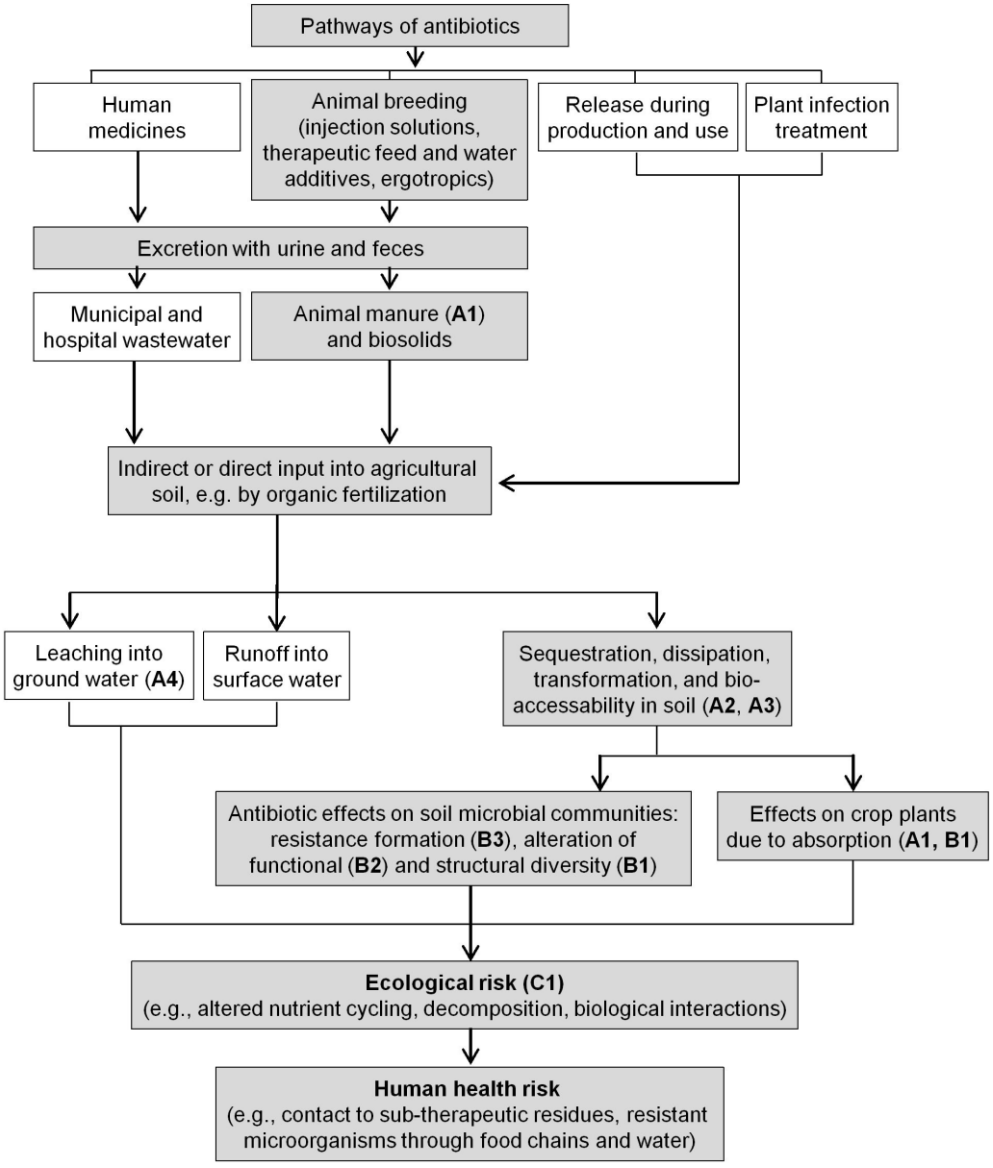

Figure 1-1. Pathways of antibiotics (adapted from Du and Liu, 2012). Grey boxes mark the pathway focused on by the DFG research unit (FOR566). The respective subprojects are indicated by abbreviations (A1-4, B1-3, and C1) as described below.

On this basis, the DFG research unit FOR566 “Veterinary Medicines in soils, basic research for risk analysis” was founded in 2005 and disbanded in 2014. The present manuscript relates to the 2nd and 3rd phase of the DFG unit FOR566. The research unit investigated the fate and effects of pharmaceutical antibiotics in arable soil with special regard to sulfadiazine

(SDZ) of the sulfonamide (SA) and difloxacin (DIF) of the fluoroquinolone (FQ) antibiotic class.

The FOR566 research unit comprised the following subprojects (cf. Figure 1-1):

Dynamics of veterinary medicines in soil (A-groups)

(A1) Prof. Dr. M. Spiteller: fate and metabolism

(A2) Prof. Dr. A. Schäffer and Dr. B. Schmidt: degradation and residue dynamics

(A3) Prof. Dr. W. Amelung, Dr. V. Laabs, Dr. J. Siemens: sequestration and dissipation

(A4) Prof. Dr. H. Vereecken and Dr. Groeneweg: transport from soils to groundwater

Effects of veterinary medicines in soils (B-groups)

(B1) Prof. Dr. S. Thiele-Bruhn: effects on prokaryotic structural diversity

(B2) Prof. Dr. B.-M. Wilke and Prof. Dr. M. Schloter: effects on the functional diversity

(B3) Prof. Dr. K. Smalla: effects on the abundance/transfer of antibiotic resistance genes

Modelling of the environmental fate of veterinary medicines (C-group)

(C1) Prof. Dr. Matthies and Dr. Klasmeier: modeling the chemical dynamics and effects

1.2 Occurrence of natural antibiotics in soil

Based on secondary metabolites of natural resources, new pharmaceuticals were discovered and often served as blueprints for the development of synthetic antibiotics (Newman and Cragg, 2007). Natural antimicrobial production is widely distributed in microbial communities of soil and plant roots (Gottlieb, 1976; Mavrodi et al., 2012; Raaijmakers and Mazzola, 2012), often comprising fungal, actinomycete, and pseudomonad species (Raaijmakers et al., 2002; Butler and Buss, 2006; Newman and Cragg, 2007). The antibiotic production in natural microbial communities is suggested to promote microbial defense, fitness, competiveness, signaling, and gene regulation (Mavrodi et al., 2012). Hence, antibiotics are part of the disease regulation in soil. In nutrient-limited soil, the detection and quantification of natural antimicrobials are often prevented by concentrations near the detection limit, fast degradation, and strong sorption to the soil matrix (Thomashow, et al. 1997; Mavrodi et al., 2012). In nutrient-rich rhizosphere soil of wheat plants, however, the natural broad-spectrum antibiotic phenazine-1-carboxylic acid was successfully quantified at concentrations up to 1.6 µg g^{-1} root fresh weight (Mavrodi et al., 2012). Natural antibiotic concentrations of 0.02-5.0 µg g^{-1} were reported in rhizosphere samples of plants (Thiele-Bruhn, 2003a, Thomashow, et al., 1995; Mavrodi et al., 2012).

The exposure to such autochthonous antimicrobial compounds is suggested to be a key driver for the evolution of antibiotic resistance in natural microbial communities. Such exposure might also alter the response to synthetic antibiotics from anthropogenic sources

(Aminov et al., 2007). Soil microbial communities are probably composed of more and less antibiotic-susceptible strains (Bevill, 1989), suggesting co-adaptions also to some synthetic antimicrobials.

1.3 Occurrence of anthropogenic antibiotics in soil

Contamination with synthetic VAs, particularly of aquatic and terrestrial soil environments, is a wide-spread problem and begins with the excretion by the animals (Boxall, 2004, 2010). Most of VAs have been designed to be readily excreted after medication with rather short half-lives, for example 0.1-26 h for SA antibiotics (Bevill, 1989) and 1.5-16 h for FQ antibiotics (Picó and Andreu, 2007). Of the applied drug, 44% SDZ (SA) was excreted unchanged as parent compound, with acetyl conjugates (26%) and hydroxylated compounds (19%) as major metabolites (Lamshöft et al., 2007, 2010). Of the applied DIF (FQ), approximately 96% were excreted as parent compound, with sarafloxacin as the major metabolite (Lamshöft et al., 2010). SA concentrations in contaminated manure typically range from 1 to 10 mg kg^{-1}, occasionally up to 235 mg kg^{-1} fresh weight (Kumar et al., 2005; Hamscher and Mohring, 2012). Manure monitoring in Austria discovered SAs, tetracyclines, and FQs with concentrations up to 91, 46, and 8 mg kg^{-1}, respectively (Martínez-Carballo et al., 2007). Feedlot pig manure from China contained tetracycline and FQ concentrations up to 60 and 47 mg kg^{-1}, respectively (Zhao et al., 2010). Hence, large quantities of antibiotic compounds are entering agricultural soils with contaminated animal manure (Sarmah et al., 2006; Sukul and Spiteller, 2006; Martínez-Carballo et al. 2007).

Under long-term conventional farming practice, a tetracycline concentration of 200 µg kg^{-1} was extractable from soil (Hamscher et al., 2002). Farmland soils of southern China had FQ and SA concentrations of even 1,537 µg kg^{-1} and 321 µg kg^{-1}, respectively (Li et al., 2011). SDZ concentrations up to 90 µg kg^{-1} were reported in soil of wheat-planted and manure-fertilized agricultural landscapes (Grote et al., 2007).

Mixtures of different antibiotics such as SAs, FQs, and tetracyclines may even increase the overall antibiotic concentration in field soil, as indicated in the northern Marmara region of Turkey (Karcı and Balcıoğlu, 2009). Real antibiotic concentrations in soil are probably underestimated due the low extraction efficiencies of many compounds (Thiele-Bruhn, 2003b; Hamscher and Mohring, 2012).

1.4 Antibiotic fate in the soil environment

Antibiotics are structurally diverse, composed of polar functional groups attached to unipolar, aromatic or heterocyclic cores; they exhibit many possibilities to interact with soil matrices (Juhel-Gaugain et al., 2000; Thiele-Bruhn et al., 2003b). Overall, VAs are more or less water

soluble, ionize depending on medium pH, and are thus sensitive to strong acids and bases and hardly volatilize (Thiele-Bruhn, 2003b; Wolters and Steffens, 2005; Picó and Andreu, 2007). The behavior of antibiotics in soil depends on molecular size, shape, structure, hydrophobicity, solubility, speciation of the antibiotic, as well as on soil properties such as pH, ionic strength, texture, cation exchange capacity, content and quality of organic matter, clay, and pedogenic oxides (Thiele-Bruhn, 2003b; Picó and Andreu, 2007; Doretto et al., 2013). Sorption mechanisms are likely based on hydrophobic interactions, ionic bonds, hydrogen bonds, and complexation on particles surfaces (Tolls, 2001). It is suggested that the effectiveness of antibiotics on soil microorganisms diminishes after the antibiotic adsorbs or binds to soil colloids (Halling-Sørensen, et al., 2003; Thiele-Bruhn et al. 2003a; Lv et al., 2013). Sorption coefficients such as the K_d values are indicative for the soil-water phase distribution of an antibiotic at equilibrium; expressed by K_{oc} values, the distribution is often normalized on the basis of soil organic carbon as sorbent (Picó and Andreu, 2007). Depending on the texture and pH, the K_{oc} values of the SA ranged from 48 to 323 L kg^{-1} (Thiele-Bruhn et al., 2003b). This indicates SAs as being potentially more mobile and bio-accessible in soil compared to FQs with K_{oc} values up to 15,800 L kg^{-1} (Picó and Andreu, 2007).

FQs such as DIF (see below, Figure 1-2) are characterized by a recalcitrant aromatic molecule structure, with large resistance to hydrolysis and temperature changes, strong sorption, and sequestration in soil (Midtvedt, 2001; Thiele-Bruhn, 2003b; Picó and Andreu, 2007; Rosendahl et al., 2012). They form hardly extractable, strongly bound residues (Picó and Andreu, 2007; Rosendahl et al. 2012). The FQs ionize at two functional groups, the acid 3-carboxyl group (pK_{a1} = 5.9-6.3) and the basic N4 of the piperazine substituent (pK_{a2} ~ 8). Hence, ion bridging of zwitter ionic species, ion-pairing, and related interactions with soil sorbents have been reported (Picó and Andreu, 2007; Figueroa-Diva et al., 2010). Nonetheless, cation exchange processes are important for the cationic FQ species that dominate in acid tropical soils (Vasudevan et al. 2009; Wang et al., 2010; Leal et al., 2013).

FQs are therefore very persistent in soil, as abiotic and biotic degradation is slow and probably incomplete (Picó and Andreu, 2007). Nonetheless, photodegradation of FQs by UV radiation in the topmost 0.5 mm of soil is possible, as well as near the surface of aquatic bodies and manure (Thiele-Bruhn, 2003b; Picó and Andreu, 2007, Sturini et al., 2012). The metabolization of DIF primarily yields sarafloxacin, having the same antibiotic potential as DIF (Granneman et al., 1986; Marengo et al., 1997; Lamshöft et al., 2010; Rosendahl et al., 2012). In principle, complete biodegradation by fungal metabolism pathways is possible (Wetzstein, 2001, 2006, 2012; Picó and Andreu, 2007; Parshikov and Sutherland, 2012). Importantly, those basidiomycete species needed to initiate cleavage of the aromatic FQs cores are not very abundant in agricultural soils. Approximately 80% of an investigated set of

microbes were not able to degrade danofloxacin (FQ; Chen et al., 1997). After application of ^{14}C-labeled ciprofloxacin with pig slurry, only a very weak microbial mineralization of 0.01% occurred (Mougin et al., 2013).

SA antibiotics are more often used as model antibiotics than FQs. SDZ, which belongs to the SA class, has been used in soil experiments as a representative for the whole group of SAs. N4-acetyl-conjugate metabolites of SDZ are less stable in the environment (Nouws 1988; Mohring et al., 2009), reacting back to the active parent compound (Lamshöft et al., 2010). SDZ has two dissociation constants at pK_{a1} = 2-3 and pK_{a2} = 5-11, indicating a potential to be protonated or de-protonated at the amino group or the N1-nitrogen of the $R_1SO_2NHR_2$ moiety (Figure 1-2; Ingerslev and Halling-Sørensen, 2000; Sukul and Spiteller, 2006). At weak acid and neutral pH conditions, as typical for most agricultural soils, neutral and positive-charged SA species are abundant (Bevill, 1989). Particularly neutral molecule species of SDZ can sorb onto soil constituents by hydrophobic interactions (Tolls, 2001; Sukul et al., 2006, Leal et al., 2013).

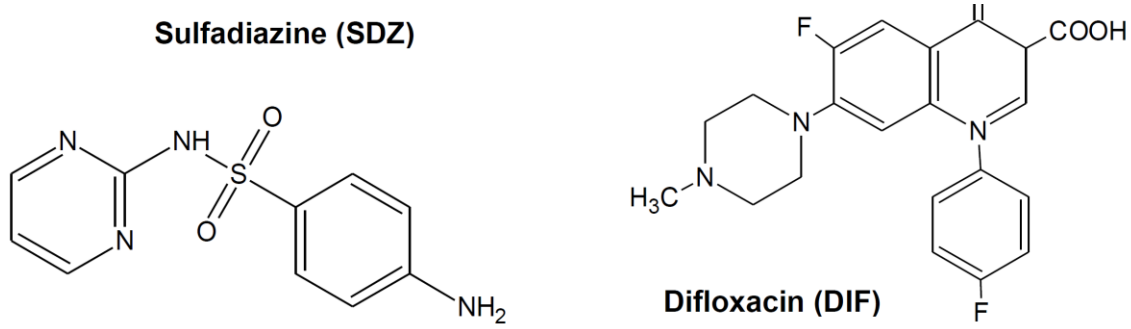

Figure 1-2. Molecular structures of the model antibiotics used in this study.

Ionic species of SA antibiotics can adsorb on charged sites of clay minerals, pedogenic oxides, and organic substances as the main sorbent (Thiele-Bruhn, 2003a; Essington et al., 2010; Gao and Pedersen, 2010; Leal et al., 2013). Sorption of SAs is probably altered in the presence of manure with high loads of ammonia due to pH changes, feeding back on molecule and sorbent properties (Boxall et al., 2002). Furthermore, dissolved organic matter probably competes with SA molecules for sorption sites (Thiele-Bruhn, 2003a; Thiele-Bruhn and Aust, 2004; Haham et al., 2012), while on the long term organic matter provides additional sorption sites in soil (Kahle and Stamm, 2007). SA sorption to soil is not completely reversible and results in a rapid dissipation by the loss of SDZ extractability (Rosendahl et al, 2011, 2012; Müller et al., 2013; Leal et al., 2013). Diffusion into hardly accessible pores, formation of surface complexes, and covalent bonds to functional groups are related to sequestration and non-extractable residue formation processes, demonstrated

by an adsorption-desorption hysteresis (Pignatello and Xing, 1996, Gao and Pedersen, 2010; Schwarz et al., 2012; Müller et al., 2013). Overall, this makes also SAs persistent in soil (Förster et al., 2009; Rosendahl et al., 2011, 2012) and provides protection against major abiotic and biotic degradation (Gavalchin and Katz, 1994). Again, UV radiation efficiently degrades SAs, but this is restricted to the topmost soil and manure layer (Miller and Donaldson, 1994; Wolters and Steffens, 2005; Thiele-Bruhn and Peters, 2007). Degradation by hydrolytic processes is less effective (Yang et al., 2009a), while oxidation at reactive mineral surfaces (Bialk et al., 2005) and in the presence of ozone (Wolters and Steffens, 2005) has been reported. Biodegradation of SA molecules is probably catalyzed by enzymes such as oxidoreductases (Schwarz et al., 2010). Microbial adaptation to the antibiotic compound positively influences the biodegradation of SAs in manure (Ingerslev and Halling-Sørensen, 2000). In soil, however, experiments with ^{14}C-labeled SAs revealed that only ~1% of the applied amount was mineralized (Kreuzig and Höltge, 2005). Nonetheless, members of the *Microbacterium* genus have been reported to efficiently mineralize SDZ after adaptation in soil (Tappe et al., 2013; Topp et al., 2013). *Microbacterium lacus* mineralizes and assimilates parts of the SDZ molecule, which explained the high mineralization of 35% of the initially applied ^{14}C-labeled SDZ within three years (Tappe et al., 2013).

For both antibiotics (SDZ and DIF) an accelerated dissipation has been reported in soil near plant roots, locally decreasing the extractable antibiotic concentration in soil (Lin et al., 2010, Rosendahl et al., 2011, 2012). In contrast to FQs, more mobile SA antibiotics can be distributed with soil water, diminishing the local concentrations, but also increasing the risk of water body and drinking water contamination (Hirsch et al., 1999; Kay et al., 2004, 2005a, b, c; Aust et al., 2008, 2010, Arenz-Leufen, 2012, Ostermann et al., 2013).

1.5 Antibiotic effects in the soil environment

Antibiotics act on target structures of lipid biosynthesis (e.g., plantesimycin), protein synthesis at the level of tRNA (mupirocin, puromycin), 30S ribosomes (tetracyclines, streptomycin), 50S ribosomes (erythromycin/macrolides, chloramphenicol), on DNA-directed RNA polymerase (rifampicin), RNA elongation (actinomycin), cell wall synthesis (penicillin, vancomycin), cytoplasmic membrane structure and function (polymyxins, daptomycin), DNA gyrase (quinolones such as FQ), and folic acid metabolism (trimethroprim, SAs; Madigan and Brock, 2009). Antibiotics frequently determined in agricultural soils are bacteriostatic tetracyclines, SAs and bactericidal FQs (Boxall et al., 2004; Picó and Andreu, 2007). SDZ, as a bacteriostatic compound, impairs bacterial growth of infectious Gram$^+$ and Gram$^-$ bacteria by competitively inhibiting the enzymatic conversion of p-aminobenzoic acid during folic acid metabolism (Brown, 1962). Also DIF impairs growth of infectious bacteria due to bactericidal

interactions with topoisomerase-II in Gram$^-$ and topoisomerase-IV in Gram$^+$ bacteria (Drlica and Zhao, 1997). Adverse effects of antibiotics on plant roots as a result of uptake from soil are also reported, but depended on soil, plant, and antibiotic properties (Jjemba, 2002; Liu et al., 2009; Michelini et al., 2012). Even less mobile enrofloxacin (FQ) and trimethroprim were incorporated by carrot roots from manure-amended soil (Boxall et al., 2006). SDZ uptake into wheat plants of 0.5% was demonstrated after applying ^{14}C-labeled molecules to soil (Grote et al., 2007). Accumulation of SA antibiotics within plant roots affect root geotropism, number of lateral roots, and water uptake by plants (Migliore et al., 1995, 1998, Liu et al., 2009; Migliore et al., 2010; Michelini et al., 2012). This effect has not yet been reported for FQ antibiotics (Picó and Andreu, 2007). Furthermore, SA and FQ antibiotics had weak toxic effects on algae (Lützhøft et al., 1999) since target structures are not identically found in cells of flora and fauna (Thiele-Bruhn, 2003a; Boleas et al., 2005). Sorption to soil colloids reduces the effectiveness of antibiotics, particularly of FQ antibiotics (Picó and Andreu, 2007). Nonetheless, clear effects of DIF on microbial soil nitrogen turnover, microbial biomass, respiration, bacteria-to-fungi ratio, and microbial catabolic diversity have been demonstrated over the short term after application to soil (Kotzerke et al., 2011; Rosendahl et al., 2012; Cui et al., 2013).

The effects of more mobile SA antibiotics on soil microbial structural and functional diversity are usually much longer-lasting (Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008, Kotzerke et al., 2008; Schauss et al., 2009a), despite the rapid dissipation of the potentially bioaccessible fraction (Hammesfahr et al., 2008; Rosendahl et al., 2011). Thus, small quantities of previously inactivated antibiotic compounds are probably released from the soil matrix over the long term. Hence, changes in humus content, pH and cation exchange mediate these long-term effects of SAs on soil microorganisms (Chander et al., 2005; Zarfl et al., 2007, 2009; Focks et al., 2010).

Broad-band antibiotics such as bacteriostatic SAs are particularly effective against Gram$^+$ and Gram$^-$ bacteria that are metabolically active (Gräfe, 1993, Madigan and Brock, 2009). Hence, research frequently demonstrated that increasing bacterial activity potentiates the effectiveness of bacteriostatic SA antibiotics in soil (Thiele-Bruhn and Beck, 2005; Zielezny et al., 2006, Demoling et al., 2009). Reported SA effects on soil microorganisms are thus mostly based on co-application of substrates that stimulate soil microbial activity. SAs co-applied with glucose, manure, or other substrates reduced the soil respiration and soil microbial Fe(III) reduction activity over the short term, following a dose-response relationship (Vaclavik et al., 2004; Thiele-Bruhn, 2005; Zielezny et al., 2006; Kotzerke et al., 2008; Liu et al., 2009). No effects were observed in soil without organic amendments. Occasionally, SAs reduced certain microbial-derived enzymatic activities such as those involved in N-cycling (Kotzerke et al., 2008; Gutiérrez et al., 2010; Hammesfahr et al., 2011c; Toth et al., 2011).

SA effects on *nir*K, *nir*S, and *nos*Z key genes and transcripts of N-cycling processes in soil have been reported in bulk and rhizosphere soil (Kleineidam et al., 2010; Ollivier et al., 2010). In SA-contaminated soils, functional redundancy is equally important and was indicated by structurally different microbial communities, maintaining the same functions (Kotzerke et al., 2008; Hammesfahr et al., 2008; Schauss et al., 2009a). Impaired bacterial ammonia-oxidation can therefore be partly compensated by ammonia-oxidizing archaea in antibiotic-contaminated soil (Schauss et al., 2009b). This indicates that some soil microorganism are less susceptible to antibiotic actions than others. Soil amendments with manure or easily available root exudates increased the pollution-induced community tolerance to SDZ (Schmitt et al., 2004; Brandt et al., 2009; Demoling et al., 2009). This shows that some specific antibiotic effects depend on co-factors. Also, resistance to antibiotics can be acquired by soil microorganisms due to the presence and accumulation of resistance genes in soil with each application of antibiotic-contaminated manure (Götz and Smalla, 1997; Heuer and Small, 2007; Binh et al., 2008, Heuer et al. 2011a, b). Most of the previously described antibiotic effects come along with phenotypic and genotypic shifts within soil microbial community structures. Genotypic analyses of 16S rRNA gene fragment patterns on DGGE very sensitively indicate structural shifts within microbial communities by band loss and appearance after applying SDZ-contaminated manure to bulk and rhizosphere soil (Zielezny et al., 2006; Hammesfahr et al., 2011a; Chapter 3). Genotypic responses to SDZ contamination are often confirmed by phenotypic analyses of microbial-derived phospholipid fatty acids (PLFA) of bacteria and fungi. Moreover, microbial biomass-C analyses often indicated a suppressed microbial biomass in soils amended with SDZ (Hammesfahr et al., 2008, 2011a). PLFA-analyses further revealed that SDZ effects on $Gram^+$ and $Gram^-$ soil bacteria are almost equivalent, which is in line with the broad-spectrum antibiotic action of SDZ (Hammesfahr et al., 2008, 2011b, Chapter 3). Finally, microbial responses to SDZ in soil can vary with the time of manure storage and amount applied to soil (Hammesfahr et al, 2011c). Reduced bacterial competitiveness was often followed by growth of fungi in SDZ-contaminated soil. This was reflected by decreased bacteria-to-fungi PLFA ratios or increased ergosterol concentrations (Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008; Demoling et al., 2009; Gutiérrez et al., 2010).

2. Motivation, influencing Factors, and Hypotheses

Antibiotic effects on soil microbial biomass, activity, resistance, as well as functional and structural diversity have been reported (Section 1.5). In the 1st phase of the FOR566 project, group B1 demonstrated that manure-stimulated antibiotic effects on soil microorganism are more pronounced at the structural than on the functional community level. This phenomenon remains over the long term in bulk soil, showing apparent concentration-independent effects (Hammesfahr et al., 2011a). The 2nd and 3rd project phase, as reported in this manuscript, were designed to evaluate the following aspects: (1) Are mid- to long-term effects of single antibiotic applications also relevant in rooted soil and field soils, where soil heterogeneity, the influence of plant species, and weather conditions add to the major effect of soil type on soil biological diversity? (2) Are turnover and effects of pharmaceutical antibiotics amplified in specific soil microhabitats of increased biological density and activity such as the rhizosphere, outer layers of soil aggregates, and earthworm channels? (3) Is the apparent concentration-independent action of antibiotics governed by the spatial proximity of agents and organisms, and hence increased in structured soil microhabitats compared to homogenized samples? (4) Is the replacement of antibiosis-inhibited bacteria compensated by other bacteria, indicating functional redundancy? (5) Are antibiotic effects enhanced or disguised in a field situation as altered by additional natural stressors, e.g. soil moisture changes? (6) The following sections provide insight into the nature of these potential influencing factors, leading to the central hypotheses.

2.1 Influence of manure

Manure is a mixture of livestock urine and faeces containing water, dung, urine, bedding material, and nutrients. Its composition depends on animal species, growth stage, supplied diet, and manure processing (Chadwick and Chen, 2002). Soil fertilization with manure delivers large amounts of substrates that stimulate soil microbial activities and growth (Larkin et al., 2006; Bünemann et al., 2006; Guerrero et al., 2007; Chakraborty et al., 2011). Hence, fertilization-derived effects on autochthonous soil microbial communities were indicated by an altered bacterial diversity, e.g., structural shifts within $Gram^-$ and $Gram^+$ bacteria proportions and *Actinobacteria* communities (Bünemann et al., 2006; Ge et al., 2008; Jangid et al., 2008; Zhong et al., 2010). PLFA-derived $Gram^-$ bacteria markers increased after applying farmyard manure to soil (Böhme et al., 2005; Larking et al., 2006). Effects of organic amendments on functional diversity were reported based on higher enzymatic activity levels of dehydrogenase, ß-glycosidase, alkaline phosphatase, or protease (Böhme et al., 2005; Gomez et al., 2006; Hernandez et al., 2007). Manure also changes microbial habitat properties such as soil aggregation, porosity, or pH (Bünemann et al., 2006; Yagüe et al.,

2012). This enhances the competitiveness of manure-derived non-pathogenic and pathogenic microorganism such as *Escherichia coli* strains within autochthonous soil microbial communities for weeks (Stoddard et al., 1998; Williams et al., 2007 Van Elsas et al., 2011). Nonetheless, in combination with antibiotic contamination, stimulating effects of manure on soil microbial activities are suppressed and respective adverse effects on autochthonous soil microbial diversity are potentiated (cf. Chapter 1.5; Hammesfahr et al., 2008; Kotzerke et al., 2008; Demoling et al., 2009; Kleineidam et al., 2010).

2.2 Influence of soil habitats

Field soil is structured, resulting in diverse microbial habitats. An estimated 90% of microbial activities are concentrated on a soil volume of 10% (Gobat et al., 2004, p. 433). Beare et al. (1995) named five hot spots that account for most of the microbial activity in soil: porosphere, detritusphere, aggregatosphere, drilosphere of earthworms, and rhizosphere. The latter three microhabitats were selected to evaluate specific antimicrobial effects and are described in the following sub-sections.

2.2.1 Rhizosphere as microbial habitat

Root architecture, growth, and function are controlled by genetic and environmental factors of the plant (Hodge et al., 2009). Roots are characterized by different regions: a secretion zone between root cap and sites of cell elongation/differentiation, an absorption zone with root hairs, and zones of lateral root emergence (Gobat et al., 2004, pp. 80-83; Hodge et al., 2009). Roots contribute to soil porosity and aggregation, are sinks of nutrients and water, as well as sources of acidifying compounds and beneficial rhizodeposits (Hinsinger et al., 2009). These physicochemical influences expand from the root surface a few millimeters into bulk soil and considerably alter microbial community structures and functions (O'Donnell et al., 2001; Kandeler et al., 2002; Hinsinger et al., 2005). The quantity and quality of these root influences depend on multiple factors such as soil type, water and nutrient status, season, organic amendments, plant development stage, plant species, as well as infection with beneficial rhizobacteria, mycorrhiza or antagonistic pathogens (Hawkes et al., 2007; Buée et al., 2009). The influences of low- and high-molecular rhizodeposits on root penetration through soil and microbial biomass growth and activity are huge (Berin et al., 2003; Hartmann et al., 2009). Rhizodeposits account for approximately 10 to 40% of the assimilated C (Sørensen and Sessitsch, 2007), but their stimulating effects on soil microbial communities vary at different root zones (Yang and Crowley, 2000; Shi et al., 2013). The “rhizosphere effect” often describes an accumulation of microorganisms in root-influenced soil (Hinsinger et al., 2005). This is reflected by a one and more magnitudes higher microbial biomass and activities compared to bulk soil (Buée et al., 2009; Hartmann et al., 2009;

Compant et al., 2010). In detail, yeasts and filamentous saprophytic or symbiotic fungi of the Ascomycota and Basidiomycota taxa are important in the rhizosphere and make up one- to two-thirds of the microbial biomass (reviewed by Buée et al., 2009). Nevertheless, copiotroph and oligotroph soil bacteria of Alpha- and Betaproteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, as well as Cytophaga-Flavobacterium-Bacteroides often dominate the rhizosphere (Buée et al., 2009). Many are $Gram^-$ bacteria that efficiently degrade easily available root exudates (Buée et al., 2009), but 10 to 23% of the microorganisms are also $Gram^+$ bacteria, as determined by sequencing of the total rhizosphere communities (Hawkes et al., 2007, pp. 4-5). Rhizosphere habitats are important for N-cycling and comprise nitrogen-fixation by bacterial strains of e.g. *Rhizobium* and *Frankia*, nitrification by ammonia-oxidizing archaea such as Crenarchaeota and bacteria (e.g. *Nitrobacter* and *Nitrosomonas*), as well as denitrification by others such as *Pseudomonas* (Leininger et al, 2006; Hawkes et al., 2007, p. 11). In addition, the rhizosphere selects for microorganisms that also degrade recalcitrant rhizodeposits with chemical structures analogous to organic pollutants. This leads to an increased degradation potential for such compounds in rhizosphere soil (reviewed by Shaw and Burns, 2003; Chaudry et al., 2005; Compant et al., 2009). Indeed, dissipation of SDZ and DIF antibiotics was faster in rooted soil, yet without direct proof of microbial transformation processes (Rosendahl et al., 2011, 2012). Nonetheless, this also implies that rhizospheres contain microorganisms that are less susceptible to antimicrobials, e.g. antimicrobial-producing *Pseudomonas* (Chapter 1.2, Bergsma-Vlami et al., 2005; Costa et al., 2006; Brandt et al., 2009; Buée et al., 2009; Raaijmakers et al., 2009). Antibiotic resistance can be also acquired by soil bacteria, particularly in active rhizospheres, due to available genetic information contained in antibiotic-contaminated manure (Chapter 1.5; Schwaner and Kroer, 2001; Kopmann et al., 2013).

2.2.2 Earthworm burrows as microbial habitat

Earthworms are “ecosystem engineers” that affect the whole soil, influencing soil porosity, aggregation, organic matter decomposition, nutrient cycling and availability, plant growth, along with the size and regulation of microbial populations (reviewed by Edwards, 2004; Blouin et al., 2013). The drilosphere defines soil habitats directly influenced by earthworm activity such as casts and burrows (Brown et al., 1995). Earthworms ingest soil with fungi and bacteria, which then are dispersed along with substrate and nutrient-enriched excrements in drilosphere soil (reviewed by Bohlen et al., 2004; Brown et al., 2004a). For instance, nitrogen and phosphorous availability is enhanced in fresh earthworm burrows and casts (reviewed by Brown et al., 2004a, p. 32). A ten or even more times larger microbial biomass was reported in earthworm burrows, particularly if lined with mucus and organic matter (Lavelle et al., 1995; Edwards, 2004). For instance, anecic *Lumbricus terrestris* (L.) create mostly vertically oriented burrows that provide aerated microbial habitats (reviewed by

Bohlen et al., 2004; Brown et al., 2004a). Organic-rich earthworm burrows are inhabited by bacterial and fungal strains growing on easily degradable substrates, e.g. *Penicillium* and *Pseudomonas,* cellulose- and hemicellulose-degrading microbial strains such as *Trichoderma* and *Bacillus*, as well as white rot fungi known to even degrade recalcitrant compounds such as lignin, polyphenols, and organic pollutants (reviewed by Brown and Doube, 2004b, p. 217b). Indeed, dissipation of benzo[a]pyrene was increased four-fold in earthworm burrows with an autochthonous microbial community (Hernández-Castellanos et al., 2013). Mineralization of ^{14}C-labeled ciprofloxacin antibiotic was also increased in soil with earthworms; their activity allocated about 40% of the radioactivity within the soil profile (Mougin et al., 2013). The exposure to introduced antibiotics likely favors the competitiveness of tolerant microorganisms, e.g. *Streptomyces* strains that occur naturally in earthworm casts (Kumar et al., 2012b). Other microorganisms such as denitrificants, inhabiting earthworm intestines, are adversely affected by soil contamination with antibiotics: this induces tenfold reduced gene copy numbers (Kotzerke et al., 2010).

Earthworm-derived soil macropores have wide diameters, facilitating water infiltration, soil drying (Ernst et al., 2009), and dispersion of manure constituents, microorganisms (Joergensen, 1998; Chadwick and Chen, 2002), herbicides (Stehouwer et al., 1994), and veterinary antibiotics in soil (Kay et al., 2004). Diffuse reflectance infrared Fourier transformed (DRIFT) spectral analysis showed stronger hydrophobicity along earthworm burrows compared to adjacent soil (Leue et al., 2011). The higher amount and different composition of organic matter also affects the sorption of pollutants (Stehouwer et al., 1994; reviewed by Shipitalo and Le Bayon, 2004, p. 193). Lowered concentrations of the hydrophobic alachlor and moderately polar atrazine in leachates indicated the retention of pollutants in earthworm burrows (Edwards et al., 1992).

2.2.3 Soil aggregates as microbial habitat

Soil aggregates are important building-blocks of soil structure and not discrete units by nature. Aggregates are more or less connected with each other, pervaded by a network of pores (Young and Ritz, 2005, pp. 32-33; Jasinska et al., 2006; Wang et al., 2012). Different types of aggregates can be defined according to size and physicochemical properties: small (2-20 µm) and large microaggregates (20-250 µm) are very stable and built from compounds such as bacterial polysaccharides, clays, and highly aromatic organic matter; small (250-2000 µm) and larger macroaggregates (>2 mm to several centimeters) are less stable and built-up from preceding aggregates that are linked by bacterial, fungal, root, and other organic constituents (Gobat, 2004, pp. 48-51; Briar et al., 2011). Manure amendments (Chapter 2.1), root exudation (Chapter 2.2.1), earthworm activity (Chapter 2.2.2), and agricultural management practice can influence soil aggregation (Six et al, 2000). Microbial communities of aggregate outer surface and interior fractions differ in their activities,

structures, and functions (Mummey et al., 2004, 2006). This reflects small-scale gradients of pH, water, gases such as O_2, and organic matter as well as predation (Standing and Killham, 2007; Davinic et al., 2012; Mueller et al., 2012). Microbial biomass and activity are increased particularly at aggregate surfaces with connections to pores; this makes them more easily accessible substrates compared to soil macroaggregate interiors (e.g., Jasinska et al., 2006; Helgason et al., 2010).

Sequencing of total community DNA revealed that particularly Proteobacteria such as Rhizobiales, Pseudomonadales, Burkholderiales, as well as $Gram^+$ Actinobacteria dominate at the aggregate level (Mummey et al., 2004; Davinic et al., 2012; Bailey et al., 2013). PLFA analyses revealed that $Gram^+$ bacteria are more abundant in soil of inter-aggregate spaces (surface fraction) than in aggregate interiors (Mummey et al., 2004; Briar et al., 2011). These bacteria included *Actinomyces* members, which are able to produce own antibiotics (Baltz, 2008; Dantas et al., 2008). Microorganisms at aggregate surfaces are more readily exposed to natural and anthropogenic stresses. This is because diffusion, for example of pesticides, into the interior of aggregates is time-dependent (Van Beinum et al., 2005). SDZ concentrations in field soil also gradually declined towards the interiors of aggregates, which was attributed to the incorporation by soil rearrangements after remoistening or thawing (Rosendahl et al., 2011). Particularly recurring drying-rewetting events promote the sequestration of xenobiotics within aggregate nano-pores (Kottler et al., 2001).

2.3 Influence of soil moisture

Different microbial populations have different moisture optima that satisfy their environmental demands best by positively influencing activity and degradation processes (Davet, 2004; Schroll et al., 2006; Bouseba et al., 2009; Moyano et al., 2013). Soil moisture alters motility, sensing, access to microhabitats, growth substrates, and chemical agents (e.g., Young and Ritz, 2005, pp. 36-38). Soil moisture generally influences microbial activity (Orchard and Cook, 1983) such as heterotrophic soil respiration that, as a result of different microbial moisture optima, shows a parabolic relationship (Moyano et al., 2013). Even slight soil drying from -0.01 to -0.02 MPa lowered the microbial activity by 10%; rewetting of dry soils by changes of 5 MPa induced microbial activity over the short term by up to 40 times (Orchard and Cook, 1983). In contrast to bacteria, fungi are less sensitive to dynamic soil moisture changes and can grow even during drying or rewetting events (Davet, 2004; Gordon et al., 2008; Singh et al., 2009; Bapiri et al., 2010). Shifts of bacterial structures are less pronounced when the community has already adapted to drying-rewetting dynamics (Fierer et al., 2003; Evans and Wallenstein, 2012). $Gram^+$ bacteria are thought to be protected against drying due their thick cell walls, whereas $Gram^-$ bacteria synthesize protective polysaccharide compounds to withstand moisture stress (reviewed by Fierer et al., 2003;

Davet, 2004; Paul, 2007, p. 48). Species of *Azotobacter* and *Nitrosomonas* are particularly sensitive to dynamic soil moisture changes, while *Actinomyces* and ammonifying *Clostridium* and *Penicillium* are more tolerant, indicating different susceptibility to moisture stress (Davet, 2004, Paul, 2007, p. 48). Rewetting temporarily improves the release of substrates that stimulate microbial activity, in turn fostering the degradation of organic compounds in soil (Orchard and Cook, 1983, Wu and Brookes, 2005; Iovieno and Bååth, 2008; Xiang et al., 2008). Hence, soil moisture dynamics promote the dissipation of organic pollutants (Baughman and Shaw, 1996; García-Valcárcel et al., 1999; Saison et al., 2010) and help remobilize formerly non-extractable fractions of pollutants such as herbicides in soil (Pätzold and Brümmer, 2003).

2.4 Resulting objectives and hypotheses

In the 1st phase the following aims were pursued. (i) What is the extent of antibiotic effects on soil microbial biomass, activity, as well as structural, and functional diversity? (ii) Is functional redundancy a typical response to antibiotic contamination of soil? (iii) Do these effects depend on a co-application of manure as a microbial activity stimulating inoculum? (iv) Are mid- and long-term SDZ effects on soil microbial community structures concentration independent? These general aims were also the objectives of this study, designed to provide further proof and evidence.

The further aims of the 2nd and 3rd phase, reported in this cumulative PhD-thesis, were as follows. (v) Are effects of manure contaminated with antibiotics increasing upon repeated manure application? Field soil is structured into diverse soil microhabitats such as rhizosphere, earthworm burrows, and soil macroaggregates that, under agricultural practice, additionally are influenced by manures with varying organic composition and variable soil moisture. This led to the formulation of the following central hypotheses (H_1-H_5):

H1: Effects of antibiotic pharmaceuticals on soil microbial communities are characteristic for bulk and rhizosphere soil, but become masked by manure from medicated pigs with different organic composition. This refers to Chapter 3: antibiotic effects on manure and microbial rhizosphere composition.

H2: The antibiotic fate and effects are different and more pronounced in soil microbial hot-spots such as soil macroaggregate surfaces, earthworm burrows, and rhizosphere microhabitats of structured soil. This refers to Chapter 4: microbial responses to manure with SDZ in diverse soil microhabitats.

H3: The effects of SDZ on microbial communities are more pronounced in soils which undergo periodic changes in soil moisture by drying-rewetting dynamics compared to soils without such moisture fluctuations. This refers to Chapter 5: effects of SDZ-spiked manure under different soil moisture regimes.

H4: SDZ is taken up by plants and adversely affects the plant and root development and function. This refers to Chapter 6 and was also part of the PhD-thesis of Michelini (2013): SDZ effects on *Salix fragilis* L. and *Zea maize* L. plant and roots.

H5: SDZ effects are indirectly controlled by antibiotic effects on plant roots and increase with proximity to the root as a source of microbial activity stimulation. This refers to Chapter 7: SDZ effects on plant roots and microbes at different distance to roots.

3. Antibiotic Effects on Manure and microbial Rhizosphere Composition

Publication I

Effects of slurry from sulfadiazine- (SDZ) and difloxacin- (DIF) medicated pigs on the structural diversity of microorganisms in rhizosphere soil

Reichel R., Rosendahl I., Peeters E.T.H.M., Focks A., Groeneweg J., Bierl R., Schlichting A., Amelung W., Thiele-Bruhn S. (2013).

Soil Biology and Biochemistry 62, 82–91.
DOI: 10.1016/j.soilbio.2013.03.007

ABSTRACT

Conventional farming still consumes considerable amounts of antibiotics such as sulfadiazine (SDZ) or difloxacin (DIF) to protect livestock from infectious diseases. Consequently, slurries from medicated animals are applied to arable soils. Antibiotics, co-applied with pig slurry, are increasingly reported to change soil microbial community structures in un-rooted bulk soil. The effects in rhizosphere soil, as well as the medication-derived direct and indirect effects of an altered slurry composition are poorly investigated. We evaluated the response of microorganisms to slurry of SDZ- and DIF-medicated pigs in a 63-d mesocosm experiment, considering the natural complexity of a typical agricultural pig slurry amendment and developing *Zea mays* L. root systems. Slurry-derived fecal bacteria were still present in mesosocosm soil 14 days after amendment. Medication with DIF and SDZ further altered the molecular-chemical pattern of the pig slurry, confounding the precise antibiotic effect. This has to be considered when investigating antimicrobial effects under ecological relevant conditions. Effects on the microbial community in mesocosm bulk soil widely matched results from previous studies on directly spiked soil. Effects were also found in the mesocosm rhizosphere soil, but not more pronounced than in bulk soil. This was also verified under laboratory conditions after application of artificially SDZ-spiked control slurry.

1 INTRODUCTION

Increasing amounts of veterinary antibiotics are administered to livestock to protect from or cure infectious diseases. A large proportion of these antibiotics are readily excreted unchanged or as metabolite, and thus enter the environment (Boxall et al., 2004; Sarmah et al., 2006).

Some of the antibiotic classes frequently found in agricultural soils are bacteriostatic, such as sulfonamides; others are bactericidal, such as fluoroquinolones (Boxall et al., 2004; Picó and Andreu, 2007). Sulfadiazine (SDZ), a sulfonamide often used in pig husbandry (Sarmah et al., 2006), reduces the reproduction of Gram-positive ($Gram^+$) and Gram-negative ($Gram^-$) bacteria by competitively inhibiting the dihydropteroate synthesis in the folic acid pathway (Focks et al., 2010). Difloxacin (DIF), a fluoroquinolone, has a bactericidal mode of action causing the inhibition of topoisomerase-II in $Gram^-$ and topoisomerase-IV in $Gram^+$ bacteria (Drlica and Zhao, 1997).

Typically, antibiotics reach agricultural soil either directly with the excreta of grazing livestock or indirectly through the spreading of contaminated manure used as organic fertilizer (Sarmah et al., 2006). With manure application, a mixture of different nutrients and organic matter returns to soil. Manure-derived nitrogen and phosphorus as well as easily degradable organic compounds are desirable for plant growth (Chadwick and Chen, 2002, Aust et al., 2009). Uncontaminated manure also stimulates the microbial activity and growth in soil,

however, it also adds a considerable amount of fecal microorganisms (van Elsas et al., 2011), including potential pathogens (Chadwick and Chen, 2002) and antibiotic-resistant strains (Heuer et al., 2011).

Beneficial effects of manure on the soil microflora, however, are substantially reduced when antimicrobial pollutants such as SDZ are co-applied (Hammesfahr et al., 2008). The effectiveness of growth-inhibiting, bacteriostatic antibiotics such as sulfonamides and tetracyclines is even increased in the presence of manure as a nutrient substrate that promotes microbial activity and growth (Schmitt et al., 2004; Hammesfahr et al., 2008). Hence, antibiotic effects on microbial growth and activities such as respiration, enzyme and reductive activities as well as the community-level physiological profile (CLPP) and the pollution induced community tolerance (PICT) have been reported (Schmitt et al., 2004; Zielezny et al., 2006; Demoling et al., 2009; Gutiérrez et al., 2010, Kotzerke et al., 2011, Rosendahl et al., 2012). Furthermore, effects on the structural diversity of soil microbial communities were reported using phospholipid fatty acid analysis (PLFA) and denaturating gradient gel electrophoresis (DGGE) of PCR amplified 16S rDNA (Schmitt et al., 2004; Thiele-Bruhn and Beck 2005; Zielezny et al., 2006; Hammesfahr et al., 2008; Gutiérrez et al., 2010). In addition, the abundance of *sul* resistance genes increased in soil on a mid-term following application of manure containing SDZ (Heuer and Smalla, 2007; Heuer et al., 2011).

Fluoroquinolones such as DIF bind strongly to the soil matrix, and thus persist longer in the soil environment than sulfonamides (Rosendahl et al., 2012). The rapidly formed strongly bound residues are only extractable using harsh methods like ASE (Förster et al., 2008), while the mild-solvent extractable fraction, operationally defined as bioaccessible quickly vanishes (Rosendahl et al., 2012). Nonetheless, a lacking extractability with mild solvents is not necessarily linked to lacking antimicrobial effects. Antimicrobial effects of sulfonamides even increased during weeks while the bioaccessible fraction determined through mild-solvent extraction declined within days to concentrations below the limit of detection (Hammesfahr et al., 2008, 2011), leading to apparent concentration independency.

Furthermore, soil microflora and decomposition processes can substantially change depending on the applied manure quality, which was clearly related to the feeding strategy of animals (Jost et al., 2011). Medicating livestock with antibiotics can cause shifts in intestine microflora (Raun, 1990), changes in animals' food digestion and utilization efficiency, and consequently alter manure composition (Klopfenstein et al., 1964; Elmund et al., 1971; Patten et al., 1980; Tedeschi et al., 2003). Thus, it must be expected that excreta from medicated animals have a different quality and microbial community structure, potentially supporting the survival of fecal pathogens and the spread of resistance genes in soil (van Elsas et al., 2011; Heuer et al., 2011). Manure of medicated pigs not only contains the

unchanged parent antibiotic, but also biologically inactive or active metabolites, which are formed by biotransformation processes within the pig's body (Lamshöft et al., 2007). Major metabolites are 4-hydroxyl-SDZ (4-OH-SDZ) and N-acetyl-SDZ (N-Ac-SDZ) for SDZ (Rosendahl et al., 2011), and sarafloxacin (SAR) for DIF (Rosendahl et al., 2012). Altogether, the evaluation of antibiotic effects on the soil microbial community under agricultural relevant conditions has to integrate preceding alternations of the pig slurry composition, its microflora, the excreted antibiotic parent compounds and metabolites, which is not reflected by artificially spiking soil with pure chemicals.

Moreover, antibiotic effects may be different in diverse soil microcompartments and especially in the rhizosphere as a soil compartment of divergent properties and microbial activity. Physical or chemical properties such as pH, and quality and quantity of root exudates are important drivers for microbial activity and the development of a plant-specific rhizosphere microflora (Smalla et al., 2001; Buée et al., 2009; Compant et al., 2010). For instance, *Pseudomonas* are especially abundant in rhizosphere soil (Costa et al., 2006). This includes strains able to produce natural antimicrobial compounds (Bergsma-Vlami et al., 2005; Costa et al., 2006). These compounds can be found in rhizosphere and often range at the level of micrograms per kilogram soil, while concentrations of synthetic antibiotics co-applied with manure can be more than a thousand times higher. Hence, oxytetracycline artificially spiked to the rhizosphere of wheat plants changed the microbial community structure (Yang et al., 2009b). The abundance pattern of genes involved in the N-cycle evidently changed in clover and maize rhizosphere soil after application of manure from SDZ medicated pigs (Ollivier et al., 2010). Pharmaceutical antibiotics can also adversely affect plant growth and yield (Michelini et al., 2012). Additionally, the chemical fate of antibiotics might be different in rhizosphere compared to bulk soil. For example, SDZ and DIF were reported to dissipate more quickly in rhizosphere than in bulk soil (Rosendahl et al., 2011, 2012), whereby the reasons and microbial consequences remain poorly understood.

With this study we intended to evaluate antibiotic effects using slurry from medicated animals to simulate a realistic slurry composition and microflora as well as a natural pattern of the excreted antibiotic parent compound and metabolites. This should help to integrate the different direct and indirect antibiotic effects, drawing an environmental relevant picture of responses in the bulk and rhizosphere microflora of field soil.

The overall objective of this study was to identify effects on the structural diversity of microorganisms under environmental relevant conditions in planted soil mesocosms. In more detail this was done using slurry from pigs medicated either with SDZ or DIF, thus showing partly metabolized parent antibiotic compounds, an altered microflora and molecular composition of slurry. The pyrolysis-field ionization mass spectrometry (Py-FIMS) was used to evaluate antibiotic-induced changes of slurry composition and was previously applied to

determine the molecular composition of organic matter from pig slurry fractions (Aust et al., 2009). Effects on the soil microbial structural diversity were determined by 16S rRNA gene denaturing gradient gel electrophoresis (DGGE) and phospholipid fatty acids (PLFA) analysis in both bulk and rhizosphere soil of maize plants in a 63-d mesocosm experiment. Additionally, sequencing data of extracted *Pseudomonas* DGGE bands were obtained to further separate the direct antibiotic effects and the indirect effects from changes in slurry composition and microflora, we conducted a parallel laboratory experiment and could refer to previous studies where the same soil, the same antibiotics and manure from the same source have been used, however, with artificial spiking of antibiotics to unaltered slurry (e.g. Heuer and Smalla, 2007; Hammesfahr et al., 2008, 2011; Kotzerke et al., 2008, 2011; Kleineidam et al., 2010).

2 MATERIAL AND METHODS

2.1 Medication of pigs and pyrolysis-field ionization mass spectrometry of pig slurries

To obtain pig slurry with a realistic composition of organic substances, antibiotic compounds, and intestine microorganisms, 5 mg DIF kg^{-1} and 30 mg SDZ kg^{-1} bodyweight were intramuscularly applied each to five randomly selected pigs on four consecutive days, following the recommended dosage. Preparations were Dicural injectable solution (50 mg DIF ml^{-1}, Fort Dodge, Würselen, Germany) and SDZ injectable solution (200 mg ml^{-1}), supplied by Vetoquinol Biowet (Gorzow Wielkopolski, Poland). All pigs were fed with the same food mixture over the whole sampling period. Antibiotic-polluted slurry was collected cumulatively over 10 days after the first application. Control slurry was collected from the same pigs before they received the antibiotics. The pigs were held in groups at the Agricultural Experimental Station for Livestock Science of the University of Bonn (Germany), and thus non-homogenized, composite samples, but no independent replicates were collected. For Py-FIMS analyses, 1 L of un-homogenized fresh slurry was sampled and directly frozen at -20°C to overcome storage-derived changes. Three subsamples of 40 ml were randomly selected, individually dried by lyophilization, and ground to powder. Slurry of each treatment was investigated by Py-FIMS in triplicate. A modified Finnigan high-performance MAT 900 mass spectrometer (Finnigan MAT, Bremen, Germany) was used for Py-FIMS analyses of slurry samples. From each sample, three internal parallels of 0.47 (±0.14) mg (mean ± standard deviation) were weighed in quartz crucibles and placed into the micro-heater of a high-temperature-probe. The probe was finally introduced into the ion source and the sample thermally degraded by stepwise heating (increment = 10 K) from 50 to 650°C in high vacuum. For each temperature step a magnetic scan was recorded in the mass range from *m/z* 15 to *m/z* 900 (single spectra). In total, 62 scans were recorded per sample and analysis; these were combined to obtain one thermogram of total ion intensity

(TII) and an averaged mass spectrum. All samples were analyzed in triplicate and TII related to mg sample weight. For further details on the Py-FIMS methodology and the statistical evaluation of TII data, see Schulten (1999) and Schulten and Leinweber (1999).

2.2 Experimental properties and sampling

The mesocosm experiment was performed at the Forschungszentrum Jülich (Jülich, Germany). Before application to soil, pig slurries were kept in the dark at 15°C for acclimatization. The slurries then were thoroughly homogenized and mixed with soil at a ratio of 1:25 (w/dw), equivalent to an application of 40 ml slurry per kg soil. Dry matter content of slurry and pH of the slurries were characterized by the LUFA NRW (Münster, Germany; Chapter 11.1, Tab. S3). The Luvisol topsoil from an arable field at Jülich-Merzenhausen, Germany (50°55'51.1"N, 6°17'47.8"E) had an organic carbon content of 1.2%, a pH ($CaCl_2$) of 6.3, a CEC of 11.4 $cmol_c\ kg^{-1}$ (measured at pH 8.1), 16% clay, 78% silt, and 6% sand (Förster et al., 2009). For mesocosm experiments 500 kg soil was filled into each of twelve 0.5 m^3 containers (140 cm x 80 cm x 40 cm). Nominal concentrations of applied antibiotic compounds (respectively of metabolites) calculated on soil dry weights were 256 µg kg^{-1} SDZ, 168 µg kg^{-1} 4-OH-SDZ, 168 µg kg^{-1} N-Ac-SDZ, 452 µg kg^{-1} DIF, and 164 µg kg^{-1} SAR (Chapter 11.1, Tab. S1). Subsequently, maize plants (*Zea mays* L., cultivar PR39K13, Pioneer Hi-Bred, Buxtehude) pre-grown for 14 d were transplanted in rows at a planting distance of 20 cm. Each mesocosm container was illuminated by two full spectrum halogen lamps (400 W) in a day/night cycle of 16:8 h. The experiment was kept at a constant temperature of 21 (± 1) °C, soil moisture was monitored using TDR probes, and water losses were replenished three times per week using a garden sprayer. Each treatment comprised four independent replicates. Composite bulk soil samples were collected between maize rows at incubation times 0, 7, 14, 28, 42 and 63 d. The average above-ground biomass of single maize plants was almost equal for all treatments and steadily increased from 7 g (14 d), 12 g (28 d), 55 g (42 d), to finally 129 g fresh weight at day 63.

The supplementary laboratory experiment was established in a greenhouse at the University of Trier (Germany) under comparable conditions, however, pig slurry was artificially spiked with SDZ sodium salt (> 99.0%, Sigma Aldrich, Germany) to reach final soil concentrations of 0 (control), 1, and 10 mg kg^{-1}. The contaminated slurry (pH 6.0, 8.2% dry matter) was mixed with soil (1:25, w/dw) and transferred to Kick-Brauckmann pots (25.5 cm height and 28.5 cm external diameter). Pots were split into two separate chambers, transplanting one pre-grown maize plant to each chamber. Bulk and rhizosphere soil sampling was performed 63 days after the slurry application. At this time, the average above-ground maize plant biomass of the three treatments was 92 g (control, 0 mg kg^{-1}), 93 g (1 mg kg^{-1}) and 75 g (10 mg kg^{-1}).

Generally, each of four independent bulk soil replicates was obtained by mixing three subsamples, which were taken between the maize plants using a soil sampling cylinder (5.6 cm inner diameter * 12 cm height). Substrate still adhering to roots after shaking was defined as rhizosphere soil (Costa et al., 2006). The rhizosphere soil was sampled according to Rosendahl et al. (2011) after the root system had established at incubation times 14, 28, 42 and 63 d. Four independent rhizosphere soil replicates per treatment were obtained. For one replicate, roots of three plants were mixed to obtain a composite rhizosphere sample, which then was sieved gently ≤ 2 mm to remove plant residues. Each independent replicate was further split in subsamples to parallelize the results of different measurements. Samples were stored at -20°C until further processing.

2.3 Determination of antibiotic concentration

Mild-solvent extractable concentrations of SDZ and DIF compounds were determined using fresh soil samples corresponding to 10 g dry weight, using 25 ml 0.01 M $CaCl_2$ solution. The suspensions were processed in an end-over-end shaker for 24 h and centrifuged at 3000 x *g* for 15 min. After decanting the supernatant, the centrifugation pellet was re-suspended and extracted for 4 h with 25 ml methanol. The residual (more strongly bound) SDZ fraction was subsequently extracted from the soil pellet by exhaustive microwave extraction using 50 ml (20:80, v/v) acetonitrile:water (Förster et al., 2008, 2009). The DIF residual fraction was extracted by accelerated solvent extraction (ASE 350, Dionex, Idstein, Germany) with ethylacetate:methanol:25% ammonia solution (63:25:12, v/v/v). The ASE was operated at 100°C and 100 bar with a preheat time of 5 min, a flush volume of 50% and 2 cycles with a static time of 30 min each. One milliliter of the resulting supernatants was transferred to HPLC vials, spiked with 50 µl (1 mg L^{-1}) of internal standard $^{13}C_6$-SDZ or $^{13}C^{15}N$-DIF solution (Institute of Environmental Biology and Chemodynamics, RWTH Aachen University, Germany) and measured by HPLC-MS/MS as described for SDZ and DIF by Rosendahl et al. (2011, 2012).

2.4 Determination of phospholipid fatty acid patterns

Lipids were extracted from 10 g soil and cleaned-up according to Zelles and Bai (1993). The extracted fatty acid methyl ester fraction was dissolved in 200 µl hexane containing 9.2 µg C19:0 internal standard (methylene nonadecanoate, Sigma-Aldrich, Taufkirchen, Germany) for quantification. Peaks were identified using a bacterial acid methyl ester mix (BAME, Sigma-Aldrich Chemie GmbH, Munich, Germany) as external standard. PLFA was analyzed using an HP 6890 gas chromatograph (Agilent, Böblingen, Germany), equipped with a 30 m x 0.4 mm x 0.2 µm fused silica capillary column (Optima 5 MS, Macherey-Nagel, Düren, Germany) and a mass spectrometer (Hewlett Packard MSD 5973, Palo Alto, CA,

USA). Helium was used as carrier gas with a flow rate of 1.5 ml min^{-1}. Initial oven temperature was 60°C for 1 min, ramped to 150°C at 10°C min^{-1}, then increased to 320°C at a rate of 5°C min^{-1}, and finally held for 25 min.

Quantitative effects on soil microbial community structure were determined using selected PLFA fatty acid markers (Hammesfahr et al., 2008, 2011): 14:0 (all bacteria), i-15:0, a-15:0, i-16:0, i-17:0 ($Gram^+$ bacteria), cy17:0, cy19:0, 18:1n7c ($Gram^-$ bacteria), 18:2n6, 18:1n9c (fungi). Microbial biomass was indicated by total concentration of all above mentioned PLFA markers ($PLFA_{tot}$). The principal response curves further include 18:1n9t as additional, but less common fungal marker, 16:0 and 18:0, that occur in eukaryotes and bacteria (Hellmann et al., 1997), as well as 15:0, and 17:0, which are also found in plant materials (Olsson et al., 1996).

Additional to the microbial total PLFA content, C_{mic} and the metabolic quotient (qCO_2) were determined according to Joergensen and Emmerling (2006) in the supplementary laboratory experiment to confirm overall antibiotic-related microbial alterations in bulk and rhizosphere soil.

2.5 Determination of DGGE band patterns

Qualitative effects on soil microbial community structures were analyzed using DNA from slurry and soil samples, extracted according to Heuer and Smalla (2007). The 16S rRNA gene fragments were amplified using total community primer set F984GC and R1378 (Heuer et al., 1997). The 16S rRNA genes of *Pseudomonas* were specifically amplified prior to PCR-DGGE using primer pairs F311Ps/R1459Ps (Milling et al., 2005). The PCR products, differing in melting properties, were separated using a DCode System for DGGE (Bio-Rad Laboratories GmbH, Munich, Germany). The PCR samples were loaded onto polyacrylamide gel (6-9%, w/v) in 1 x TAE buffer. Gels were prepared with denaturing gradients ranging from 26 to 58% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was run at 58°C for 6 h at 220 V. Subsequently, gels were silver stained and photographed on an UV-transillumination table (BioDocAnalyze, Biometra, Germany). Gels were analyzed using image analysis BIOGENE software (Vilber-Lourmat, Marne-la-Vallée, France). Comparisons were based on relative molecular weight calculations, which were derived from a defined standard lane. Band patterns were linked together using the BIOGENE database and exported as binary data for further statistical analysis.

2.6 Sequence analysis of selected *Pseudomonas* DGGE bands

For sequencing, the most prominent bands and those contributing most to the differentiation among uncontaminated and antibiotic-polluted soil treatments were selected. The successfully recovered 16S rRNA gene fragments were amplified using PCR primer pair

F984GC/R1378 without GC-clamp (Milling et al., 2005). Fifty nanograms of the resulting template were used for ligation into pCR®2.1 vector (TA Cloning® Kit, Invitrogen Corporation, Darmstadt, Germany). Transformation into chemically competent *Escherichia coli* cells (One Shot® TOP10, Invitrogen GmbH, Darmstadt, Germany) was done according to the manufacturer's protocol. DNA of the randomly picked clones was extracted and re-amplified with GC-clamp. Re-amplification products were compared with corresponding lanes of original *Pseudomonas* DGGE gel, using the BIOGENE software package (Vilber-Lourmat, Marne-la-Vallée, France). Subsequently, plasmids containing prominent bands were selected and sequenced by Eurofins MWG Operon GmbH (Ebersberg, Germany) using the standard M13uni(-21) primer. The sequence data was evaluated with Chromas 1.5 (Technelysium Pty Ltd, Brisbane, Australia) and exported for the BlastN procedure on the Greengenes database (http://greengenes.lbl.gov).

2.7 Accession numbers

Sequence data of *Pseudomonas* 16S rRNA gene fragments were submitted to GenBank® (Bethesda, Maryland, USA) with accession numbers JN217132, JN217133, JN217134, JN217135 and JN217136.

2.8 Data analysis

Results were expressed as mean values (± standard deviation) of four independent replicates calculated on an oven-dry weight (dw) basis (105°C, 48 h). Plants and associated rhizosphere developed over time, thus enabling sampling of sufficient amounts of rhizosphere soil from day 14. The Py-FIMS data of pig slurry treatments were analyzed using the *t*-test and discriminant analysis. Univariate Wilks' Lambda testing combined with a multiple *F*-test allowed the selection of mass signals of highest discriminating power. The significance between antibiotic concentration and quantitative microbial data was repeatedly tested by one-way ANOVA with Tukey-B or T2 as post-hoc tests. The overall influences of compartment-affiliation, treatment and time on PLFA development were evaluated by a three-way ANOVA (SPSS Statistics 20.0, IBM Deutschland GmbH, Ehningen, Germany). Detrended correspondence analysis (CA) of DGGE gels was performed using the software packages PC-ORD Version 5.02 (MjM Software, Gleneden Beach, Oregon, USA), and principal response curves (PRC) were calculated by CANOCO for Windows 4.5 software (Microcomputer Power, Ithaca, New York, USA). Using PRC, the deviation (canonical coefficient, C_{dt}) of fatty acid composition referenced to control was modeled over time. The importance of an individual marker fatty acid for displayed canonical coefficients (C_{dt}) was identified by b_k-statistics (statistical weight). The fatty acids with highest b_k-values contribute the most to displayed deviations. For the PRC data, the significance of time and treatment

was evaluated by Monte Carlo permutation tests. Additional information about the PRC approach can be found in van den Brink and ter Braak (1999).

3 RESULTS

3.1 Physicochemical and microbial properties of the pig slurries

Slurry from pigs treated with SDZ contained the typical pattern (Lamshöft et al., 2007) of intra-corporal transformation products: 6.4 mg l^{-1} SDZ, 4.2 mg l^{-1} 4-OH-SDZ, and 4.2 mg l^{-1} N-Ac-SDZ and were recalculated for soil spiking concentration (Chapter 11.1, Tab. S1). Slurry of DIF treated pigs contained 11.3 mg l^{-1} DIF and 4.1 mg l^{-1} SAR as the major metabolite (Chapter 11.1, Tab. S1). The control slurry had a dry matter content of 9.8% and a pH of 6.8, while dry matter content and pH were 12.2% and 6.9 for slurry from SDZ medicated pigs and 7.3% and 7.1 for the DIF slurry (Chapter 11.1, Tab. S3).

The organic matter composition of pig slurries used in the mesocosm experiment was evidently altered after medication with antibiotics as evaluated by discriminant analysis (Fig. 1) of pyrolysis-field ionization mass spectra (Chapter 11.1, Fig. S1a, b, c). During pyrolysis, on average 63.5% (w/w) of the freeze-dried slurry samples were volatilized. The detected total ion intensities (TIIs) ranged from 80.4 to 275.0 $\times 10^6$ counts mg^{-1}, with the highest mean value (± standard deviation) for the SDZ slurry (210.5 ±69.4 $x10^6$ counts mg^{-1}), followed by the DIF slurry (169.6 ±28.1 $\times 10^6$ counts mg^{-1}) and the control slurry (96.5 ±14.0 $\times 10^6$ counts mg^{-1}). The thermograms of TII (Chapter 11.1, Fig. S1a, b, c, inserts upper right) indicated different overall thermal stabilities. Generally, three intensity maxima were observed, which indicated fractions of low (T_{max} at around 230 °C), medium (T_{max} at around 340 °C) and high (T_{max} at around 500 °C) thermostability. The molecular-chemical composition of the analyzed slurries was characteristic for pig slurry (Chapter 11.1, Fig. S1a, b, c). Based on an in-depth data evaluation and the publications of Aust et al. (2009), Dinel et al. (1998) and Jardé et al. (2009), mass signals of higher abundance (> 1.0% of TII in at least one of the samples) were assigned to the molecular ions of acetic acid (*m/z* 60), indole (*m/z* 117), n-fatty acids (*m/z* 242, 256, 280, 282, 284), sterols (388, 396, 398, 408, 414, 416, 426) and n-diol lipids (*m/z* 370, 384). Further mass signals (*m/z* 61, 257, 281, 283, 285, 371, 389 and 399) were assigned to isotope peaks and/or protonated molecules of the above-listed marker signals. The most pronounced differences were determined for summed absolute ion intensities of marker signals for the compound classes of sterols, lipids and free n-fatty acids. Thus, the ion intensity of sterols in the SDZ slurry was significantly higher ($p < 0.05$, 70.1 $\times 10^6$ counts mg^{-1}) by approximately three orders of magnitude compared to the other samples. Lipids showed significant differences between the untreated control and the SDZ slurry, whereas the free n-fatty acids were significantly more abundant in the DIF slurry versus the untreated control slurry. Nonetheless, no significant differences were

observed for the summed relative ion intensity abundances (% TII) of the classes. Based on the discriminant analysis of the whole *m/z*-signal pattern (Fig. 1), all slurry samples were clearly separated by the score plot of the first two DA components, explaining 57% (DA1) and 31% (DA2) of the total variance. Mass signals of highest discriminating power were selected by univariate Wilks' Lambda testing combined with a multiple *F*-test. Accordingly, a number of 7, 24 and 70 *m/z*-signals significantly contributed to the differences between the samples at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively. Most of these *m/z*-signals can be assigned to n-fatty acids and sterols and other lipids, respectively. The most significant differences, calculated by the *t*-test, were found between the SDZ slurry and the other two treatments. Thus, the relative abundances of n-fatty acids, in particular palmitic (*m/z* 256), stearic (*m/z* 284) and oleic acid (*m/z* 282), were significantly lower in the SDZ slurry. Significant antibiotic effects, indicated by differences between the untreated control slurry and the two other slurries (SDZ and DIF slurry), were assigned to increased proportions of ion intensities for *m/z* 81 (methylpyrrole), class overlapping mass signals for lignin dimers and lipids (*m/z* 270, 272, 300, 326, 328), and unassigned mass signals (*m/z* 247, 406, 420). In conclusion, the organic matter composition of the pig slurries used in the mesocosm experiment was considerably altered by medication with antibiotics. The total PLFA content in pig slurry was 459.3 (±160.3) nmol g^{-1} dw for the control slurry, 581.4 (±113.0) nmol g^{-1} dw for the DIF slurry, and with 1,110.0 (±248.7) nmol g^{-1} significantly increased ($p < 0.05$) in the SDZ slurry (Tab. 1). The PLFA-derived bacteria:fungi and $Gram^+$:$Gram^-$ ratios ranged from 5.8 to 6.5 and 19.2 to 28.6, respectively, with no significant differences among treatments (Tab. 1). Slurry-specific structural changes, however, were indicated by total bacterial community DGGE banding patterns (Chapter 11.1, Fig. S2), as indicated by higher total band intensities as well as by the significant appearance (SDZ slurry) or loss (DIF slurry) of certain DGGE bands.

3.2 SDZ and DIF concentrations in bulk and rhizosphere soil

After amending soil with SDZ-contaminated slurry (0 d), 21.0 (±1.5) µg kg^{-1} (SDZ), 70.8 (±1.1) µg kg^{-1} (N-Ac-SDZ) and 7.9 (±0.9) µg kg^{-1} (4-OH-SDZ) of the co-applied concentration was found as mild-solvent extractable and 98.0 (±25.8) µg kg^{-1} (SDZ), 72.6 (±14.0) µg kg^{-1} (4-OH-SDZ) and 9.9 (±1.5) µg kg^{-1} (N-Ac-SDZ) as residual fraction (Chapter 11.1, Tab. S1). During the 63 days, the mild-solvent extractable fractions strongly dissipated to low concentrations of < 2 µg kg^{-1} (SDZ and 4-OH-SDZ), while N-Ac-SDZ diminished to undetectable traces. After the same period, the residual concentrations of SDZ and 4-OH-SDZ were slightly reduced, while N-Ac-SDZ residues again were not detectable even one day after slurry application. In the period from incubation day 14 to 63, mild-solvent extractable and residual SDZ concentrations of SDZ and 4-OH-SDZ were lower in

rhizosphere compared to the bulk soil (Chapter 11.1, Tab. S1). However, this was significant ($p < 0.05$) only for the mild-solvent extractable SDZ at incubation time 28 d and 63 d.

After applying DIF-contaminated slurry to soil, the mild-solvent extractable DIF and SAR instantaneously dropped to unquantifiable traces. However, 284.3 (±51.4) µg kg^{-1} (DIF) and 6.8 (±1.2) µg kg^{-1} (SAR) of the applied concentration was determinable as residual fraction (Chapter 11.1, Tab. S1). During incubation, this residual concentration declined slightly to 258.4 (±42.6) µg kg^{-1} (DIF) and 6.3 (±1.1) µg kg^{-1} (SAR) in bulk soil until day 63. The residual concentrations of DIF were significantly lower ($p < 0.05$) in rhizosphere compared to bulk soil at incubation time 42, 63 d, and for SAR at 63 d.

No mild-solvent extractable or residual concentrations of SDZ and DIF were determined in slurry of unmediated animals and after application to soil, respectively.

3.3 Impact on PLFA in bulk and rhizosphere soil

The three-way ANOVA indicated that the factors time (*F*-value 31.7), treatment (*F*-value 72.0) and compartment-affiliation (*F*-value 138.9) significantly influenced ($p < 0.001$) the $PLFA_{tot}$ development in soil and interacted with each other (*F*-value 6.6), explaining 37% of the total variance (data not shown). After applying slurry to soil, $PLFA_{tot}$ concentrations of control-, SDZ-, and DIF-slurry treatment generally increased until the end of incubation (Tab. 1). The $PLFA_{tot}$ concentration of the rhizosphere soils usually was about one third larger than that of the bulk soils. Compared to the control-slurry treatment, the $PLFA_{tot}$ concentration was even significantly larger ($p < 0.05$) in antibiotic contaminated bulk soil at incubation time 63 d (SDZ-slurry treatment), 7 and 14 d (DIF-slurry treatment), and in rhizosphere soil at day 14 (DIF-slurry treatment; Tab. 1).

Additionally, PLFA, C_{mic} and qCO_2 data of the supplementary laboratory experiment revealed explicit antibiotic-related changes in soil after amendment with control pig slurry that was artificially spiked with SDZ (Chapter 11.1, Tab. S2). Total PLFA concentration and microbial biomass C were particularly lower in rhizosphere soil of SDZ treatments, but differences were only significant ($p < 0.05$) at the 10 mg kg^{-1} spiking level. At the low antibiotic spiking level of 1 mg kg^{-1} a larger ($p < 0.05$) qCO_2 indicated an additional stress response of microbial communities to antibiotics in rhizosphere soil.

The application of slurry from antibiotic-medicated pigs to the mesocosms resulted in temporal shifts to the $Gram^-$ bacterial populations, indicated by decreased PLFA-derived $Gram^+$:$Gram^-$ ratios. These shifts were significant ($p < 0.05$) in bulk soil of the SDZ- and DIF-slurry treatment at incubation time 14, 63 d, and in rhizosphere soil of the DIF-slurry treatment at day 14 (Tab. 1). In contrast, the supplementary laboratory experiment did not reveal any statistically significant responses of the $Gram^+$:$Gram^-$ ratio to the rhizosphere compartments or to the 1 and 10 mg kg^{-1} SDZ treatment (Chapter 11.1, Tab. S2).

Soils with slurry from medicated pigs occasionally showed shifts to fungi, as characterized by lowered PLFA-derived bacteria:fungi ratios. Significant changes ($p < 0.05$) occurred in bulk soil at incubation time 14 d (SDZ-slurry treatment), 42 d (DIF-slurry treatment) as well as in rhizosphere soil at day 14 (SDZ- and DIF-slurry treatment), and 42 d (DIF-slurry treatment). The supplementary laboratory experiment only indicated rhizosphere-specific shifts to the fungal population, but a statistically significant response to the 1 and 10 mg kg^{-1} SDZ spiking concentration was not determined (Chapter 11.1, Tab. S2).

The somewhat inconsistent time-courses of the PLFA responses to slurry of medicated pigs were evaluated using PRC statistics. For PLFA of bulk soil (Fig. 2a), 66% of the total variance could be explained by treatment (43%), time (33%) and the difference between the replicates (24%). Monte Carlo permutation tests confirmed with $p = 0.005$ the significance of the first PRC (Fig. 2a, b) and revealed that the PLFA composition significantly changed ($p < 0.05$) with time. Significant responses to the slurry treatment regime were revealed at individual incubation times 7, 14 d (DIF-slurry treatment), 63 d (SDZ-slurry treatment) in bulk soil, and at day 14 (DIF-slurry treatment) in rhizosphere soil. The response (C_{dt}) of PLFA composition to the application of DIF- and SDZ-contaminated slurry showed a contrasting development with temporal maxima at day 7, 14 and 63 of incubation. The C_{dt} values of all bulk soils were mostly affected by alterations of specific fatty acids, as indicated by their high b_k values. These were a-15:0 ($Gram^+$ bacteria) and 18:1n9t, while the influence of the common fungal markers 18:2n6 and 18:1n9c on C_{dt} values was less pronounced.

The rhizosphere soil receiving SDZ-contaminated slurry continuously showed smaller deviations relative to the control (Fig. 2b). The C_{dt} value in rhizosphere soil treated with DIF-contaminated slurry again showed a contrasting deviation compared to the SDZ-slurry treatment at incubation time 14 d, but thereafter changes were not significant. According to the PRC analysis of these, 90% of the total variance was explained by treatment (40%), time (31%) and differences between replicates (29%). The b_k values identified PLFA markers assigned to fungi (18:1n9c), 18:0, and $Gram^+$ bacteria (a-15:0, i-16:0) that had the strongest impact on the C_{dt} values of rhizosphere soils.

3.4 Impact on total bacterial 16S rRNA gene DGGE profiles

Total community (TC) 16S rRNA gene fragment banding patterns of bulk and rhizosphere soil were captured on DGGE (Chapter 11.1, Fig. S3a-f) and were analyzed by detrended CA (Fig. 3a, b). The results showed that the total community band profile of untreated soil (day -1, without slurry and SDZ) considerably changed after slurry application (0 d). The samples of control- and SDZ-slurry treatment clustered separately but mostly by time and compartment (bulk and rhizosphere soil) affiliation (Fig. 3a). TC-DGGE profiles of DIF-slurry-treated bulk and rhizosphere soils were analyzed on the selected incubation times 14 and

28 d (Fig. 3b). The ordination revealed clearly separated clusters of the DIF- and control-slurry treatment. Additionally, clusters of the DIF-slurry treatment were less strongly shifted by time and, also, the differences between the compartment affiliation (rhizosphere vs. bulk soil) were less pronounced. In any case, the DGGE results showed parallels to findings of PLFA (Fig. 2a, b).

3.5 *Pseudomonas* DGGE fingerprint and sequence analysis of selected bands

Antibiotic effects in bulk and rhizosphere soil were more pronounced on the specific *Pseudomonas* community level, which is illustrated for incubation time 14 d (Chapter 11.1, Fig. S4). Letters were assigned to bands exhibiting the most substantial qualitative or quantitative changes upon antibiotic treatment. Some of the most prominent bands were successfully sequenced (^{s4}e, ^{s4}f, ^{s4}g, ^{s4}h and ^{s4}j) and assigned to the *Pseudomonas* operational taxonomic unit (OTU) 3227. Accession numbers and several closely related *Pseudomonas* strains are listed in Tab. 2. Band (a) was specific for bulk and rhizosphere compartments of control- and SDZ-slurry treatments, and completely disappeared in DIF-slurry-amended soil. Bands labeled (b), (d) and (^{s4}g) were specific to all SDZ-slurry-supplied soils. Band (^{s4}g) showed close relation to *Pseudomonas* strains isolated from swine-effluent-amended soils (*Pseudomonas* sp. BBTR25) or pig slurry (*Pseudomonas* sp. str. 91S1), and to *Pseudomonas* sp. str. d130, which was described to be found in antibiotic-(oxytetracycline) contaminated wastewater. Recessional bands (c) and (^{s4}j) exclusively appeared within rhizosphere soil of the SDZ-slurry treatment. Band (^{s4}j) was associated with a strain (*Pseudomonas resinovorans* str. c87) mentioned for antibiotic wastewater. Bulk and rhizosphere soil band (^{s4}h) was found only in SDZ- and control-slurry treatments. The corresponding sequence was similar to strains already described for band (^{3}g). All bands mentioned so far were completely lacking in DIF-slurry-amended bulk and rhizosphere soils. The significant band (i) occurred only in soil treated with DIF slurry, but was not captured by sequencing. However, sequenced bands (^{s4}e) and (^{s4}f) emerged in all treatments, but were more dominant in rhizosphere than bulk soil. Sequence analysis of (^{s4}f) revealed a close relation to *Pseudomonas* strains harboring special degradation abilities for organic compounds (i.e. terpene, quinolone, polycyclic aromatic hydrocarbons). Band (^{s4}e) was most closely related to the growth-promoting strains *Pseudomonas* sp. str. G52 and str. G62. The strains of both latter bands seemed to be not susceptible to DIF- and SDZ-slurry treatment.

4 DISCUSSION

The organic composition (Fig. 1) and microbiological parameters of pig slurry were altered depending on antibiotic medication (Tab. 1, Chapter 11.1, Fig. S2). Slurry quality and its microbial community depend on the feeding management (Jost et al., 2011), and might also

change after medication with antibiotics. Patten et al. (1980) reported a changed composition of slurry from cattle supplied with chlortetracycline and oxytetracycline as reflected by its higher degradability (carbon dioxide evolution) in soil. A changed organic matter composition of the three slurries was documented by Py-FIMS mass spectra. The n-fatty acids contributed most to the overall TII and were mostly attributed to microbial origin (Aust et al., 2009). Correspondingly, TII and $PLFA_{tot}$ (Tab. 1) contents of the slurry samples followed the same order: SDZ > DIF > control slurry. Hence, intestinal microorganisms strongly responded to the antibiotic regime with a higher abundance in slurry, which can be related to the suggested increase of easily degradable organic compounds in feces of antibiotic-treated animals (Klopfenstein et al., 1964; Elmund et al., 1971, Patten et al., 1980).

Specific antibiotic-derived changes in slurry composition were indicated by an altered relative abundance of sterols in slurries of medicated pigs. Sterols are either markers for an increased fungal biomass (e.g. ergosterol, *m/z* 396) and also occur in animals and plants (Dinel et al., 1998). Since all pigs received the same fodder, changed relative sterol abundances in slurry indicate a modified intestinal metabolism. Changes in the digestive system seem likely, since shifts in the bacteria:fungi ratios of slurries from medicated pigs were not confirmed by PLFA markers.

The inhibition potential of slurry from medicated pigs on soil microorganisms might be potentiated or reduced by an altered abundance of specific compounds, in special by sterols. The slurry of the SDZ-medicated pigs had the largest and that of the DIF-medicated pigs the lowest relative abundance (% TII) of sterols, which may have led to the contrasting PLFA response in bulk and rhizosphere soil (Fig. 2a, b). Heumann et al. (2011) and Negassa et al. (2011) have shown that higher proportions of sterols in soil adversely affected biochemical activities such as the N-mineralization. Both hypothesized that these effects are due to an inhibition of microbial activity by phytosterols, as reported earlier by Bouhadjera et al. (2005) and Khan et al. (2001). However, it remains unknown if the different amount of sterols in slurry of antibiotic-medicated pigs was sufficient to initiate any microbial response in soil.

Furthermore, parent compounds and metabolites of both antibiotics evolved from the intestinal metabolism of the medicated pigs and showed a different fate in bulk and rhizosphere soil. The mild-solvent extractable SDZ fraction ranged below the operationally defined risk assessment trigger value of 100 µg kg^{-1} (EMEA 2008; Chapter 11.1, Tab. S1). Also it was smaller and in part significantly smaller in rhizosphere compared to bulk soil. Rosendahl et al. (2011) related this to an accelerated dissipation in rhizosphere soil due to plant-enhanced microbial biotransformation processes. Mild-solvent extractable fractions of DIF and it major metabolite SAR were neither detected in rhizosphere nor in bulk soil, which is a result of the strong sorption of fluoroquinolones to soil matrices (Rosendahl et al., 2012). The concentration level of SDZ and DIF and major metabolites in mesocosms were similar

and in parts even lower than that reported under field-relevant conditions (Rosendahl et al., 2011, 2012). The residual concentrations of the SDZ and DIF parent compound, which in contrast to the metabolites were proved to be antimicrobial active, declined only gradually over 63 days. Hence, although being not easily available, a long-term released reservoir of antibiotics was present in mesocosm soils.

Correspondingly, the effects of slurry from DIF- and SDZ-medicated pigs on the soil microbial community structure such as time-dependent significant shifts in PLFA and derived microbial ratios were not significantly related to the mild-solvent extractable antibiotic fraction (correlation coefficients $r < 0.20$). Previous studies likewise reported significant changes of microbial community structures and the appearance of an apparent concentration independency of effects after adding antibiotic-spiked slurry to bulk soil (Hammesfahr et al., 2008, 2011). This is explained by a continuous remobilization of antibiotic residues by microorganisms associated with the same sorptive soil surfaces. Thus matrix-attached microorganisms may be affected after absorption of the remobilized antibiotic compounds, while mild-solvent extractability of absorbed antibiotics is still low. The long-term release of small quantities of residual SDZ was also suggested by Zarfl et al. (2009), posing a long-term source of antibiotic concentrations and potential microbial responses in soil. With this in mind, the mild-solvent extractable fraction, operationally defined as bioaccessible, may be not as relevant to evaluate or predict microbial responses to antibiotics under practical conditions.

Within the 63-d mesocosm trial we demonstrated effects of slurries from differently antibiotic-medicated pigs in soil planted with *Zea mays* L. and in the respective rhizosphere soil. At single incubation times, PLFA-derived $Gram^+$:$Gram^-$ ratios of SDZ- and DIF-slurry treated soils were significantly shifted towards the $Gram^-$ bacteria group in bulk and also in rhizosphere soil of the DIF-slurry treatment (Tab. 1). Similar shifts were reported for sulfonamide antibiotics spiked to soil (Gutiérrez et al., 2010). In contrast, no shifts in $Gram^+$:$Gram^-$ ratios were determined in this and other studies when SDZ or DIF were added to soil with artificially spiked slurry (Hammesfahr et al., 2008; Kotzerke et al., 2011). It is suggested that mixed effects of the antibiotic and the specific slurry composition together promoted the shift towards $Gram^-$ bacteria, but also led to the inconsistent responses over time. This group of bacteria prefers easily decomposable substrates (Fierer et al., 2003), which can be especially introduced to soil by manure of antibiotic treated pigs (Klopfenstein et al., 1964; Elmund et al., 1971, Patten et al., 1980).

After application of slurry from SDZ- and DIF-medicated pigs to mesocosm soil, the PLFA-derived bacteria:fungi ratio was temporarily shifted towards the fungal population in bulk and rhizosphere soil (Tab. 1). Similar shifts to fungi were reported for unplanted soil after co-application of substrates spiked with SDZ (Thiele-Bruhn and Beck, 2005; Hammesfahr et al.,

2008) or DIF (Kotzerke et al., 2011), confirming that bacteria are usually more susceptible to pharmaceutical antibiotic action than fungi.

Microbial responses to slurry of SDZ- and DIF-medicated pigs generally were less pronounced in rhizosphere soil compared to bulk soil under the complex mesocosm conditions. Under field conditions, rhizospheres of plants are frequently inhabited by microorganisms, capable of producing own antimicrobial compounds (Bergsma-Vlami et al., 2005; Costa et al., 2006), which may render them less susceptible to the introduced synthetic antibiotics. Root exudates can promote the tolerance of the soil microbial community to antibiotics (Brandt et al., 2009), but also the acquisition of genetic resistance after application of contaminated manure (Heuer et al., 2011). Hence, the availability of easily degradable root deposits did not additionally promote the antibiotic effects in rhizosphere compartments as reported in bulk soil after co-application of readily available C-substrates such as milled maize straw, glucose or manure (Thiele-Bruhn and Beck, 2005; Zielezny et al., 2006, Hammesfahr et al., 2008). Even more, concentrations of SDZ and DIF declined stronger in rhizosphere compared to bulk soil, which was likely promoted by a stronger biodegradative activity in the rhizosphere. The strong effect on rhizosphere microorganisms that was determined at high soil spiking concentrations of 10 mg kg^{-1} is attributed to additional adverse effects on the plants. This was likewise reported for SDZ-spiked soil with nominal concentrations of ≥ 10 mg kg^{-1}, altering root exudation, growth, and morphology of maize plants (Michelini et al., 2012). However, no such effects on plants appeared at the antibiotic concentrations tested in the mesocosm experiment.

The mentioned response of $Gram^-$ bacteria to the antibiotic treatments were evaluated in more detail on the *Pseudomonas* population level (Chapter 11.1, Fig. S4) and supported by sequencing data of extracted DGGE bands (Tab. 2). Application of slurry from medicated pigs significantly altered *Pseudomonas* band patterns in bulk and especially in rhizosphere soil. The emergence of additional bands in SDZ-contaminated soil was likewise reported by Zielezny et al. (2006) for total bacteria. Some sequences (Tab. 2) occurred in bulk and rhizosphere soil and indicated *Pseudomonas* that have been previously isolated from slurry and likewise amended soils. The survival of such fecal bacteria in soil environments is not unusual, e.g., *Escherichia coli* O157:H7 survived under natural soil conditions even up to 14 weeks (van Elsas et al., 2011). It further indicates that medicated-induced changes of the intestinal microflora have the potential to remain in bulk and rhizosphere soil. This might facilitate the exchange of resistance genes with the autochthonous soil microflora, since considerable amounts of antibiotic resistance carrying bacteria are added to agricultural soils (Heuer et al., 2011).

Other members of the *Pseudomonas* group were clearly enriched in rhizosphere soil and closely related to *Pseudomonas* strains with degradative enzymatic or plant growth

promoting activities (Costa et al., 2006; Compant et al., 2010). Such root-inhabiting *Pseudomonas*, naturally unsusceptible or with acquired tolerance to antibiotics (Bergsma-Vlami et al., 2005; Brandt et al., 2009; Heuer et al., 2011), may preferentially proliferate in rhizosphere soil, replacing redundant members and thus may maintain crucial soil microbial functions in antibiotic contaminated soil.

5 CONCLUSION

Microbial responses in planted soil mesocosms to the application of field-relevant concentrations of SDZ and DIF with slurry from medicated pigs mostly matched the previously reported effects after application of antibiotic-spiked manure to soil. Our results indicate that medication with DIF and SDZ evidently altered the molecular-chemical pattern of the slurry, confounding the detection of consistent microbial responses. Despite the mixed effects of medication-derived changes in composition of excreta, the excreted microflora and the partly metabolization of the parent antibiotic, effects frequently referred to the action of the antibiotics. For example, shifts of DGGE and PLFA patterns were determinable in bulk soil and occasionally were weaker in rhizosphere soil. The strong sorption of DIF to soil matrix did not eliminate the effect of slurry from DIF-medicated pigs on the microbial community composition. It is concluded that the assessment of responses of the soil microflora to slurry from medicated pigs requires considering the mixed and interacting effects in the excreta and soil. While in this study, the antibiotic effect alone was determined in an independent experiment, in practice, mixed antibiotic effects may hinder a precise differentiation between responses to slurry composition or excreted antibiotic compounds.

Acknowledgements

We thank Elvira Sieberger and Petra Ziegler for practical support and all our colleagues involved. This project was funded by the German Research Foundation (DFG) within the Research Unit FOR 566 “Veterinary medicines in soil: basic research for risk assessment”.

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Tables and Figures of Chapter 3

Table 1. Concentrations of total PLFA ($PLFA_{tot}$; nmol g^{-1} dw) and respective PLFA ratios of Gram-positive-to-Gram-negative bacteria ($Gram^+$:$Gram^-$) and of bacteria-to-fungi (bacteria:fungi) in the soil with control slurry from pigs without medication (control-slurry treatment) and in soils receiving slurry from pigs medicated with sulfadiazine (SDZ-slurry treatment) or difloxacin (DIF-slurry treatment). Shown are mean values of four independent bulk and rhizosphere soil replicates (standard deviations in parentheses).

Treatments of the mesocosm experiment		Incubation time [days]							
		0	7	14	42	63	14	42	63
		Pig slurry	Bulk soil				Rhizosphere soil		
Control-slurry treatment	$PLFA_{tot}$ nmol g^{-1}	459.3 ^{a} (160.3)	16.8 ^{a} (1.0)	14.8 ^{a} (1.2)	17.8 ^{a} (1.4)	23.7 ^{a} (1.3)	23.2 ^{a} (1.6)	22.8 ^{a} (1.6)	24.0 ^{a} (3.7)	32.1 ^{a} (2.5)
	Bacteria:fungi	5.8 ^{a} (0.5)	4.3 ^{a} (0.2)	3.4 ^{a} (0.3)	4.8 ^{a} (0.3)	4.2 ^{a} (0.1)	3.7 ^{a} (0.3)	2.9 ^{a} (0.4)	3.5 ^{a} (0.5)	2.8 ^{a} (0.7)
	$Gram^+$:$Gram^-$	28.1 ^{a} (7.6)	4.5 ^{a} (0.3)	3.1 ^{a} (0.3)	4.6 ^{a} (0.5)	3.6 ^{a} (0.3)	5.5 ^{a} (0.4)	3.9 ^{a} (0.1)	3.5 ^{a} (1.0)	4.3 ^{a} (0.5)
SDZ-slurry treatment	$PLFA_{tot}$ nmol g^{-1}	1,110.0 ^{b} (248.7)	18.5 ^{a} (1.1)	15.7 ^{a} (0.8)	14.9 ^{a} (1.7)	21.2 ^{a} (2.8)	28.7 ^{b} (1.5)	19.5 ^{a} (1.7)	24.1 ^{a} (2.1)	29.0 ^{a} (5.2)
	Bacteria:fungi	5.9 ^{a} (2.4)	4.3 ^{a} (0.0)	3.6 ^{a} (0.3)	4.0 ^{b} (0.2)	3.9 ^{a} (0.4)	3.3 ^{a} (0.8)	1.7 ^{b} (0.2)	2.9 ^{ab} (0.4)	2.8 ^{a} (1.1)
	$Gram^+$:$Gram^-$	19.2 ^{a} (4.8)	4.5 ^{a} (0.4)	4.0 ^{a} (0.8)	3.8 ^{b} (0.3)	4.7 ^{a} (0.7)	3.1 ^{b} (0.8)	4.0 ^{a} (0.1)	4.3 ^{a} (1.1)	3.8 ^{a} (0.2)
DIF-slurry treatment	$PLFA_{tot}$ nmol g^{-1}	581.4 ^{a} (113.0)	18.8 ^{a} (2.5)	22.5 ^{b} (1.4)	23.2 ^{b} (3.4)	24.0 ^{a} (1.9)	22.6 ^{a} (0.8)	40.9 ^{b} (3.5)	28.2 ^{a} (2.0)	35.8 ^{a} (2.1)
	Bacteria:fungi	6.5 ^{a} (0.6)	4.6 ^{a} (1.2)	3.5 ^{a} (0.9)	3.8 ^{a} (1.2)	2.5 ^{b} (0.0)	3.2 ^{a} (1.5)	1.6 ^{b} (0.1)	2.5 ^{b} (0.2)	2.6 ^{a} (0.6)
	$Gram^+$:$Gram^-$	28.6 ^{a} (5.8)	4.6 ^{a} (1.2)	3.7 ^{a} (0.0)	3.5 ^{b} (0.1)	3.4 ^{a} (0.1)	3.5 ^{b} (0.1)	3.2 ^{b} (0.1)	4.1 ^{a} (1.0)	3.1 ^{a} (0.9)

Means of $PLFA_{tot}$, $Gram^+$:$Gram^-$ and bacteria:fungi, respectively, in columns followed by the same letter are not significantly different at $p < 0.05$

Table 2. Sequence analysis and tentative phylogenetic affiliation of indexed *Pseudomonas* DGGE bands (Chapter 11.1, Fig. S4) at incubation time 14 d.

DGGE band (accession no.[a])	Most closely related *Pseudomonas* otu_3227 sequence(s)	% Identity	Accession no[b]	Supplied database information[c]
s4e (JN217136)	*Pseudomonas* sp. str. G52	100.0	FN547408.1	Growth-promoting rhizobacteria
	Pseudomonas sp. str. G62 (unclassified)	100.0	FN547413.1	Growth-promoting rhizobacteria
s4f (JN217134)	*Pseudomonas pseudoalcaligenes* str. W-20	100.0	EU187489.1	Quinoline degrading
	Pseudomonas pseudoalcaligenes str. JM2	100.0	FJ472854.1	PAHs degrading
	Pseudomonas sp. str. PASS3-tpna	100.0	EU043333.1	Terpene degrading
s4g (JN217132)	*Pseudomonas* sp. BBTR25	97.7	DQ337603.1	Swine effluent amended soil
	Pseudomonas sp. str. 91S1	97.7	EU370417.1	Pig manure
	Pseudomonas sp. str. d130	97.3	FJ950669.1	Oxytetracycline production wastewater
s4h (JN217133)	*Pseudomonas* sp. BBTR25	97.5	DQ337603.1	Swine effluent-amended soil
	Pseudomonas sp. str. 91S1	97.5	EU370417.1	Pig manure
	Pseudomonas sp. str. 98S1	97.5	EU370416.1	Pig manure
s4j (JN217135)	*Pseudomonas resinovorans* str. c87	99.5	FJ950593.1	Oxytetracycline production wastewater

a GenBank® accession numbers of own sequences

b GenBank® accession number of related bacterial sequence (date of database: 2011-04-13)

c Supplied database information on Greengenes database (http://greengenes.lbl.gov)

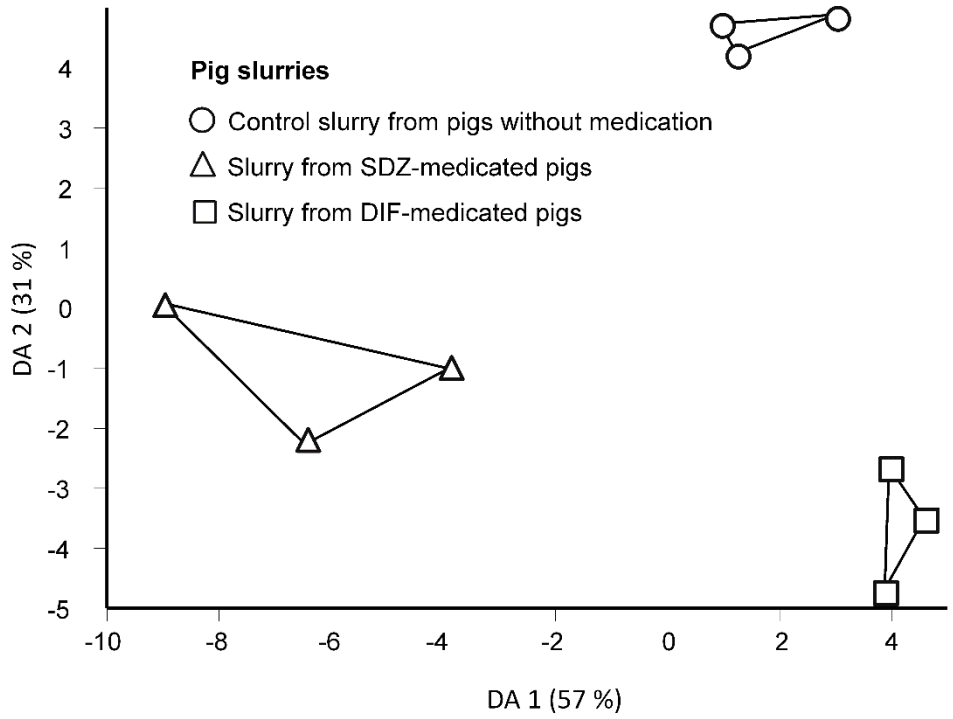

Figure 1. Discriminant analysis of the organic slurry composition determined by pyrolysis-field ionization mass spectrometry (Py-FIMS) of control slurry from pigs without medication (control slurry) and slurry after applying sulfadiazine (SDZ slurry) or difloxacin to pigs (DIF slurry). The DA displays 88% of the variance.

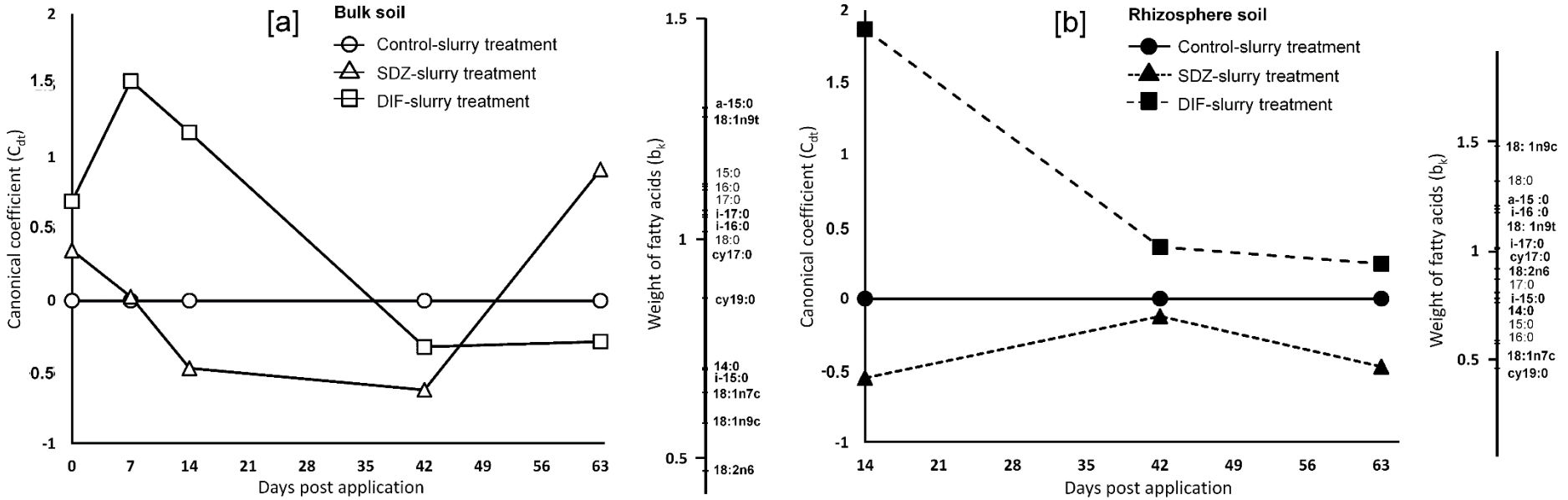

Figure 2. Principal response curves (PRC) of PLFA fingerprints from [a] bulk soil and [b] rhizosphere soil of maize-planted mesocosms treated with slurry from pigs medicated with sulfadiazine (SDZ-slurry treatment) and difloxacin (DIF-slurry treatment) referenced to the soil with slurry from pigs without medication (control-slurry treatment) at incubation times 7, 14, 42 and 63 d. The b_k-statistic shows the statistical weight of individual marker fatty acids for the displayed canonical coefficients (C_{dt}). The PRC displays 66% [a] and 90% [b] of the variance and was explained by treatment ([a] 43%, [b] 40%), time ([a] 33%, [b] 31%) and the differences between replicates ([a] 24%, [b] 29%).

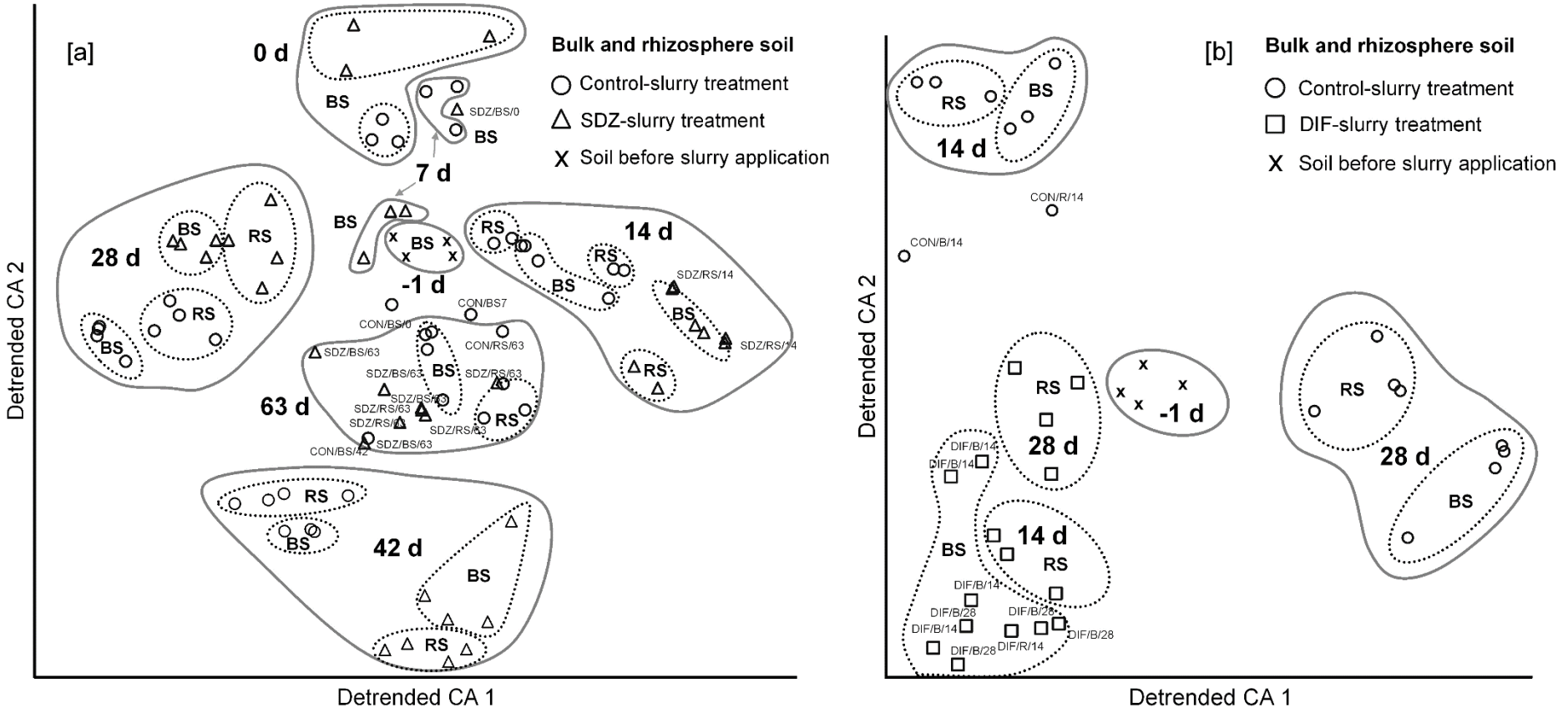

Figure 3. Detrended CA of total bacterial community 16S rRNA gene DGGE mesocosm profiles from rhizosphere soil (RS) and bulk soil (BS) with slurry from pigs without medication (control-slurry treatment) and treatment receiving slurry from pigs medicated with [a] sulfadiazine (SDZ-slurry treatment) at incubation times -1, 0, 7, 14, 28, 42 and 63 d or [b] difloxacin (DIF-slurry treatment) at incubation times -1, 14 and 28 d. Samples not contributing to individual clusters are marked with small captions.

4. Microbial Response to Manure with SDZ in diverse Soil Microhabitats

Publication II

Soil microbial community responses to sulfadiazine-contaminated manure in different soil microhabitats

Reichel, R., Patzelt, D., Barleben, C., Rosendahl, I., Ellerbrock, R.H., Thiele-Bruhn, S. (in press).

Applied Soil Ecology
DOI: 10.1016/j.apsoil.2014.03.010

ABSTRACT

Veterinary antibiotics such as sulfadiazine (SDZ) are applied with manure to agricultural soil. Antimicrobial effects of SDZ on soil microbial community structures and functions were reported for homogenized bulk soils. In contrast, field soil is structured. The resulting microhabitats are often hot spots that account for most of the microbial activity and contain strains of different antibiotic sensitivity or resilience. We therefore hypothesize that effects of SDZ are different in diverse soil microhabitats. We combined the results of laboratory and field experiments that evaluated the fate of SDZ and the response of the microbial community in rhizosphere, earthworm burrow, and soil macroaggregate microhabitats. Microbial communities were characterized by phenotypic phospholipid fatty acid (PLFA) and genotypic 16S rRNA gene patterns (DGGE) and other methods. Data was evaluated by principle component analyses followed by two-way ANOVA with post-hoc tests. Extractable SDZ concentrations in rhizosphere soil were not clearly different and varied by a factor 0.7-1.2 from those in bulk soil. In contrast to bulk soil, the extractable SDZ content was two-fold larger in earthworm burrows, which are characterized by a more hydrophobic organic matter along the burrow surface. Also, extractable SDZ was larger by up to factor 2.6 in the macroaggregate surface soil. The rhizosphere effect clearly increased the microbial biomass. Nonetheless, in the 10 mg SDZ kg^{-1} treatment, the biomass deceased by about 20% to the level of uncontaminated bulk soil. SDZ contamination lowered the total PLFA concentrations by 14% in the rhizosphere and 3% in bulk soil of the field experiment. Structural shifts represented by *Pseudomonas* DGGE data were about one third larger in SDZ-contaminated earthworm burrows compared to bulk soils. In the laboratory experiment, a functional shift was indicated by a four-fold reduced acid phosphatase activity in SDZ-contaminated burrows compared to bulk soil. Structural and functional shifts after SDZ contamination were larger by a factor of 2.5 in the soil macroaggregate surface versus interior, but this relation reversed over the long-term under field conditions. Overall, the combined effects of soil microhabitat, microbial community composition, and exposure to SDZ influenced the microbial susceptibility towards antibiotics under laboratory and field conditions.

1 INTRODUCTION

Bactericidal or bacteriostatic antibiotics are widely used in agricultural practice to protect or cure livestock from infectious diseases by disrupting the propagation of undesirable bacteria. Global agricultural antibiotic use is estimated to be 50,000 - 200,000 tons/year (Ok et al., 2011; Du and Liu, 2012; Kumar et al., 2012). Antibiotics of the sulfonamide class are among the most prescribed veterinary antibiotics in North America and Europe (Sarmah et al., 2006). Large antibiotic amounts are excreted unchanged or as metabolites and released into the soil environment by farmyard manure (Thiele-Bruhn, 2003; Sarmah et al., 2006).

Sulfadiazine (SDZ) is an often used sulfonamide that inhibits the enzymatic conversion of *p*-aminobenzoic acid during folic acid metabolism (Brown, 1962). SDZ, as a bacteriostatic broad-band antibiotic, inhibits the growth of both $Gram^+$ and $Gram^-$ bacteria (Brown, 1962). Antibiotic effects on soil microbial community activities have been clearly documented for bulk soil samples. They include soil respiration and Fe(III) reduction (Thiele-Bruhn and Beck, 2005), functional aspects (e.g., microbial-derived exoenzymes, nitrification; Gutiérrez et al., 2010; Kotzerke et al., 2008), and structure (16S rRNA gene, phospholipid fatty acid patterns; Hammesfahr et al., 2008; Reichel et al., 2013). Dose-response relationships of the microbial community to SDZ were documented in bulk soil (Thiele-Bruhn, 2003; Liu et al., 2009). Despite the rapid dissipation of the operationally defined bio-accessible SDZ fraction (Rosendahl et al., 2011), long-term effects of SDZ in soil have been determined (Hammesfahr et al., 2008; Reichel et al., 2013). This might be explained by a remobilization of small quantities of SDZ over the long-term from the more strongly bound and persistent SDZ residues (Zarfl et al., 2009; Rosendahl et al., 2011). Soil, however, is typically not a homogeneous bulk soil but separated into microhabitats that might be hot spots of microbial activity.

Beare et al. (1995) named five hot spots that account for most of the microbial activity in soil: porosphere, detritusphere, aggregatosphere, drilosphere of earthworms, and rhizosphere. The latter three microhabitats were selected in this study to evaluate antimicrobial effects.

The rhizosphere habitat sums up many different influences of soil type, pH changes, water and nutrient status, organic amendments, plant species and development stage (Hawkes et al., 2007; Buée et al., 2009). The release of low- and high-molecular rhizodeposits is particularly relevant for the growing microbial community (Bertin et al., 2003; Shi et al., 2013). Rhizosphere soil is inhabited by various saprophytic and mycorrhizal fungi along with beneficial bacteria or antagonistic pathogens (Buée et al., 2009). Dominant bacteria are Proteobacteria such as *Pseudomonas* (Hawkes et al., 2007) that often occur in higher abundance in the rhizosphere, comprising also some members that produce antimicrobials (Bergsma-Vlami et al., 2005; Costa et al., 2006). Accordingly, Mavrodi et al. (2012) determined an accumulation of the natural antibiotic phenazine-1-carboxylic acid in the rhizosphere of dryland cereals of up to 1.6 μg g^{-1} fresh roots. Importantly, contamination by synthetic antibiotics applied with manure often ranges at a higher level of μg kg^{-1} to mg kg^{-1} dry soil (Thiele-Bruhn, 2003). Only few reports are available on the effects of anthropogenic antibiotics on rhizosphere microbial communities. SDZ-contaminated manure changed the gene patterns of N-cycling microbes in rhizospheres of clover and maize (Ollivier et al., 2010), and affected the microbial community structure in rhizosphere soil (Reichel et al., 2013). Bio-accessible SDZ dissipated faster near roots than in bulk soil

(Rosendahl et al., 2011), but also altered root architecture and function in highly contaminated soil (Michelini et al., 2012).

Earthworm activity often stimulates the biological activity in soil due to beneficial influences on soil porosity, aggregation, nutrient and substrate availability, and bioturbation (Edwards, 2004; Blouin et al., 2013). The earthworm drilosphere is characterized by structures such as casts and burrows (Brown, 1995). Burrow walls can be lined with a mixture of soil, mucus and organic matter, temporarily stimulating the microbial biomass and activity up to ten or more times (Lavelle et al., 1995; Brown and Doube, 2004). Anecic earthworms such as *Lumbricus terrestris* (L.) create vertical burrows that facilitate the infiltration of water from the soil surface (Ernst et al., 2009). These macropores can also promote the transport of dispersed or dissolved manure compounds, microorganisms (Joergensen et al., 1998; Chadwick and Chen, 2002), as well as veterinary antibiotics into the soil profile (Kay et al., 2004). Xenobiotics bind onto the burrow walls and can accumulate (Edwards et al., 1992). Such transport and accumulation of antibiotics in earthworm burrows will have further consequences for dose-related adverse effects on microorganisms. Kotzerke et al. (2010) found that denitrificants (gene copies) were reduced tenfold by the exposure to SDZ in earthworm guts. Forty percent of the radioactivity of ^{14}C-ciprofloxacin was transported down the soil profile by earthworm activity (Mougin et al., 2013).

Soil aggregates are part of the soil structure and are operationally defined units of soil that are revealed after mechanical disruption along zones of weakness or pores (Young and Ritz, 2005). Different aggregate types are defined according to size as well as physical or chemical characteristics: small (2-20 µm) and large microaggregates (20-250 µm) are very stable and formed by e.g., bacterial polysaccharides, clays, and highly aromatic organic matter; small (250-2000 µm) and larger macroaggregates (>2 mm up to several centimeters) are less stable and built-up from preceding aggregates that are linked by bacteria, fungi, roots, and organic matter (Gobat et al., 2004). Microbial communities of different aggregate fractions (outer surface and interior) differ in their composition, functions, and activity (Mummey and Stahl, 2004; Mummy et al., 2006) due to small-scale gradients of pH, water, gases such as O_2, and organic matter as well as predation (Standing and Killham, 2007; Davinic et al., 2012; Mueller et al., 2012). Microbial biomass and activity are higher at pore system-connected aggregate surfaces with a better accessibility to growth substrates compared to the interior of soil macroaggregates (Jasinska et al., 2006). SDZ concentrations also showed small-scale gradients and gradually declined towards the interiors (Rosendahl et al., 2011). Time-dependent diffusion into the interior of aggregates was previously documented for pesticides by Van Beinum et al. (2005). We hypothesized that antibiotic fate and effects are different and more pronounced in soil microbial hot spots such as soil macroaggregate surfaces, earthworm burrows, and rhizosphere microhabitats of

structured soil. This was evaluated by investigating SDZ fate and effects in different soil microhabitat samples from laboratory and field experiments on the same Luvisol topsoil.

2 MATERIAL AND METHODS

2.1 Experimental

Soil material from the Ap horizon of a silt loam Luvisol was sampled from an arable field at Jülich-Merzenhausen, Germany (50°55'51.1"N, 6°17'47.8"E). The same soil was used for the laboratory and field experiments. The soil had the following properties: pH ($CaCl_2$) 6.3; clay 16%; silt 78%; sand 6%; C_{org} 1.2%; maximum water holding capacity (WHC_{max}, w/w) 45.8% (Förster et al., 2009). The pig manures were cumulatively collected from a group of pigs at the Agricultural Experimental Station for Livestock Science of the University of Bonn (Germany). The animals were fed with the same food mixture over the whole sampling period. For the field experiment, the same group of pigs was medicated intramuscularly with 30 mg SDZ kg^{-1} bodyweight on four consecutive days, following the recommended dosage for the SDZ injectable solution (200 mg ml^{-1}), supplied by Vetoquinol Biowet (Gorzow Wielkopolski, Poland). Pig manures of the field experiment were stored in dark at 15°C. Aliquots of the uncontaminated manure were stored at -20°C, acclimatized over one week, and sieved before conducting the laboratory experiments with and without spiking in artificial sulfadiazine sodium salt (99.0% minimum, CAS: 547-32-0, Sigma-Aldrich, Germany). The processed manure was characterized by pH 6.0; C:N 7.0, and 1.2% dry matter (dm). Generally, the laboratory experiments were conducted at a manure-to-soil ratio of 1:25 (w/dm). The supplementary information (Chapter 11.2) of this manuscript provides more detailed method descriptions and result data.

2.1.1 Experimental design and sampling of laboratory experiments

2.1.1.1 Rhizosphere soil. Effects of SDZ-spiked manure in rhizosphere soil were investigated using inert polypropylene Kick-Brauckmann pots (25.5 cm height, 28.5 cm external diameter), filled with field-moist soil corresponding to 6 kg dry soil. Nominal SDZ soil concentrations of 0 (control), 1 and 10 mg SDZ kg^{-1} dm were obtained. Maize plants (*Zea maize* L.) of cultivar PR39K13 Pioneer Hi-Bred (Buxtehude, Germany) were pre-grown in tap water until emergence of first roots and leaves. Afterwards, plants were transplanted into Kick-Brauckmann pots and cultivated for 63 d in a greenhouse, illuminated by two full spectrum halogen lamps (400 W) in a day:night cycle of 16:8 h and 21:15 °C ± 1°C. Water losses were replenished three times per week by spraying. Each treatment comprised four independent soil replicates. After removing the root ball, bulk soil was sampled from parts without root influence. Rhizosphere soil of each replicate was obtained after vigorously shaking the root ball. Samples were stored at -20°C until further processing.

2.1.1.2 Earthworm burrows. Soil 2D-terraria (cuvettes) with *L. terrestris* (L.) individuals and horse manure as nutrient supply were prepared according to Felten and Emmerling (2009). Each cuvette was split into two independent halves, filled with uncontaminated soil (ø 2 mm), wetted to 70% of the WHC_{max}, and repacked to 1.3 g cm^{-3} bulk density. The cuvettes were sealed by an air-permeable cover to keep the soil moist. Pre-incubation at 10°C was conducted for 30 days until sufficient vertical earthworm burrows were built (Chapter 11.2, Fig. S1). Earthworms were removed from soil before top-application of uncontaminated (control) or SDZ-spiked manure equivalent to a nominal concentration of 0.3 mg SDZ kg^{-1}. Fourteen days after pig manure application, earthworm burrows were sampled at >5 cm below soil surface. Each of four replicates of control and SDZ treatment was sampled using a sterile scalpel and spatula. Samples were taken from the lining and boundary soil layer (0-5 mm) and the distant bulk soil >15 mm. Samples were stored at -20°C until further processing. For the diffuse reflectance infrared Fourier transformed (DRIFT) spectral analysis of the organic matter composition at the earthworm burrow cross-sections (Fig. 3), undisturbed and air-dried samples of earthworm burrow surfaces were prepared according to Leue et al. (2011).

2.1.1.3 Soil macroaggregates. Soil macroaggregates (ø 2.5 cm) were obtained from the field site. Macroaggreagtes were either sprayed with uncontaminated pig manure (control) or with SDZ-spiked manure, equivalent to 4 mg SDZ kg^{-1} dm. The macroaggregates of each treatment were incubated in the dark for 14 days at 15°C and 85% relative humidity. Four independent soil replicates of control and SDZ treatments were obtained from the first millimeter of the exterior and after dissection from the interior part of ≥10 randomly selected macroaggregates. Samples were stored at -20°C prior to analysis.

2.1.2 Experimental design and sampling of field experiment

Eight experimental maize and two fallow plots (3 m x 6 m) were established in a randomized block design under representative agricultural conditions (cf. Rosendahl et al., 2011). Weeds were repeatedly removed from the plots by hand. Four plots were amended with uncontaminated pig manure; the other four received manure from SDZ-medicated pigs. Maize plots and unplanted fallow plots received two manure applications, equivalent to 30 m^3 ha^{-1} at day 0 and 10 m^3 ha^{-1} at day 49 (maize plots) or 133 d (fallow plots), respectively, following agricultural practice. After the first application, manure was incorporated into topsoil by tillage (cf. Rosendahl et al., 2011).

2.1.2.1 Field rhizosphere soil. Rhizosphere soil and bulk soil were sampled before and after the second manure application at day 48 and 132, respectively. For each rhizosphere

soil replicate, root balls of ≥3 maize plants were extracted from soil. Bulk soil was obtained from the plant interspaces using soil sampling cylinders (cf. Rosendahl et al., 2011). The samples were processed as described for the laboratory experiment (Section 2.1.1.1).

2.1.2.2 Field earthworm burrows. Earthworm burrows were obtained 252 days after the second manure application to the fallow plots. Burrows were excavated from topsoil (0-15 cm depth). Soil material was obtained from two discrete soil zones: surface plus boundary layer of the burrow (0-5 mm) and distant bulk soil (10-15 mm). Three to four independent replicates were achieved and stored at -20°C (cf., Section 2.1.1.2).

2.1.2.3 Field soil macroaggregates. Undisturbed soil cores were sampled from fallow plots 252 days after the last application of uncontaminated or SDZ-contaminated manure. The macroaggregates were obtained by gently dissecting the sampled soil cores. Four independent replicates per treatment were obtained from macroaggregate exterior and interior and were processed as described for the laboratory experiment (Section 2.1.1.3).

2.2 Extraction and analysis of sulfadiazine (SDZ)

2.2.1 Laboratory rhizosphere soil, earthworm burrows and macroaggregates

SDZ was extracted from moist burrow, soil macroaggregate, and rhizosphere soil samples corresponding to 5 g dm using accelerated solvent extraction (ASE 350, Dionex, Idstein, Germany) according to Michelini et al. (2012) with Milli-Q water (Millipore Corporation, Billerica, USA), 100 bar and two cycles at 100 °C (mild extraction of potentially bioaccessible SDZ; EAS fraction) or 200°C (harsh extraction of strongly bound residues; RES fraction). EAS and RES fractions were determined separately for rhizosphere experiments. The total SDZ (SDZ_{tot}) concentration of burrow and macroaggregate experiments comprises EAS + RES. The LC-MS/MS analysis was done according to Michelini et al. (2012) using a Shimadzu LC-20 HPLC (Shimadzu, Duisburg, Germany) coupled to an API 3200 LC-ESI-MS/MS (Applied Biosystems/MDS Sciex Instruments, Toronto, Canada) with a limit of detection (<LOD) of 5 µg kg^{-1} and limit of quantification of 10 µg kg^{-1} (<LOQ).

2.2.2 Rhizosphere soil of the field experiment. The rhizosphere soil samples of the field experiment were analyzed according to Rosendahl et al. (2011). SDZ was extracted from moist soil samples corresponding to 10 g dm with 25 ml 0.01 M $CaCl_2$ solution (mild extraction; EAS). The corresponding suspensions were processed in an end-over-end shaker for 24 h and centrifuged at 3000 x g for 15 min. After decanting the supernatant, the centrifugation pellet was re-suspended and extracted again with the 0.01 M $CaCl_2$ solution. The more strongly bound residual SDZ fraction was subsequently extracted from the soil

pellet by exhaustive microwave extraction using 50 ml (20:80, v/v) acetonitrile:water (harsh extraction; RES). The resulting extracts were processed and analyzed by HPLC-MS/MS with limits of quantification (LOQ) of 1.25 µg kg^{-1} for EAS and 2.5 µg kg^{-1} for RES (Rosendahl et al., 2011).

2.3 Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectral analysis

The DRIFT-mapping analysis was performed according to Ellerbrock et al. (2009). The DRIFT mapping analysis is defined here as a collection of DRIFT data from measurements along transects of intact earthworm burrows obtained by combining a DRIFT device with an XY-positioning table (Resultec Analytic Equipment, Illerkirchberg, Germany). The revealed ratios of hydrophobic (i.e., aliphatic, region A in FTIR spectra) to hydrophilic (i.e., carbonyls, etc., region B in FTIR spectra) functional groups may be used as a measure of the potential wettability of the soil organic matter along undisturbed soil transects (Ellerbrock et al., 2005). Extended details of the DRIFT-mapping procedure are available under supplementary information (Chapter 11.2, Section A).

2.4 Analyses of microbial-derived soil exoenzyme activities

Exoenzyme activities in moist soil samples corresponding to 0.5 g dm were analyzed as described by Michelini et al. (2012). For the measurement, 50 µl soil suspension, 50 µl of the dilution buffer (pH 6.1 for methylumbelliferone (MUB) and pH 7.8 for 7-amino-4-methylcoumarin (AMC) substrates), and 100 µl buffer with the MUB- or AMC-coupled substrate were used: MUB-α-D-glucopyranoside for α-glucosidase (EC 3.2.1.3), MUB-N-acetyl-β-D-glucosaminide for chitinase (EC 3.2.1.14), MUB-β-D-xylopyranoside for ß-xylosidase (EC 3.2.1.37), MUB-β-D-cellobioside for β-cellobiohydrolase (EC 3.2.1.91), L-leucin-AMC for leucine-aminopeptidase (EC 3.4.11.1), and MUB-phosphate for acid phosphatase (EC 3.1.3.2). The α-glucosidase, chitinase, ß-xylosidase, and β-cellobiohydrolase are associated with C-cycling, wherease the leucine-aminopeptidase and acid phosphatase activity are involved in N- and P-cycling, respectively. Exoenzyme activity was determined in multi-well plates after 60 min incubation and is expressed as nmol MUB g^{-1} h^{-1} or nmol AMC g^{-1} h^{-1}.

2.5 Determination of phospholipid fatty acid patterns

PLFAs were extracted from moist soil equivalent to 10 g dm using a mixture of 50 ml methanol, 25 ml chloroform, and 20 ml 0.05 M phosphate buffer (pH 7.4). The extracts were processed according to the protocol of Zelles and Bai (1993). The revealed fatty acid methyl esters (FAMEs) were analyzed using an Agilent 6890 series GC system (Agilent, Böblingen, Germany), equipped with a 30 m x 0.4 mm x 0.2 µm fused silica capillary column (Optima 5

MS, Macherey-Nagel, Düren, Germany) and a mass spectrometer (Hewlett Packard MSD 5973, Palo Alto, USA). The carrier gas was helium with a flow rate of 1.5 ml min^{-1}. Initial oven temperature was 80°C for 2 min, ramped to 290°C at 5°C min^{-1}, and finally held for 10 min. PLFA fatty acid markers were indicated as described by Reichel et al. (2013) with a focus on markers such as i-15:0, a-15:0, i-16:0, i-17:0 ($Gram^+$ bacteria), cy17:0, cy19:0, 18:1ω7c ($Gram^-$ bacteria), and 18:2ω6c and 18:1ω9c (fungi). In graphics we used "n" for "ω". The two latter were inter-correlated and thus confirmed as representatives of the fungal biomass. Microbial biomass was indicated by the total concentration of all measured microbial PLFA markers ($PLFA_{tot}$). PLFA-derived ratios of Gram-negative-to-Gram-positive ($Gram^+$:$Gram^-$) bacteria and bacteria-to-fungi (bac:fungi) were calculated using the summed markers of the corresponding microbial group.

2.6 Determination of DGGE band patterns

Effects on Betaproteobacteria and *Pseudomonas* as representatives of large parts of the total bacterial communities were analyzed after the total community DNA of 0.5 g soil was extracted using the Fast DNA® Spin Kit for soil and subsequent purification by the Geneclean® Spin Kit (MP Biomedicals, Heidelberg, Germany). The 16S rRNA gene fragments were amplified using a nested PCR approach. In a first step the specific primer pairs F311Ps/R1459Ps (Milling et al., 2005) or F948β/R1494 (Gomes et al., 2001) were used. In a second step the universal primer set F984GC and R1378 (Heuer et al., 1997), which contained the GC clamp was applied. The PCR products, differing in melting properties, were separated using a DCode System for denaturing gradient gel electrophoresis (DGGE; Bio-Rad Laboratories GmbH, München, Germany). PCR templates were loaded onto polyacrylamide gel (6-9%, w/v) in 1 x TAE buffer. Gels were prepared with denaturing gradients ranging from 26 to 58% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was run at 58°C for 6 h at 220 V. Silver stained gels were photographed on a UV-transillumination table (Biometra GmbH, Göttingen, Germany). The DGGE gels (Chapter 11.2, Figs. S4-S7) were analyzed using image analysis BIOGENE software (Vilber-Lourmat, Marne-la-Vallée, France). Shifts in band patterns among replicates were indicated by the loss or appearance of bands. Comparisons were based on relative molecular weight calculations, which were derived from a defined standard lane. If necessary, band patterns were linked together using the BIOGENE database and exported as binary data for further statistical analysis (cf., Reichel et al., 2013).

2.7 Microbial biomass C and additional analyses of bulk and rhizosphere soil

2.7.1 Laboratory experiment. Microbial biomass C (C_{mic}) was determined from 10 g moist soil using the chloroform fumigation extraction method (Vance et al., 1987). The analyses of

metabolic quotients (qCO_2), colony forming units (CFU), and bacterial activities (FISH) are available from the supporting information (Chapter 11.2, Section B).

2.8 Data analysis

Results were calculated on an oven-dry weight (dm) basis (105°C, 48 h) and are expressed as mean values ± standard deviation (S.D.) of four independent replicates. Three replicates were used for the SDZ_{tot} measurement in earthworm burrows and soil macroaggregates due to the limited sample amount. The bi-factorial effects of habitat and SDZ were analyzed by a two-way ANOVA with Tukey-B as post-hoc tests (SPSS Statistics 20.0, IBM, Ehningen, Germany). Prior to this, Levene's test was used to assess the equality of variances ($p > 0.05$). In case of equality the ANOVA and post-hoc test was applied at a significance level of $p < 0.05$. In case of a violated Levene's test the significance level was set to $p < 0.001$ to protect from type-one errors. Eta^2 indicates the size of the factors' effect (e.g., large at > 0.14) and gives the proportion of the variance explained by an ANOVA-factor. The sum of eta^2 of all factors can exceed one. Principal component analyses (PCA) were conducted on parameters with multiple input data such as exoenzyme activities, PLFA-derived FAMEs, and binary 16S rRNA gene DGGE fragment patterns to reduce the complexity to two dimensions. If necessary, data were standardized before applying the PCA. The sample scores of the principle components PC1 and PC2 were extracted from the output data and further analyzed by two-way ANOVA and post-hoc test; this enabled testing the effects of habitat and SDZ and the significance also for qualitative gene pattern data. This evaluation was conducted by CANOCO for Windows 4.5 software (Microcomputer Power, Ithaca, USA).

3 RESULTS

3.1 Laboratory experiment results

3.1.1 Rhizosphere versus bulk soil. The EAS SDZ concentrations were <LOD (0 mg SDZ), 10.9 µg kg^{-1} (1 mg SDZ), and 415.2 µg kg^{-1} (10 mg SDZ) in bulk soil; and <LOD (0 mg SDZ), 1.4 µg kg^{-1} (1 mg SDZ), and 426.1 µg kg^{-1} (10 mg SDZ) in rhizosphere soil (Chapter 11.2, Table S1). The EAS concentration of rhizosphere soil was significantly lower ($p < 0.05$) than that of bulk soil in the 1 mg treatment. In contrast, RES SDZ concentrations were not significantly different ($p < 0.001$; Chapter 11.2, Table S1a). The two-way ANOVA confirmed that the SDZ distribution was significantly ($p < 0.001$) related to the treatment (Fig. 1; Chapter 11.2, Table S1a). No SDZ contamination was detected in soils of the control (0 mg SDZ) treatment.

The microbial biomass (C_{mic}) of the 0 and 1 mg SDZ treatment was >33% larger in rhizosphere versus bulk soil, but significantly decreased ($p < 0.05$) in rhizosphere soil of the

10 mg kg^{-1} SDZ treatment (Chapter 11.2, Table S1a). The ANOVA confirmed that microbial biomass (C_{mic}) responded significantly ($p < 0.05$) to the habitat > treatment (Chapter 11.2, Table S1a). The total PLFA concentrations of the 0 and 1 mg SDZ treatment were 2-fold larger in rhizosphere than in bulk soil (Chapter 11.2, Table S1a). These significant differences ($p < 0.05$) between bulk and rhizosphere soil disappeared at the nominal SDZ level of 10 mg kg^{-1} (Chapter 11.2, Table S1a). Altogether, $PLFA_{tot}$ concentrations were significantly ($p < 0.05$) influenced by the factor habitat > habitat x treatment interaction > treatment (Chapter 11.2, Table S1a). A significant response ($p < 0.05$) to the habitat > treatment > habitat x treatment was determined for the PLFA-derived bac:fungi ratio and was represented by a 35% lowered ratio in rhizosphere soil of the 0 and 1 mg SDZ kg^{-1} treatment relative to the bulk soil; this was not, however, the case in samples of the 10 mg SDZ treatment (Chapter 11.2, Table S1a). No responses to the habitat and treatment were indicated by the $Gram^{+}$:$Gram^{-}$ bacteria ratios (Chapter 11.2, Table S1a). The PCA of PLFA pattern (83% VarE; data not shown) and the derived PC1 scores additionally indicated structurally different ($p < 0.05$) microbial communities in bulk and rhizosphere soil of the 0 and 1 mg SDZ treatment (Chapter 11.2, Table S1a); rhizosphere soil samples of the 10 mg SDZ treatment showed comparable PLFA patterns to bulk soil (Chapter 11.2, Table S1a). The ANOVA of PCA scores of PC1 (75% VarE) confirmed that the community structure was influenced by habitat > habitat x treatment > treatment (Chapter 11.2, Table S1a). Scores of PLFA pattern PC2 (8% VarE) indicated no additional differentiation among the groups. The standardized data of C_{mic} and the additional microbial analyses (qCO_2, CFU, FISH) were subjected to a PCA that accounted for 88% of the total VarE (Fig. 2.1a; Chapter 11.2, Table S1a). The rhizosphere groups of the 0 and 1 mg SDZ treatments were significantly differentiated ($p < 0.05$) from other treatments and verified the previous results (Chapter 11.2, Table S1a). The PC1 scores (62% VarE) of the PCA pattern derived from additional microbial analyses confirmed the significant response ($p < 0.001$) to habitat > treatment > habitat x treatment (Chapter 11.2, Table S1a). The scores of PC2 (26% VarE) significantly ($p < 0.05$) responded to the habitat x treatment > treatment (Chapter 11.2, Table S1a). The displayed correlated factors in PCA (Fig. 2.1a) additionally indicated that the plant and root biomass responded to the different SDZ treatment levels (Fig. 2.1a).

Overall, microbial responses to the habitat were dominant (Chapter 11.2, Table S1a). SDZ effects differed in the rhizosphere and bulk soil, and this difference tended to increase with SDZ concentration. At nominal concentrations of 10 mg SDZ kg^{-1}, however, the rhizosphere effect disappeared (Chapter 11.2, Table S1a).

3.1.2 Earthworm burrow versus bulk soil.

DRIFT spectra of the undisturbed earthworm burrows were taken along representative transects in cross direction (Fig. 3). The DRIFT-derived A:B ratio of soil organic matter was significantly ($p < 0.001$) larger for the linings in earthworm burrows (0.014 ±0.006 A:B) than for the adjacent bulk soil (0.008 ±0.001 A:B).

The SDZ_{tot} concentrations in burrow soil (80.5 µg kg^{-1}) were on average 2-fold larger than in bulk soil, but not significant (41.7 µg kg^{-1}; Fig. 1; Chapter 11.2, Table S1.1b). The ANOVA showed that the SDZ distribution was significantly related to the SDZ treatment ($p < 0.001$; Chapter 11.2, Table S1.1b).

The exoenzyme activity pattern of bulk and earthworm burrows was significantly ($p < 0.05$) differentiated on PC1 (61% VarE; Chapter 11.2, Fig. S2.1b), and the latter microhabitat was further significantly separated into control and SDZ treatment ($p < 0.05$). This particularly related to the significantly lowered ($p < 0.05$) acid phosphatase activity in SDZ-contaminated burrow soil compared to the control treatment (Chapter 11.2, Table S2a). The PC1 scores confirmed the significant influence of habitat > treatment > habitat x treatment ($p < 0.05$; Chapter 11.2, Table S1b). The PC2 scores (38% VarE) indicated no additional differentiation (Chapter 11.2, Table S1b).

The scores of *Pseudomonas* PC1 (60% VarE) significantly differentiated the community structure into control and SDZ treatment (Fig. 2.1b). The community structures of *Pseudomonas* were further differentiated by habitat on PC2 (18% VarE), while the significance ($p < 0.05$) of this differentiation was annulled in the presence of SDZ (Fig. 2.1; Chapter 11.2, Table S1b). The ANOVA confirmed that the scores of PC1 responded significantly ($p < 0.05$) to treatment > habitat x treatment > habitat, and PC2 scores to habitat (Chapter 11.2, Table S1b). The PCA of the Betaproteobacteria genetic profiles revealed a different clustering into control vs. SDZ treatment as well as into bulk vs. burrow habitat (Chapter 11.2, Fig. S2.1c). Post-hoc tests of the scores of PC1 (53% VarE) and PC2 (22% VarE) confirmed the significance ($p < 0.05$) of this differentiation (Chapter 11.2, Table S1b). The ANOVA confirmed that PC1 responded significantly to treatment > habitat ($p < 0.001$) and PC2 to habitat > habitat x treatment > treatment ($p < 0.05$; Chapter 11.2, Table S1b).

Overall, the habitat and SDZ treatment dominated the microbial responses and differentiated the microbial communities (Chapter 11.2, Table S1.1b). The genetic community structure in soil of control and SDZ treatments was further differentiated into burrow and bulk soil (e.g., Fig. 2.1b). Microbial functions were altered particularly in SDZ-contaminated burrow soil (Chapter 11.2, Table S2a).

3.1.3 Macroaggregate interior versus surface.

The SDZ_{tot} concentration of the macroaggregate interior was 500.2 µg kg^{-1} and thus significantly ($p < 0.05$) lower compared to 1220.3 µg SDZ kg^{-1} in samples from the surface fraction (Fig. 1; Chapter 11.2, Table S1c).

The $PLFA_{tot}$ concentration of the control and SDZ treatment showed a 67% respectively 77% larger ($p < 0.001$) microbial biomass in samples of macroaggregate surface compared to interior (Chapter 11.2, Table S1c). The PCA of PLFA-derived microbial markers confirmed that the microbial community structures also clustered into surface and interior soil on PC1 (62% VarE; Chapter 11.2, Fig. S2.1d), but this was significant only for the SDZ treatment ($p < 0.001$; Chapter 11.2, Table S1c). The ANOVA confirmed that $PLFA_{tot}$ as well as PLFA patterns (PC1 scores) responded significantly to the habitat factor ($p < 0.001$; Chapter 11.2, Table S1c). The bacteria:fungi ratio as well as the $Gram^+$:$Gram^-$ bacteria ratio showed no significant response (Chapter 11.2, Table S1c). The PCA of microbial-derived exoenzyme activities indicated that the structural response of the PLFA pattern was also reflected by the functional resonses (Chapter 11.2, Fig. S2.1d, e). Thus the PC1 (61% VarE) scores of the exoenzyme pattern significantly separated ($p < 0.05$) the macroaggregate interior and surface samples of the control and SDZ treatment (Chapter 11.2, Table S1c). This was assigned to significantly larger ($p < 0.05$) β-xylosidase, α-glucosidase, chitinase, leucin-aminopeptidase, and to overall 33% (control treatment) and 27% (SDZ treatment) larger total exoenzyme activity in the surface relative to interior soil samples (Chapter 11.2, Table S2c). The respective ANOVA confirmed that the PC1 scores were also significantly ($p < 0.05$) influenced by habitat (Chapter 11.2, Table S1c). The PC2 scores (4% VarE) only weakly responded to the interacting factors habitat x treatment ($p < 0.05$; Chapter 11.2, Table S1c). The Betaproteobacteria PCA revealed clustering into different soil macroaggregate habitats (interior vs. surface) and treatments (control vs. SDZ; Fig. 2.1c); this was confirmed as being significant by the scores of PC1 (29% VarE) and PC2 (22% VarE; Chapter 11.2, Table S1c). The ANOVA confirmed a significant response ($p < 0.05$) of the community structure (PC1 scores) to habitat > treatment = habitat x treatment; and PC2 to habitat x treatment > treatment (Chapter 11.2, Table S1c). The PCA of the *Pseudomonas* community indicated similar responses (data not shown) and significantly separated ($p < 0.001$) the community of surface samples on PC1 (40% VarE) into control and SDZ treatment (Chapter 11.2, Table S1c); a differentiation into different microhabitats was indicated only for the control treatment on PC2 (29% VarE). The ANOVA verified that PC1 scores responded significantly ($p < 0.001$) to treatment; and PC2 scores significantly ($p < 0.05$) to habitat > habitat x treatment (Chapter 11.2, Table S1c).

Overall, functional and structural microbial responses to SDZ were influenced by habitat (Chapter 11.2, Table S1c). The SDZ effects on community structure were different and more obvious at the soil macroaggregate surface than interior (e.g., Fig. 2.1c).

3.2 Field experiment results

3.2.1 Rhizosphere versus bulk soil. The pig manure applied to soil contained 30.3 ±1.7 (0 d) and 131.1 ±12.0 mg SDZ kg^{-1} (48 d). The EAS SDZ fraction almost completely dissipated and was indicated only in bulk soil by 4.7 µg kg^{-1} at day 48 (Chapter 11.2, Table S3a). In bulk and rhizosphere soil, 154 and 104 µg kg^{-1} (48 d) and 93 and 111 µg kg^{-1} (132 d) of the RES SDZ fraction was discovered (Chapter 11.2, Table S3a), but the differences were not significant (Fig. 1; Chapter 11.2, Table S3a). No SDZ contamination (<LOD) was found in soils of the control treatment.

The 1.7-fold larger ($p < 0.001$) $PLFA_{tot}$ concentrations in rhizosphere soil confirmed the influence of habitat in both treatments, but the values were significantly lowered ($p < 0.001$) by 14% in combination with SDZ in rhizosphere soil of day 132 (Chapter 11.2, Table S3a). The ANOVA confirmed that the $PLFA_{tot}$ at day 48 d ($p < 0.05$) and 132 d ($p < 0.001$) mainly responded to habitat (Chapter 11.2, Table S3a). No such responses were indicated for the bac:fungi ratio (48, 132 d) or $Gram^+$:$Gram^-$ ratio at 48 d (Chapter 11.2, Table S3a). Only in SDZ-treated rhizosphere soil did the $Gram^+$:$Gram^-$ ratio respond to habitat x treatment > habitat (132 d); thus the ratio was lower ($p < 0.05$) compared to the control rhizosphere treatment (Chapter 11.2, Table S3a). The further analysis of the PLFA pattern (Chapter 11.2, Fig. S2.2a), particularly on PC1 (78% VarE), revealed – separately for bulk soil (48, 132 d) and rhizosphere soil (132 d) – significant clustering ($p < 0.05$) into control and SDZ treatment (Chapter 11.2, Table S3a). The ANOVA of PC1 scores confirmed that PLFA patterns responded significantly ($p < 0.05$) to habitat > (treatment; 132 d) > habitat x treatment; PC2 scores responded only to habitat x treatment (Chapter 11.2, Table S3a).

Genotypic shifts within the *Pseudomonas* DGGE data were displayed by PCA at day 48 and 132 (Fig. 2.2a). The scores of PC1 (32% VarE) and PC2 (19% VarE) indicated a significant differentiation ($p < 0.05$) of the bulk and rhizosphere community (Chapter 11.2, Table S3a). The community structure further differentiated into control and SDZ treatment at incubation time 48 d (Fig. 2.2a). The ANOVA on PC1 scores confirmed that the *Pseudomonas* community structure significantly responded to habitat > treatment ($p < 0.05$) at incubation time 48 d; while the PC2 scores indicated responses to habitat > treatment (> habitat x treatment; 48 d). The PCA of the Betaproteobacteria DGGE data showed that the community clustered separately according to the bulk and rhizosphere habitat at both incubation times (Chapter 11.2, Fig. S2.2b). This was confirmed by significantly different PC1 (70% VarE) score values ($p < 0.001$; Chapter 11.2, Table S3a). The scores of

PC2 (14% VarE) indicated SDZ effects on the community structure of bulk soil at day 48 ($p < 0.05$) and rhizosphere soil at day 132 (Chapter 11.2, Table S3a). The respective ANOVA confirmed the significant response of the Betaproteobacteria to habitat (PC1 scores) and treatment > habitat x treatment (PC2 scores).

Overall, the habitat dominated the microbial responses (Chapter 11.2, Table S3a). Time-dependent SDZ effects on microbial community structure occurred particularly in rhizosphere soil and were less pronounced in bulk soil (Chapter 11.2, Table S3a).

3.2.2 Earthworm burrow versus bulk soil. The pig manure applied to soil contained 30.3 ±1.7 mg kg^{-1} (0 d) and 82.1 ±12.7 mg kg^{-1} fresh weight (133 d). The SDZ_{tot} concentration was approximately 2-fold larger in the earthworm burrow (99.8 µg kg^{-1}) than in bulk soil (51.3 µg kg^{-1}; Fig. 1). The ANOVA confirmed the significant influence ($p < 0.001$) of the interacting factors treatment and habitat on the SDZ_{tot} distribution (Chapter 11.2, Table S3b).

The PCA of the exoenzyme activity pattern and the corresponding score values of PC1 (56% VarE) indicated significant responses to habitat, which roughly separated the exoenzyme activity patterns of the control treatment into bulk and burrow soil (Chapter 11.2, Fig. S2.2c).

The PCA of the *Pseudomonas* DGGE data and the scores of PC1 (45% VarE) and PC2 (15% VarE) confirmed that the *Pseudomonas* community of the control and SDZ treatment clustered separately only in the earthworm burrow microhabitat ($p < 0.05$; Fig. 2.2b; Chapter 11.2, Table S3b). The ANOVA confirmed the significant influence ($p < 0.05$) of habitat > habitat x treatment > treatment on the *Pseudomonas* community (Chapter 11.2, Table S3b). The PCA-derived scores of Betaproteobacteria PC1 (68% VarE) and PC 2 (15% VarE) were significantly influenced ($p < 0.001$) by (habitat; PC1) > habitat x treatment (Table 2b). This supports the strong separation of the Betaproteobacteria community into control and SDZ treatment and into earthworm burrow and bulk soil (Chapter 11.2, Fig. S2.2f; Table S3b).

Overall, and beside the habitat influence, characteristic effects of SDZ on the microbial community structure were stronger in burrow versus bulk soil, while functional shifts were not significant (Chapter 11.2, Table S2b, S3b).

3.2.3 Macroaggregate interior versus surface. The same manure and SDZ loads were applied as described in Section 3.2.2. The SDZ_{tot} concentration extracted from the macroaggregate surface fraction (94.4 µg kg^{-1}) was on average 1.6-fold larger compared to samples from the interior (60.9 µg kg^{-1}), but this was not significant (Fig. 1; Chapter 11.2, Table S3c).

In the macroaggregate interior, the control and SDZ treatment significantly ($p < 0.05$) changed the PCA exoenzyme activity pattern (PC1, 89% VarE; Chapter 11.2, Fig. S2.2e;

Table S3c). This was related to the significantly larger ($p < 0.05$) exoenzyme activity of β-xylosidase, cellobiohydrolase, leucin-aminopeptidase, and acid phosphatase in the macroaggregates' interior of the control relative to the corresponding SDZ treatment (Chapter 11.2, Table S2d). The ANOVA confirmed the significant influence ($p < 0.05$) of habitat > habitat x treatment on the exoenzyme activity pattern (Chapter 11.2, Table S3c).

The PCA of *Pseudomonas* DGGE data indicated a clustering of the community structure into control and SDZ treatment (Fig. 2.2c; Chapter 11.2, Table S3c), which was proved to be significant by PC1 scores ($p < 0.05$). On PC2 a significant separation into interior and surface microhabitat was evident only for the control treatment (Chapter 11.2, Table S3c). The ANOVA of PC1 (34% VarE) and PC2 (27% VarE) scores were significantly influenced by treatment ($p < 0.05$; PC1) > habitat ($p < 0.001$) > habitat x treatment (PC1). The PCA of Betaproteobacteria DGGE data with PC1 (51% VarE) and PC2 (13% VarE) responded only to the control and SDZ treatment (ANOVA of PC1 scores, $p < 0.05$) with corresponding differentiations in the community profile (Chapter 11.2, Fig. S2.2f, Table S3c).

Overall, the microbial communities differentiated into surface and interior microhabitats, particularly in control treatments, although these differentiations diminished in SDZ-contaminated soil. In the interior microhabitat of the latter, microbial functions were also altered (Chapter 11.2, Table S4c).

3.3 Comparison of laboratory and field experiment data

Further analyses showed that 56% of the data from the laboratory and field experiments significantly ($p < 0.05$) responded to the factor habitat, 35% to treatment, and even 41% to an interaction of habitat and treatment (Chapter 11.2, Tables S1 and S3). On average the habitat explained 80 ±20% (n = 37), treatment 67 ±21% (n = 23), and the interaction of habitat x treatment 56 ±22% (n = 27) of the variance deduced from the calculated eta^2 values of significant ANOVA factors (Chapter 11.2, Tables S2 and S4). The median values indicated that the microbial responses to microhabitat and treatment were similar in laboratory and field experiments, while the interaction between habitat x treatment was more pronounced under controlled laboratory conditions (Chapter 11.2, Fig. S3).

4 DISCUSSION

The microhabitat influence on microbial community composition was strong (Section 3.3), providing the basis for evaluating microbial susceptibility to SDZ in the rhizosphere, earthworm burrows, macroaggregate fractions and bulk soil. The SDZ concentrations applied (Table 1) and extracted from soil (Chapter 11.2, Table S1, S3) ranged from μg kg^{-1} to mg kg^{-1}, covering eco-toxicologically relevant contamination levels found in field soils (Grote et al. 2004; Schmitt et al. 2005; Rosendahl et al., 2011). We found comparable SDZ

fates and effects on soil microorganisms in different microhabitats of laboratory and field experiments (Section 3.3). This confirmed that SDZ residues, and hence related effects, remain in soil on a longer-term, which agrees with other mesocosm and field experiments (Rosendahl et al., 2011; Reichel et al., 2013).

4.1 Rhizosphere versus bulk soil

In contrast to RES SDZ (Fig. 1), the EAS SDZ concentration was lowered in rhizosphere compared to bulk soil of laboratory (1 mg kg^{-1} treatment) and field experiments (Sections 3.1.1 and 3.2.1). Accordingly, the dissipation of EAS SDZ, and temporarily also of RES SDZ, fractions was increased in the rhizosphere compared to bulk soil under field conditions (Rosendahl et al., 2011). Pot experiments with maize and willow plants further revealed that SDZ accumulates in roots and affects root geotropism, number of lateral roots, and water uptake by plants (Michelini et al., 2012). Hence, sequestration, transformation, and translocation processes are assumed to promote SDZ dissipation, particularly in rhizosphere soil (Rosendahl et al., 2011).

SDZ treatment effects on microbial biomass (C_{mic}, $PLFA_{tot}$), community structure (e.g., phenotypic PLFA and genotypic 16S rRNA gene patterns), as well as on bacterial activity (qCO_2, FISH) were determined in rhizosphere soil (Sections 3.1.1 and 3.2.1). Similar SDZ effects have been reported from earlier laboratory experiments with homogenized bulk soil (Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008, 2011a, b) and structured soil with growing rhizospheres under less controlled mesocosm conditions (Reichel et al., 2013). The root-derived activation of the microbial community clearly promoted significant SDZ effects in rhizosphere versus bulk soil, which is also documented by treatment x habitat interactions (Sections 3.1.1 and 3.2.1). Easily available substrates stimulating microbial activity have been reported to accelerate SDZ effects on diverse microbial parameters in bulk soil (Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008). Hence, after manure was consumed, the stimulating effects of rhizosphere deposits on microbial activities might have promoted the SDZ effects in rhizosphere versus bulk soil under laboratory and particularly under field conditions (Sections 3.1.1 and 3.2.1). Such combined influences of activating root exudates and SDZ on microbial communities might have also promoted the establishment of SDZ-resistant strains, as reported after application of realistic rates of artificial root exudates to soil (Brandt et al., 2009). The soil experiment with maize and representative manure and SDZ loads confirmed that the rhizosphere microhabitat feeds back on the SDZ effects on the soil microbial community structure (Fig. 2.2a). Particular SDZ effects related to rhizosphere microhabitat have been reported. These include N-cycling functions (Ollivier et al., 2010), structural community shifts, and the establishment of strains that are adapted to rhizosphere microhabitat and antimicrobial actions (Bergsma-Vlami et al., 2005; Costa et al., 2006;

Reichel et al., 2013). The second scenario refers to the strong responses discovered in rhizosphere soil of the 10 mg SDZ treatment (Fig. 2.1a; Section 3.1.1). We assume that indirect adverse interactions of SDZ on plant roots (Michelini et al., 2012), and thus on microhabitat properties, combined with microbial SDZ effects reduced or altered the typically reported large microbial biomass, activity, and specific microbial community structures (Smalla et al., 2001; Hinsinger et al., 2005) in highly contaminated rhizosphere; ultimately, these parameters equaled that of the bulk soil (Section 3.1.1). Overall, we discovered that rhizosphere microhabitat selects for specific microbial communities that respond differently to SDZ exposure than those of bulk soil.

4.2 Earthworm burrows versus bulk soil

The earthworm burrows in our laboratory and field experiment (Section 2.1.1.2) – and in nature – are connected to the soil surface and thus facilitate the infiltration of water (Ernst et al., 2009), manure and the microorganisms it contains (Joergensen et al., 1998), as well as antibiotics (Kay et al., 2004) from the surface into the soil along bio-macropores. In the field soil, earthworms were not extracted before applying SDZ-contaminated manure, in contrast to the laboratory experiment (Sections 2.1.1.2 and 2.1.2.2). Hence, active transport of antibiotics down the soil profile by earthworms might have occurred in the field, as was reported for radioactively marked ciprofloxacin after application to soil (Mougin et al., 2013). This, in addition to passive infiltration, might have fostered the SDZ accumulation on burrow linings in the field experiment (Fig. 1; Section 3.2.2). In this context and in accordance with Leue et al. (2010), 2D-DRIFT-derived A:B ratios along transects of undisturbed soil surfaces spanning earthworm burrows (laboratory experiment) were indicative for a more hydrophobic organic matter along burrow surfaces compared to bulk soil (Fig. 3; Section 3.1.2). The hydrophobicity of soil organic matter strongly relates to the sorption of antibiotics and is the main sorbent of SDZ in soil (Thiele-Bruhn, 2003). Thus, also moderately polar atrazine accumulates in earthworm burrows, as indicated by reduced concentrations in remaining leachates (Edwards et al., 1992).

In the laboratory and field experiments, the SDZ-uncontaminated earthworm burrows contained microbial communities that differed structurally from those of bulk soil (Figs. 2.1b and 2.2b; Sections 3.1.2 and 3.2.2). Earthworm activity enriches the burrow walls with a mixture of mucus and organic matter, stimulating microbial growth, activity and the establishment of specific microbial community structures (Lavelle et al., 1995; Beare et al., 1995; Tiunov and Scheu, 1999). In contrast to the field experiment, dissimilarities within the exoenzyme activity pattern, particularly of the phosphatase, were indicated only in rather fresh burrows of the laboratory experiment (Chapter 11.2, Table S2a, b). Exoenzyme activity such as phosphatase was reported to increase in young casts

and linings, before it diminishes over several weeks in unused burrows (Le Bayon and Binet, 2006). This helps explain its absence in the field experiment. Hence, our laboratory and field experiments indicate that the earthworm burrow microhabitat feeds back over the long-term on the establishment of characteristic microbial community structures and on SDZ fate and effect (Figs. 2.1b and 2.2b; Sections 3.1.2 and 3.2.2). Note that SDZ effects were only reported in the gut microflora of earthworms, indicated by an evident reduction of functionally relevant denitrifiers (Kotzerke et al., 2010). In the laboratory experiment, after incubation with SDZ-spiked manure, we detected shifted functions in the form of reduced phosphatase activity, particularly in burrow microhabitats (Chapter 11.2, Table S2a). Phosphatase activity is normally stimulated in fresh earthworm burrows in uncontaminated soil (Le Bayon and Binet, 2006). Overall, the interaction of microhabitat and treatment also in the burrow microhabitat (Sections 3.1.2 and 3.2.2) induced characteristic microbial communities with specific responses to SDZ contamination.

4.3 Macroaggregate interior versus surface

Field soil is never 'bulk soil', but rather is structured by soil aggregates. Such aggregates were used in our laboratory and field experiments (Sections 2.1.2.3 and 2.1.1.3). Macroaggregates are pervaded by a network of pores (Young and Ritz, 2005). Hence, SDZ transport from macroaggregate surface to interior sorption sites is probably supported by the pore system, leading to substantial lower SDZ concentrations in interior versus surface fractions (Fig. 1; Sections 3.1.3 and 3.2.3). Comparable gradients involving lower SDZ concentrations towards aggregate interiors have been reported after sampling of SDZ-contaminated, undisturbed soil cores from field soil (Rosendahl et al., 2011). Furthermore, SDZ may become incorporated into the aggregate interior under representative field conditions, as in our field experiment, by soil rearrangements such as remoistening or thawing (Rosendahl et al., 2011). Time-dependent diffusion into the soil aggregate interior, as reported for pesticides in clay-loam soil, may also play a role (Van Beinum et al., 2005).

Our results (e.g., Figs. 2.1c and 2.2c) support other studies showing that surface and interior microhabitats of soil aggregates are inhabited by dissimilar microbial communities (Chenu et al., 2001; Mummey and Stahl,, 2004, Mummey et al., 2006). This was reflected in our data by different structural and, as indicated in the field experiment, functional responses to SDZ at the surface versus interior microhabitat of soil macroaggregates (Chapter 11.2, Tables S1c and S3c). Since soil preparation such as sieving alters microbial structures (Thomson et al., 2010), environmentally representative community responses to SDZ, as in macroaggregate surface and interior soil samples (Chapter 11.2, Tables S2c and S3c), cannot be deduced from experiments with homogenized soil substrate (e.g., Hammesfahr et al., 2008, 2011a, b).

5 CONCLUSIONS

Based on the polar antibiotic SDZ, we show that the distribution of pollutants varies between soil microhabitats in structured field soil. SDZ accumulates at the surfaces of earthworm burrows and soil macroaggregates, while the distribution in the rhizosphere is highly variable compared to bulk soil. All these microhabitats host most of the microbial biomass and activity in natural soils. Hence, the behavior of a pollutant in structured field soil as well as the true exposure of the community to the contaminant can hardly be reflected by experiments with homogenized soil samples. Rather, pollutants and microorganisms accumulate in the same hot spots. The microhabitat properties dominated the microbial community composition and, reflected by the community's different responsiveness towards SDZ, also the occurrence of more or less antibiotic-sensitive and -resilient strains. Experiments with homogenized bulk soil, in the worst case, may therefore underestimate the true microbial susceptibility of the microflora towards antibiotics in structured field soil. Overall, we confirmed the existence of combined effects of soil microhabitat, microbial community composition, and SDZ exposure on the responsiveness to the pollutant under laboratory and field conditions.

Acknowledgements

We thank Dr. Joost Groeneweg from the Agrosphere Institute (Forschungszentrum Jülich), Dr. Marc-Oliver Aust (Thünen Institute of Fisheries Ecology), and Dr. Andreas Focks (Aquatic Ecology and Water Quality Management, Wageningen UR) for scientific support and Elvira Sieberger and Petra Ziegler for technical assistance. This project was funded by the German Research Foundation (DFG) within the Research Unit FOR 566 "Veterinary medicines in soil: basic research for risk assessment".

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Tables and Figures of Chapter 4

Table 1. Experimental setup (replicates, soil volume, calculated nominal SDZ concentration, type of SDZ application, number of manure applications, and day of sampling after the last (or first) application of SDZ uncontaminated and SDZ contaminated manure to soil of rhizosphere, earthworm burrow, and soil macroaggregates laboratory and field experiments (scale).

Experimental setup	**Rhizosphere soil**		**Earthworm burrows**		**Soil aggregates**	
Scale	Laboratory	Field	Laboratory	Field	Laboratory	Field
Replicates [n]	4	4	3	4	4	4
Soil volume [dm^3 per replicate]	4	3000	2	750	2	750
Nominal SDZ [mg kg^{-1}]	1 and 10	0.4 / 0.6	0.3	0.4 / 0.4	4	0.4 / 0.4
Origin of manure contamination (manure-to-soil ratio of 1:25)	Spiking	Medicated pigs	Spiking	Medicated pigs	Spiking	Medicated pigs
No. of manure applications	1	2	1	2	1	2
Sampling after the last (first) SDZ-manure application [days]	63	83 (132)	14	252 (385)	14	252 (385)

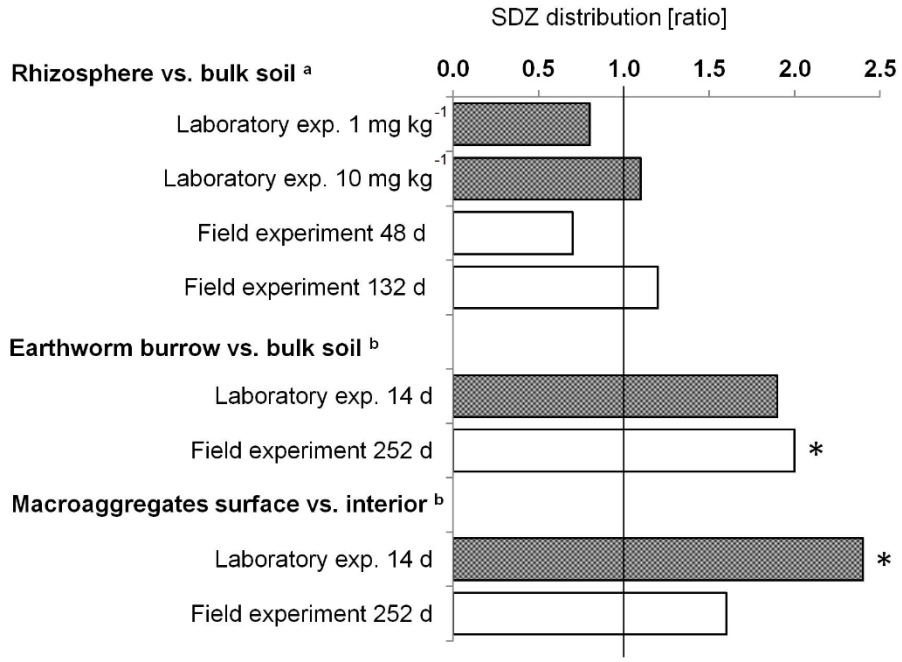

Fig. 1. SDZ distribution ratios derived from residual SDZ[a] of rhizosphere soil or total extractable SDZ[b] of earthworm burrow and soil macroaggregate surface relative to bulk soil or macroaggregate interior of laboratory (grey bars) and field experiments (white bars). Significances are indicated at $p < 0.05$ by asterisk (*).

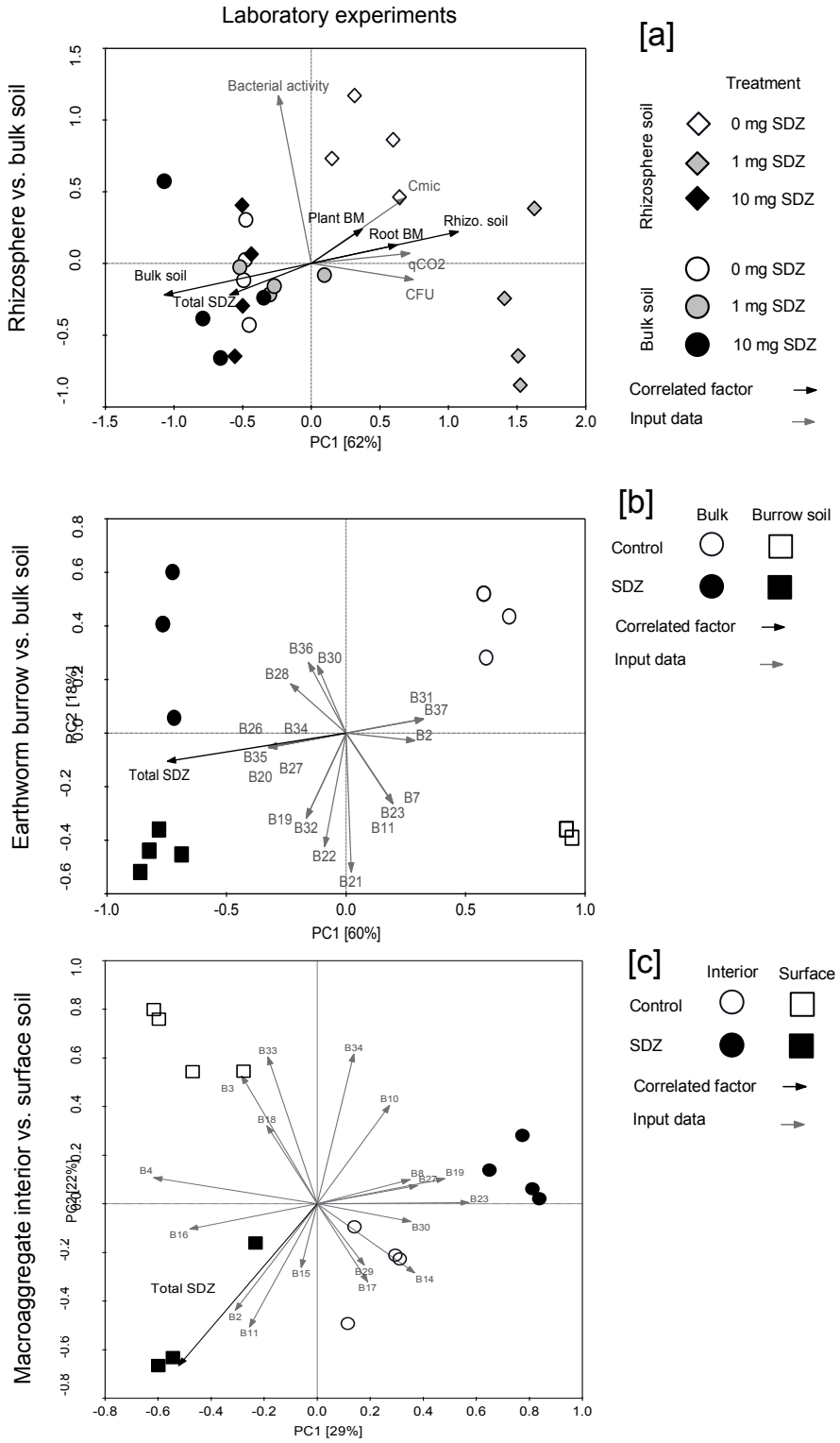

Fig. 2.1. Laboratory experiments. [a] PCA of additional microbial analyses (standardized values of C_{mic}, qCO_2, CFU, and FISH) of rhizosphere vs. bulk soil; [b] *Pseudomonas* 16S rRNA gene fragment DGGE data of earthworm burrow vs. bulk soil; [c] Betaproteobacteria 16S rRNA gene fragment DGGE data of soil macroaggregate interior vs. surface. Symbols of treatments (control vs. SDZ) and habitats are defined by each legend.

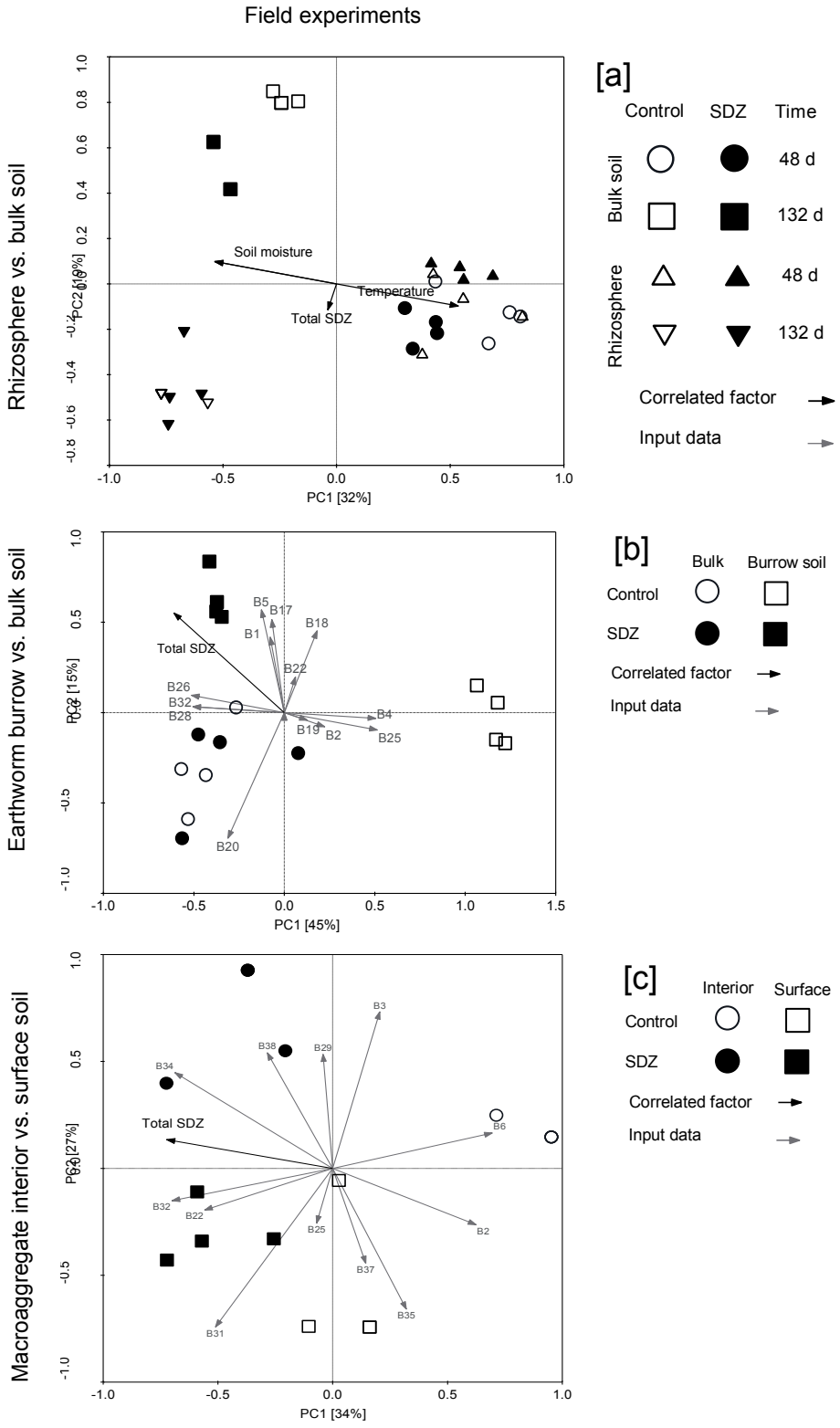

Fig. 2.2. Field experiments. Principal component analysis (PCA) of *Pseudomonas* 16S rRNA gene fragment DGGE data of [a] rhizosphere soil vs. bulk soil, [b] earthworm burrow vs. bulk soil, and [c] macroaggregate interior vs. surface. Symbols of treatments (control vs. SDZ) and habitats are defined by each legend.

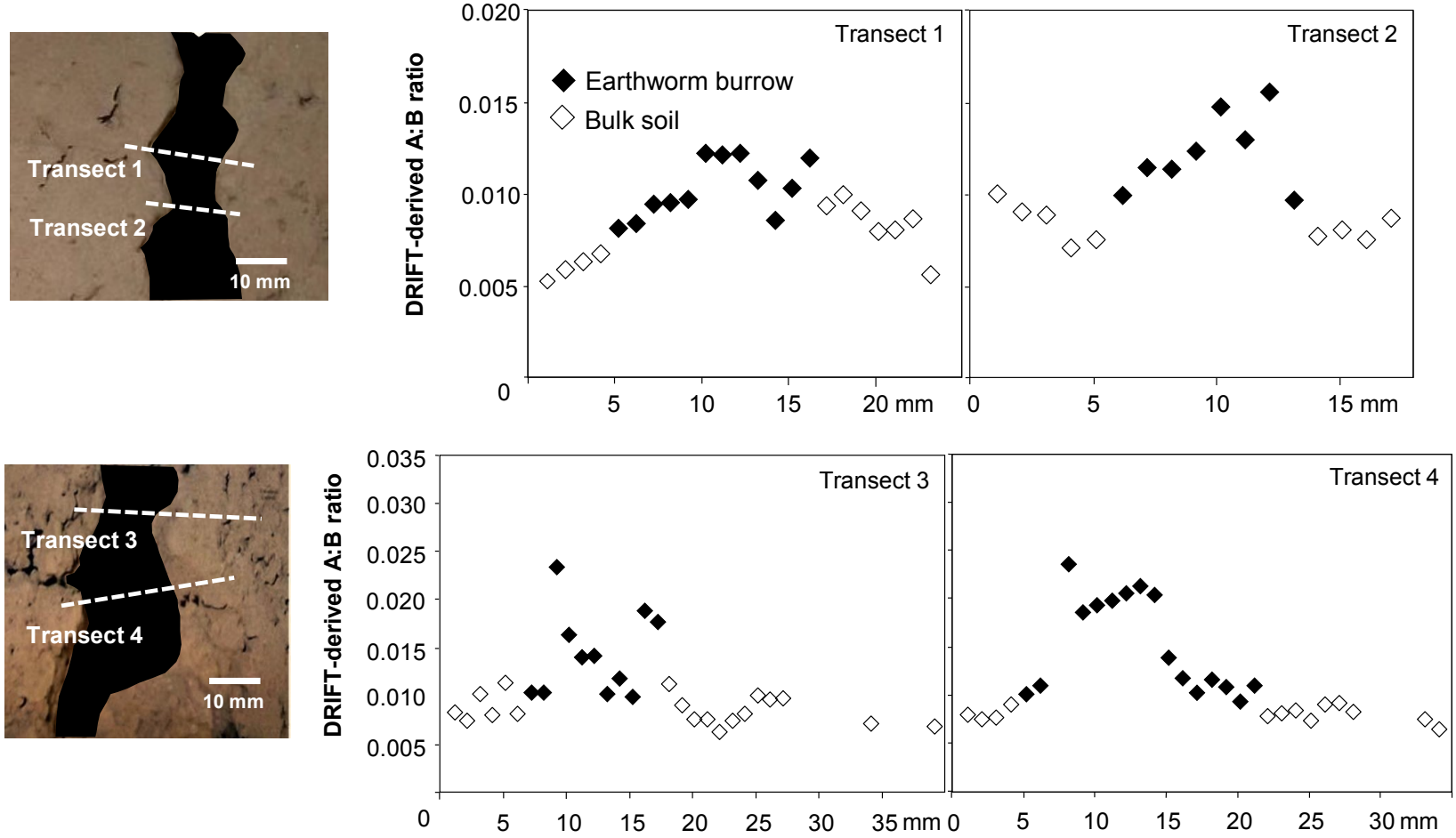

Fig. 3. Diffuse reflectance infrared Fourier transformed (DRIFT) spectroscopy of organic matter properties along individual cross-sections (transect 1-4) of earthworm burrows (left pictures). The A:B ratio of hydrophobic to hydrophilic functional groups used as measure of the potential wettability of the soil organic matter along undisturbed surfaces of air-dried earthworm burrows (filled diamonds) and bulk soil (open diamonds).

5. Effects of SDZ-spiked Manure under different Soil Moisture Regimes

5.1 Effects of soil moisture on SDZ effects under laboratory conditions

Publication III

Soil microbial community responses to antibiotic-contaminated manure under different soil moisture regimes

Rüdiger Reichel, Viviane Radl, Ingrid Rosendahl, Andreas Albert, Wulf Amelung, Michael Schloter, Sören Thiele-Bruhn (in press).

Applied Microbiology and Biotechnology
DOI: 10.1007/s00253-014-5717-4

ABSTRACT

Sulfadiazine (SDZ) is an antibiotic frequently administered to livestock, and it alters microbial communities when entering soils with animal manure, but understanding the interactions of these effects to the prevailing climatic regime has eluded researchers. A climatic factor that strongly controls microbial activity is soil moisture. Here, we hypothesized that the effects of SDZ on soil microbial communities will be modulated depending on the soil moisture conditions. To test this hypothesis, we performed a 49-day fully controlled climate chamber pot experiment with soil grown with *Dactylis glomerata* (L.). Manure-amended pots without or with SDZ contamination were incubated under a dynamic moisture regime (DMR) with repeated drying and rewetting changes of >20 % maximum water holding capacity (WHC_{max}) in comparison to a control moisture regime (CMR) at an average soil moisture of 38 % WHC_{max}. We then monitored changes in SDZ concentration as well as in the phenotypic phospholipid fatty acid and genotypic 16S rRNA gene fragment patterns of the microbial community after 7, 20, 27, 34, and 49 days of incubation. The results showed that strongly changing water supply made SDZ accessible to mild extraction in the short term. As a result, and despite rather small SDZ effects on community structures, the PLFA-derived microbial biomass was suppressed in the SDZ-contaminated DMR soils relative to the CMR ones, indicating that dynamic moisture changes accelerate the susceptibility of the soil microbial community to antibiotics.

1 INTRODUCTION

The usage of antibiotics is common practice in livestock farming for prophylaxis or to cure infectious diseases. Large amounts of the administered antibiotics are excreted unchanged (Halling-Sørensen, 2001) and released to agricultural soils with manure (Sarmah et al., 2006). Despite the rising public awareness and the EU-wide prohibition of antibiotics as growth promoters, no indication is given that consumption declines (Ok et al., 2011). In soils, antibiotics are thus increasingly detected (Sarmah et al., 2006), which may support the spread of antibiotic resistance to humans (Marshall and Levy, 2011), change the soil microbial diversity (Hammesfahr et al., 2008), and therewith potentially interfere with microbial performance on, e.g., organic matter decomposition and mineralization (Kumar et al., 2005).

Sulfonamides, an antibiotic class frequently used in livestock breeding, can reach field soil concentrations of 500 µg kg^{-1} after application of manure from medicated pigs (Schmitt et al., 2005; Rosendahl et al., 2011). However, the extractability and bioaccessibility of sulfonamides declines rapidly in soil (Rosendahl et al., 2011). A commonly used sulfonamide is sulfadiazine (SDZ). This antibiotic acts against infectious Gram-positive ($Gram^+$) and Gram-negative ($Gram^-$) bacteria by competitively inhibiting the enzymatic

conversion of *p*-aminobenzoic acid during folic acid metabolism, impairing bacterial growth (Brown, 1962). When co-applied with manure, the antibiotic effectiveness can be potentiated and SDZ partly suppresses the manure-induced growth-stimulation of soil microorganisms (Schmitt et al., 2005; Hammesfahr et al., 2008). Hence, there is an increasing number of studies that documented adverse effects of SDZ on soil microbial growth, respiration and exoenzyme activities (e.g., Schmitt et al., 2004; Zielezny et al., 2006; Demoling et al., 2009; Gutiérrez et al., 2010), on the abundance of resistance genes in soil (e.g, Heuer et al., 2011; Kopmann et al., 2013), as well as on the soil microbial community structure (e.g., Hammesfahr et al., 2008; Gutiérrez et al., 2010, Reichel et al., 2013).

Most of the available studies on the fate and effects of SDZ in soil have been conducted in the laboratory, using constant and optimum environmental conditions for microbial growth. Results from mesocosm and field experiments that did not control these conditions showed that the fate of SDZ responded to soil temperature, whereas the effects of SDZ were partly ambiguous (e.g., Rosendahl et al., 2011; Reichel et al., 2013). In part, this has been attributed to a yet undefined influence of soil moisture changes upon the fate and effects of SDZ.

Increasing soil moisture (Walker et al., 1992) and drying-rewetting events were reported to promote the dissipation of herbicides of different polarity (Baughman and Shaw, 1996; García-Valcárcel et al., 1999). Thus microbial responses to organic pollutants might vary with soil moisture due to changes of their bioavailability (Baughman and Shaw, 1996). Furthermore, also direct effects of changing water availability on soil microbial communities and their functions have been found (e.g., Fierer et al., 2003; Bapiri et al., 2010).

Soil moisture content influences the microbial activity (Orchard and Cook, 1983). The relationship of microbial activity and water availability is parabolic (Moyano et al., 2013). Fluctuating water contents, in turn, might stimulate microbial growth due to the release of dissolved C after rewetting of dry soil (Wu and Brookes, 2005; Iovieno and Baath, 2008, Xiang et al., 2008). Hence, we postulated that the effect of SDZ on microbial communities might be more pronounced in soils which undergo periodic changes in soil moisture by drying-rewetting dynamics compared to soils without such moisture fluctuations. To test this hypothesis a 49-d fully controlled climate chamber experiment with orchardgrass-planted soil pots was conducted. The pots have been augmented with SDZ contaminated manure. Thereafter, we monitored the dissipation of SDZ as well as its effects on microbial community structure, based on the phenotypic phospholipid fatty acid and genotypic 16S rRNA gene fragment patterns of Betaproteobacteria and Gammaproteobacteria of the genus *Pseudomonas*. Both taxa comprise antibiotic sensitive as well as resilient strains and strains that typically inhabit the rhizosphere (Milling et al., 2005; Costa et al., 2006).

2 MATERIAL UND METHODS

2.1 Climate chamber experiment and sampling

The randomized climate chamber pot-experiment was carried out at Helmholtz Zentrum München, Germany. Uncontaminated Luvisol topsoil was obtained from an arable field located in Merzenhausen, Germany (50°55´48,77´´ N, 6°17´20,02´´ E). The soil had an organic carbon content of 1.2%, a pH ($CaCl_2$) of 6.3, a cation-exchange capacity (CEC) of 11.4 $cmol_c$ kg^{-1} (measured at pH 8.1), 16% clay, 78% silt, 6% sand, and a maximum water holding capacity (WHC_{max}, w/w) of 45.8% (Förster et al., 2009).

Uncontaminated pig manure was produced one month before the start of the experiment at the Agricultural Experimental Station for Livestock Sciences Frankenforst (University of Bonn, Germany) and kept in the dark at 15°C. The manure was characterized by 10.8 (±0.9)% dry mass (dm), a pH ($CaCl_2$) of 7.7 (±0.1), and a C:N ratio of 5.7 (Chapter 11.3, Table S1).

88 pots (9 x 9 x 20 cm^{-3}) were filled with 1.45 kg of soil (dm) at a bulk density of 1.2 g cm^{-3}, sowed with *Dactylis glomerata* (L.) and kept in a greenhouse for 11 weeks until a dense root mass was achieved. Stock solutions of SDZ were mixed with manure (56:56 ml). A 116 ml-volume of this mixture was applied carefully to the soil surface of each pot, avoiding plant contamination with SDZ and manure. This corresponded to a typical manure load and nominal SDZ concentrations of 0 (SDZ 0) or 4 mg kg^{-1} soil dm (SDZ 4). The pots were transferred to the climate chambers with 70% humidity, 16 h daylight, 20±1°C; 950 µmol m^{-2} s^{-1} photosynthetically active radiation (400-700 nm), 18.0 W m^{-2} UV-A radiation (315-400 nm), 0.43 W m^{-2} UV-B radiation (280-315 nm). The radiation was measured using a double monochromator system TDM300 (Bentham, Reading, UK).

For both SDZ 0 and SDZ 4 treatments, half of the pots were daily watered in order to maintain moist soil conditions without soil moisture fluctuations > 5% WHC_{max}. With the growth of the plants, keeping absolute variations of water content at zero was not possible, and also these samples showed moisture variations; however, they never dried out below 27% WHC_{max} (Table 1). These treatments at an average soil moisture of 38% WHC_{max} have been termed as “control moisture regime” (CMR). The remaining pots were subjected twice to 7-day periods without watering to achieve soil moisture of approximately 10% WHC_{max}, followed by 21 d of daily watering to 40% WHC_{max}. Resulting samples with repeated drying and rewetting and moisture changes >20% WHC_{max} have been then termed as “dynamic moisture regime” (DMR). The drying phases of 7 days were defined, based on pre-experiments, in order to reach almost air-dry soil conditions, but avoiding the wilting point of grass. Soil samples were taken from the upper 5 cm, before (-1 d) and immediately after manure application (0 d), as well as at the end of each drying (7, 34 d) and rewetting

(20, 27, 49 d) period. Four replicates were taken per sampling date, split into subsamples and stored at -20°C.

2.2 SDZ extraction and measurement

Soil samples were processed according to Rosendahl et al. (2011), and extracted with 25 ml 0.01 M $CaCl_2$ solution (mild solvent extraction) added to moist soil equivalent to 10 g dm. After processing the suspensions in an end-over-end shaker for 24 h and centrifugation at 3000 x *g* for 15 min, supernatants were sampled. The remaining pellet was re-suspended and extracted again with 25 ml 0.01 M $CaCl_2$ solution. Subsequently, the stronger bound residual SDZ fraction was extracted from the soil pellet by harsh, exhaustive microwave extraction using 50 ml (20:80, v/v) acetonitrile:water (residual fraction). The resulting extracts were processed and analyzed by HPLC-MS/MS. For further details see Rosendahl et al. (2011). The limits of quantitation (LOQ) were 1.25 µg kg^{-1} for SDZ in the $CaCl_2$-extracts and 2.5 µg kg^{-1} in the residual fraction (Rosendahl et al., 2011).

2.3 Determination of microbial community structure based on phospholipid fatty acids

PLFAs were extracted from field moist soil equivalent to 10 g dm, using a mixture of 50 ml methanol, 25 ml chloroform, and 20 ml 0.05 *M* phosphate buffer (pH 7.4). The further processing of the extract was conducted according to the protocol of Zelles and Bai (1993). For the analysis we used an Agilent 6890 gas chromatograph (Agilent, Böblingen, Germany), equipped with a 30 m x 0.4 mm x 0.2 µm fused silica capillary column (Optima 5 MS, Macherey-Nagel, Düren, Germany) and a mass spectrometer (Agilent MSD 5973, Agilent, Böblingen, Germany). The helium carrier gas had a flow rate of 1.5 ml min^{-1}. The oven temperature initially was 80°C with 2 min static time, ramped at 5°C min^{-1} to 290°C and finally held for 10 min. The identification of individual PLFA markers was performed as described by Reichel et al. (2013): 14:0 (all bacteria), i-15:0, a-15:0, i-16:0, i-17:0 ($Gram^+$ bacteria), cy17:0, cy19:0, 18:1ω7c ($Gram^-$ bacteria), 18:2ω6c, 18:1ωn9c (fungi). Microbial biomass was indicated by total concentration of all PLFA markers ($PLFA_{tot}$). PLFA-derived ratios of $Gram^+$:$Gram^-$ bacteria and bacteria:fungi were calculated using summed marker concentrations for each microbial group. The calculated ratios of cyclopropyl-to-precursor fatty acids indicate starvation stress in soil (Bossio and Scow, 1998; Hammesfahr et al., 2008) and was derived from the ratio of cy17:0+cy19:0 to 18:1ω7c (16:1ω7c, was excluded since not safely identified).

2.4 Determination of bacterial communities based on 16S rRNA gene fingerprinting

Total community DNA was extracted from 0.5 g soil sample, using the Fast DNA® Spin Kit for soil and the Geneclean® Spin Kit for purification (MP Biomedicals, Heidelberg, Germany).

The 16S rRNA gene fragments of Betaproteobacteria and Gammaprotobacteria of the genus *Pseudomonas* were amplified using a nested PCR approach. In a first step the specific primer pairs F311Ps/R1459Ps (Milling et al., 2005) respectively F948β/R1494 (Gomes et al., 2001) were used. In a second step the universal primer set F984GC and R1378 (Heuer et al., 1997), which contained the GC clamp was applied. The PCR products, differing in melting properties, were separated using a DCode System for denaturing gradient gel electrophoresis (DGGE; Bio-Rad Laboratories GmbH, München, Germany). PCR templates were loaded onto polyacrylamide gel (6-9%, w/v) in 1 x TAE buffer. Gels were prepared with denaturing gradients ranging from 26 to 58% (where 100% denaturant contains 7 *M* urea and 40% formamide). Electrophoresis was run at 58°C for 6 h at 220 V. Silver stained gels were photographed on a UV-transillumination table (Biometra GmbH, Göttingen, Germany). Analyses of gels were done with the BIOGENE software (Vilber-Lourmat, Marne-la-Vallée, France). Comparisons were based on relative molecular weight calculations, which were derived from a defined standard lane. Band patterns were linked together using the BIOGENE database and exported as binary data for further statistical analysis.

2.5 Data analysis

Mean values ± standard deviation (sd, Chapter 11.3, Table S2) were calculated from replicates. Individual replicates that did not match the same moisture class of the others, resulting from different plant performance, were excluded, reducing the independent replicates to a minimum of three (Chapter 11.3, Table S2). All results were calculated on oven-dry mass basis (dm; 105°C, 48 h). A two-way ANOVA with post-hoc test (Tukey-B) was used to evaluate the significance among the influencing factors moisture regime, treatment, and different treatments at individual incubation times (Table 1) or whole incubation period (Chapter 11.3, Table S2). To avoid false decisions in case of a violated Levene's test ($p < 0.05$; see Table 2), significance levels were increased from $p < 0.05$ to $p < 0.001$. Statistics were performed by the SPSS Statistics 20.0 software (IBM Deutschland GmbH, Ehningen, Germany). Principle component analysis (PCA) was applied to the binary DGGE data using the CANOCO for Windows 4.5 software (Microcomputer Power, Ithaca, New York, USA). The sample scores of the first (PC1) and second principal component (PC2) were extracted from the output file to calculate a two-way ANOVA with post-hoc tests.

3 RESULTS

3.1 Soil moisture

The two different soil moisture regimes CMR and DMR resulted temporarily in significantly different moisture status of the soils, as intended (Table 1). The ANOVA indicated that the soil moisture treatment ($F = 110.9$), time ($F = 10.1$), as well as the interaction of both factors

(F = 79.9) had significant ($p < 0.001$) influence on the soil moisture content at different sampling points (Table 2). In soil of DMR, naturally air-dry soil conditions were determined at incubation times 7 and 34 d with an average of 11.5% and 8.9% WHC_{max}, respectively (Table 2). Soil moisture was significantly lower ($p < 0.05$) in DMR compared to CMR at these incubation times and higher upon rewetting, resulting in increased moisture gradients in DMR soils (Table 2).

3.2 Fate of the SDZ parent compound

No SDZ (MES + RES) was discovered in SDZ 0 CMR and DMR soil (<LOQ; Table 1). In SDZ 4 samples, the mild-solvent extractable SDZ (MES) dissipated over time ($p < 0.001$) from 2,459 and 775 µg kg^{-1} (7 d) to 97 and 66 µg kg^{-1} (49 d) in CMR and DMR soil, respectively. The MES fraction of the SDZ 4 treatment significantly ($p < 0.001$) interacted with the moisture regime (Table 1, 2). This was reflected by MES concentrations being significantly larger ($p < 0.05$) by a factor of 3.2 (7 d), 1.7 (27 d), and 1.8 (34 d) in DMR compared to CMR soils (Table 1), i.e., already initial soil drying obviously provided the antibiotic in mild-extractable forms.

The extractable SDZ residues (RES) decreased significantly ($p < 0.001$) though substantially less than MES over time from 2,434 and 2,350 µg kg^{-1} (7 d) to 1,330 and 1,144 µg kg^{-1} (49 d) under DMR and CMR conditions (Table 1, 2). The trend of higher SDZ concentrations in the RES fraction in DMR compared to CMR soil was significant ($p < 0.05$) at incubation time of 34 d, i.e., SDZ became increasingly sequestered as incubation time proceeded in DMR.

3.3 Phospholipid fatty acid analyses

The $PLFA_{tot}$ concentrations significantly decreased ($p < 0.001$) by a factor of >2.1 from incubation time 7 to 49 d (Table 1, 2). At individual incubation times, the SDZ treatment significantly increased ($p < 0.05$) the $PLFA_{tot}$ concentrations by a factor of 1.6 (20 d) and 1.5 (27 d) in SDZ 4/CMR soils compared to the SDZ 0/CMR (Table 1). After 34 days of incubation, SDZ significantly lowered the $PLFA_{tot}$ concentrations 2.3-times in SDZ 4/CMR compared to the SDZ 0/CMR soils (Table 1).

The microbial community responded to the moisture regime; in SDZ 0 samples we detected 1.5-fold larger $PLFA_{tot}$ concentrations ($p < 0.05$) in rewetted DMR soils compared with the moist CMR ones after 20 d (Table 1). In contrast, when SDZ was present, we found 1.4- (20, 49 d) and 4.4-times (27 d) lowered $PLFA_{tot}$ concentrations ($p < 0.05$) in SDZ 4/DMR compared to SDZ 4/CMR soils (Table 1). Hence, the microbial responses to the interacting factors treatment x moisture regime were significant and most pronounced after soil wetting at prolonged time of incubation ($p < 0.001$; Table 2). In contrast, no such responses were indicated during the drying periods at incubation time 7 and 34 d.

The bacteria-to-fungi (bac:fungi) ratio responded significantly to the factor incubation time ($p < 0.01$), but not to SDZ treatment or moisture regime (Table 2). The time-dependence was reflected by a 1.1-times (SDZ 0/DMR, SDZ 0/CMR), 1.2-times (SDZ 4/DMR), and 1.8-times (SDZ 4/CMR) lowered bac:fungi ratio between incubation time 7 and 49 d (Table 1). Similarly, the ratios of Gram-positive-to-Gram-negative bacteria ($Gram^+$:$Gram^-$) indicated significant shifts ($p < 0.05$) towards the $Gram^+$ bacteria from 1.4 (-1 d) to 2.1 and 2.3 (0 d) after soil application of SDZ uncontaminated and SDZ contaminated manure, respectively (Table 1). The $Gram^+$:$Gram^-$ ratios were influenced by time ($p < 0.001$) and continuously decreased by a factor of 1.6 to 1.7 between incubation times 7 and 49 d, but they were unaffected by the presence of SDZ (Table 1, 2).

The calculated stress PLFA ratios showed significant responses to time ($p < 0.001$) and decreased by a factor of 1.2 to 1.7 from day 7 until 49 (Table 1, 2). The stress ratio was influenced by the different moisture regimes ($p < 0.01$). The first drying event at incubation time 7 d significantly lowered ($p < 0.05$) the stress ratio in DMR soils relative to the CMR soils in both the SDZ 0 and SDZ 4 treatment (Table 1). This difference was repeated when SDZ was present and when samples were rewetting at day 20 and 49 of incubation, showing that the PLFA-derived stress ratio was lowered ($p < 0.05$) by a factor of 1.2 and 1.3 in SDZ 4 DMR relative to the respective CMR soil (Table 1). Also at sufficient moisture supply 27 d after incubation, the DMR trials showed significant lower PLFA-derived stress ratios than the CMR trials (Table 1). When the soils dried out, these differences diminished (day 34), and though the SDZ treatment had lowered the PLFA-derived stress ratio ($p < 0.05$) by a factor of 1.7 (DMR) to 2.0 (CMR) relative to the corresponding SDZ 0 treatments (Table 1). Overall, there was a different time-dependency of the PLFA-derived stress ratio in our samples and moisture regime (Table 2). The interaction term with SDZ treatment was not significant, but after substituting the factor moisture regime by the direction of moisture change (drying/rewetting), the interaction with treatment was significant ($F = 5.4$; $p < 0.05$).

3.4 DGGE analyses of the 16S rRNA gene

Genotypic shifts within the Betaproteobacteria and *Pseudomonas* community were indicated by band appearance or loss (Chapter 11.3, Figure S1a, b). The according principal component analyses with PC1 and PC2 explained 28% (Betaproteobacteria) and 34% (*Pseudomonas*) of the total variance (Figure 1a, b). ANOVA analyses of the PC2 score values (see material and methods, Section 2.5) showed that the Betaproteobacteria community significantly ($p < 0.05$) responded to time and moisture regime (Table 1). Significant shifts ($p < 0.05$) related to the DMR and CMR moisture regime were determined within the Betaproteobacteria community profile at incubation times 7, 27 d (PC2 scores) and 34 d (PC1 scores) of the SDZ 0 treatment and at 7, 34 d (PC1 scores) and 27, 34 d (PC2

scores) of the SDZ 4 treatment, respectively (Table 1, 2). An SDZ treatment-related shift ($p < 0.05$) was detected within the Betaproteobacteria community profiles of CMR and remoistened DRM soils at day 20 (PC1). The *Pseudomonas* community responded significantly to the factors time ($p < 0.01$, PC1+2) and to the treatment ($p < 0.05$, PC2). This was reflected by significant shifts in *Pseudomonas* community structure to the moisture regime SDZ was present ($p < 0.05$) in CMR soil at incubation time 7 (PC1), 34 d (PC2), and in remoistened DMR soil at day 20 (PC2; Table 1, 2). Besides, this was also sensitive to the moisture treatments at day 20 and 34, though changes occurred again with different sign, therewith escaping the significance test of ANOVA. Again, the replacement of moisture regime by drying-rewetting showed that significant shifts in Betaproteobacteria (PC1: $F = 8.4$, $p < 0.01$; PC2: $F = 5.6$, $p < 0.05$) and *Pseudomonas* (PC1: $F = 4.3$, $p < 0.05$) community structure depended on the direction of soil moisture change and even an interaction with treatment was revealed (*Pseudomonas*, PC2: $F = 5.3$, $p < 0.05$).

4 DISCUSSION

Drying and rewetting periods were exclusively induced in the DMR soils (Table 1), providing the basis for the evaluation of SDZ sorption behavior and effects on soil microbial communities under different moisture regimes. The DMR soils reached almost air-dry conditions at incubation time 7 and 34 d (Table 1), simulating a typical drought situation (Landesman and Dighton, 2011). The consecutive rewetting of DMR soils completed each of two drying-rewetting cycles (Table 1); such cycles are known to alter the soil microbial status (Xiang et al., 2008).

This climate chamber experiment now showed that extractable SDZ fractions were repeatedly larger in DMR relative to CMR soils (Table 1), providing a stronger pulse on soil microbial community development. The SDZ dissipation is strongly related to soil organic matter (SOM) as main sorbent of the antibiotic (Thiele-Bruhn, 2003). The rigidity of the supramolecular structure and the accessibility of polar sorption sites of SOM are substantially affected by moisture changes, feeding back on its sorptive properties (Schneckenburger et al., 2012). This holds true for the accessibility of SOM-sorption sites for SDZ and the formation of hardly and non-extractable residues (Thiele, 2000; Rosendahl et al., 2011). Hence, dissipation of SDZ through retention on SOM is possibly accelerated in permanent moist soil without drying and rewetting, thus rapidly reducing the bioavailability of SDZ over time (Table 1).

Application of manure altered the microbial community structure ($PLFA_{tot}$, bac:fungi, $Gram^+$:$Gram^-$ ratio) in all soil treatments and induced time-related microbial dynamics (Table1, 2), which similarly were reported after application of manure with and without SDZ to unplanted and planted bulk soil (Hammesfahr et al., 2008, 2011a; Reichel et al., 2013).

In contrast, the responses of the microbial parameters to the moisture regime were lower or even absent (Table 1, 2). Larkin et al. (2006) already demonstrated that microbial responses to gradual drying and rewetting are relatively small compared to the influence of manure soil amendments on the microbial community. Similarly, the bac:fungi or $Gram^+$:$Gram^-$ ratios did not respond to the dynamic moisture changes (Table 1). Fungi are less sensitive to soil moisture changes (e.g., Gordon et al., 2008; Singh et al., 2009) than bacteria (Fierer et al., 2003; Evans and Wallenstein, 2012) and remain growing under drying-rewetting stress (Bapiri et al., 2010). Furthermore, $Gram^+$ bacteria are thought to be protected against drying due their thick cell walls (e.g., reviewed by Fierer et al., 2003; Davet, 2004), while $Gram^-$ bacteria invest into the synthesis of protective saccharide compounds and thus both are able to withstand moisture stress (Miller et al., 1986). Additionally, microbial communities can be pre-adapted to soil drying and rewetting (Fierer et al., 2003; Evans and Wallenstein, 2012), which might explain the absence of changes of the bac:fungi, $Gram^+$:$Gram^-$ ratios, but also the low responsiveness of the genotypic community structure and total microbial biomass in SDZ uncontaminated DMR soil (Figure 1; Table 2).

Typical SDZ effects were indicated by the trend to lower bac:fungi ratios, the unchanged $Gram^+$:$Gram^-$ ratios, as well as genetic shifts within the 16S rRNA gene patterns (Table 1, Figure 1). These particular SDZ effects have been reported in experiments with unplanted bulk soil (e.g., Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008, 2011a). The total microbial biomass development was more variable in SDZ-treated compared to SDZ uncontaminated CMR soils (Table 1) indicating an inconsistent time-course as reported under mesocosm conditions with, masking additional influences of the manure composition and, similar to our soils, growing rhizospheres (Reichel et al., 2013). SDZ applied with manure typically reduces $PLFA_{tot}$ concentrations in soil (Hammesfahr et al., 2008), while a temporarily increased microbial biomass is related to cryptic growth of still active, resilient microorganisms using the affected part of the biomass as source of nutrients (Thiele-Bruhn, 2005) or on N- and C-compounds derived from the SDZ molecule itself when incubation time had proceeded (Tappe et al., 2013). And indeed, also SDZ dissipated faster in these soils.

The PLFA-derived stress ratio was lowered in SDZ uncontaminated DMR soils due to the dynamic moisture changes (Table 1, 2). The stress ratio was introduced to monitor the level of bacterial starvation in soil (Bossio and Scow, 1998) and here it indicated lower starvation stress in DMR soils at several sampling dates (Table 1). Hence, we assume a temporarily enhanced availability of growth substrates frequently reported after rewetting of soil (Wu and Brookes, 2005; Iovieno and Baath, 2008; Xiang et al., 2008). With the addition of SDZ, the PLFA-derived stress ratio continued to decline, as described by Hammesfahr et al. (2008), but soil microbial biomass was lowered, particularly in combination with wet-dry cycles (Table 1, 2). The latter finding is in line with observations that antibiotics are most effective

under conditions that tend to facilitate the activity of microorganisms, as also observed here for DMR soils (e.g., Schmitt et al., 2005; Thiele-Bruhn, 2005; Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008). Depending on the moisture optima of different microbial populations of the total community (Moyano et al., 2013), drying and rewetting of soil additionally increases or decreases the microbial activity and thus also potential SDZ effects. Apparent contradictions between lowered PLFA-derived biomass and decrease of stress levels (Table 1a) are resolved when assuming that PLFAs of dead microorganisms are readily degraded in soil and thus lower stress ratios are representing only the improved nutritional status of the community not affected by the combined stress of drying-rewetting and SDZ contamination. This implies that SDZ effects in soil are not always bacteriostatic as expected (Brown 1962). Overall, moisture interfered with effects of SDZ on total microbial biomass, but less with its effect on specific microbial community structures (Figure 1, Table 1a, b). This finding reflects that even microorganisms, pre-adapted to soil moisture changes (Fierer et al. 2003) and to natural antibiotic actions, typically found in rhizospheres (Mavrodi et al. 2012) are affected by the combined moisture and SDZ stress.

In summary, this study demonstrated that soil moisture feeds back on the SDZ fate and effects, which improves the interpretation of SDZ effects between experiments conducted at a variety of different moisture contents of 19 up to 60 % WHC_{max} (Hammesfahr et al. 2008, 2011a, b; Kotzerke et al. 2008, 2010; Ollivier et al. 2010; Kopmann et al. 2013).

Acknowledgements

We thank Elvira Sieberger and Petra Ziegler for practical support. This project was funded by the German Research Foundation (DFG) within the Research Unit FOR 566 “Veterinary medicines in soil: basic research for risk assessment”.

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Tables and Figures of Chapter 5.1

Table 1. Mean values of $PLFA_{tot}$ concentration [nmol g^{-1} dm], bacteria-to-fungi ratio (bac:fungi), Gram-positive-to-Gram-negative bacteria ratio ($Gram^+$:$Gram^-$) and cyclopropyl-to-precursor PLFA ratio (stress), sample scores of the first and second principal component (PC1 and 2) of the Betaproteobacteria and *Pseudomonas* DGGE data, mild solvent extractable SDZ (MSE), residual SDZ (RES) fraction [µg kg^{-1}], and soil water content [%WHC_{max}] in SDZ uncontaminated (SDZ 0) and SDZ contaminated (SDZ 4) soil samples, influenced by the dynamic moisture regime (drying, ↓DMR; rewetting, ↑DMR) or control moisture regime (CMR). Significant differences between treatments at incubation time -1 (before manure application), 0 (after manure application), 7, 20, 27, 34, and 49 d are indicate with different letters ($p < 0.05$ or $p < 0.001$, in case of violated Levene's test, cf. Tab. 1).

Time	**-1 d**	**0 d**		**7 d**				**20 d**				**27 d**				**34 d**				**49 d**			
Treatment	-	SDZ 0	SDZ 4	SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4	
Moisture regime	-	-	-	↓DMR	CMR	↓DMR	CMR	↑DMR	CMR	↑DMR	CMR	↑DMR	CMR	↑DMR	CMR	↓DMR	CMR	↓DMR	CMR	↑DMR	CMR	↑DMR	CMR
Soil water [%WHC_{max}]	9	66^{a}	71^{a}	12^{a}	58^{b}	11^{a}	58^{b}	33^{ab}	30^{a}	37^{b}	27^{a}	33^{ab}	27^{a}	38^{b}	29^{a}	8^{a}	45^{b}	9^{a}	49^{b}	31^{a}	28^{a}	33^{a}	27^{a}
PLFA analyses:																							
$PLFA_{tot}$ [nmol g^{-1}]	34.3	107.4^{a}	95.5^{a}	98.7^{b}	105.6^{b}	75.0^{a}	90.8^{ab}	63.9^{b}	43.0^{a}	48.0^{a}	67.3^{b}	64.6^{b}	56.0^{b}	19.1^{a}	83.3^{c}	42.3^{b}	46.7^{b}	23.0^{a}	20.0^{a}	43.2^{b}	40.6^{ab}	31.5^{a}	44.0^{b}
Bac:fungi ratio	2.7	2.7^{a}	2.6^{a}	2.3	2.6	2.8	3.1	2.1	1.8	2.0	1.9	1.5	1.8	1.5	1.4	2.1	1.9	1.5	1.6	2.2	2.2	2.3	1.7
$Gram^+$:$Gram^-$ ratio	1.4	2.1^{a}	2.3^{a}	1.7	1.6	1.7	1.7	1.4	1.4	1.3	1.1	1.0	1.0	1.2	1.0	0.9	0.9	1.0	0.9	1.0	1.0	1.0	1.0
Stress [PLFA ratio]	-	-	-	4.2^{ab}	5.3^{c}	3.8^{a}	4.8^{bc}	4.6^{ab}	4.3^{ab}	3.9^{a}	4.7^{b}	4.1	4.3	4.5	6.6	3.4^{b}	3.9^{b}	2.0^{a}	2.0^{a}	3.6^{b}	3.2^{ab}	2.8^{a}	3.5^{b}
Betaproteobacteria:																							
Scores of PC1	-	-	-	-0.22^{ab}	-0.29^{ab}	-0.15^{b}	-0.38^{a}	-0.69^{a}	-0.32^{a}	0.11^{b}	0.10^{b}	-0.07	-0.05	-0.21	-0.01	0.01^{b}	-0.44^{a}	0.56^{c}	-0.23^{ab}	-0.35^{a}	-0.25^{ab}	-0.11^{b}	-0.22^{ab}
Scores of PC2	-	-	-	-0.14^{a}	0.16^{b}	0.00^{ab}	0.09^{b}	-0.33	-0.38	-0.30	-0.35	-0.43^{a}	0.40^{c}	-0.14^{b}	-0.43^{a}	0.09^{b}	-0.2^{b}	0.03^{b}	-0.25^{a}	-0.26	-0.16	-0.28	-0.28
Pseudomonas:																							
Scores of PC1	-	-	-	0.70^{ab}	0.55^{a}	0.61^{ab}	0.77^{b}	-0.56	-0.31	-0.24	-0.42	-0.36	-0.37	-0.35	-0.40	-0.22	-0.26	-0.30	-0.35	-0.48	-0.37	-0.38	-0.48
Scores of PC2	-	-	-	-0.26^{a}	-0.01^{b}	-0.26^{a}	-0.12^{ab}	-0.03^{b}	-0.05^{b}	-0.45^{a}	-0.09^{b}	-0.38	-0.32	-0.42	-0.45	0.25^{a}	0.62^{b}	0.25^{a}	0.13^{a}	0.05	0.14	0.06	0.20
SDZ analyses:																							
MSE [µg kg^{-1}]	-	-	-	<LOQ	<LOQ	2459^{a}	775^{b}	<LOQ	<LOQ	124	112	<LOQ	<LOQ	107^{a}	62^{b}	<LOQ	<LOQ	106^{a}	58^{b}	<LOQ	<LOQ	97	66
RES [µg kg^{-1}]	-	-	-	<LOQ	<LOQ	2434	2350	<LOQ	<LOQ	1433	1605	<LOQ	<LOQ	1907	1,575	<LOQ	<LOQ	1526^{a}	1079^{b}	<LOQ	<LOQ	1330	1144

LOQ: limits of quantitation: 1.25 µg kg^{-1} for SDZ in the $CaCl_2$-extracts (MES) and 2.5 µg kg^{-1} in the residual (RES) fraction

Table 2. Two-way ANOVA for the factors moisture regime (CMR/DMR), treatment (SDZ 0/SDZ 4), interaction of moisture regime x treatment, incubation time as co-variable and for the dependent variables: $PLFA_{tot}$ concentration [nmol g^{-1} dm], bacteria-to-fungi ratio (bac:fungi), Gram-positive-to-Gram-negative bacteria ratio ($Gram^+$:$Gram^-$), cyclopropyl-to-precursor PLFA ratio (stress), and the sample scores of the first (PC1) and second (PC2) axis of the principal component analyses of Betaproteobacteria and *Pseudomonas* DGGE data, mild solvent extractable SDZ (MSE), and residual SDZ (RES) fraction [µg kg^{-1}]. Shown are the *F*-values and the significances: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). In case of a violated Levene's test ($p < 0.05$), only factors with $p < 0.001$ are expected as significant.

Factors / parameter	$PLFA_{tot}$	Bac:fungi	$Gram^+$:$Gram^-$	Stress	Betaproteobacteria		*Pseudomonas*		SDZ concentration	
					PC1	PC2	PC1	PC2	MES	RES
Levene's test	0.497	0.809	0.376	0.083	0.017	0.444	0.396	0.415	0.018	0.000
Incubation time	89.4***	8.6**	82.5***	22.1***	0.0	4.0*	65.6***	8.6**	202.1***	18.1***
Moisture regime	7.9**	0.0	1.2	6.8**	7.5	6.1*	0.0	0.7	64.6***	0.4
Treatment	6.7*	0.4	0.2	1.6	6.6	0.1	0.1	5.1*	-	-
Moisture regime x treatment	11.3***	0.1	1.3	2.6	1.6	3.8	0.7	1.3	52.0***	2.0

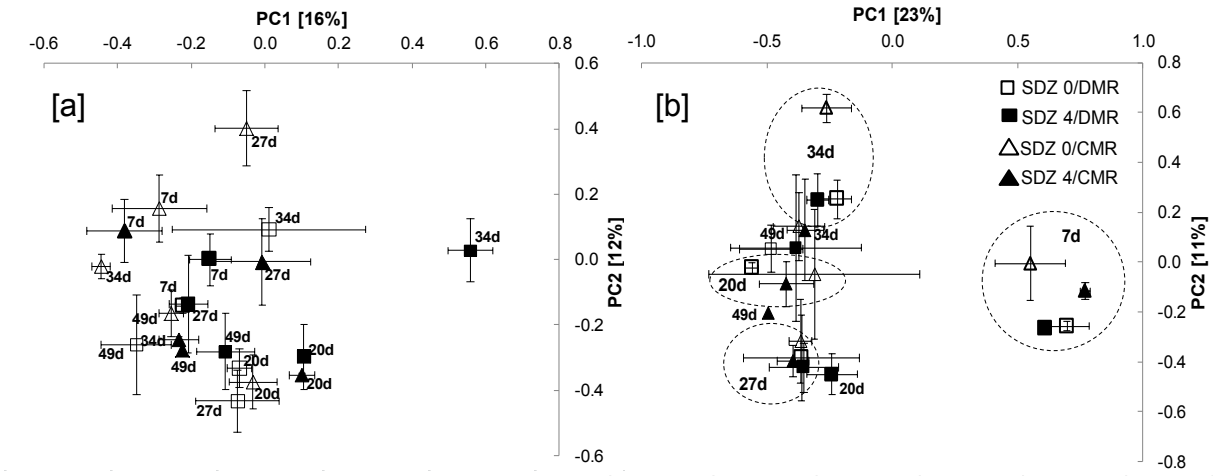

Figure 1. Community shifts displayed by principal component analysis (PCA) of [a] Betaproteobacteria and [b] *Pseudomonas* 16S rRNA gene fragment DGGE data of the SDZ uncontaminated (SDZ 0) and SDZ contaminated (SDZ 4) soil samples, influenced by the dynamic (DMR) or control moisture regime (CMR). Displayed are the mean PCA scores with standard deviation for each treatment at incubation times 7, 20, 27, 34, and 49 d. The PCA explains [a] 28% and [b] 34% of the total variance of the DGGE raw data.

5.2 Effects of soil moisture on SDZ effects under field conditions

MATERIAL AND METHODS

The field experiment was conducted at a field site near Jülich-Merzenhausen, Germany (50°55'51.1"N, 6°17'47.8"E) under representative field conditions with 700 mm annual precipitation and a mean temperature of 9.9°C (see Rosendahl et al., 2011). The soil was a Haplic Luvisol (Förster et al., 2009) and soil moisture was monitored during the experiment using TDR sensors (INFIELD 7b, UMS, München, Germany) and recalculated on the basis of maximum water holding capacity (%-WHC_{max}). Eight experimental plots (18 m²) with *Zea mays* (L.) were established in a randomized block design with four replicates that received pig manure from un-medicated or SDZ-medicated pigs. Uncontaminated manure was collected cumulatively from a group of pigs fed with the same food mixture. SDZ-contaminated manure was collected from randomly selected pigs after medication with the recommended dosage of 30 mg SDZ kg^{-1} bodyweight intramuscularly on four consecutive days. The SDZ injectable solution (200 mg ml^{-1}) was supplied by Vetoquinol Biowet (Gorzow Wielkopolski, Poland). Manures were produced separately before each of the three consecutive field applications. These were equivalent to a manure top-application of 30 m^3 ha^{-1} (day 0), 10 m^3 ha^{-1} (49 d), and 10 m^3 ha^{-1} (133 d; cf. Rosendahl et al., 2011). Soil was obtained at incubation times of 14, 48, 56, 132, 140 d from un-rooted soil between plant rows using soil sampling cylinders (5.6 cm inner diameter x 12 cm height). Soil samples were stored at -20°C before analyses were conducted on parallel subsamples of each replicate. For additional methodological aspects such as the characterization of the soil microbial community and statistics, see Chapter 5.1.

RESULTS

Soil moisture and temperature were influenced by the season and thus showed a natural variability over time (Table 5.2-1; Rosendahl et al., 2011). The two-way ANOVA analysis confirmed the strong influence of time (F = 755.9; p < 0.001) and especially temperature (F = 6,754.6; p < 0.001) on the representative field soil moisture dynamics (Table 5.2-1, Table 5.2-2). Both of these factors were set as co-variables. The subsequent ANOVA, however, revealed that only the factor moisture difference had a predominant influence on soil moisture content (F = 5,675.8; p < 0.001; Table 5.2-2). This factor defines if soil moisture decreased or increased from one sampling date to another. The soil moisture differences were related with rainfall events and temperature changes (Rosendahl et al., 2011). The easily accessible SDZ (EAS) fraction increased after each of three consecutive applications of SDZ-contaminated manure to soil and, compared to the residual SDZ (RES) fraction, rapidly dissipated in interim periods (Table 5.2-1). The PLFA-derived ($PLFA_{tot}$) biomass

responded significantly to time (F = 80.0; p < 0.001) and especially temperature (F = 755.9; p < 0.001; Table 5.2-2). The data showed that applying SDZ-uncontaminated and contaminated manure did not exclusively explain the occasionally lowered PLFA-derived microbial biomass at incubation times 56 and 132 d (Table 5.2-1, Table 5.2-2). Interestingly, these $PLFA_{tot}$ concentrations were lower after soil moisture changed from dry to wetter conditions (Table 5.2-1). The ANOVA confirmed that total microbial biomass responded significantly to the factor moisture difference (F = 24.6; p < 0.001), more than to the treatment with or without SDZ-contaminated manure (F = 11.5; p < 0.01) or to interactions of both factors (F = 7.5; p < 0.05; Table 5.2-2). The $PLFA_{tot}$ concentrations were particularly low in soil samples of the SDZ treatment at incubation time 56 d and related to the combined effect of soil moisture, manure application, and an increased easily accessible SDZ concentration (EAS; Table 5.2-1, Table 5.2-2). The microbial community structure significantly (p < 0.05) shifted towards fungal biomass, indicated by a lower bac:fungi ratio, particularly at sampling time 56 d, when EAS concentrations were high (Table 5.2-1). In contrast, shifts between Gram-positive and Gram-negative bacteria were not significant at p < 0.05 (Table 5.2-1).

DISCUSSION

Under field conditions, dry and wet periods occurred (Table 5.2-1), as simulated in the laboratory climate chamber experiment, yet without reaching air dry soil conditions. The field $PLFA_{tot}$ data showed that the microbial biomass was especially low in SDZ-contaminated soil after a period with increased moisture (Table 5.2-1). Hence, in accordance with the laboratory experiment (Chapter 5.1), the antibiotic effects on soil microorganisms were accelerated by moisture changes (Moyano et al., 2013) in combination with a possible substrate release (Iovieno and Bååth, 2008; Xiang et al., 2008) and the increased accessibility of SDZ due to remobilization. Fungal biomass increased relative to bacteria in SDZ-contaminated soil (Table 5.2-1). Shifts to fungi are well known as a characteristic response to SDZ in soil with substrates that stimulate microbial activity (Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008; Demoling et al., 2009; Gutiérrez et al., 2010). Hence, the combined effects of soil moisture and SDZ are probably related to the lowered bacterial biomass, which is the target of the SDZ action (Brown, 1962). $Gram^+$:$Gram^-$ ratios did not respond to the SDZ treatment or to the factor moisture difference (Table 5.2-2). $Gram^+$ and $Gram^-$ bacteria withstand moisture stress either due to their thick cell walls (reviewed by Fierer et al., 2003; Davet, 2004) or by synthesizing protective compounds such as saccharides (Miller et al., 1986). Furthermore, SDZ equally acts against both infectious $Gram^+$ and $Gram^-$ bacteria (Brown, 1962). Hence, microorganisms sensitive to combined effects of moisture dynamics and SDZ stress diminished, while those better adapted to moisture and antibiotic stress clearly remained viable (Fierer et al., 2003; Bergsma-Vlami et

al., 2005; Costa et al., 2006). Overall, the findings are in line with the climate chamber laboratory experiment (Chapter 5.1), emphasizing an increased sensitivity of the soil microflora to combined stress of soil moisture and SDZ also under field conditions.

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Tables and Figures of Chapter 5.2

Table 5.2-1. Mean values with standard deviations (±sd) of soil moisture content [%-WHC_{max}], total PLFA concentration [nmol g^{-1} dry soil] and PLFA-derived Gram-positive-to-Gram-negative bacteria ratio ($Gram^+$:$Gram^-$) and bacteria-to-fungi ratio (bacteria:fungi), easy accessible SDZ (EAS) and residual SDZ (RES) fraction [µg kg^{-1}] in uncontaminated (control) and SDZ contaminated (SDZ) soil of the field experiment. ANOVA/post-hoc tests were used to determine the significant differences between the parameters over the incubation times 14, 48, 56, 132, and 140 d and separately for the summary. The soil moisture content was determined in-situ at sampling dates and are indicated below each incubation time [%-WHC_{max}].

	14 d (31% WHC_{max})				48 d (26% WHC_{max})				56 d (35% WHC_{max})				132 d (31% WHC_{max})				140 d (37% WHC_{max})				Summary Bulk soil	
	Control		SDZ		Control		SDZ		Control		SDZ		Control		SDZ		Control		SDZ		Control	SDZ
Replicates	4	sd	4	sd	4	sd	4	sd	4	sd	4	sd	4	sd	4	sd	4	sd	4	sd	20	20
$PLFA_{tot}$	16.6^{cd}	0.7	15.1^{bcd}	0.4	18.5^{d}	2.2	19.1^{d}	3.2	11.8^{b}	2.2	4.6^{a}	0.7	26.7^{e}	0.3	23.0^{f}	1.8	41.1^{g}	1.0	38.5^{g}	2.0	22.9^{a}	20.1^{a}
Bac:fungi ratio	5.1^{cd}	0.7	3.4^{abc}	0.5	4.5^{bcd}	2.1	3.2^{abc}	1.6	6.1^{d}	1.5	2.8^{abc}	0.6	3.2^{abc}	0.6	3.2^{abc}	0.9	3.2^{abc}	0.3	2.9^{abc}	0.1	4.4^{a}	3.1^{b}
$Gram^+$:$Gram^-$	0.9^{ab}	0.2	0.8^{a}	0.1	1.1^{abc}	0.3	1.0^{abc}	0.2	1.4^{c}	0.3	1.0^{abc}	0.3	1.0^{abc}	0.0	1.0^{abc}	0.1	0.9^{ab}	0.1	1.0^{abc}	0.1	1.0^{a}	0.9^{a}
EAS [µg kg^{-1}]	<LOQ	-	77^{a}	61	<LOQ	-	5^{b}	3	<LOQ	-	157^{a}	59	<LOQ	-	<LOQ	-	<LOQ	-	110^{a}	14	-	-
RES [µg kg^{-1}]	<LOQ	-	216^{a}	24	<LOQ	-	154^{c}	18	<LOQ	-	305^{ab}	99	<LOQ	-	93^{d}	29	<LOQ	-	380^{b}	28	-	-

LOQ: limits of quantitation: 1.25 µg SDZ kg^{-1} in 0.01M $CaCl_2$-extracts and 2.5 µg kg^{-1} in the residual fraction

Table 5.2-2. Two-way ANOVA of the field experiment parameters conducted for soil moisture content [%-WHC_{max}], total PLFA concentration [nmol g^{-1} dry soil], PLFA-derived Gram-positive-to-Gram-negative bacteria ratio ($Gram^+$:$Gram^-$), and bacteria-to-fungi ratio (bac:fungi). Time and temperature (field experiment) were set as co-variable. Shown are *F*-values of the factors time, temperature, moisture difference, treatment, and the interaction of the latter two factors (AxB). Significances are marked with asterisks: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

	Field experiment			
Factors / parameter	%-WHC_{max}	$PLFA_{tot}$	Bac:fungi	$Gram^+$:$Gram^-$
Time (co-variable)	755.9***	80.0***	2.8	0.1
Temperature (co-variable)	6,754.6***	113.3***	0	3.9
(A) Moisture difference	5,675.8***	24.5***	1.0	0.6
(B) Treatment (+/- SDZ)	0	11.5**	12.1***	2.5
Factor A x B	0	7.5*	1.0	0.0

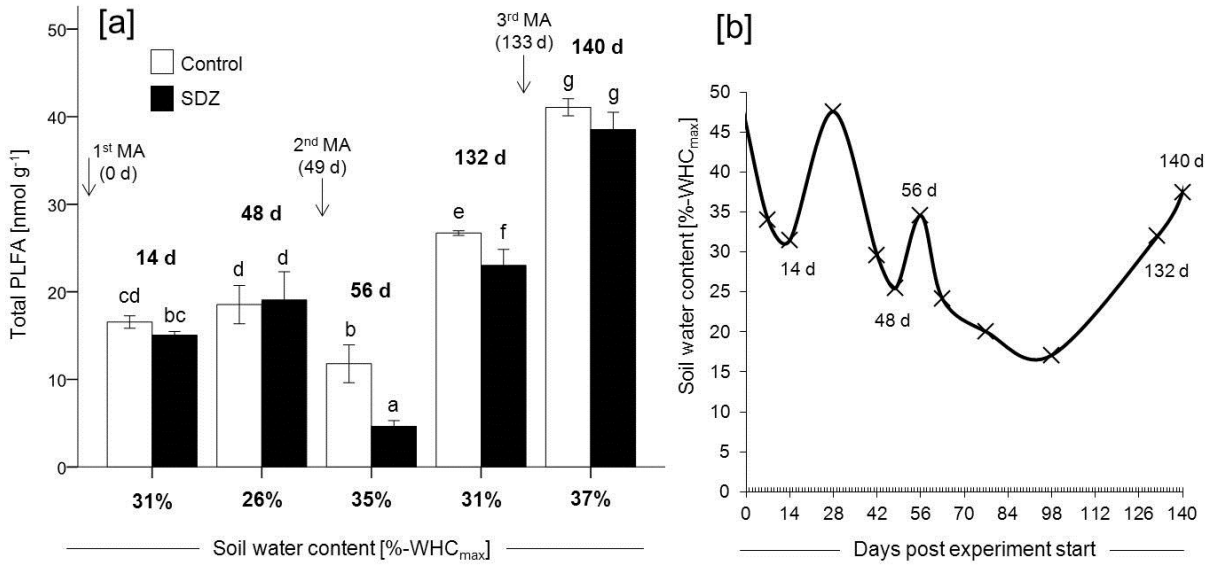

Figure 5-1. [a] Mean total PLFA ($PLFA_{tot}$) concentrations [nmol g^{-1} dry soil] and standard deviations of uncontaminated (control, unfilled bars) and SDZ-polluted (SDZ, filled bars) soil of the field experiment at day 14, 48, 56, 132, and 140 d with the soil moisture content measured *in-situ* at the date of sampling [%-WHC_{max}]. The three consecutive manure applications (1st, 2nd, 3rd MA) are marked with arrows. ANOVA/post-hoc tests were used to determine the significant differences ($p < 0.05$) among $PLFA_{tot}$ and are indicated above the bars by deviating letters. [b] Development of soil moisture content [%-WHC_{max}] during the days post experiment start with the dominant soil moisture change by moderate drying (down-arrow) or rewetting (up-arrow) before the sampling at day 14, 48, 56, 132, and 140 d.

6. SDZ Effects on *Salix fragilis* L. and *Zea maize* L. Plant and Roots

Publication IV

Sulfadiazine uptake and effects on *Salix fragilis* L. and *Zea maize* L. plants

Michelini L., Reichel R., Werner W., Ghisi R., Thiele-Bruhn S. (2012).

Water, Air and Soil Pollution 223, 5243-5257.
DOI: 10.1007/s11270-012-1275-5

ABSTRACT

Frequently, sulfonamide antibiotic agents reach arable soils via excreta of medicated livestock. In this study accumulation and phytotoxicity indicators were analyzed to evaluate the effects of sulfonamides on plants. In a greenhouse experiment willow (*Salix fragilis* L.) and maize (*Zea maize* L.) plants were grown for 40 days in soil spiked with 10 and 200 mg kg^{-1} sulfadiazine (SDZ). Distribution of SDZ and major metabolites among bulk and rhizosphere soil, roots, leaves and stems was determined using accelerated solvent extraction and LC-MS/MS analysis. Accumulation of SDZ was stronger in willow. The antibiotic was mainly stored inside roots and 4-OH-SDZ presence increased with the administered SDZ concentration. In both species SDZ altered root geotropism, increased the lateral root number and affected plant water uptake. The high concentration caused serious stress in willow (e.g., reduced C:N ratio and total chlorophyll content) and the death of maize plants. Even at environmentally relevant soil concentrations (10 mg kg^{-1}), SDZ exhibited adverse effects on root growth while at artificially high concentrations (200 mg kg^{-1}) it showed a strong potential to impair plant performance and biomass. Willow, a fast growing tree species, showed potential for possible phytoremediation purposes.

Abbreviations SDZ sulfadiazine, dm dry mass, fm fresh mass, 4-OH-SDZ 4-hydroxy-sulfadiazine, 5-OH-SDZ 5-hydroxy-sulfadiazine, N-AC-SDZ N-acetyl-sulfadiazine

1 INTRODUCTION

The medication of livestock with pharmaceutical antibiotics represents a normal practice in conventional animal production (Halling-Sørensen et al. 1998; Jjemba 2002; Jørgensen and Halling-Sørensen 2000). In particular, sulfonamides are widely used for the prevention of infectious diseases due to their broad spectrum antibacterial and anticoccidial activity (De Liguoro et al. 2007). However, following administration up to 90% of the parent compound is quickly excreted (Sarmah et al. 2006). As a consequence, manuring agricultural soils with excreta results in soil contamination with pharmaceutical antibiotics. In field studies, sulfonamide antibiotic agents were detected in extractable concentrations of up to 500 mg l^{-1} in pig slurry (Grote et al. 2004; Hölzel et al. 2010) and 0.5 mg kg^{-1} in field soil (Grote et al. 2004; Schmitt et al. 2005). Christian et al. (2003) and Aust et al. (2008) detected extractable sulfonamide residues in soil up to one year after application with manure to agricultural fields. It must be noted that the extractability of sulfonamides quickly declines due to immobilizing processes (Förster et al. 2009; Wehrhan et al. 2010). For example, already 2 d after spiking SDZ at a concentration of 10 mg kg^{-1} to soil, <2 mg kg^{-1} remained extractable, a concentration very much resembling those reported for field soil (Grote et al. 2004; Schmitt et al. 2005). Vice versa, it must be assumed that sulfonamide

concentrations extracted from field soil originated from considerably higher initial amounts. However, a decline in extractable concentration is not due to metabolization or mineralization of the parent compound, which was shown to be subordinate (Langhammer et al 1990; Sukul & Spiteller 2006). Instead, sulfonamides tend to persist for month (Boxall et al. 2004; Aust et al. 2008).

As therapeutic agents are designed to be biologically very active chemicals, once reached the soil their activity clearly affects soil microorganisms (Thiele-Bruhn 2003; Ding & He 2010) and could also impact vegetation (Jjemba 2002). An uptake of several antibiotics into food plants and translocation within the plant was recently reported in literature (Dolliver et al. 2007; Grote et al. 2007; Ferro et al. 2010). The sulfonamide sulfamethazine was taken up from manure-amended soil by maize, lettuce and other plants (Dolliver et al. 2007) and Ferro et al. (2010) reported relevant sulfadimethoxine accumulation in barley roots. Moreover, Grote et al. (2007) identified the radiolabel from ^{14}C-sulfadiazine in roots and leaves of winter wheat. However, plant uptake was small with <0.1% of the applied amount for sulfonamides (Grote et al. 2007), which is in accordance with earlier findings from Langhammer et al. (1990). Furthermore, plant responses to the active molecules are not yet clear and both promoting and inhibiting effects of antibiotics on plants were determined in pot experiments (Jjemba 2002; Liu et al. 2009; Migliore et al. 1995, 1996, 1998). Recent evidence that field concentrations of fluoroquinolones might negatively influence plant growth (Boxall et al. 2006) has to be corroborated in further studies for other antibiotics. However, investigations concerning plant uptake, distribution within the plant and subsequent effects on vegetal physiology remain still limited.

Consequently, the presented study had a twofold aim, (i) to investigate the plant uptake of a sulfonamide antibiotic from soil and its possible utilization in phytoremediation and (ii) to determine adverse effects on crop plants. To investigate this, willow (*Salix fragilis* L.) and maize (*Zea maize* L.) plants were exposed for 40 d (i.e., minimum time required to obtain enough plant material to perform the analyses described below) to sulfadiazine (SDZ), a sulfonamide antibiotic that is frequently applied in livestock husbandry to prevent and treat bacterial diseases (Boxall et al. 2004). Maize was chosen as it is an agricultural plant typically receiving high manure fertilization and respective antibiotic loads. Willow was investigated because it is a representative plant for phytoremediation purposes and short rotation plantations (Kuzovkina and Quigley 2005). Plants were grown in soil containing 10 mg SDZ kg^{-1}, corresponding to an upper concentration level that can be expected in soil, when considering the rapidly declining extractability of sulfonamides in soil (Thiele-Bruhn 2003), and 200 mg SDZ kg^{-1}, yet being an unusual high concentration, to show the potential of uptake and effects. Analyses of plant growth, physiological parameters and the concentration of SDZ and major metabolites in plant tissues and soil sections were carried out, with particular attention to the root apparatus as it was supposed to be the main site of antibiotic accumulation and effects (Michelini et al. 2012).

2 MATERIALS AND METHODS

2.1 Experimental design

Eighteen *Salix fragilis* L. cuttings (20-cm long and 1-cm diameter), taken from a selected tree in the experimental farm of the University of Padova at Legnaro (Italy), and eighteen *Zea maize* L. seeds (cultivar PR39K13 Pioneer Hi-Bred Buxtehude) were pre-grown in tap water for 10 d to allow first root and leaf development. Plants were then transferred to soil. Soil material was obtained from the Ap horizon (0-30 cm) of an Orthic Luvisol silt loam from an arable field at Jülich-Merzenhausen, Germany (50°55'51.1"N, 6°17'47.8"E). The soil was not previously treated with manure and pharmaceutical antibiotics. The main soil properties are: pH ($CaCl_2$) 6.3; clay 15.4%; silt 78.2%; sand 6.4%; OC 2.1%; CEC 11.4 $cmol_c\ kg^{-1}$; maximum water holding capacity 45.8% (w/w). The air-dried soil was sieved through a 4 mm screen, to ensure physical homogeneity. Soil was mixed with 50 g m^{-2} of NPK fertilizer before planting, thus ensuring unimpeded plant nutrition but without affecting further soil properties. Kick-Brauckmann pots (25.5 cm height and 28.5 cm external diameter), made of polypropylene inert material, and ensuring the catchment of percolating water and its re-use by the plant, were filled with 7 kg soil. Each pot was split in two halves by a PE sheet and one plant per half was grown. Soil was spiked with sulfadiazine sodium salt (99.0% minimum, CAS: 547-32-0, Sigma Aldrich, Germany) with resulting final concentrations of 0 (control), 10 and 200 mg kg^{-1}. Antibiotics were added without manure to not bias antibiotic effects with those of nutrients. The experiment was conducted with six independent replicates per treatment group. In parallel, one pot per SDZ treatment was maintained without plants. Plants were cultivated in a greenhouse under natural photoperiod for 40 d (from 7 April to 16 May 2011) and at an average temperature of 25±5 °C during the day and 20±5 °C in the night. Light was not artificially provided, thus depended on the meteorological conditions characterized by sunny weather during the experimental period. Pots were irrigated twice per week with the same amount of water ranging from 200 to 500 ml water per pot. Soil and plant sampling was performed 40 d after the beginning of the SDZ exposure; each of the six independent replicates was treated separately. Bulk soil samples (the fraction of soil not influenced by roots) were collected and the entire root apparatus of every plant was vigorously shaken by hand in order to collect the rhizosphere soil, defined as the fraction of soil adhering to roots. Samples of roots, leaves, stems and bulk and rhizosphere soils were stored at –20 °C prior to further analyses. Dry masses of soil samples were determined after drying at 105 °C for 24h and at 60 °C, until complete dryness, for plant material. Root morphology of both plant species and the different treatments was documented with digital photos (SONY, Cyber-shot, DSC-S930, 10.1 megapixels).

2.2 Biometrics and soil moisture

Biometric measures were recorded weekly for each plant until few days before harvest. In particular, the stem length and the total leaf number were documented for both species and for the maize also the length of the second to fifth leaf. At the end of the 40 d cultivation period root areas, root volumes and total root lengths for both species were recorded through a scanner-based image analysis system (WinRHIZO Basic, Reg and Pro 2007a, Regent Instruments, Inc., Quebec, Canada). Additionally, the soil moisture was determined twice per week in order to get any difference in the water uptake from control and treated plants. To this intent, an ECH_2O EC-5 (Decagon Devices, Inc, Pullman, Washington, USA) probe inserted at 10 cm soil depth was used together with a TDR device (INFIELD 7b, UMS, München, Germany).

2.3 Antibiotic extraction procedure

After collecting adhering rhizosphere soil and thoroughly cleaning the roots with running water (Grote et al. 2007) until they were visually free from adhering soil, root samples (0.5 g of fresh material) were sonicated in 50 ml of deionized water for 15 min to extract the fraction attached to the rhizoplane of SDZ and respective metabolites. A 1-ml-aliquot of this washing solution was transferred to a 1.5 ml amber glass vial and 10 µl of sulfamethazine (500 ng ml^{-1} in methanol), which has very similar properties to SDZ, were added as internal standard. However, correction of LC-MS data with internal standard was not required. Subsequently, all plant tissues (i.e., willow and maize leaves, roots and stems) were ground < 0.125 mm in liquid nitrogen prior to accelerated solvent extraction (ASE 350, Dionex, Idstein, Germany) to determine the concentration of SDZ and major metabolites in the plant cortex. A similar procedure was applied for soil. To this end, plant samples (0.5 g fresh mass) or soil (5 g field moist soil) were mixed with 1.5 g or 1 g of diatomaceous earth, respectively, to prevent clogging of the extraction cells. Five (in case of shortage in plant material) to six replicates were extracted from each sample. The solvents used for antibiotic extraction (i) from plants were methanol/deionized water 1:4 (v/v) according to Förster et al. (2008) and (ii) deionized water for soil samples. These extractants proved to be most efficient in preliminary experiments. Briefly, (i) extraction yield from willow plant material using methanol/water was 1.6 times higher than that of methanol/citrate buffer pH 4.2 (3:1 v/v) and (ii) recovery rate of SDZ from spiked soil samples (1 mg kg^{-1}) was 89% (±7) using ASE water extraction compared to 75% (±19) using ASE methanol/water extraction (unpublished data). Parameters of the applied ASE-method were adjusted as follows: 9 min of preheat; two and one cycles for plants and soil, respectively; 15 min of static time; 200 °C temperature; 60% of flush; 100 bar pressure and 400 sec of N_2-purge. A 1-ml-aliquot of the extract was transferred to a 1.5 ml amber glass vial and 10 µl of the internal standard were added in order to account for matrix effects.

2.4 LC-MS/MS analysis

The concentration of SDZ and the presence of its acetyl- (N-Ac-SDZ) and hydroxy-metabolites (4-OH-SDZ, 5-OH-SDZ) in extracts from plant and soil samples were determined using a Shimadzu LC-20 HPLC (Shimadzu, Duisburg, Germany) coupled to an API 3200 LC-ESI-MS/MS (Applied Biosystems/MDS Sciex Instruments, Toronto, CA). The HPLC consisted of two LC-20AD pumps, an autosampler SIL-20AC, a column oven CTO-10ASvp and a system controller CBM-20A Lite. A Sunfire C18, 3.5 µm, 3.0×20 mm guard column and a Sunfire C18, 3.5 µm, 3.0×100 mm (Waters, Eschborn, Germany) were used for separation of SDZ and its metabolites from other matrix components. The eluent consisted of 0.1M HCOOH in water (solvent A) and 0.1M HCOOH in methanol (solvent B) which were delivered in a gradient program listed in Chapter 11.4, Table S1. For analysis the API 3200 LC-MS/MS was operated in positive ionization MRM mode with a sample injection volume of 10 µl. Nitrogen was used as nebulizer gas at 413.68 kPa and as drying gas at 482.63 kPa respectively; the latter was heated to 650 °C. Ionization voltage was set to 5.5 kV. Additional ion dependent parameters for the specific mass transitions are listed online in Chapter 11.4, Table S2. The software Analyst 1.4.2 (Applied Biosystems/MDS Sciex Instruments, Toronto, Canada) was used for analysis of the data obtained. The quantification of the parent compound was done by summarizing the signal of the different mass transitions, while the ratio of two single mass transitions was used for compound identification (Antignac et al. 2003). The minimum signal-to noise ratio for separation of a peak from baseline noise was 10. External standards containing 0, 10, 20, 50, 100, 200, 500 and 1000 µg l^{-1} SDZ were used for the calibration curve. The metabolites 4-OH-SDZ, 5-OH-SDZ, and N-Ac-SDZ were quantified relatively to SDZ using the SDZ calibration curve. The most abundant mass transition of each metabolite was compared with the sum of SDZ transition masses. In particular, for N-Ac-SDZ masses considered were *m/z* 134.2 and 198.0, while for OH-SDZ it was *m/z* 155.9; the abundance of other masses was negligible. The limit of detection (LOD) of the method was 5 µg l^{-1} and the limit of quantification (LOQ) was 10 µg l^{-1} determined using the procedure of Antignac et al. (2003). Final results are expressed in mg kg^{-1} on a dry mass (dm) basis.

2.5 Bioconcentration factor and translocation factor

To evaluate the ability of the two plant species to extract and accumulate SDZ in plant tissues the bioconcentration factor (BCF, Eq. 1) was determined for roots according to Zayed et al. (1998).

$$BCF = \frac{\text{Contaminant concentration in plant tissue at harvest (mg kg}^{-1}\text{)}}{\text{Initial concentration in the external growth medium (mg kg}^{-1}\text{)}} \quad (1)$$

Furthermore, to better define the active molecule fate after plant uptake, the translocation factor (Tf) was calculated using Eq. 2 in accordance to Zacchini et al. (2009). The Tf indicates the percentage of the accumulated pollutant that reaches the aerial part (leaves and stems) of the plant in relation to that remaining in roots.

$$Tf = \frac{\text{Contaminant concentration in the aerial parts (mg kg}^{-1})}{\text{Contaminant concentration in the roots (mg kg}^{-1})} * 100 \quad (2)$$

2.6 Element content

Samples of leaves, roots and stems were dried at 105 °C for 24 h, ball milled (Retsch MM200; Retsch, Haan, Germany) until a powder-like material was reached and transferred into tin capsules (5×9 mm; IVA Analysentechnik, Düsseldorf-Meerbusch, Germany). Total carbon and total nitrogen (percentage of dry mass) were determined after combustion using an elemental analyzer (Euro-EA 3000CNS, HEKAtech, Wegberg, Germany). Concentrations of Ca and K were determined after digesting 0.1 g of dry material per replicate at 170 °C for 6 h with 1 ml H_2O_2 30% (Merck, Darmstadt, Germany) and 3 ml HNO_3 65% (Carl Roth, Karlsruhe, Germany) in hermetically closed Teflon tubes. After this step, samples were purified with 125 mm diameter filters (Whatman, Dassel, Germany) and brought to 50 ml with deionized water. Ca and K contents were measured with atomic absorption spectroscopy (Agilent-Varian AA240FS, Mulgrave, Australia). Three replicates per group were carried out for these measurements and each sample was analyzed twice. Final data are expressed as g kg^{-1} dm of Ca or K.

2.7 Chlorophyll content

Chlorophyll content was evaluated in two different ways. At first, chlorophyll meter readings (SPAD-502, Minolta Camera Co. Ltd., Munich, Germany) were taken at the center of three full expanded leaves per plant at the end of the experiment. For each leaf six independent measurements were collected, each of which was the average of five repeated measurements. In parallel to SPAD (Soil Plant Analysis Development) values, total chlorophyll content was measured according to Lichtenthaler (1987). Leaf discs (approximately 0.1-0.2 g) were cut out with a cork-borer (1 cm diameter) from the youngest and fully expanded leaf. Discs were placed in glass tubes containing 5 ml methanol (MeOH; VWR, Darmstadt, Germany) and incubated at 60 °C for 30 min in the dark. After the material cooled down, absorbance of the solutions was measured with a UV/Vis spectrophotometer (UV-160a, Shimadzu, Duisburg, Germany) at 665 and 650 nm. Total chlorophyll (Total chl) concentrations (µg g^{-1} fm) were calculated using Eq. 3, where A665 and A650 represent the two wave lengths used in the analysis.

$$\text{Total chl} = \frac{\text{MeOH (ml)}*[(A665*4)+(A650*23.5)]\ (\mu g\ ml^{-1})}{\text{Fresh weight (g)}} \quad (3)$$

2.8 Statistical Analysis

Open source software R (R Development Core Team, 2008), with the application of “car” and “agricolae” packages, was used for statistical analyses. Significant differences ($p < 0.05$) among groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences test for comparisons. Significant differences ($p < 0.05$) between groups were assessed by Student’s t-test.

3 RESULTS

3.1 Plant biometrics and soil moisture

Willow and maize growth during the experimental time was monitored following the total number of leaves and the stem lengths per plant (Fig. 1a-d). For all the parameters analyzed the first measuring point (0 d), immediately before the beginning of SDZ exposure, did not show statistical differences among treatment groups of the two species. In the further course of the experiment, effects due to SDZ were detected for both number of leaves and length of stems following exposure to 200 mg kg^{-1} of SDZ. In contrast, the SDZ soil concentration of 10 mg kg^{-1} did not significantly affect the leaf numbers and the stem lengths of both plant species although in most cases there was a slight trend of smaller values for the plants growing in soil with 10 mg SDZ kg^{-1} (Fig. 1a-d). At the end of the exposure time willow and maize plants of control and 10 mg kg^{-1} groups reached a mean number of about 81 and 9 leaves per plant, while the stem lengths were approximately 38 and 23 cm for willow and maize, respectively (Table 1). To the opposite, 200 mg kg^{-1} of SDZ caused a drastic decrease in the leaf number (28 leaves for willow and 4 leaves for maize) and in the stem length (17 cm for willow and 5 cm for maize). The lengths development of the second to fifth leaves of maize plants (Chapter 11.4, Fig. S1a-d) showed that plants in control soil and plants exposed to 10 mg kg^{-1} had a similar mean length development for all leaves at all measurement points, while leaves evolved within 7 d (2^{nd} and 3^{rd} leaves), 11 d (4^{th} leaf) and 24 d (5^{th} leaf) in the treatments with 200 mg SDZ kg^{-1} exhibited a significantly ($p < 0.05$) reduced leaf length. The difference to leaf length and development from maize plants of control and 10 mg SDZ kg^{-1} treatments further increased with leaf number and maize plants exposed to 200 mg SDZ kg^{-1} did not develop a fifth leaf.

Similar results were obtained for the leaf and stem mass, root areas, root volumes and total root lengths for both species and root fresh mass in the case of maize after 40 d of exposure to SDZ (Table 1), where plant tissue development was inhibited by 200 mg SDZ kg^{-1}. Root volume and total length of maize roots and willow root area tended to be larger in the 10 mg SDZ kg^{-1} treatment compared to the control. Even more, this increase in root biometrics was significant ($p < 0.05$) for the fresh mass and area of maize roots. However, for willow plants an effect of 10 mg SDZ kg^{-1} was only found for the total root length (Table 1).

The percentage of the dry mass content was evaluated in roots, leaves and stems (Table 1). For both species, the dry mass content of roots and leaves was not statistically different for plants exposed to the SDZ concentrations. In contrast, a change in root structure became evident from the specific root length (SRL, root total length per unit root dry mass, in cm g^{-1} dm). This parameter increased for the spiked SDZ concentration. Willow SRL data were 2604, 3534 and 5122 for control and treatments 10 and 200 mg kg^{-1}, while for maize SRL values were 619, 675 and 1520, respectively (Table 1). The dry mass content of roots and stems of willow and maize plants exposed to 200 mg SDZ kg^{-1} was substantially altered. Dry mass content was mostly and in the case of willow stems even significantly reduced, while dry mass content of aerial parts was substantially increased to a mean of 79% for maize plants. It must be noted that the latter dry mass data represent both leaves and stems, since the singular tissues were too small for separate sampling and analysis due to strong SDZ effects. Maize plants even wilted and died off.

With the aim to identify possible effects on the plant physiology following SDZ exposure, soil moisture was recorded and readjusted if necessary twice per week for every pot. In soil without plants the average moisture after the first days of the experimental time remained around 40-45% (Fig. 2a), resembling the maximal water holding capacity, and with no effect of SDZ on the water content as expected. In soil without SDZ moisture was substantially reduced in the presence of plants, due to water uptake by maize and willow, beginning from day 7 (Fig. 2b-c). Reduction in soil moisture by willow plants was similar to controls for pots with 10 mg SDZ kg^{-1} soil, while soil moisture was significantly higher in respective pots with maize. This indicated some kind of inhibition of plant functions/metabolism, which was even stronger in treatments with 200 mg SDZ kg^{-1} soil. There, no statistical difference was found to bare control soils, indicating almost complete inhibition of plant water uptake.

3.2 SDZ content in plants and soil

Within the experimental duration of 40 d the total extractable soil concentration of SDZ considerably decreased to a range from 1.2 to 16.5% of the spiking level in both bulk and rhizosphere soil of treatments 10 and 200 mg kg^{-1} (Table 2). No SDZ was detected in untreated soil samples (data not shown). Differences between samples with and without plants indicated a

presumable direct or indirect (i.e., through action on microbial population) plant effect on the dissipation of SDZ in or from soil at the spiking concentration of 10 mg SDZ kg^{-1} soil, with mean antibiotic bulk soil concentration of 0.12-0.13 mg kg^{-1} in planted pots and 0.34 mg kg^{-1} in pots containing only soil. Furthermore, SDZ concentrations were by a factor of 2 to 3 higher in rhizosphere soil compared to bulk soil of 10 mg kg^{-1} treatments. However, such difference was not detected at a soil spiking level of 200 mg SDZ kg^{-1}.

In plants of all control pots no antibiotic was detected, as expected, while SDZ was taken up by plants from spiked soil and was found in several vegetal tissues (Table 2). Also SDZ was adhering to the rhizoplane as determined by ultrasound-assisted water extraction of intact roots, when plants had been exposed to 200, but not to 10 mg SDZ kg^{-1} soil. However, the majority of the active molecule was found inside roots, showing large differences between willow and maize at the lower SDZ treatment (10 mg SDZ kg^{-1} soil).

From the data, BCFs were calculated. The highest BCF of 33.3 was determined for willow plants exposed to the low SDZ concentration, while maize exhibited a BCF of 2.6. The BCF were similar for plants treated with 200 mg kg^{-1} SDZ with mean values of 27.3 and 26.7 for willow and maize plants, respectively. The Tf data, which were calculated only for plants exposed to 200 mg kg^{-1} as for the low SDZ soil concentration the parent compound was not detected in leaves, showed higher values for maize plants (13.3) compared to willow plants (7.12).

Finally, the occurrence of two major SDZ metabolites was investigated. Specifically, the presence of OH-SDZ (mostly 4-OH-SDZ) was detected in all plant tissues ($\leq$46 mg kg^{-1}) and soil samples ($\leq$2.05 mg kg^{-1}) with the concentration increasing with the SDZ spiking one (Table 2). However, it was not detected in aerial parts of plants exposed to 10 mg SDZ kg^{-1} soil. The OH-SDZ was also detected in pots containing only soil spiked with SDZ. The second metabolite *N*-acetyl-SDZ was detected only at trace levels ($\leq$0.02 mg kg^{-1}) in a few willow leaves from pots treated with 200 mg kg^{-1} (data not shown).

3.3 Element content in plants

In Table 3 data are presented on the total carbon and nitrogen contents in leaves, roots and stems collected at the end of the exposure period of 40 d. Results show that plants exposed to SDZ at 10 mg kg^{-1} had similar ability to assimilate C and N as plants grown in control soil without SDZ, since values detected for root, leaf and stem tissues were almost equal. In contrast, in the presence of SDZ spiking concentrations of 200 mg kg^{-1} some significant differences were determined for total C in stems (maize) and leaves (maize, willow), and total N in roots (maize, willow), stems (maize, willow) and leaves (maize) (Table 3). Differences were even more pronounced for C:N ratios and significant for all plant tissues grown at the high SDZ spiking level except for C:N ratio of the leaves

of willow plants. Some alterations in the Ca and K contents were also found, in particular for the leaves of both plant species (Table 3). In fact, most leaf samples from 200 mg kg^{-1} treatments showed increased K and Ca concentrations in comparison with plants from control treatments. More evident was the effect on the ratio of K and Ca. The K:Ca ratio was higher in aerial parts especially of maize plants compared to roots. On average of all three plant tissues investigated, the ratio declined with increasing SDZ spiking concentrations to 0.9- and 0.7-fold for willow and 0.6- and 0.2-fold for maize of the control values. This clearly indicated a shift from K to Ca uptake in the plants in the presence of SDZ.

3.4 Chlorophyll content

In this study SPAD values and total chlorophyll content of leaves from plants grown in SDZ-contaminated soil were determined (Table 3). SPAD values did not reveal large differences between the species and the SDZ treatments, with average values around 36 for all samples. This parameter revealed a slight decrease in willow plants treated with 200 mg kg^{-1}, but the difference was not statistically significant. Instead, looking at the final contents of total chlorophylls it appeared that plants were able to maintain normal levels of photosynthetic pigments in both willow and maize, even in the presence of 10 mg kg^{-1} SDZ. However, a substantial reduction in chlorophyll was recorded in willow plants exposed to 200 mg SDZ kg^{-1}. Furthermore, no SPAD and total chlorophyll values of maize leaves exposed to 200 mg kg^{-1} were determined since plants suffered severely from SDZ so that not enough leaf material could be sampled for analyses at the end of the experiment. This high spiking concentration caused chlorotic and yellow areas in willow leaves (Chapter 11.4, Fig. S2) and the death of maize plants.

3.5 Morphological root alterations

After 40 d of SDZ exposure, plants exhibited a disturbed morphology in the root system. In fact, substantial root alterations occurred in plants exposed to both 10 and 200 mg kg^{-1} of SDZ. In particular, the antibiotic promoted an abnormal root tip geotropism in maize exposed to 10 mg kg^{-1} compared to root orientation in control soil (Fig. 3a-b). Furthermore, few millimeters behind the root tips, a largely increased number of lateral roots was found for willows exposed to 200 mg kg^{-1} (Fig. 3d-f).

4 DISCUSSION

4.1 SDZ in soil

Soil spiking concentrations of SDZ strongly declined within 40 d and the soil extractable SDZ diminished to concentrations that are frequently detected in arable soil (e.g., Hamscher et al. 2003; Höper et al. 2002). The resulting formation of non-extractable residues was previously reported (Kreuzig and Höltge 2005) and was possibly linked to chemical incorporation into humic substances through covalent cross-coupling mediated by soil oxidoreductases (Bialk et al. 2005; Schwarz et al. 2010). Even more, sorption and diffusion processes most likely contributed to the sequestration of SDZ (Förster et al. 2008) that, with a $pK_{a,1}$ of 6.5 ± 0.30 (Sukul and Spiteller 2006), predominantly occurred as neutral (55%) and acidic species (44%). Thus, polar bonds as well as hydrophobic interactions with soil organic matter and mineral surfaces will have been the reason for the sorption and observed sorption non-linearity of SDZ (Chiou et al. 2000; Thiele-Bruhn et al. 2004). Taking into consideration the total mass balance of SDZ recovered in the whole plant biomass grown in a single pot, it was calculated that within 40 d willow removed 0.16% of the total amount of SDZ spiked to soil at the low and 1.35% of SDZ at the high spiking level, while uptake by maize equaled 0.003% and 0.04%, respectively. These findings closely matched data from Dolliver et al. (2007), who found sulfamethazine accumulation in maize, lettuce and potatoes being less than 0.1% of the initial amount applied to soil. The mild solvent extractable fraction of SDZ from soil planted with willow equaled 1.7% of the low and 14.2% of the high spiking concentration, while for maize values were 2.5% and 13.4%. These results highlight that more than 85% of the applied SDZ was incorporated into the soil matrix. It was previously shown that plants may affect the non-extractable fraction of xenobiotics by enhancing the transformation and bound residue formation (Pilon-Smits 2005).

Based on similar findings, the application of phytoremediation to tetracyclines and sulfonamides was recently proposed (Boonsaner and Hawker 2010; Ferro et al. 2010). However, from our findings SDZ total uptake was low, which was probably aggravated by the young plant age and a relatively low plant number per soil volume in the pot experiments. Furthermore, in our study the SDZ concentration in planted pots with 10 mg SDZ kg^{-1} was higher in the rhizosphere soil compared to bulk soil, probably owing to passive transport with water moving towards roots. Similar contaminant migration to plant rhizosphere was reported, e.g. for polycyclic aromatic hydrocarbons (Gerhardt et al. 2009).

4.2 SDZ in plants

In plant samples the antibiotic was much more abundant inside roots than at the rhizoplane level. Accordingly, Ferro et al. (2010) showed that root cell wall preparations of barley sorbed much less

sulfadimethoxine and sulfamethazine than the fresh roots. The determined SDZ concentrations in plant parts were clearly higher, especially at a soil spiking concentration of 200 mg kg^{-1} (up to 5464 mg kg^{-1} dm for roots and up to 708 mg kg^{-1} dm for leaves), compared to sulfamethazine concentrations of 0.1 to 1.2 mg kg^{-1} dm in maize, lettuce and potato after 45 d exposure to 2.5 mg sulfamethazine kg^{-1} soil (Dolliver et al. 2007). As for soil, also for plant samples the extracted SDZ was not linearly related with the spiking concentration, which is in agreement with previous findings (Michelini et al. 2012). In fact, root concentrations and BCF values of the 200 mg kg^{-1} treatment clearly highlighted that the maximum uptake of SDZ in willow and maize was reached, probably because of the high stress and hampered water uptake experienced by the plants.

Only in willow and maize plants exposed to 200 mg kg^{-1} SDZ was transported to the leaves, corresponding to the decreased translocation of sulfadimethoxine from roots to shoots of crops (*Panicum miliaceum* L., *Pisum sativum* L., *Zea mays* L. and *Hordeum distichum* L.) and weeds (*Amaranthus retroflexus* L., *Plantago major* L. and *Rumex acetosella* L.) (Migliore et al. 1995, 1996, 1998). The antibiotic movement was probably driven by diffusion and/or advection with the transpiration stream, the main processes of the passive uptake of organic pollutants such as chlortetracycline (Kumar 2005; Pilon-Smits 2005; Trapp et al. 1990). Therefore, the low SDZ concentration in leaves could have been due to an inhibited transpiration, which was reflected by the soil moisture data recorded. Vice versa, decreases in transpiration might have been related to leaf damages induced by SDZ (Chapter 11.4, Fig. S2).

It is assumed that after plant uptake toxic contaminants, in this case SDZ, are subsequently sequestered in places where they could do the least damage to essential cellular processes (Pilon-Smits 2005), such as vacuole or cell wall (Burken 2003; Li et al. 1997). However, investigating this was beyond the scope of this study. Detoxification of organic contaminants in plants is mostly driven by cytochrome P-450 enzymes catalyzed transformation reactions (Barrett 1995), which frequently involve hydroxylation (Trapp and Karlson 2001). In our study OH-SDZ was the most prominent metabolite in both plants and soil, which exhibits a strongly reduced antibiotic potential (Hammesfahr et al. 2008). The OH-metabolites were found in both planted and unplanted soils, confirming that abiotic/and or biotic degradation processes in soil contribute to SDZ metabolism (Schwarz et al. 2010). However, the ratio of SDZ:OH-SDZ differed between soil and plant and was mostly lower in roots but higher in aerial plant parts compared to soil (Table 2). Hence, from the data it remains unclear, whether OH-SDZ in plants originated from plant metabolism or root uptake from soil.

4.3 Effects on plants

Biometric analyses evidenced that SDZ has the potential to adversely affect *Salix fragilis* L. and *Zea maize* L. plants even within a short exposure period. This potential was clearly observed at a

spiking concentration of 200 mg SDZ kg^{-1} soil, which is highly above what can be typically expected in agricultural soil (e.g., Aust et al. 2008; Christian et al. 2003; Hamscher et al. 2002). However, even at an environmentally relevant soil concentration of 10 mg kg^{-1} SDZ led to alterations in root morphology. Correspondingly, sulfadimethoxine had similar effects on *Salix fragilis* L. roots (Michelini et al. 2012). According to Sartorius et al. (2009), a growth regulator disturbance could be the reason of the abnormal root geotropism and leaf pigmentation noticed. In fact, sulfonamide antibiotics inhibit the synthesis of folic acid (Stokstad and Jukes 1987; Thiele-Bruhn 2003), a phytohormone precursor. If this pathway is hampered by the drug, abnormal cell division and differentiation can occur (Boonsirichai et al. 2002; Migliore et al. 1995). Since the architecture of a root system determines its exploration of the soil (Lynch 1995), the modified root morphology (i.e., weight and area reduction), combined with an indicated reduced transpiration, adversely affected the plant water uptake. The drought stress was obviously reflected by substantially increased dry matter contents of plant tissues. In agreement with these results, Sartorius et al. (2009) found evident decreases in leaf and root length development when plants were grown in liquid medium containing 300 mg l^{-1} of sulfadimethoxine. Also, Mikes and Trapp (2010) noticed decreased transpiration of *Salix viminalis* L. exposed for a few days to trimethoprim at 100 mg l^{-1}. Contrary, the 10 mg kg^{-1} concentration tested in our study did not reduce the plant development, while, in some cases, it even enhanced root growth. A similar hormetic answer was described for the aerial parts of *Lythrum salicaria* L. treated with sulfadimethoxine nominal concentrations in a range between 0.005 and 50 mg l^{-1} (Migliore et al. 2010).

The high SDZ concentration caused serious disequilibria in the nutrient contents. The C:N ratio was lower in both roots and stems of the two species exposed to 200 mg SDZ kg^{-1}. This is at least partly explained by SDZ effects on photosynthesis that were evidenced by a reduced biomass production, while N uptake appeared to be unaffected. Normally N uptake of juvenile plants starts before C assimilation begins. Assimilated C then dilutes the N concentration to normal C:N ratios (Marschner 2012) which was not the case in the presence of SDZ. It is suggested that the decreased water uptake caused the particularly concentrated nutrient content in willow and maize leaves treated with 200 mg SDZ kg^{-1}. Even more, the K:Ca ratio clearly showed that with more SDZ in soil relatively more Ca was taken up by the plants. Although the bulk of the K and Ca uptake is notoriously passive (Schachtman and Schroeder 1994; Taiz and Zeiger 2009), K is also absorbed through the ionophoric protein systems (Pressman et al. 1967). As numerous single-carbon-transfer reactions are altered following interference of folic acid synthesis, it is plausible that these lipid-soluble membrane molecule formation and/or regulation were disturbed. Consequently, lack of K probably compromised plant nutrition, growth, tropism, enzyme homeostasis and osmoregulation (Schachtman and Schroeder 1994; Taiz and Zeiger 2009).

5 CONCLUSIONS

This study focused on some of the ecological consequences of antibiotic contaminated waste application on agricultural lands. The overall physiological parameters tested in this study, i.e. water uptake, nutrient accumulation and photosynthetic pigments, clearly showed the potential of the sulfonamide antibiotic SDZ to adversely affect the growth and yield of important agricultural crops such as maize. In particular, the lower concentration tested may be expected in arable soils as the upper level of sulfonamide contamination. Additionally, it must be considered that more than one pharmaceutical antibiotic is often used for livestock at a time and thus may end up in soil. On the other hand willow, which is like other fast growing tree species preferred for phytoremediation purposes, proved to withstand and take up higher SDZ concentrations. Also in view of possible phytoextraction and/or phytodegradation aims, toxic effects of SDZ to vegetal organisms deserve further investigation, certainly with longer term works, considering the peculiar interactions between the soil matrix and the tested antibiotic.

Acknowledgements

This work was partly funded by a grant of “Ing. Aldo Gini” Foundation and through the financial support of Veneto Agricoltura Agency and of the German Research Foundation DFG. The support and assistance of M.-O. Aust, E. Sieberger and P. Ziegler is highly appreciated. We thank B.-M. Wilke and two anonymous reviewers for valuable comments on the manuscript.

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Tables and Figures of Chapter 6

Table 1. Final biometrics and dry matter contents (percent) of willow and maize plants.

Sample	Stem length	Leaves	Leaves and stems	Roots				Dry matter			
					Area	Volume	Total length	Root	Leaf	Stem	SRL
	cm	number	g fm	g fm	cm^2	cm^3	cm	%	%	%	cm g^{-1} dm
Willow											
Control	37.0 ± 4.1[a]	80.8 ± 4.7[a]	22.5 ± 1.3[a]	1.6 ± 0.3	87.1 ± 9.7[a]	2.1 ± 0.9[a]	416.7 ± 81.0[ab]	9.96 ± 1.3	20.98 ± 0.5	25.98 ± 0.3[a]	2,604
SDZ 10	38.4 ± 3.3[a]	81.2 ± 3.2[a]	22.8 ± 1.6[a]	1.6 ± 0.3	97.1 ± 16[a]	1.3 ± 0.3[a]	600.8 ± 77.5[a]	10.38 ± 1.1	20.77 ± 0.7	27.08 ± 0.4[a]	3,534
SDZ 200	16.9 ± 4.3[b]	28.0 ± 4.8[b]	3.2 ± 0.8[b]	0.9 ± 0.1	40.2 ± 4.6[b]	0.5 ± 0.1[b]	256.1 ± 22.2[b]	5.57 ± 3.2	21.63 ± 1.5	17.23 ± 2.8[b]	5,122
Maize											
Control	23.5 ± 0.5[a]	9.2 ± 0.2[a]	102.2 ± 4.2[a]	6.8 ± 0.7[b]	156.7 ± 28.6[b]	6.8 ± 1.4[a]	371.5 ± 98.2[a]	8.77 ± 2.0	12.58 ± 0.2[b]	6.34 ± 0.1[b]	619
SDZ 10	22.5 ± 0.6[a]	8.8 ± 0.3[a]	97.0 ± 6.4[a]	9.9 ± 1.4[a]	252.9 ± 38.5[a]	9.0 ± 1.7[a]	580.7 ± 73.0[a]	8.68 ± 0.7	13.62 ± 0.3[b]	6.16 ± 0.5[b]	675
SDZ 200	5.2 ± 0.2[b]	4.5 ± 0.2[b]	0.2 ± 0.0[b]	0.8 ± 0.1[c]	17.1 ± 1.2[c]	0.4 ± 0.0[b]	60.8 ± 4.9[b]	5.69 ± 2.1	78.53 ± 9[a,2]		1,520

Control, 10 and 200 denote treatments 0, 10 and 200 mg SDZ kg^{-1} soil. Values denote mean ± SE; HSD post-hoc test. Letters, when present, indicate significant differences among SDZ treatments ($p < 0.05$).

(fm) fresh mass; (SRL) specific root length, centimeter per gram dry mass (dm)

[2] significant difference to the respective sum of other treatments (control and SDZ 10).

Table 2 Concentrations of SDZ, of two hydroxyl-metabolites and SDZ:OH-SDZ ratio in bulk and rhizosphere soil, at the rhizoplane and in plant tissues (mg SDZ kg^{-1} dry mass) and resulting bioconcentration factor (BCF) and translocation factor (Tf %) after 40 days of experiment.

Sample	SDZ	Soil		Rhizoplane	Plant			BCF	Tf (%)
	soil conc.[1]	Bulk	Rhizosphere	(mg kg^{-1})	Roots	Stems	Leaves		
				SDZ					
Maize	10	0.13 ± 0	0.39 ± 0	<LOD[2]	26.48 ± 10	<LOD	<LOD	2.6	<LOD
	200	33.06 ± 4	21.1 ± 3	699.03 ± 141	5331.17 ± 210	707.70 ± 184[5]		26.7	13.3
Willow	10	0.12 ± 0	0.23 ± 0	<LOD	333.03 ± 68	<LOD	<LOD	33.3	<LOD
	200	29.44 ± 3	27.95 ± 1	600.38 ± 52	5464.19 ± 233	113.5 ± 82.2	664.65 ± 131	27.3	7.12
Control	10	0.34 ± 0							
	200	22.39 ± 2							
				OH-SDZ[3,4]					
Maize	10	0.02 ± 0.0	0.03 ± 0.0	<LOD	0.1 ± 0.0	<LOD	<LOD		
	200	2.05 ± 0.1	1.65 ± 0.2	0.4 ± 0.1	1.4 ± 0.1	46.03 ± 3.7[5]			
Willow	10	0.06 ± 0.0	0.04 ± 0.0	<LOD	0.9 ± 0.1	<LOD	<LOD		
	200	1.73 ± 0.2	1.31 ± 0.2	0.2 ± 0.0	1.6 ± 0.1	7.45 ± 1.6	0.5 ± 0.1		
Control	10	0.07 ± 0.0							
	200	1.71 ± 0.1							
				SDZ:OH-SDZ					
Maize	10	6.5 ± 0.2	7.4 ± 0.3	<LOD	12.7 ± 0.3	<LOD	<LOD		
	200	16.5 ± 0.5	19.5 ± 0.3	7.3 ± 0.2	13 ± 0.2	62.3 ± 4.4[5]			
Willow	10	6.2 ± 0.2	6.9 ± 0.3	<LOD	4.7 ± 0.2	<LOD	<LOD		
	200	16.3 ± 0.5	18.3 ± 0.7	10.8 ± 0.3	11.1 ± 0.2	31.1 ± 2.5	66.3 ± 1.1		
Control	10	5.1 ± 0.2							
	200	13.1 ± 0.4							

Values denote mean ± SE.

[1] Soil spiking concentration (mg kg^{-1})

[2] <LOD = below limit of detection

[3] Relative quantification based on calibration for SDZ

[4] Sum of 4-OH-SDZ and 5-OH-SDZ

[5] Leaves and stems together

Table 3 Total C and N (%), C:N ratio, amount of K, Ca (g kg^{-1} dm), K:Ca ratio, SPAD values and total chlorophyll (µg g^{-1} fm) in willow and maize tissues. Control, 10 and 200 correspond to soils treated with 0, 10 and 200 mg SDZ kg^{-1}.

		Willow			**Maize**		
		Control	**10**	**200**	**Control**	**10**	**200**
C total	Roots	39.72 ± 0.6	38.51 ± 0.1	38.92 ± 0.6	36.92 ± 1	36.09 ± 0.8	37.58 ± 0.8
	Stems	41.59 ± 0.1	42.36 ± 0.1	41.42 ± 0.5	35.21 ± 0.3^{a}	35.97 ± 0.8^{a}	32.58 ± 0.2^{b}
	Leaves	41.57 ± 0.2^{a}	41.01 ± 0.3^{ab}	40.52 ± 0.4^{b}	39.77 ± 0.2^{ab}	40.27 ± 0.2^{a}	38.75 ± 0.0^{b}
N total	Roots	1.86 ± 0.1^{b}	1.71 ± 0.0^{b}	2.83 ± 0.0^{a}	0.99 ± 0.1^{b}	0.91 ± 0.1^{b}	2.14 ± 0.2^{a}
	Stems	1.39 ± 0.1^{b}	0.93 ± 0.1^{b}	3.83 ± 0.5^{a}	0.92 ± 0.1^{b}	1.24 ± 0.4^{b}	6.00 ± 0.3^{a}
	Leaves	3.52 ± 0.0	3.33 ± 0.0	3.40 ± 0.1	2.66 ± 0.1^{b}	3.10 ± 0.3^{b}	4.84 ± 0.0^{a}
C:N	Roots	21.6 ± 0.9^{a}	22.6 ± 0.5^{a}	13.8 ± 0.5^{b}	40.1 ± 5.2^{a}	40.6 ± 2.5^{a}	18.4 ± 2.0^{b}
	Stems	30.5 ± 1.9^{b}	46.7 ± 3.4^{a}	11.5 ± 1.3^{c}	42.4 ± 6^{a}	27.5 ± 7.4^{a}	5.5 ± 0.3^{b}
	Leaves	11.8 ± 0.2	12.3 ± 0.2	12.0 ± 0.5	15.0 ± 0.3^{a}	13.6 ± 1.3^{a}	8.0 ± 0.0^{b}
K	Roots	12.1 ± 0.2	13.6 ± 2.9	n.a.[1]	16.1 ± 1.1	14.4 ± 1.1	n.a.
	Stems	12.1 ± 0.3	11.2 ± 0.4	9.6 ± 1.5	63.7 ± 2.7^{a}	57.6 ± 4.7^{a}	43.5 ± 2.4^{b}
	Leaves	21.4 ± 0.1^{a}	17.4 ± 0.6^{b}	23.4 ± 0.9^{a}	27.4 ± 0.3^{b}	28.5 ± 2^{b}	55.1 ± 0. 7^{a}
Ca	Roots	4.6 ± 0.1	5.3 ± 0.9	n.a.	2.4 ± 0.2	2.6 ± 0.2	n.a.
	Stems	3.9 ± 0.3	3.3 ± 0.3	4.8 ± 0.7	2.1 ± 0.1^{b}	2.7 ± 0. 2^{b}	8.6 ± 0.5^{a}
	Leaves	9.7 ± 0.4^{b}	10.5 ± 0.5^{b}	15.8 ± 0.5^{a}	0.6 ± 0.1^{c}	2.4 ±0. 2^{b}	8.1 ± 0.0^{a}
K:Ca	Roots	2.6 ± 0.1	2.5 ± 0.2	n.a.	6.8 ± 0.2	5.6 ± 0.2	n.a.
	Stems	3.2 ± 0.1^{ab}	3.5 ± 0.1^{a}	2.1 ± 0.2^{b}	30.7 ± 0.4^{a}	21.7 ± 0.3^{b}	5.2 ± 0.2^{c}
	Leaves	2.2 ± 0.1^{a}	1.7 ± 0.1^{b}	1.5 ± 0.1^{b}	43.3 ± 1.2^{a}	12.7 ± 0.4^{b}	6.8 ± 0.1^{b}
SPAD	Leaves	35.6 ± 0.7	36.9 ± 0.6	31.4 ± 3.3	39.5 ± 1.1	36.4 ± 1.2	n.a.
Total chlorophyll		2870.1 ± 147^{ab}	3129.7 ± 160.3^{a}	2013.9 ± 482.1^{b}	1510.6 ± 26.5	1309.1 ± 144.8	n.a.

Values denote mean ± SE; HSD post-hoc test ($p < 0.05$). Control, 10 and 200 denote treatments 0, 10 and 200 mg SDZ kg^{-1} soil. Values denote mean ± SE; HSD post-hoc test. Letters, when present, indicate significant differences among SDZ treatments ($p < 0.05$).

[1] n.a. = not analyzed due to shortage in sample material resulting from strongly inhibited plant development

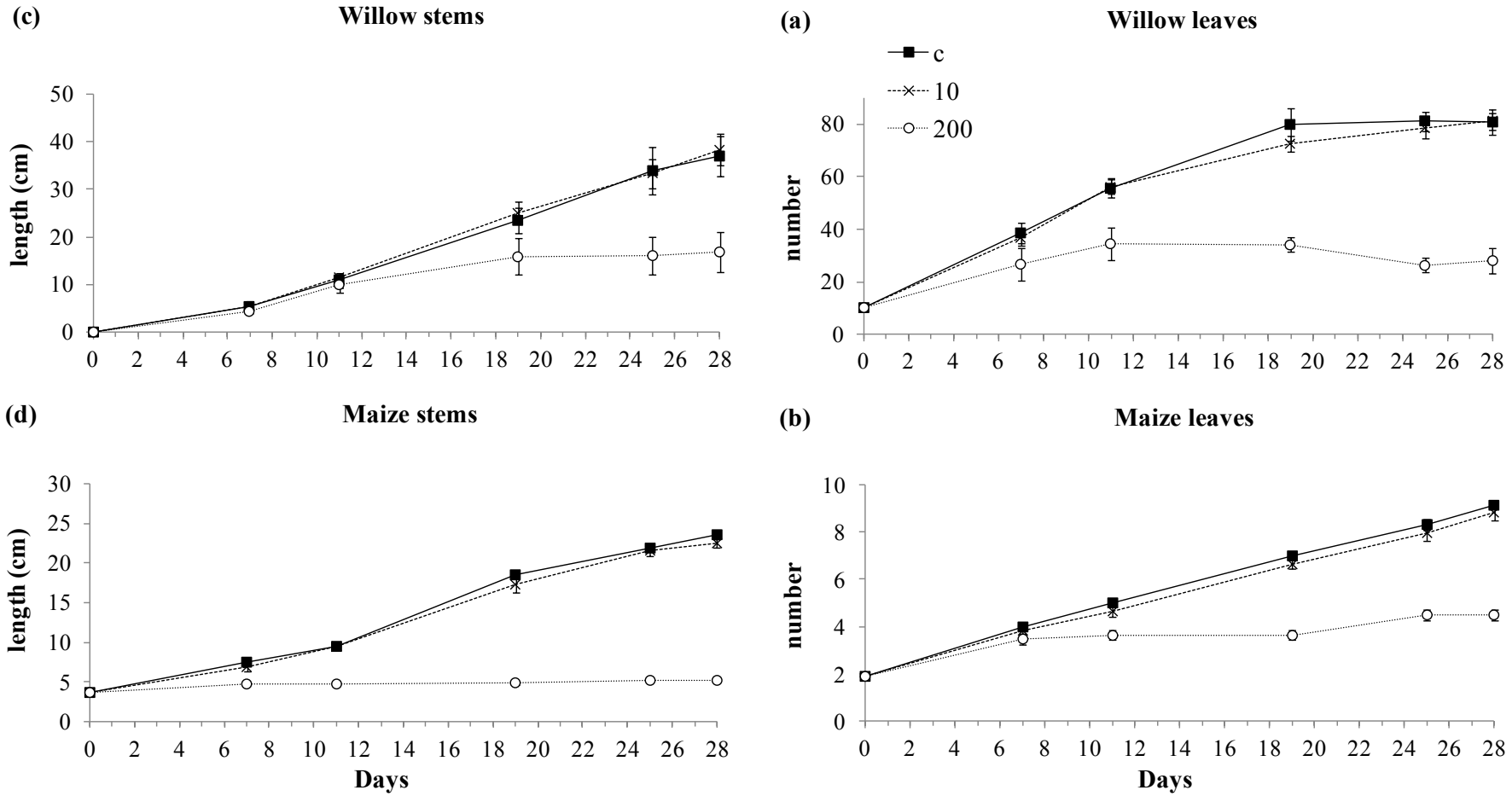

Fig. 1 Leaf number development (a, b) and stem lengths (c, d) during the experimental time in willow (a, c) and maize plants (b, d) exposed to 0 (control), 10 (10) and 200 (200) mg kg^{-1} SDZ. Values denote mean ± SE. Error bars not shown are smaller than symbols.

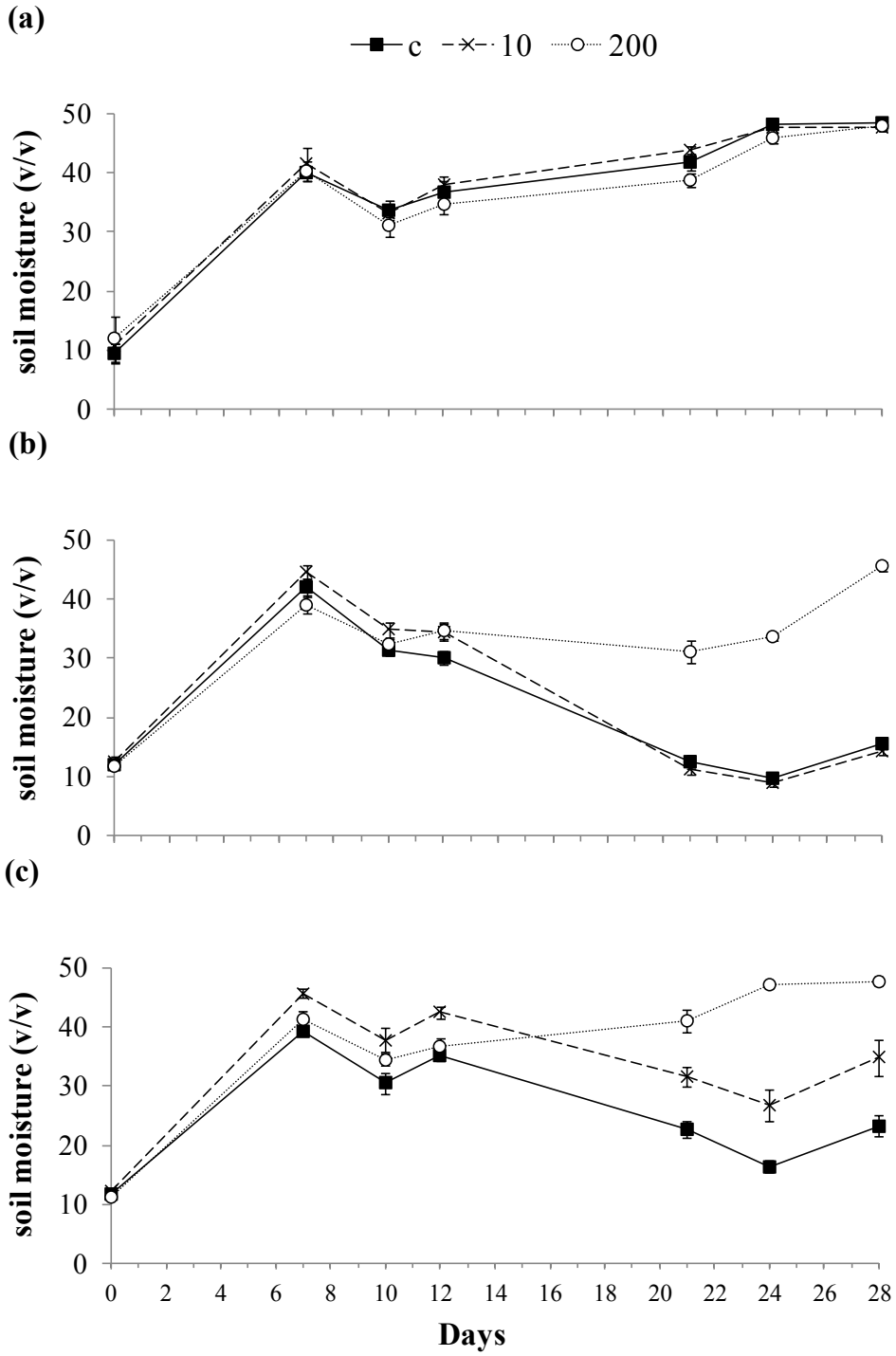

Fig. 2 Soil moisture (v/v) in pots without plants (a), and with willow (b) and maize plants (c). C, 10 and 200 denote treatments with 0, 10 and 200 mg kg^{-1} SDZ. Values are mean ± SE. Error bars not shown are smaller than symbols.

Fig. 3 Root tips from *Zea mays* L. (A-C) and *Salix fragili*s L. (D-F) plants exposed to soil concentrations of 0 (A, D), 10 (B, E) and 200 (C, F) mg kg^{-1} SDZ. White lines correspond to 2 cm.

7. SDZ Effects on Plant Roots and Bacteria at different Distance to Root

Publication V

Soil microbial community responses to sulfadiazine at different distances to plant roots

Rüdiger Reichel, Lucia Michelini, Rossella Ghisi, Sören Thiele-Bruhn

Draft for submission to

Journal of Plant Nutrition and Soil Science

ABSTRACT

Sulfonamide antibiotics reach soil via manure and adversely affect microbial diversity. Clear effects of these bacteriostatic, growth-inhibiting antibiotics occur in the presence of a parallel input of manure, stimulating microbial activity and growth. Natural hot spots with already increased soil microbial activity are located in the rhizosphere, containing microorganism such as *Pseudomonas* with important influence on plant health and growth. The hypothesis was therefore that the antibiotic activity of sulfonamides is promoted in the rhizosphere even in the absence of manure, disturbing the integrity of the plant-specific microbial community structure. This was evaluated by a laboratory experiment with *Salix fragilis* L. and *Zea mays* L. Sampling was done at different distances to plant roots after 40 days of incubation. Mild-solvent extraction revealed bioaccessible SDZ concentrations of 0.2 and 28 mg kg^{-1}. Soil microbial responses were investigated using 16S rRNA gene fragment patterns of total bacteria community, *Pseudomonas,* and selected exoenzymes of N-, P-, and C-cycling. The results showed that the plant species had the largest influence on the bacterial community structure and soil exoenzyme activity patterns. This was also reflected by an up to 1.5-fold higher acid phosphatase activity in samples from maize-planted soil. The structural shifts within the bacterial community increased with the SDZ spiking concentration and declined with the distance to the plant root. Antibiotic effects on the microbial community structure were more pronounced close to roots. In highly contaminated soil, strong SDZ effects on the soil microbial community were additionally derived from adverse influences on the integrity of the rhizosphere microhabitat.

1 INTRODUCTION

Veterinary antibiotics are agents to prevent and treat animal diseases. After the administration to livestock, most antibiotics are excreted by animals in a still bioactive form (*Sarmah* et al., 2006). For pasture animals, the excreta are released directly to soil, whereas for intensively reared animals, the main route of entry is through slurry used as fertilizer (*Vaclavik* et al., 2004). Consequently, the mean extractable concentrations of sulfonamides in field soils can reach 0.5 mg kg^{-1} (*Grote* et al., 2004; *Schmitt* et al., 2005). In addition, many pharmaceuticals such as sulfonamides are characterized by a low biodegradability and tend to form non-extractable residues in soil (*Förster* et al., 2008), which increases their persistence in the environment (*Halling-Sørensen* et al., 1998). Sulfonamides such as sulfadiazine (SDZ) impair bacterial growth of infectious bacteria of the Gram-positive and Gram-negative group by competitively inhibiting the enzymatic conversion of p-aminobenzoic acid during folic acid metabolism (*Brown*, 1962). Altered effects on soil functioning are expected because diverse soil microbial communities are a prerequisite for ecosystem stability and services such as decomposition of organic compounds and nutrient cycling

(*Westergaard* et al., 2001; *Thiele-Bruhn* et al., 2012; *Ding* et al., 2014). To date, a number of studies have documented adverse effects of SDZ on microbial respiration, enzymatic activities (*Zielezny* et al., 2006; *Kotzerke* et al., 2008; *Hammesfahr* et al., 2011b), and other shifts within functional diversity (*Schmitt* et al., 2004; *Demoling* et al., 2009, *Liu* et al., 2012). These shifts are accompanied by changes in the structural community composition (*Hammesfahr* et al., 2008; *Schauss* et al., 2009; *Kleineidam* et al., 2010; *Ding* et al., 2014), development of community tolerance and an increasing resistance level (*Schmitt* et al., 2004; *Demoling* et al., 2009; *Heuer* et al., 2011). *Rosendahl* et al. (2011) reported a faster dissipation of bioaccessible SDZ in rhizosphere compared to bulk soil. In highly contaminated soil, SDZ clearly altered the root architecture and function (*Michelini* et al., 2012). Effects of SDZ on the soil microbial community structure in rhizosphere soil have been indicated, but additionally were masked by different manure compositions derived from pigs with and without previous antibiotic medication (*Reichel* et al., 2013). Patterns of genes and transcripts associated with microbial N-cycling, however, clearly demonstrated effects of SDZ-spiked manure in root-rhizosphere complexes (*Ollivier* et al., 2010). Sequencing of total community DNA 16S rRNA gene fragments clearly confirmed that SDZ contamination decreased the stability of bacterial communities in soil, enriched potential pathogenic genera, and reduced the abundance of beneficial bacteria such as *Pseudomonas* (*Ding* et al., 2014).

As is well known, plants strongly influence the structural and functional diversity of soil microbial consortia due to root exudation and rhizodeposition (*Sørensen* 1997; *Smalla* et al., 2001; *Zahar Haichar* et al., 2008). The composition of molecules released from roots shapes the microbial rhizosphere community, depending on the plant species, cultivar, and physiological status (*Sørensen* 1997; *Heuer* et al., 2002). Furthermore, microbial community structures and functions change at different distance to and locations along the roots (*Baudoin* et al., 2001; *Marschner* et al., 2004). Prominent models of plant-microbe interactions are *Pseudomonas*, comprising functional versatile members with beneficial effects on plant health, as well as strains adapted to natural produced antimicrobials (*Berg* et al. 2014).

This study examines the SDZ effects on soil microbial structures and functions at different distances to roots of a woody and herbaceous plant. For this purpose, *Salix fragilis* L. and *Zea mays* L. plants – with different uptake potential for SDZ (*Michelini* et al., 2012) – were grown in SDZ-spiked topsoil of an Orthic Luvisol. Antibiotic effects on soil microorganisms are based on co-application of substrates that stimulate soil microbial activity (*Hammesfahr* et al., 2008; *Hammesfahr* et al., 2011a). Hence, we hypothezised that also plant root influence alone promotes SDZ effects in soil. The microbial responses were evaluated using

activity patterns of selected exoenzymes of C-, N-, and P-cycling and 16S rRNA gene fragment DGGE patterns of total bacteria and *Pseudomonas* as important root colonizers.

2 MATERIAL UND METHODS

2.1 Experimental setup and sampling

For this study, two different plant species were selected: (i) the monocotyledon *Zea mays* L., widely grown as a fodder plant for animal breeding; it is often grown in soil that previously received some kind of manure that potentially contains pharmaceutical antibiotics; (ii) *Salix fragilis* L., a dicotyledon plant with high potential for soil phytoremediation and use in short rotation coppice or forestry for fast woody biomass production. Details on plant growth conditions and the soil are reported by *Michelini* et al. (2012). Briefly, genetically identical *Salix fragilis* L. cuttings (about 20 cm long and 1 cm of diameter) from the experimental farm of the University of Padova at Legnaro (Italy) and *Zea mays* L. seeds (cultivar PR39K13 Pioneer Hi-Bred, Buxtehude, Germany) were pre-grown in tap water for 10 d before transplanting them into sieved (4 mm screen) Orthic Luvisol topsoil substrate. Nutrient deficiency of plants was compensated by adding 28 mg N, 14 mg P, and 34 mg K per kg^{-1} dm (dry mass). The soil was collected from an arable field at Merzenhausen, Germany (50°55'51.1"N, 6°17'47.8"E), which received no organic amendments in the last decade. It consisted of clay 15.4%; silt 78.2%; sand 6.4%; with a pH ($CaCl_2$) of 6.3; the total organic carbon was 2.1%; and the maximum water holding capacity (WHC_{max}, w/w) was 45.8% (*Förster* et al., 2009). Each vessel (polypropylene Kick-Brauckmann pots, 25.5 cm height and 28.5 cm external diameter) contained one plant and fresh soil equivalent to 3.5 kg dm, which was spiked with SDZ sodium salt (99.0% minimum, CAS: 547-32-0, Sigma Aldrich, Germany) at nominal concentrations of 0 (SDZ0), 10 (SDZ10), and 200 mg SDZ kg^{-1} dm (SDZ200). The greenhouse cultivation was conducted over 40 days under natural photoperiod (7 April to 16 May 2011) and an average temperature of 25±5 °C during the day and 20±5 °C at night. The experiment with willow and maize was conducted with six independent replicates for each soil spiking concentration (treatment). Water losses were replenished twice per week with the same amount of tap water. Soil moisture was monitored using time domain reflectometer (TDR) devices (INFIELD 7b, UMS, München, Germany), showing that soil moisture varied considerably from 10 to 50%. A detailed presentation of soil moisture development during the trial, of the plants, SDZ effects thereon, and extractable SDZ was given by *Michelini* et al. (2012). At the end of the exposure period, samples of bulk soil were collected from soil fallen off after vigorously shaking the root ball. Soil loosely adhering to the root represented the rhizosphere soil. Root samples were directly obtained from the root cortex. For all samples we used sterilized material such as scalpel, tweezers, and spatula. Before sampling, soils were re-moistened to 55% of the maximum water holding

capacity to ensure similar conditions for subsequent analyses of enzyme activities. Root and soil samples were stored at -20°C until analyses. Dry mass of soil was determined after drying at 105°C for 24 h.

2.4 Soil exoenzyme activities

Moist samples equivalent to 0.5 g dm were processed as described by *Michelini* et al. (2012). Before measurement, 50 µl suspension, 50 µl dilution buffer (pH 6.1 for methylumbelliferone (MUB) and pH 7.8 for 7-amino-4-methylcoumarin (AMC) substrates), and 100 µl buffer with the MUB- or AMC-coupled substrate were added: MUB-α-D-glucopyranoside for α-glucosidase (EC 3.2.1.3), MUB-N-acetyl-β-D-glucosaminide for chitinase (EC 3.2.1.14), MUB-β-D-xylopyranoside for ß-xylosidase (EC 3.2.1.37), MUB-β-D-cellobioside for β-cellobiohydrolase (EC 3.2.1.91), L-leucin-AMC for leucine-aminopeptidase (EC 3.4.11.1), and MUB-phosphate for acid phosphatase (EC 3.1.3.2). Multi-well plates were kept at 30°C in the dark for 120 min and the fluorescence was measured using a Victor 3 MultiLabel Reader (Perkin Elmer, Germany) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Final enzyme activities are expressed as nmol MUB g^{-1} h^{-1} or nmol AMC g^{-1} h^{-1}, respectively. The α-glucosidase, chitinase, ß-xylosidase, β-cellobiohydrolase are associated with C-cycling such as decomposition of more or less recalcitrant root deposits. Leucine-aminopeptidase and acid phosphatase activity are involved in N- and P-cycling, important for plant nutrition.

2.4 Bacterial 16S rRNA gene patterns

From 500 mg fresh sample, total community DNA was extracted using the Fast DNA® Spin Kit for soil and the Geneclean® Spin Kit for purification (MP Biomedicals, Heidelberg, Germany). The 16S rRNA gene fragments of total bacteria community (TC) were amplified using the universal primer set F968GC/R1378, which contained the GC clamp (*Heuer* et al., 1997). For the *Pseudomonas* community the specific primer pair F311Ps/R1459Ps was used (*Milling* et al., 2005), followed by an amplification with the already described universal primer set. PCR products were separated on gels by their different melting properties using the DCode System for denaturing gradient gel electrophoresis (DGGE; Bio-Rad Laboratories GmbH, München, Germany) as described in *Reichel* et al. (2013). Silver-stained gels were scanned on a UV-transillumination table (Biometra GmbH, Göttingen, Germany) and analyzed using the BIOGENE software (Vilber-Lourmat, Marne-la-Vallée, France). The appearance and absence of gene fragment bands were detected and related to relative molecular weights of a standard lane. The binary data was exported after linking the band patterns together using the BIOGENE database.

2.5 Data analysis

All results were calculated on oven-dry mass basis (dm; 105°C, 48 h). Results are expressed as mean values with standard deviation (± S.D.) or are available within the figures. Analyses of variance (ANOVA) and post-hoc test (Tukey-B) were applied to evaluate the influence of plant species (willow vs. maize), soil microhabitat (distance to root), and antibiotic treatment (SDZ0, 10, 200) on soil microbial properties. In case of a violated Levene's-test ($p < 0.05$) and to avoid false decisions, significance levels were partly increased from $p < 0.05$ to $p < 0.001$. Statistics were performed using the SPSS Statistics 20.0 software (IBM Deutschland GmbH, Ehningen, Germany). Principle component analysis (PCA) was applied to standardized DGGE and exoenzyme activity data using the CANOCO for Windows 4.5 software (Microcomputer Power, Ithaca, New York, USA). The obtained sample scores of the first (PC1) and second principal component (PC2) were extracted from the output file and further evaluated by ANOVA with post-hoc test. The total explained variance of PCA is displayed on the axes of PC1 and PC2 in Figure 1.

3 RESULTS

3.1 SDZ concentrations and plant responses

The fate of SDZ in soil and uptake by maize and willow plants of this experiment has been already reported by *Michelini* et al. (2012). The authors showed that the bioaccessible SDZ fraction decreased to 12 and 14% of the initially applied 10 and 200 mg SDZ kg^{-1} soil after 40 days. *Michelini* et al. (2012) further extracted SDZ concentrations of 0.33 (SDZ10) and 5.46 mg g^{-1} dm (SDZ200) from willow root samples; the maize root samples contained 0.03 (SDZ10) and 5.3 mg SDZ g^{-1} dm (SDZ200).

In soil of the SDZ0 treatment, root weights of maize were about 4-fold larger ($p < 0.05$) compared to willow (Table 1), but not in soil of the SDZ10 and SDZ200 antibiotic treatment. Within each plant species, root weights were significantly lowered ($p < 0.05$) when SDZ spiking concentrations were increased to 200 mg kg^{-1}. The C:N ratio was significantly different ($p < 0.05$) between both plant species in the SDZ0 and SDZ10 soil treatment (Table 1). In soil of the SDZ200 treatment, however, the C:N ratios were significantly lowered ($p < 0.05$) by a factor of 1.6 (willow) and 2.2 (maize) compared to the SDZ0 treatment. The ANOVA confirmed significant interactions ($p < 0.001$) of the plant and the SDZ treatment on the plant properties (Table 2).

3.2 Soil microbial responses

Root weight and microbial properties of bulk soil were correlated (Pearson correlation, $p < 0.01$): *Pseudomonas*, $r = 0.609$ (PC1 scores), total bacteria, $r = 0.638$ (PC1 scores), and

exoenzyme activity pattern, $r = 0.800$ (PC1 scores). Accordingly, even bulk soil was not entirely unaffected by root influences.

The ANOVA with post-hoc test clearly confirmed that plant species dominated the response of the total bacteria (TC) community, of *Pseudomonas*, and of exoenzyme activities ($p < 0.001$; Table 1, 2). In willow-planted soil, the *Pseudomonas* community was differentiated by the soil microhabitat (Figure 1a, b). The *Pseudomonas* community (PC1 and PC2 scores, $p < 0.05$) of the root microhabitats was significantly different compared to the rhizosphere and bulk soil (Table 1). The ANOVA confirmed that the distance of the soil microhabitat to the root was the second most significant factor for the differentiation of *Pseudomonas* community structures ($p < 0.001$; Figure 1a; Table 2).

Additionally, the *Pseudomonas* community within the different microhabitats of willow-planted soil was shifted by the SDZ treatment (Figure 1a). The significances among the *Pseudomonas* community PCA scores of the untreated SDZ0 versus SDZ10 and SDZ200 treatment increased in proximity to roots (rhizosphere < root; Table 1). In detail, significant community responses to the SDZ treatment were observed in rhizosphere samples ($p < 0.001$, PC2 scores; Table 1) and were even increased at root level of the SDZ10 and SDZ200 treatment ($p < 0.05$, PC1 scores; Table 1). In bulk soil, significant *Pseudomonas* community shifts occurred only at the highest SDZ-spiking concentration of 200 mg kg^{-1} (PC1, $p < 0.05$; Table 1). The ANOVA confirmed that the SDZ treatment was the third most influential factor for the bacterial community differentiation ($p < 0.001$; Table 2). Clear significant interactions between the influencing factors plant species, soil microhabitat, and SDZ treatment were revealed at $p < 0.05$, in some cases also $p < 0.001$ (Table 2).

The significant total bacterial community responses to SDZ were larger in the root microhabitat compared to bulk and rhizosphere soil (PC1 and PC2 scores, $p < 0.001$; Figure 1a; Table 1). Again, the ANOVA of the total bacteria PC1 and PC2 score values confirmed the significant influence of the interacting factors plant species, soil microhabitat, and SDZ treatment on the bacterial community structures ($p < 0.05$; Table 2). Hence, the previously described responses of *Pseudomonas* in soil with willow and maize are representative for the whole bacterial community (Figure 1a, b).

The analysis of the exoenzyme activity patterns was conducted on samples of bulk soil and rhizosphere microhabitats (Table 1). Exoenzyme patterns in soil of willow and maize plants were significantly different (Table 1; Figure 1c). The acid phosphatase activity was generally 1.4- to 1.5-times higher in maize- versus willow-planted pots (Chapter 11.5, Table S1). Within each plant species, the exoenzyme activity patterns were less affected by the soil microhabitat (bulk soil and rhizosphere) and SDZ0 and SDZ10 treatment. Significant responses ($p < 0.05$) to the treatment were only detected at the highest spiking concentration of 200 mg kg^{-1} in bulk soil samples of willow (PC1 and PC2 scores) and maize (PC1;

Table 1). In the maize bulk soil, most exoenzyme activities were significantly lowered ($p < 0.05$) in soil of the SDZ200 treatment. Significantly lower activities of exoenzymes involved in C-cycling were documented in rhizosphere samples of maize-planted soil ($p < 0.05$; Chapter 11.5, Table S1). The ANOVA confirmed that microbial exoenzyme activity patterns responded significantly to the plant species and in second place to the treatment, whereas interactions of both factors were not observed (Table 2).

4 DISCUSSION

The initial spiking levels of SDZ of 10 and 200 mg kg^{-1} were higher than concentrations expected in the environment (*Grote* et al., 2004; *Schmitt* et al., 2005). In rhizosphere soil of the SDZ10 and SDZ200 treatment, however, mild solvent-extractable SDZ concentrations of 0.2 (maize) and 0.4 mg SDZ kg^{-1} (willow), as well as 21 (maize) and 28 mg SDZ kg^{-1} (willow) were reached after 40 days (cf. Section 2.1), approaching moderate and high antibiotic loads occasionally found in arable field soil (*Kim* et al., 2011).

The influence of the plant species was dominant and clearly reflected by the associated microbial exoenzyme activity and bacterial community structure patterns (Table 1, 2). This is in line with reports of plant-species induced structural and functional shifts within the soil microbial community (*Smalla* et al., 2001; *Kourtev* et al., 2003; *Garbeva* et al., 2008; *Berg* and *Smalla*, 2009). In feedback with the environment, plant-derived influences on the root-associated microflora are related to differences in root exudation patterns (*Walker* et al., 2003). Adverse antibiotic effects on plant roots in soil with a large bioaccessible SDZ concentration of 28 mg kg^{-1} were reported here and by *Michelini* et al. (2012) and might also alter the exudation pattern and thus indirectly the soil microbial community.

The distance of the microhabitat to roots was the second most important factor influencing the *Pseudomonas* and total bacteria community structure (Figure 1; Table 1). Root-derived influences such as exudation increase near plant roots and at different root zones, stimulating the soil microbial biomass, activity, and differentiation of the microbial community (*Smalla* et al., 2001; *Marschner* et al., 2004; *Bais* et al., 2006; *Cheng* 2009; *Compant* et al., 2010).

Besides the plant species, interactions between microhabitat and SDZ treatment were revealed (Table 2). These were reflected by genotypic responses of the *Pseudomonas* and total bacteria community to SDZ, which were more abundant near willow and maize roots of the SDZ10 treatment (Figure 1; Table 1). Sulfonamides directly affect soil microbial diversity, as indicated by characteristic shifts within and among groups of bacteria, fungi, and archaea (*Zielezny* et al., 2006; *Hammesfahr* et al., 2008; *Schauss* et al., 2009; *Gutiérrez* et al., 2010). A prerequisite for the potency of SDZ in soil, however, is the activation of the soil microorganisms by adding organic substrates (*Schmitt* et al., 2005; *Thiele-Bruhn* and *Beck*,

2005; *Demoling* et al., 2009; Hammesfahr et al., 2008, 2011a, b; Reichel et al., 2013). Microbial activity can also be stimulated by easily degradable and carbon-rich exudates or deposits typically found close to roots (*Kuzyakov* and *Domansky*, 2000). This explains the larger number of direct antibiotic effects near roots of the SDZ10 treatment (Table 1). SDZ effects on the *Pseudomonas* and total bacteria composition were even more pronounced in maize- and willow-planted soil with large bioaccessible SDZ concentrations of 28 mg kg^{-1} (Figure 1; Table 1, 2). Related findings showed that community structure shifts depend on the SDZ spiking concentration (*Schmitt* et al., 2006; *Hammesfahr* et al., 2008; *Demoling* et al., 2009). Additionally, the SDZ effects on root-associated bacterial communities were indirectly enhanced by adverse effects on the plant roots, which cannot be compensated by root growth in highly SDZ-contaminated soil (Michelini et al., 2012). Hence, the soil microbial community at the root-soil interface is exposed to indirect and direct SDZ effects, which together govern the community response characteristics. Antibiotic dissipation is accelerated in rhizosphere soil (*Rosendahl* et al., 2011) and more antibiotic insensitive strains are present in this microhabitat (*Bergsma-Vlami* et al., 2005; *Costa* et al., 2006; *Reichel* et al., 2013). Nonetheless, this did not mask antibiotic effects in root-associated microhabitats, which were detected in this study.

From a functional point of view, SDZ effects were also revealed in bulk soil and rhizosphere with nominal spiking concentrations of 200 mg SDZ kg^{-1} (Table 1, 2). This clearly relates to the previously described indirect SDZ effects on the soil microbial community due to adverse effects on the root microhabitat itself (Table 1, Figure 1c; *Michelini* et al., 2012). In contrast, only minimal functional responses to SDZ were detected in rhizosphere soil of the SDZ10 treatment (Table1a; Chapter 11.5, Table S1). This has been frequently related to functional redundancy in unplanted soils with environmentally relevant sulfonamide concentrations (Kotzerke et al., 2008; Hammesfahr et al., 2008; Schauss et al., 2009).

5 CONCLUSION

The results showed that effects of antibiotics in rooted soil are particularly promoted if indirect antibiotic effects on the root-associated microhabitat integrity and direct antibiotic compound effects on soil microorganisms are combined. In soils without additional organic amendment, direct antibiotic effects of bacteriostatic pharmaceuticals are more abundant close to roots. It remains open whether an additional or repeated soil amendment with manure that potentially contains more than one antibiotic pharmaceutical would further increase these effects. This study also alerts about the adverse effects of highly antibiotic-contaminated soils on nutrient cycling and crop yields.

Acknowledgements

We thank Mrs. Elvira Sieberger for her technical assistance in conducting the DGGE and enzymatic activities measures and Prof. Willy Werner, Geobotany, University of Trier, for generous support with the greenhouse experiments. This work was partially based on a grant of “Fondazione Ing. Aldo Gini” and on the financial support of Veneto Agricoltura Agency and the DFG FOR566.

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Tables and Figures of Chapter 7

Table 1. Mean values of root weight, C:N ratios, scores of the first (PC1) and second (PC2) PCA axis of *Pseudomonas*, total bacteria DGGE, and exoenzyme activity patterns. Plant species willow and maize with the (micro-)habitat: bulk soil (BS), rhizosphere soil (RZ), and root sample (RT). Each soil microhabitat contains three treatments: SDZ0 (0 mg SDZ kg^{-1}), SDZ10 (10 mg SDZ kg^{-1}), and SDZ200 (200 mg SDZ kg^{-1}). Standard deviation is displayed as ±S.D. or available in Figure 1. Significant differences between plant species are marked with an asterisk (*), those within each plant species by deviating letters ($p < 0.05$). In case of Levene's test ($p < 0.05$) violation, significance level was set to $p < 0.001$ (cf., Table 2).

		Root weight [g fm]	C:N ratio	*Pseudomonas*		Total bacteria		Exoenzyme activity	
Willow-planted pots				PC1	PC2	PC1	PC2	PC1	PC2
Habitat	**Treatment**								
BS	SDZ0	-	-	-0.73^{a*}	-0.43^{a*}	0.94^{cd*}	0.03^{a*}	0.47^{a*}	-0.31^{a*}
BS	SDZ10	-	-	-0.68^{a*}	-0.54^{a*}	0.98^{d*}	0.05^{a*}	0.46^{a*}	-0.31^{a*}
BS	SDZ200	-	-	-0.48^{b*}	-0.64^{a*}	0.98^{d*}	0.05^{a*}	1.65^{b*}	0.37^{b}
RZ	SDZ0	-	-	-0.76^{a*}	-0.39^{a}	0.88^{c*}	-0.09^{a*}	0.69^{a*}	-0.36^{a*}
RZ	SDZ10	-	-	-0.83^{a*}	-0.01^{b*}	0.83^{c*}	-0.15^{a*}	0.39^{a*}	-0.29^{a}
RZ	SDZ200	-	-	-0.77^{a*}	0.01^{b*}	0.76^{c*}	-0.11^{a*}	1.12^{ab*}	0.01^{ab}
RT	SDZ0	1.6±0.3^{a*}	21.6±0.9^{a*}	-0.40^{b*}	0.57^{c*}	-0.57^{a*}	0.61^{b}	-	-
RT	SDZ10	1.6±0.3^{a*}	22.6±0.5^{a*}	-0.11^{c}	0.42^{c*}	0.83^{c*}	-0.15^{a*}	-	-
RT	SDZ200	0.9±0.1^{b}	13.8±0.5^{b}	-0.04^{c}	0.31^{c*}	0.76^{c*}	-0.11^{a*}	-	-
Maize-planted pots									
Habitat	**Treatment**								
BS	SDZ0	-	-	0.78^{cd*}	-0.22^{a*}	-0.55^{ab*}	-0.52^{a*}	-1.11^{ab*}	-0.01^{a*}
BS	SDZ10	-	-	0.90^{d*}	-0.18^{a*}	-0.48^{ab*}	-0.51^{a*}	-0.99^{ab*}	0.04^{a*}
BS	SDZ200	-	-	0.93^{d*}	-0.22^{a*}	-0.47^{ab*}	-0.56^{a*}	0.01^{c*}	0.46^{a}
RZ	SDZ0	-	-	0.77^{cd*}	0.01^{b}	-0.60^{a*}	-0.44^{a*}	-1.05^{ab*}	0.15^{a*}
RZ	SDZ10	-	-	0.65^{c*}	-0.17^{a*}	-0.50^{ab*}	-0.59^{a*}	-1.26^{a*}	0.01^{a}
RZ	SDZ200	-	-	0.84^{d*}	-0.30^{a*}	-0.42^{bc*}	-0.56^{a*}	-0.38^{bc*}	0.25^{a}
RT	SDZ0	6.8±0.7^{b*}	40.1±5.2^{a*}	-0.10^{a*}	0.72^{d*}	-0.28^{cd*}	0.62^{b}	-	-
RT	SDZ10	1.6±0.3^{a*}	40.6±2.5^{a*}	-0.07^{ab}	0.50^{c*}	-0.16^{d*}	0.34^{b*}	-	-
RT	SDZ200	0.9±0.1^{c}	18.4±2.0^{b}	0.07^{b}	0.55^{c*}	-0.37^{bc*}	0.47^{b*}	-	-

Table 2. ANOVA of the factors plant (maize or willow species), microhabitat (bulk soil, rhizosphere soil, root sample), treatment (SDZ0, control; SDZ10, 10 mg SDZ kg^{-1}; SDZ200, 200 mg SDZ kg^{-1}), and their interactions with the factors root weight, C:N ratio, scores of the first (PC1) and second (PC2) PCA axis of *Pseudomonas*, total bacteria DGGE, and exoenzyme activity patterns. Shown are *F*-values at significances of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). In case of Levene's test ($p < 0.05$) violation, significance level was set to $p < 0.001$.

Factors / parameters	Root weight	C:N	*Pseudomonas*		Total bacteria		Exoenzyme activity	
			PC1	PC2	PC1	PC2	PC1	PC2
Levene's-test	0.026	0.137	0.139	0.001	0.001	0.001	0.051	0.058
Plant	671***	297***	2,787***	77***	4,947***	75***	252***	22***
Microhabitat	-	-	44***	952***	52***	263***	3	2
Treatment	281***	157***	23***	10***	116***	28***	36***	18***
Plant x treatment	202***	43***	1	9**	57***	3	0	1
Microhabitat x treatment	-	-	6**	26***	83***	17***	3	3
Plant x microhabitat x treatment	-	-	3*	26***	107***	8***	1	0.5

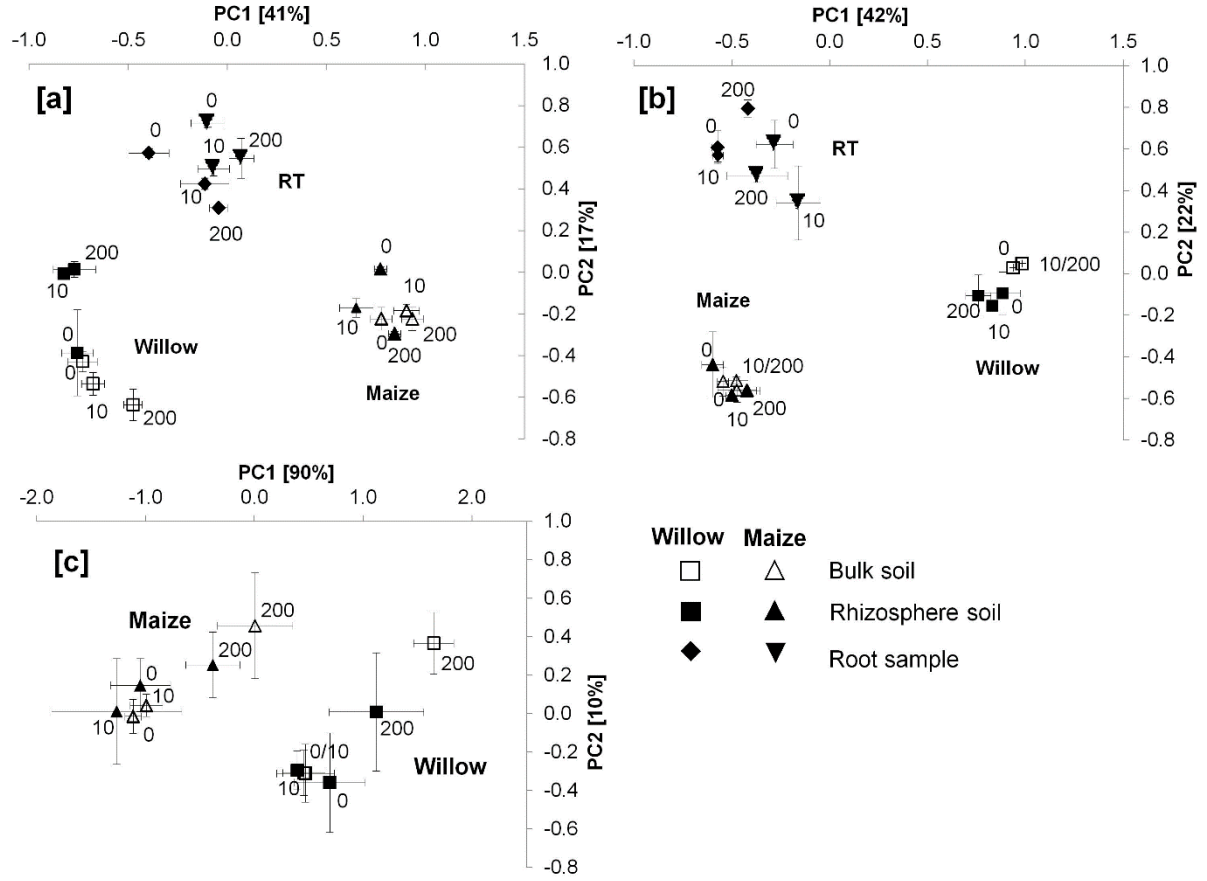

Figure 1. Principle component analyses of [a] *Pseudomonas*, [b] total bacteria (TC) 16S rRNA gene fragment DGGE data, and [c] activity data of selected soil exoenzymes for willow and maize, the soil microhabitats (bulk soil, rhizosphere, root) and treatments (0 = 0 mg SDZ kg^{-1}; 10 = 10 mg SDZ kg^{-1}; 200 = 200 mg SDZ kg^{-1}).

8. Synthesis and Conclusions

The major environmental pathway of antibiotics begins with the administration of antibiotics as therapeutic injection solutions, feed and water additives, as well as ergotropics (Chapter 1.1, Figure 1-1). Afterwards, most of the active parent compound is readily excreted with urine and feces and enters agricultural soil with manure. In soil, dissipation, sequestration, and transformation control the fate of antibiotics and their potency to alter microbial community structures and ecologically relevant functions such as nutrient cycling, decomposition, and biological interactions. Human health risks ultimately arise due to uptake of sub-therapeutic residues from soil as well as of resistant pathogens with food and water.

Veterinary antibiotics inevitably enter the environment after they have been medicated to livestock such as pigs. The medication substantially changes the manure composition by affecting the digestive tract system. Indirect antibiotic effects on the soil microbial community also occur (Chapter 3). Thus, mixed direct and indirect antibiotic effects may hinder a precise differentiation between soil microbial responses to slurry composition or excreted antibiotic compounds. The unequivocal differentiation among indirect effects from manure composition and direct antibiotic effects would require additional laboratory experiments using manure from untreated pigs, whose manure was artificially spiked with antibiotics (Chapter 4-5). An effect of antibiotics such as SDZ and DIF on soil microorganisms, either applied as standard substance or added to soil together with artificially spiked manure, has been previously shown, and numerous publications have used the same soil sample, the same antibiotics, and manure from the same source (Hammesfahr et al., 2008, 2011b, c; Kleineidam et al., 2010; Heuer et al., 2011; Kotzerke et al., 2011). The results obtained using spiked manure can vary slightly among different studies because manure ages over time or was obtained from other animals with a different fodder supply (Chapter 4-5).

In the next pathway step, excreted antibiotics are applied onto agricultural topsoil as organic fertilizer. Manure typically remains on the soil surface until it is ploughed under. This was investigated by experiments in which manure was either placed onto structured topsoil or mixed into soil (Chapter 4-5). Laboratory experiments that consider only homogenized bulk soil are not fully representative for structured field soil. This is because antibiotic concentrations and microbial responses were more variable in structured soil (Chapter 4). Hence, risk assessments based on such laboratory experiments probably underestimate the antibiotic fate and the effects in soil microhabitats, which host most of the microbial biomass and activity. Also in structured field soil, antibiotics were detected within the whole profile, indicated by antibiotic concentrations that were higher at macroaggregate surfaces and along earthworm burrows than in bulk soil (Chapter 4). In soil without manure incorporation, antibiotic effects and fate are expected to be restricted to the topmost soil centimeters where

soil organic matter acts as main sorbent (Thiele-Bruhn, 2003b). Nonetheless, the experiments showed that antibiotics are distributed within the accessible pore system of structured field soil also when contaminated manure is applied onto the soil surface (Chapter 4).

In the next pathway step, the bioaccessible fraction of antibiotics interacts with or is incorporated by soil microorganisms and plants. Indirect and direct antibiotic influences on plant roots and the associated microbial community controlled the antibiotic effects in rhizosphere (Chapters 6 and 7). Hence, concentrations of soil pollutant below the plants' toxicity level are needed to experimentally isolate the direct antibiotic effects on rhizosphere microorganisms. In soils without additional organic amendment, effects of bacteriostatic antibiotics are concentrated at the root cortex (Chapter 7). In soil spiked with 10 mg SDZ kg^{-1}, antibiotic effects on plants and roots were particularly pronounced when that soil additionally received manure (Chapter 4 vs. 6). Here, the antibiotic effects extended more into the adjacent soil than without manure amendment (compare Chapter 4 and 7), indicating complex interactions between manure, roots, and the soil microbial community. Thus, applying manure typically mediates the soil microbial responses towards antibiotics (Chapter 4; Hammesfahr et al., 2008, 2011a, b) and probably also the adverse effects of the antibiotics on plants. No such antibiotic effects on maize plants were determined in soil spiked with manure and concentrations up to 1 mg SDZ kg^{-1} (Chapter 3-4).

Drying and rewetting increases the bioaccessibility of antibiotics and, in combination with moisture stress, also accelerates microbial responsiveness (Chapter 5). This makes the detection of consistent antibiotic fates and effects more difficult under varying weather and soil moisture conditions. Sequestration, dissipation, and transformation processes affect the concentrations and bioaccessibility of antibiotics in soil, particularly in soil with moisture dynamics (Chapter 5), and in rhizosphere (Rosendahl et al., 2011). Moisture gradients are also initiated in rhizosphere soil by plant transpiration and water uptake over the root system (Hinsinger et al., 2005). Indirect effects of moisture and root dynamics on antibiotic fate and effect are probably interconnected within the rhizosphere microhabitat. Hence, in feedback with environment variables such as plant growth and soil moisture status, antibiotic concentrations and the soil microbial community are more variable in rhizosphere soil than in soil without roots (Chapter 3-7). Soil moisture also varies more strongly at aggregate surfaces and earthworm burrows (Standing and Killham, 2007; Ernst et al., 2009). This probably influences the dissipation and bioaccessibility of antibiotics and thus also controlled the microbial responses to this pollutant class in such soil microhabitats (Chapter 4).

Ecological risks to nutrient cycling, decomposition, and biological interactions are largely compensated at the level of the whole community by functional redundancy. Nonetheless, shifts in individual functions are observed within specific soil microhabitats and microbial

populations (Chapter 4 and 7). Hence, at the level of whole soils, structural plasticity of the microbial community secures the functional stability. This minimizes the expected ecological risks to the soil environment emerging from the residual concentrations of pharmaceutical antibiotics, despite dominant antibiotic effects on soil microbial community structures and resistance levels (Chapter 3-5, Heuer et al. 2011a, b). The latter might also increase the human risk of contamination with antibiotic-resistant pathogens (Marshall and Levy, 2011).

9. Summary and Outlook

Summary. This study was based on the previous findings that veterinary antibiotics affect microbial biomass, activity, structural diversity, resistance, and specific microbial functions. Structural responses of the whole microbial community to antibiotics depended on the specific susceptibility of the involved populations such as *Pseudomonas* and members of these. The conducted experiments confirmed that antibiotic effects based on the presence of easily degradable carbon sources such as manure. In the field experiment, the antibiotic effects remained over more than one year, despite the rapid dissipation of bioaccessible SDZ and transformation into poorly or even non-extractable pools. The SDZ-derived structural community shifts were time-dependent and increased over the month-scale, while the mobile antibiotic fractions declined. This confirms the previously reported apparent concentration independency of antibiotic effects in soil. After the first application of antibiotic-contaminated manure, structural community shifts reached a certain level in field soil, while these responses were not potentiated after several consecutive manure applications as expected. The central aim of this study was to evaluate the extent of antibiotic effects in microbial hot spots such as the rhizosphere, earthworm burrows, and different soil macroaggregate fractions, in soil influenced by dynamic soil moisture changes, and in soil subjected to manure of pigs medicated with different antibiotics. The first central hypothesis (H_1) explores if the effects of antibiotic pharmaceuticals on soil microbial communities of bulk and rhizosphere are dissimilar and if manures with different composition mask the detection of microbial responses to antibiotics (Chapter 3). The conducted mesocosm experiment clearly showed that medicating pigs with DIF and SDZ altered the molecular-chemical pattern of slurries. This was determined using pyrolysis-field ionization mass spectrometry (Py-FIMS). Differences between the summed absolute ion intensities of sterols, lipids, and free n-fatty acids were most pronounced. In SDZ-slurry samples, the ion intensity of the sterol class was 3-fold larger that of untreated and DIF-medicated pigs. In DIF-slurry samples, n-fatty acids were more abundant than in slurry of untreated animals. These differences were also reflected by genotypic shifts within the manure bacterial community. The total phospholipid fatty acid ($PLFA_{tot}$) concentration in DIF-slurry samples was more than 2-fold larger than in samples from untreated and SDZ-medicated pigs. Denaturing gradient gel electrophoresis (DGGE) and sequencing analyses of the *Pseudomonas* community indicated that fecal bacteria were able to survive in soil over the mid-term (weeks). Microbial responses to the different slurry compositions were clearly reflected in bulk and rhizosphere soil over more than two months, which increased the variability and temporarily masked the antibiotic effects in soil. Temporarily, the SDZ and DIF dissipation was larger in rhizosphere compared to bulk soil, which was reflected by the microbial responses. The results clearly confirmed the hypothesis (H_1).

Hypothesis (H_2) states that antibiotic fates and effects are different and more pronounced in soil microbial hot-spots such as soil macroaggregate surfaces, earthworm burrows, and rhizosphere microhabitats of structured soil. The laboratory and field experiments indicated that extractable SDZ concentrations are more variable in rhizosphere compared to bulk soil. The extractable SDZ concentrations were 3-fold larger in earthworm burrows compared to bulk soil. In samples of soil macroaggregate surfaces, the extractable SDZ concentration was up to 2.6-fold larger than in the interior. On average, microbial biomass was larger by one-third in uncontaminated rhizosphere soil. The microbial biomass decreased by about 20% in rhizosphere soil after being spiked with 10 mg SDZ kg^{-1}. The PLFA-derived microbial biomass was lowered by 14% and 3% in antibiotic-contaminated rhizosphere as opposed to bulk soil under field conditions. Genotypic shifts within the *Pseudomonas* community structure were more pronounced in SDZ-contaminated earthworm burrows than bulk soils. This was also reflected by the acid phosphatase, which was 4-times lower in SDZ-contaminated burrows. Structural and functional shifts were about 2.5-fold larger in the soil macroaggregate surface versus interior after contamination with SDZ, but this reversed under long-term field conditions. Hence, the revealed influences of soil microhabitat, microbial community composition, and exposure to SDZ on soil microbial responses to antibiotics confirm hypothesis (H_2).

Hypothesis 3 (H_3) states that antibiotic effects on microbial communities are more pronounced in soils which undergo periodic changes in soil moisture by drying-rewetting dynamics. The results clearly showed that dissipation of SDZ was accelerated in permanent moisture soil compared to soil with strong soil moisture dynamics. In contrast, drying and rewetting increased the potentially bioaccessible SDZ concentrations by a factor of 1.7 up to 3.2. The combined stresses – SDZ and strong moisture dynamics – lowered the PLFA-derived microbial biomass by a factor of 1.4 to 4.4. A similar trend was determined under complex field conditions. The susceptibility of the soil microbial community towards SDZ was increased under strong moisture dynamics such as drying-rewetting, supporting hypothesis (H_3).

Hypothesis (H_4) states that plants take up SDZ and that adverse effects on plant physiology and morphology increase with the spiking concentration. Antibiotic uptake by willow plants from soil was calculated at 0.16% and 1.35% of the initially applied 10 and 200 mg SDZ kg^{-1}, respectively. In contrast, the SDZ uptake into maize plants was more than 30-times lower. The parent compound was mainly stored within plant roots. As demonstrated for willow in soil with 200 mg SDZ kg^{-1}, stems and leaves contained 7% and 12% of levels of the parent compound stored in roots. The antibiotic uptake was accompanied with adverse effects on plant and root growth, morphology and function. Stem lengths of willow and maize plants decreased by a factor of 2.2 and 4.5 in soil with nominal 200 mg SDZ kg^{-1}; root length was

about 40% and 80% shorter in willow and maize, respectively. Antibiotic stress increased the total root length more than 1.4-fold in soil spiked with smaller amounts of SDZ, namely 10 mg SDZ kg^{-1}. Additionally, SDZ stress on plants also was reflected by decreasing chlorophyll and root C:N ratios, particularly in soil with 200 mg SDZ kg^{-1}. Overall, hypothesis (H_4) was supported.

The hypothesis (H_5) that SDZ effects are indirectly controlled by antibiotic effects on plant roots and increase with proximity to the root (as a source of microbial activity stimulation) was deduced from the previous results. Willow and maize plant species had the most pronounced influence on the bacterial community structure and microbial-derived exoenzyme activity patterns. The acid phosphatase activity was up to 1.5-times larger in samples from soil planted with maize compared to willow. The structural community responses to SDZ increased with the SDZ spiking concentration and with decreasing distance to plant roots. While indirect SDZ effects on soil microorganisms derived from adverse influences on plants dominated, direct antibiotic effects were more pronounced in root samples. These results confirmed the hypothesis (H_5).

Outlook. Microbial biomass, activity, and community structures are influenced by multiple factors such as soil temperature, moisture, and plant growth, which affect the susceptibility of the community towards antibiotics. The variability in space and time due to weather, seasonal changes, and/ or diurnal cycles on the microbial susceptibility to pollutants probably is reflected by altered dose-response relationships of the microbial community. The details about these aspects are unknown and not covered by standard eco-toxicological tests. Further research is needed to evaluate the toxicological potency of antibiotics in representative standard soils under different static environmental conditions.

Environmental factors such as soil temperature, moisture, and pH continuously change in natural soils. This induces antagonistic processes such as drying and rewetting in soil, to which the microbial community responds differently. Such contrasting gradients might also alter the susceptibility of the soil microbial community to soil pollutants in different ways. This calls for further laboratory experiments at different time scales to evaluate whether positive or negative environmental gradients attenuate or accelerate the microbial susceptibility and thus the potency of antibiotics in soil. This might also support the interpretation of antibiotic effects in plant rhizospheres (as place of pronounced moisture and pH gradients).

The susceptibility of the microbial community towards soil pollutants is higher near roots. Experiments are needed to elucidate the antibiotic effects at different root zones, locating sites of peak microbial responses and antibiotic uptake. Molecular approaches would be very promising. Additionally, the fine-scale distribution of pollutants and microbial populations in rhizosphere are still unexplored. These aspect should be evaluated on undisturbed soil thin-

sections combined with pollutant and microbial population labeling. This approach might also help explain the often observed apparent concentration independency of antibiotic effects in soil. Molecular analytical methods such as quantitative polymerase chain reaction (qPCR), DGGE, sequencing of significant 16S rRNA gene fragments, as well as the detection of selected microbial populations by fluorescence in situ hybridization (FISH) are recommended and might be combined with soil micromorphological approaches.

10. References (Chapters 1-2 and 8)

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11. Appendix

11.1 Supplementary data of Chapter 3

Antibiotic effects on manure and microbial rhizosphere composition

Table S1. Concentrations in (µg kg^{-1}) of mild-solvent extractable and residual sulfadiazine (SDZ), 4-hydroxy-SDZ (4-OH-SDZ), N-acetyl-SDZ (N-Ac-SDZ), difloxacin (DIF) and sarafloxacin (SAR) in bulk and rhizosphere soil over an incubation time of 0 to 63 days. Mean values of four replicates and standard deviations (in parentheses) are listed.

Compound	Soil spiking concentration	Incubation time [days] 0	7	14	28	42	63
[a]Mild-solvent extractable fraction (µg kg^{-1})				Bulk soil			
SDZ	**256**[c]	21.0 (1.5)	7.8 (0.7)	3.0 (1.0)	2.0 (0.0)	1.1 (0.4)	1.0 (0.3)
N-Ac-SDZ	168[c]	70.8 (1.1)	0.7 (0.3)	0.4 (0.4)	n.d.	n.d.	n.d.
4-OH-SDZ	168[c]	7.9 (0.9)	4.1 (1.2)	3.3 (1.8)	2.6 (0.7)	1.6 (0.4)	1.7 (0.0)
DIF	**452**[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SAR	164[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
				Rhizosphere soil			
SDZ		-	-	1.8 (0.4)	0.5* (0.1)	0.9 (0.1)	0.2* (0.3)
N-Ac-SDZ		-	-	4.4* (2.7)	0.4 (0.3)	n.d.	n.d.
4-OH-SDZ		-	-	3.6 (0.8)	1.8 (0.9)	1.6 (0.5)	1.2 (0.6)
DIF		-	-	n.d.	n.d.	n.d.	n.d.
SAR		-	-	n.d.	n.d.	n.d.	n.d.
[b]Residual fraction (µg kg^{-1})				Bulk soil			
SDZ		98.0 (25.8)	96.6 (28.7)	99.0 (10.5)	89.7 (15.7)	65.3 (33.3)	75.2 (12.3)
N-Ac-SDZ		9.9 (1.5)	n.d.	n.d.	n.d.	n.d.	n.d.
4-OH-SDZ		72.6 (14.0)	71.9 (18.2)	72.3 (5.9)	72.4 (12.9)	62.6 (3.6)	63.8 (9.4)
DIF		284.3 (51.4)	275.4 (46.2)	246.6 (48.0)	235.7 (46.6)	211.6 (13.8)	258.4 (42.6)
SAR		6.8 (1.2)	6.6 (1.3)	6.3 (2.1)	5.1 (2.2)	5.0 (0.4)	6.3 (1.1)
				Rhizosphere soil			
SDZ		-	-	88.5 (43.0)	81.6 (1.9)	71.0 (5.6)	61.6 (12.2)
N-Ac-SDZ		-	-	n.d.	n.d.	n.d.	n.d.
4-OH-SDZ		-	-	69.2 (34.5)	71.3 (9.0)	59.7 (7.2)	51.4 (7.2)
DIF		-	-	242.8 (84.0)	196.5 (31.8)	172.2* (18.3)	185.3* (38.2)
SAR		-	-	5.2 (1.8)	4.6 (0.8)	4.2 (0.5)	4.4* (1.0)

* Significantly different concentration compared to the corresponding bulk soil ($p < 0.05$)

a Mild-solvent extractable antibiotic fraction extracted with 0.01 M $CaCl_2$ and MeOH

b SDZ residual fractions determined by an exhaustive microwave extraction; DIF residual fractions determined by accelerated solvent extraction (ASE)

c Soil spiking concentration calculated from the parent compound concentration in slurry

n.d. Not determinable

Table S2. Concentrations of total PLFA ($PLFA_{tot}$; nmol g^{-1} dw) and respective ratios of bacteria-to-fungi (bacteria:fungi) and of Gram-positive-to-Gram-negative bacteria ($Gram^+$:$Gram^-$), as well as the microbial biomass C (µg C_{mic} g^{-1}) and the metabolic quotient (qCO_2; mg CO_2-C (g C_{mic})$^{-1}$ h^{-1}) in the control soil (0 mg kg^{-1} treatment) and in soils receiving control pig slurry artificially spiked with 1 and 10 mg kg^{-1} sulfadiazine (SDZ), 63 days after application. Shown are mean values of four independent bulk and rhizosphere soil replicates (standard deviations in parentheses). Significances are indicated by different letters in rows (ANOVA and post-hoc Tukey-B-test; $p < 0.05$).

Laboratory experiment	**Initial SDZ concentration in soil [mg kg^{-1}]**					
	0	**1**	**10**	**0**	**1**	**10**
	Bulk soil			**Rhizosphere soil**		
C_{mic} [µg g^{-1}]	144.6 ^{ab} (11.3)	139.6 ^{ab} (15.7)	117.3 ^{b} (21.1)	192.8 (8.0) ^{c}	202.9 ^{c} (13.0)	155.0 ^{a} (13.1)
$PLFA_{tot}$ [nmol g^{-1}]	14.9 ^{ab} (1.4)	12.4 ^{a} (2.5)	17.8 ^{b} (1.5)	28.0 ^{c} (2.2)	25.3 ^{c} (3.3)	14.4 ^{ab} (1.4)
qCO_2 [mg CO_2-C (g C_{mic})$^{-1}$ h^{-1}]	1.5 ^{a} (0.2)	1.9 ^{ab} (0.2)	1.6 ^{a} (0.4)	2.4 ^{bc} (0.3)	2.9 ^{c} (0.2)	1.9 ^{ab} (0.6)
Bacteria:fungi	3.1 ^{a} (0.2)	3.0 ^{a} (0.3)	3.2 ^{a} (0.1)	2.0 ^{b} (0.1)	1.9 ^{b} (0.2)	2.3 ^{b} (0.3)
$Gram^+$:$Gram^-$	0.7 ^{a} (0.0)	0.7 ^{a} (0.2)	0.8 ^{a} (0.1)	0.6 ^{a} (0.0)	0.6 ^{a} (0.1)	0.6 ^{a} (0.1)

Table S3. Results of slurry analyses in fresh (fm) and dry weight (dm) provided by Landwirtschaftliche Untersuchungs- und Forschungsanstalt Nordrhein-Westfalen (LUFA NRW).

	Slurry from pigs without medication		**Slurry from SDZ-medicated pigs**		**Slurry from DIF-medicated pigs**	
pH ($CaCl_2$)	6.8		6.9		7.1	
	fm (%)	dm (kg/m^3)	fm (%)	dm (kg/m^3)	fm (%)	dm (kg/m^3)
Dry mass (%)	9.8		12.2		7.3	
Total-N	0.52	5.24	0.43	4.31	0.46	4.59
NH_4-N	0.26	2.62	0.21	2.12	0.25	2.49
P_2O_5	0.51	5.07	0.66	6.63	0.33	3.28
K_2O	0.31	3.12	0.25	2.50	0.34	3.40
MgO	0.31	3.12	0.41	4.15	0.20	1.97
CaO	0.43	4.27	0.69	6.92	0.21	2.10

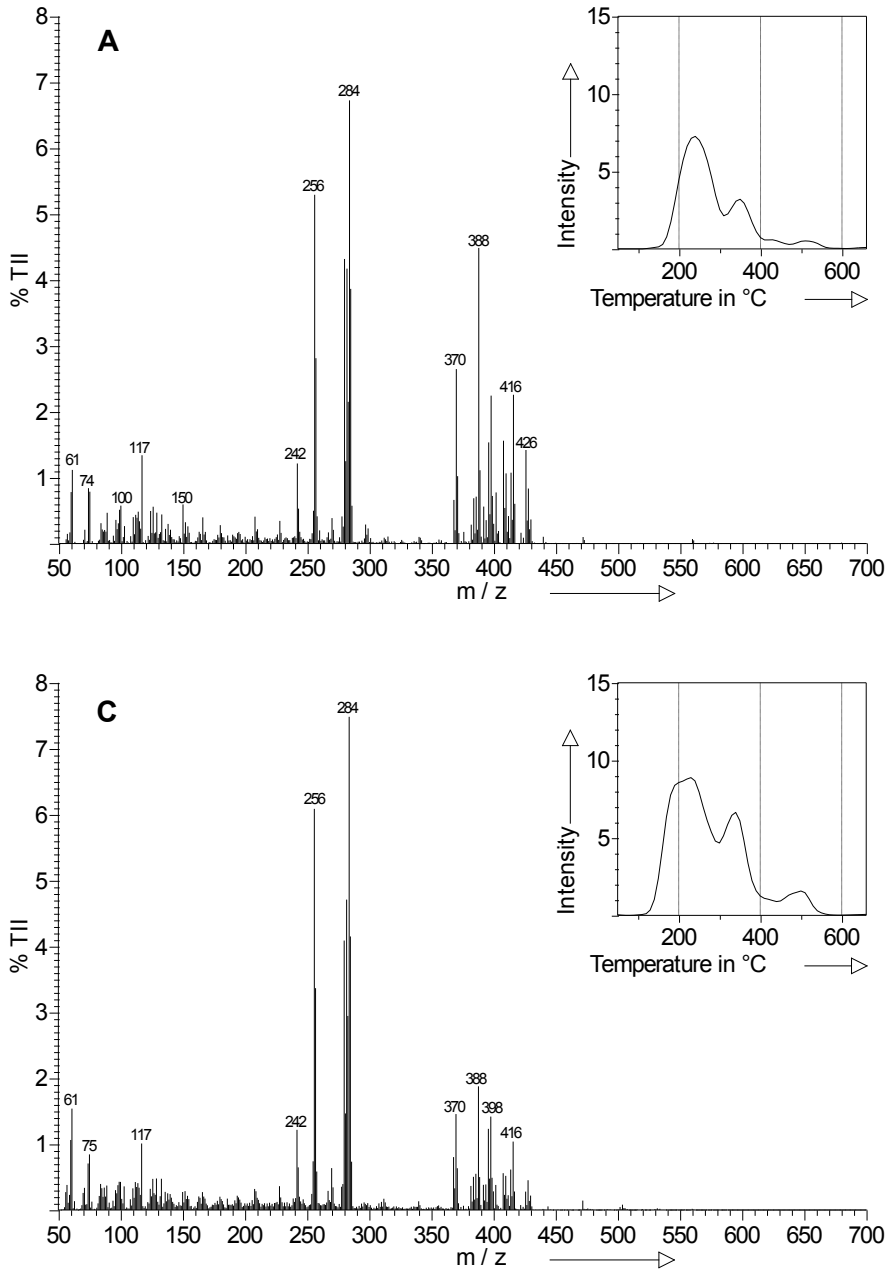


Figure S1: Slurry obtained from pigs [A] without antibiotic medication (control), [B] with SDZ and [C] DIF antibiotic medication. Thermograms of total ion intensity (TII) in 10^6 counts mg^{-1} sample (inserts upper right) and summed and averaged (n = 3) pyrolysis-field ionization mass spectra.

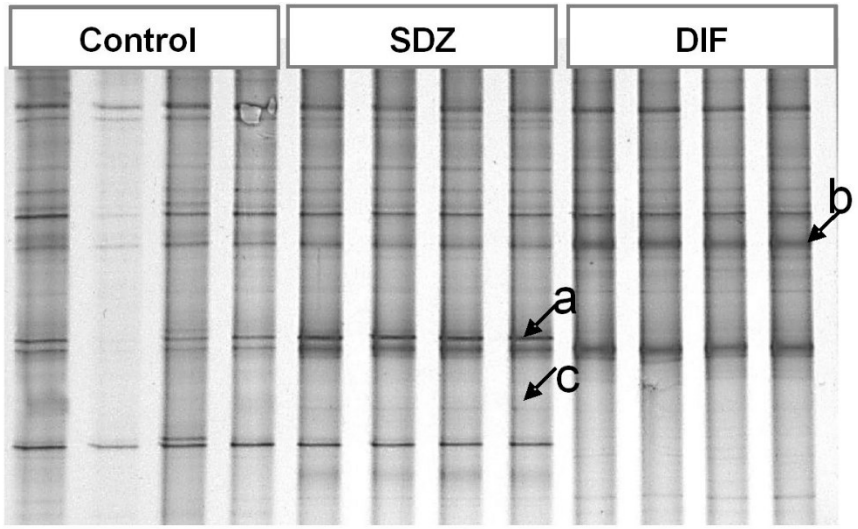


Figure S2: TC-DGGE of slurries from pigs without and after medication with SDZ or DIF. Selected differences are marked: band (a) did not appear in DIF-slurry; band (b) showed higher abundance in DIF-slurry; gene fragments of hardly seen band (c) were exclusively found in the SDZ-slurry.

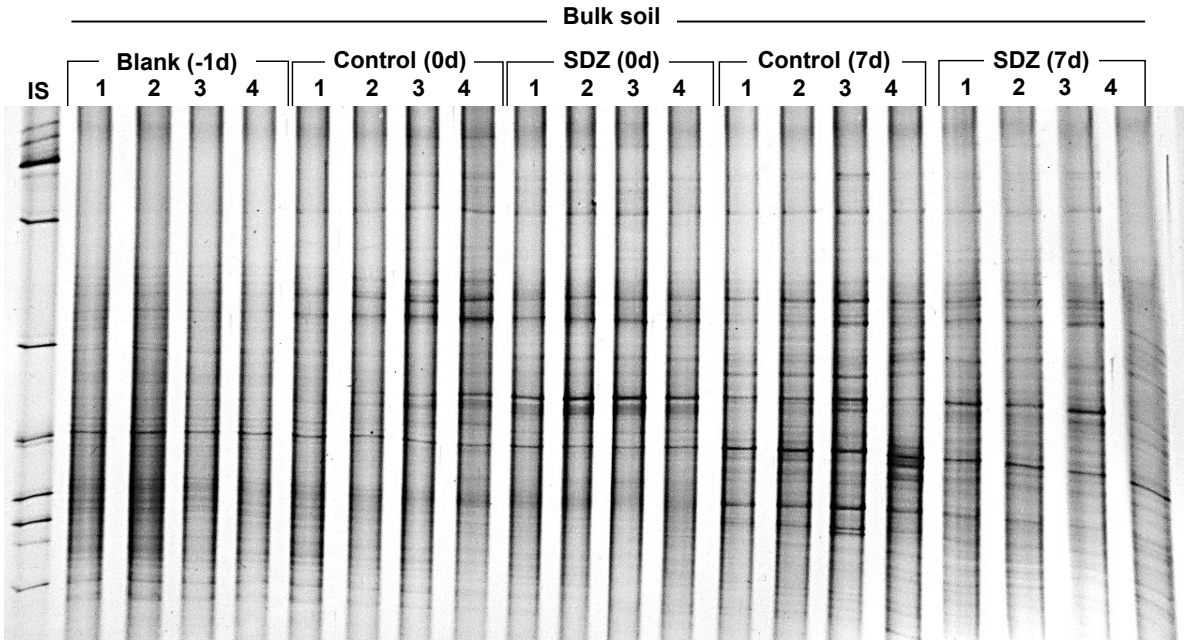
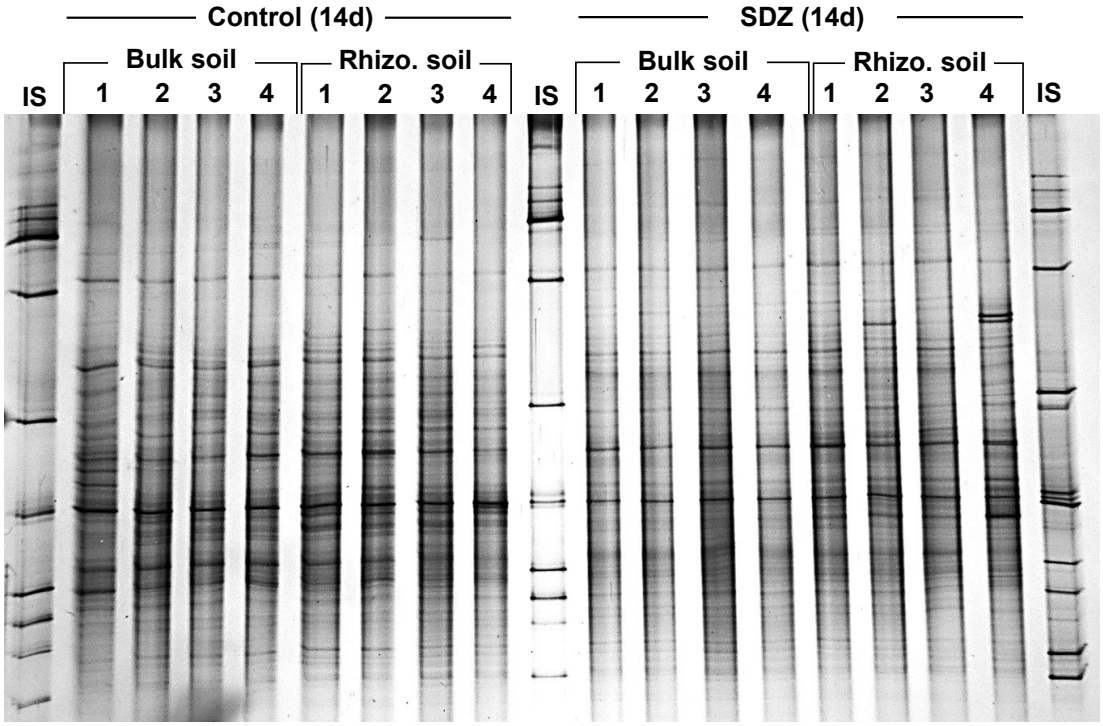
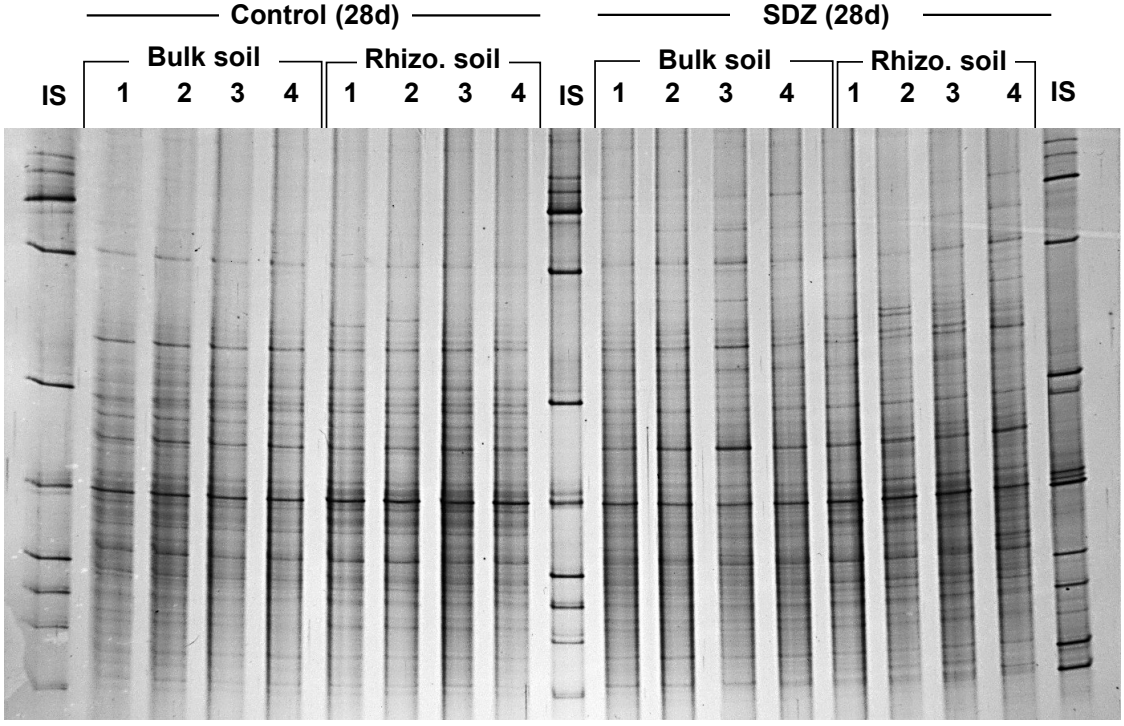


Figure S3: [a] Bulk soil TC-DGGE at day -1 (blank, without slurry), 0 and 7, receiving slurry from pigs without and with SDZ medication.

Figure S3 continued:

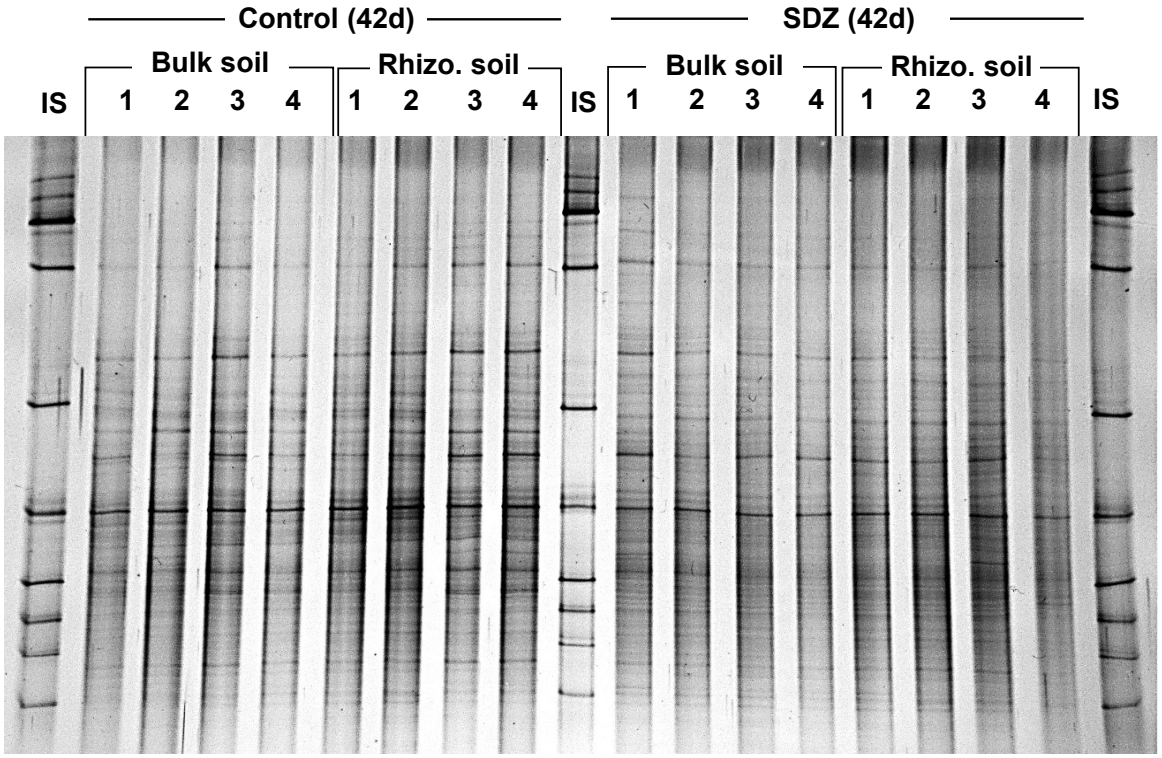


[b] Bulk and rhizosphere soil TC-DGGE at day 14, receiving slurry from pigs without and with SDZ medication

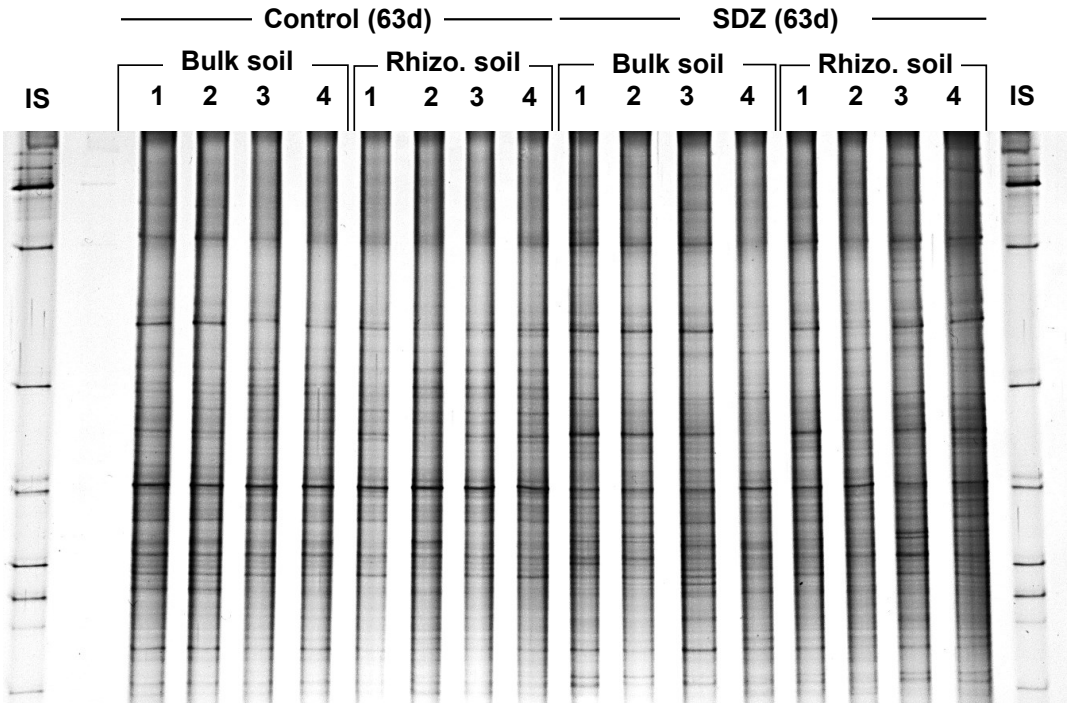


[c] Bulk and rhizosphere soil TC-DGGE at day 28, receiving slurry from pigs without and with SDZ medication.

Figure S3 continued:

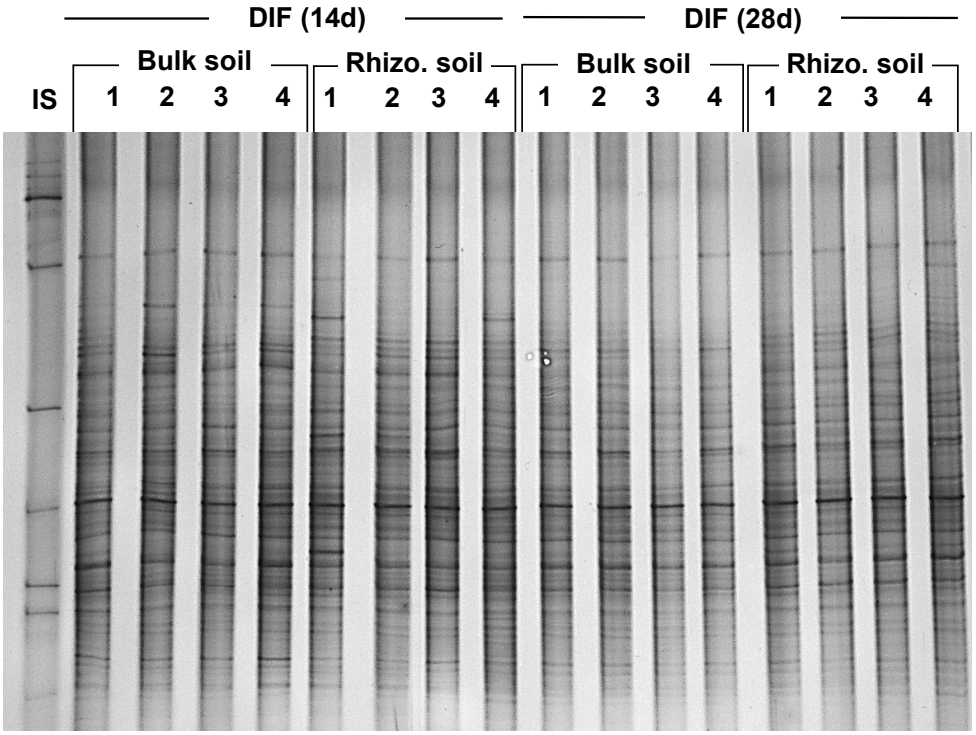


[d] Bulk and rhizosphere soil TC-DGGE at day 42, receiving slurry from pigs without and with SDZ medication.



[e] Bulk and rhizosphere soil TC-DGGE at day 63, receiving slurry from pigs without and with SDZ medication.

Figure S3 continued:



[f] Bulk and rhizosphere soil TC-DGGE at day 14 and 28d, receiving slurry from pigs with DIF medication.

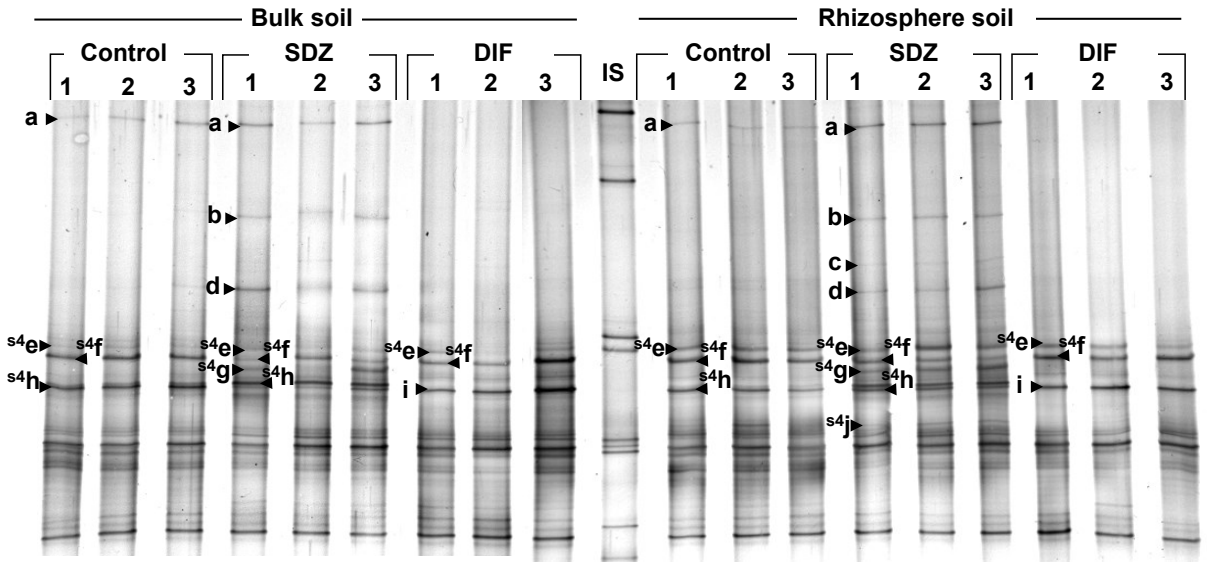


Figure S4. *Pseudomonas* 16S rRNA gene DGGE band patterns from bulk and rhizosphere soil of maize planted mesocosms at incubation time 14 d of control soil (slurry, without antibiotics) and of soils receiving slurry from pigs medicated with SDZ (sulfadiazine) or DIF (difloxacin). Significant changes of bands are marked with small letters. Bands indexed (^{3}e, ^{3}f, ^{3}g, ^{3}h and ^{3}j) were selected for subsequent sequencing (Chapter 3, Tab. 2).

11.2 Supplementary data of Chapter 4

Microbial responses to manure with SDZ in diverse soil microhabitats

Section A: Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectral analysis

The DRIFT-mapping analysis was performed according to Ellerbrock et al. (2009). The DRIFT mapping analysis is defined here as a collection of DRIFT data from measurements along transects of intact earthworm burrows obtained by combining a DRIFT device with an XY-positioning table (Resultec Analytic Equipment, Illerkirchberg, Germany). Automatic DRIFT mapping was performed across several earthworm burrow surfaces in 1.0-mm steps. For each spectrum, 16 scans were compiled to give one spectrum between wavenumbers of 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1}. The background spectra were measured by using a gold target (99%, Infragold, Labsphere, USA) installed at sample surface.

For spectra processing, the software WIN-IR Pro 3.4 (Digilab, Holliston, MA) was used. The reflectance data measured for the crack surfaces were converted to Kubelka–Munk (KM) units (Kubelka, 1948, based on Kubelka and Munk, 1931; Ciani et al., 2005) by using the standard KM transformation as implemented in the software. The spectra were smoothed with the boxcar moving average algorithm applying a factor of 25.

The absorption intensities of C–H stretch vibrations of alkyl groups within the range between 3020 and 2800 cm^{-1} are usually superimposed as a shoulder of the broad OH band reaching from 3750 to 2700 cm^{-1} (Hesse et al., 1984). Therefore the intensities of bands A were measured as the vertical distance from a local “baseline” plotted between tangential points on absorption minima in the region to each of the maxima (Capriel et al., 1995, 1997; Ellerbrock et al., 2009). The peak centers were located between 2948 and 2920 cm^{-1} for asymmetric and between 2864 and 2849 cm^{-1} for symmetric stretch vibrations. Both height values of the absorptions bands were added and denoted as band A. The heights from the baseline of the absorption bands at 1710 cm^{-1} and 1620 cm^{-1} were added and denoted as band B. Those absorption bands refer to hydrophilic groups like those with C=O double bonds that are part of ketones, carboxylic acids, or amides (MacCarthy and Rice, 1985; Hesse et al., 1984; Gottwald and Wachter, 1997). The cumulative peak heights of bands A were related to those of the bands B. The ratio of hydrophobic (i.e., aliphatic, region A in FTIR spectra) to hydrophilic (i.e., carbonyls, etc., region B in FTIR spectra) functional groups may be used as measure of the potential wettability of the soil organic matter (Ellerbrock et al., 2005).

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Section B: Additional microbial analyses of the laboratory rhizosphere experiment

Basal soil respiration was determined on sieved (<2 mm) soil equivalent to 30 g dw and used for metabolic quotient calculations (CO_2-C:C_{mic} ratio) to indicate sub-lethal stress in polluted soils (Joergensen and Emmerling, 2006).

The colony forming units (CFU) of heterotrophic bacteria were counted on R2A low-nutrient media (Difco, Becton-Dickenson, Sparks, MD) after extracting the bacteria from 5 g fresh soil with 0.85% NaCl solution and spreading 100 µl of the 10^{-3} diluted soil solution onto a Petri dish (cf. Calbrix et al., 2007). The dishes, however, were then incubated at 15°C for four days and colonies were counted using the ImageJ 1.44p software (Maryland, USA; Schneider et al., 2012).

The FISH procedure of Bertaux et al. (2007) was adapted to enumerate the total soil bacteria after staining the DNA with DAPI and to determine the metabolically active proportion

through labeling with a oligonucleotide probe EUB338I (GCT GCC TCC CGT AGG AGT) synthesized by Thermo BioSciences (Ulm, Germany). The probe was coupled at the 5´ end with fluorescein (Fluo) and not as described in the protocol with Cy3 dye. DAPI and EUB338I-Fluo signals were visualized with a Zeiss Axio Imager M1 microscope and recorded with the AxioCam MRc CCD camera (Carl Zeiss, Jena, Germany). The system was equipped with: HBO 103 W/2 Hg vapor lamp (Osram, München, Germany), fluorescein filter set 38, DAPI filter set 49, and a Plan-Neofluar 100x objective (Carl Zeiss, Jena, Germany). For each independent replicate, the average of three randomly selected microscopic images (1292x968 pixels, 2x2 binning) of the fluorescein and DAPI signal were recorded and evaluated using the ImageJ 1.44p software (Maryland, USA; Schneider et al., 2012).

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Supplementary tables

Table S1. Laboratory experiments. Two-way ANOVA of microbial parameters of [a] rhizosphere vs. bulk soil, [b] earthworm burrow vs. bulk soil, and [c] macroaggregate interior vs. surface soil. Levene's test verified the equality of variances. Equality assumed: post-hoc test-level $p < 0.05$; not assumed: $p < 0.001$. Eta^2 values indicate the size of the effect. The parameters are listed on the left with units [in brackets]. Post-hoc test results are display as mean values and standard deviation (S.D.). Significances are indicated by different latters over both habitats and treatments at the given *p*-level (test-level).

a) Rhizosphere vs. bulk soil, laboratory experiment

Two-way ANOVA					**Post-hoc tests**					
	Levene's test	*F*-value	Test-level	Eta^2		Bulk soil			Rhizosphere soil		
						0 mg SDZ	**1 mg SDZ**	**10 mg SDZ**	**0 mg SDZ**	**1 mg SDZ**	**10 mg SDZ**
EAS SDZ fraction	0.053	--->	$p < 0.05$								
[µg kg^{-1}]	Rhizosphere vs. bulk soil habitat	0.0	0.986		Means	<LOD	10.9	415.2	<LOD	1.4	426.1
	Treatment	127.8	0.000	0.93	S.D.	<LOD	3.7	49.6	<LOD	1.7	139.1
	Habitat x treatment	0.1	0.944		$p < 0.05$	a	b	c	a	a	c
RES SDZ fraction	0.001	--->	$p < 0.001$								
[µg kg^{-1}]	Rhizosphere vs. bulk soil habitat	0.1	0.786		Means	<LOD	79.3	1,351.8	0.0	75.4	1,287.8
	Treatment	108.7	0.000	0.92	S.D.	<LOD	24.1	89.9	0.0	28.2	482.6
	Habitat x treatment	0.1	0.938		$p < 0.001$	a	b	c	a	b	c
Microbial Biomass	0.309	--->	$p < 0.05$								
(C_{mic}) [µg g^{-1}]	Rhizosphere vs. bulk soil habitat	72.7	0.000	0.80	Means	144.6	139.6	117.3	192.8	202.9	155.0
	Treatment	15.0	0.000	0.63	S.D.	11.3	15.7	21.1	8.0	13.0	13.1
	Habitat x treatment	1.6	0.224		$p < 0.05$	ab	ab	a	c	c	b
Total PLFA	0.531	--->	$p < 0.05$								
[nmol g^{-1}]	Rhizosphere vs. bulk soil habitat	86.3	0.000	0.83	Mean	14.9	12.4	17.8	28.0	25.3	14.4
	Treatment	14.2	0.000	0.61	S.D.	1.4	2.5	1.5	2.2	3.3	1.4
	Habitat x treatment	39.6	0.000	0.82	$p < 0.05$	a	ab	a	bc	c	ab
Bacteria:fungi ratio	0.063	--->	$p < 0.05$								
	Rhizosphere vs. bulk soil habitat	10.8	0.004	0.38	Mean	3.1	3.0	3.2	2.0	1.9	2.3
	Treatment	49.2	0.000	0.85	S.D.	0.2	0.3	0.1	0.1	0.2	0.3
	Habitat x treatment	8.2	0.003	0.48	$p < 0.05$	a	ab	a	bc	c	ab

Table S1 continued.

Gram$^+$:Gram$^-$ ratio	0.043	--->	$p < 0.001$								
	Rhizosphere vs. bulk soil habitat	5.5	0.031		Mean	0.7	0.7	0.8	0.6	0.6	0.6
	Treatment	0.3	0.730		S.D.	0.0	0.2	0.1	0.0	0.1	0.1
	Habitat x treatment	0.5	0.590		$p < 0.001$	a	a	a	a	a	a
PLFA pattern PC1	0.909	--->	$p < 0.05$								
[score value]	Rhizosphere vs. bulk soil habitat	107.2	0.000	0.86	Mean	-0.63	-0.87	-0.28	1.29	1.00	-0.51
	Treatment	13.8	0.000	0.61	S.D.	0.24	0.36	0.21	0.21	0.34	0.28
	Habitat x treatment	38.1	0.000	0.81	$p < 0.05$	a	a	a	b	b	a
	0.002	--->	$p < 0.05$								
PLFA pattern PC2	Rhizosphere vs. bulk soil habitat	0.0	0.964		Mean	-0.01	0.07	-0.07	0.13	-0.11	-0.01
[score value]	Treatment	0.2	0.819		S.D.	0.03	0.15	0.09	0.75	0.04	0.19
	Habitat x treatment	0.5	0.605		$p < 0.05$	a	a	a	a	a	a
Additional microbial	0.04	--->	$p < 0.001$								
analyses PC1	Rhizosphere vs. bulk soil habitat	207.0	0.000	0.92	Mean	-0.50	-0.48	-0.72	0.43	1.52	-0.25
[score value]	Treatment	54.8	0.000	0.86	S.D.	0.05	0.02	0.30	0.23	0.09	0.26
	Habitat x treatment	33.1	0.000	0.79	$p < 0.05$	a	a	a	b	c	a
Additional microbial	0.227	--->	$p < 0.05$								
analyses PC2	Rhizosphere vs. bulk soil habitat	2.0	0.173		Mean	-0.12	-0.05	-0.18	0.81	-0.34	-0.12
[score value]	Treatment	4.4	0.027	0.33	S.D.	0.45	0.30	0.53	0.29	0.54	0.08
	Habitat x treatment	4.8	0.021	0.35	$p < 0.05$	a	a	a	b	a	a
Metabolic quotient	0.019	--->	$p < 0.001$								
(qCO_2) [mg CO_2-C g C_{mic}^{-1} h^{-1}]	Rhizosphere vs. bulk soil habitat	34.9	0.000	0.66	Means	1.5	1.9	1.6	2.4	2.9	1.9
	Treatment	6.7	0.007		S.D.	0.2	0.2	0.4	0.3	0.2	0.6
	Habitat x treatment	2.4	0.115		$p < 0.05$	a	ab	a	bc	c	ab
Bacterial activity											
(FISH)	0.307	--->	$p < 0.05$								
[%-EUB:DAPI]	Rhizosphere vs. bulk soil habitat	0.1	0.806		Means	0.6	0.6	0.6	0.7	0.4	0.5
	Treatment	4.6	0.025	0.34	S.D.	0.1	0.1	0.1	0.1	0.1	0.0
	Habitat x treatment	5.7	0.012	0.39	$p < 0.05$	ab	ab	ab	b	a	ab
Colony forming units											
(CFU)	0.27	--->	$p < 0.05$								
[*10^6 g^{-1}]	Rhizosphere vs. bulk soil habitat	95.3	0.000	0.84	Means	0.6	0.4	0.5	0.8	1.5	0.4
	Treatment	48.7	0.000	0.84	S.D.	0.1	0.1	0.0	0.1	0.1	0.1
	Habitat x treatment	69.7	0.000	0.89	$p < 0.05$	a	a	a	b	c	a

Table S1 continued.

b) Earthworm burrow vs. bulk soil, laboratory experiment

	Levene's test	*F*-value	Test-level	Eta^2		Bulk soil		Burrow soil	
Total SDZ	0.001	--->	$p < 0.001$			**Control**	**SDZ**	**Control**	**SDZ**
[µg kg^{-1}]	Bulk vs. burrow habitat	2.0	0.187		Mean	<LOD	41.7	<LOD	80.5
	Treatment	19.9	0.000	0.67	S.D.	-	33.5	-	45.8
	Habitat x treatment	2.0	0.187		$p < 0.001$	a	b	a	b
Enzyme pattern PC1	0.302	--->	$p < 0.05$						
[score value]	Bulk vs. burrow habitat	51.8	0.000	0.81	Mean	-0.71	-0.44	-0.04	1.19
	Treatment	22.0	0.001	0.65	S.D.	0.14	0.40	0.36	0.32
	Habitat x treatment	8.9	0.011	0.43	$p < 0.05$	a	ab	b	c
Enzyme pattern PC2	0.010	--->	$p < 0.001$						
[score value]	Bulk vs. burrow habitat	1.6	0.237		Mean	-0.06	-0.34	0.44	1.16
	Treatment	1.4	0.252		S.D.	0.18	0.20	-0.04	0.43
	Habitat x treatment	0.1	0.758		$p < 0.001$	a	a	a	a
Pseudomonas PC1	0.111	--->	$p < 0.05$						
DGGE pattern	Bulk vs. burrow habitat	34.4	0.000	0.74	Mean	0.61	-0.74	0.93	-0.79
[score value]	Treatment	4,166.2	0.000	1.00	S.D.	0.05	0.03	0.01	0.07
	Habitat x treatment	59.6	0.000	0.83	$p < 0.05$	b	a	c	a
Pseudomonas PC2	0.113	--->	$p < 0.05$						
DGGE pattern	Bulk vs. burrow habitat	152.6	0.000	0.93	Mean	0.44	0.37	-0.37	-0.44
[score value]	Treatment	1.3	0.282		S.D.	0.11	0.23	0.02	0.07
	Habitat x treatment	0.0	0.972		$p < 0.05$	b	b	a	a
Betaproteobacteria PC1	0.039	--->	$p < 0.001$						
DGGE pattern	Bulk vs. burrow habitat	208.8	0.000	0.95	Mean	-0.54	0.95	-0.85	0.09
[score value]	Treatment	2,334.2	0.000	1.00	S.D.	0.03	0.00	0.43	0.07
	Habitat x treatment	13.1	0.004		$p < 0.001$	a	b	c	d
	0.055	--->	$p < 0.05$						
Betaproteobacteria PC2	Bulk vs. burrow habitat	255.2	0.000	0.96	Mean	0.71	0.06	-0.51	-0.26
DGGE pattern	Treatment	16.7	0.002	0.58	S.D.	0.09	0.00	0.14	0.09
[score value]	Habitat x treatment	85.1	0.000	0.88	$p < 0.05$	a	b	c	d

Table S1 continued.

c) Macroaggregate interior vs. surface soil, laboratory experiment

	Levene's test	*F*-value	Test-level	Eta^2		Interior		Sufrace	
Total SDZ	0.001	--->	$p < 0.001$			**Control**	**SDZ**	**Control**	**SDZ**
[µg kg^{-1}]	Surface vs. interior habitat	4.5	0.059		Mean	<LOD	500.2	<LOD	1,220.3
	Treatment	26.0	0.000	0.72	S.D.	-	349.7	-	605.2
	Habitat x treatment	4.5	0.059		$p < 0.001$	a	b	a	c
Total PLFA	0.041	--->	$p < 0.001$						
[nmol g^{-1}]	Surface vs. interior habitat	74.2	0.000	0.86	Mean	39.3	44.9	65.5	79.4
	Treatment	7.6	0.017		S.D.	9.1	3.0	9.5	4.1
	Habitat x treatment	1.4	0.263		$p < 0.001$	a	ab	bc	c
Bacteria:fungi ratio	0.012	--->	$p < 0.001$						
	Surface vs. interior habitat	6.5	0.026		Mean	1.9	1.9	1.8	1.5
	Treatment	1.8	0.203		S.D.	0.4	0.0	0.1	0.1
	Habitat x treatment	0.6	0.448		$p < 0.001$	a	a	a	a
Gram$^+$:Gram$^-$ ratio	0.006	--->	$p < 0.001$						
	Surface vs. interior habitat	2.4	0.150		Mean	1.1	0.8	0.7	0.7
	Treatment	1.2	0.295		S.D.	0.5	0.0	0.1	0.0
	Habitat x treatment	1.8	0.201		$p < 0.001$	a	a	a	a
PLFA pattern PC1	0.004	--->	$p < 0.001$						
[score value]	Surface vs. interior habitat	54.9	0.000	0.82	Mean	-0.79	0.34	-0.53	1.05
	Treatment	8.3	0.014		S.D.	0.28	0.57	0.13	0.22
	Habitat x treatment	2.0	0.179		$p < 0.001$	a	a	ab	b
PLFA pattern PC2	0.005	--->	$p < 0.001$						
[score value]	Surface vs. interior habitat	0.0	0.966		Mean	0.21	-0.22	0.04	-0.03
	Treatment	1.2	0.288		S.D.	0.82	0.03	0.33	0.02
	Habitat x treatment	0.7	0.429		$p < 0.001$	a	a	a	a
Enzyme pattern PC1	0.142	--->	$p < 0.05$						
[score value]	Surface vs. interior habitat	34.3	0.000	0.74	Mean	-0.85	-0.81	0.89	0.77
	Treatment	0.0	0.887		S.D.	0.37	0.29	0.85	0.58
	Habitat x treatment	0.1	0.781		$p < 0.05$	a	a	b	b
Enzyme pattern PC2	0.623	--->	$p < 0.05$						
[score value]	Surface vs. interior habitat	1.4	0.256		Mean	0.09	-0.20	0.00	0.10
	Treatment	1.2	0.300		S.D.	0.18	0.10	0.24	0.17
	Habitat x treatment	4.9	0.050		$p < 0.05$	a	a	a	a

Table S1 continued.

Betaproteobacteria PC1	0.433	--->	$p < 0.05$						
DGGE pattern	Surface vs. interior habitat	212.2	0.000	0.95	Mean	0.22	0.77	-0.49	-0.49
[score value]	Treatment	16.5	0.002	0.58	S.D.	0.10	0.08	0.16	0.18
	Habitat x treatment	16.5	0.002	0.58	$p < 0.05$	b	c	a	a
Betaproteobacteria PC2	0.433	--->	$p < 0.05$						
DGGE pattern	Surface vs. interior habitat	2.2	0.163		Mean	-0.26	0.13	0.66	0.14
[score value]	Treatment	21.7	0.001	0.64	S.D.	0.17	0.11	-0.53	0.25
	Habitat x treatment	81.2	0.000	0.87	$p < 0.05$	a	b	c	a
Pseudomonas PC1	0.001	--->	$p < 0.001$						
DGGE pattern	Surface vs. interior habitat	3.5	0.086		Mean	-0.44	0.14	-0.58	0.88
[score value]	Treatment	41.7	0.000	0.78	S.D.	0.31	0.52	0.16	0.00
	Habitat x treatment	7.8	0.016		$p < 0.001$	a	ab	a	b
Pseudomonas PC2	0.028	--->	$p < 0.05$						
DGGE pattern	Surface vs. interior habitat	17.4	0.001	0.59	Mean	0.67	0.03	-0.61	-0.09
[score value]	Treatment	0.1	0.711		S.D.	0.54	0.24	0.32	0.00
	Habitat x treatment	11.8	0.005	0.50	$p < 0.05$	a	ab	b	ab

Table S2. Activities of soil exoenzymes and the calculated total activity of earthworm burrow vs. bulk soil of the laboratory experiment [a] and field experiment [b], as well as of interior and surface samples of soil macroaggregates of laboratory experiment [c] and field experiment [d] in nmol MUB (AMC) g^{-1} h^{-1}. Shown are mean values of four independent replicates (standard deviations in parentheses). Significant differences ($p < 0.05$) are indicated by different latters between the treatments and microhabitats of each laboratory and field experiment, respectively.

Exoenzyme activity	**Laboratory experiment[a]**				**Field experiment[b]**			
[nmol MUB (AMC) g^{-1} h^{-1}]	[a] Earthworm burrow		Bulk soil		[b] Earthworm burrow		Bulk soil	
	Control	SDZ	Control	SDZ	Control	SDZ	Control	SDZ
α-glucosidase	249 [a] (25)	252 [a] (8)	261 [a] (6)	255 [a] (20)	360 [a] (16)	358 [a] (18)	372 [a] (9)	388 [a] (17)
Chitinase	225 [a] (26)	259 [a] (22)	206 [a] (11)	203 [a] (16)	295 [a] (27)	280 [a] (24)	285 [a] (23)	276 [a] (12)
ß-xylosidase	178 [a] (68)	165 [a] (13)	139 [a] (7)	146 [a] (18)	229 [a] (21)	227 [a] (7)	239 [a] (28)	249 [a] (16)
Cellobiohydrolase	186 [a] (38)	188 [a] (23)	162 [a] (7)	164 [a] (16)	247 [a] (24)	235 [a] (22)	224 [a] (10)	255 [a] (25)
Leucin-aminopeptidase	2,019 [a] (233)	2,110 [a] (157)	1,794 [a] (59)	1,772 [a] (52)	2,137 [a] (54)	2,038 [a] (71)	1,973 [a] (132)	2,033 [a] (104)
Acid phosphatase	2,060 [a] (253)	1,691 [b] (29)	2,136 [a] (30)	2,023 [a] (116)	1,815 [a] (75)	1,779 [a] (61)	1,923 [a] (111)	1,916 [a] (96)
Calculated total exoenzyme activity	4,917 [a] (609)	4,665 [a] (151)	4,698 [a] (96)	4,564 [a] (152)	5,083 [a] (46)	4,916 [a] (125)	5,016 [a] (205)	5,117 [a] (180)
	[c] Macroaggregate surface		Interior		[d] Macroaggregate surface		Interior	
α-glucosidase	103 [a] (7)	105 [a] (1)	71 [b] (17)	69 [b] (6)	395 [a] (46)	413 [ab] (34)	465 [b] (13)	427 [ab] (8)
Chitinase	237 [a] (89)	254 [a] (25)	131 [b] (23)	113 [b] (16)	318 [a] (30)	313 [a] (29)	357 [a] (12)	316 [a] (5)
ß-xylosidase	103 [a] (6)	113 [a] (7)	93 [ab] (24)	81 [b] (11)	272 [a] (14)	276 [a] (19)	308 [b] (16)	280 [a] (7)
Cellobiohydrolase	146 [a] (20)	163 [a] (41)	108 [a] (29)	134 [a] (20)	272 [a] (16)	268 [a] (20)	311 [b] (9)	272 [a] (11)
Leucin-aminopeptidase	1,150 [a] (159)	1,115 [a] (124)	793 [b] (77)	800 [b] (60)	1,870 [a] (198)	1,898 [a] (118)	2,166 [b] (53)	1,872 [a] (105)
Acid phosphatase	683 [a] (69)	663 [a] (39)	626 [a] (42)	689 [a] (22)	2,217 [a] (170)	2,196 [a] (158)	2,467 [b] (94)	2,237 [a] (129)
Calculated total exoenzyme activity	2,422 [a] (330)	2,412 [a] (188)	1,822 [b] (130)	1,886 [b] (110)	5,344 [a] (424)	5,364 [a] (345)	6,073 [b] (163)	5,404 [a] (236)

[a] 14 days after the initial application of pig manure; [b] 252 days after the last of two consecutive manure applications; n.d not determined

Table S3. Field experiments. Two-way ANOVA of microbial parameters of [a] rhizosphere vs. bulk soil, [b] earthworm burrow vs. bulk soil, and [c] macroaggregate interior vs. surface soil. Levene's test verified the equality of variances. Equality assumed: post-hoc test-level $p < 0.05$; not assumed: $p < 0.001$. Eta^2 values indicate the size of the effect. The parameters are listed on the left with units [in brackets]. Post-hoc test results are display as mean values and standard deviation (S.D.). Significances are indicated by different latters over both habitats and treatments at the given *p*-level (test-level).

a) Rhizosphere vs. bulk soil, field experiment

Two-way ANOVA						**Post-hoc tests**			
	Levene's test	*F*-value	Test-level	Eta^2		Bulk soil		Rhizosphere soil	
EAS SDZ fraction	n.d.	--->	$p < 0.05$	Mann-Whitney-U		**Control**	**SDZ**	**Control**	**SDZ**
48 d [µg kg^{-1}]	Rhizosphere vs. bulk soil habitat	n.d.	n.d.		Mean	<LOD	4.7	<LOD	<LOD
	Treatment	n.d.	n.d.		S.D.	-	3.3	-	-
	Habitat x treatment	n.d.	n.d.		$p < 0.001$	a	b	a	a
RES SDZ fraction	n.d.	--->	$p < 0.05$	Mann-Whitney-U					
48 d [µg kg^{-1}]	Rhizosphere vs. bulk soil habitat	n.d.	n.d.		Mean	<LOD	154	<LOD	104
	Treatment	n.d.	n.d.		S.D.	-	18	-	68
	Habitat x treatment	n.d.	n.d.		$p < 0.05$	a	b	a	b
EAS SDZ fraction	n.d.	--->	$p < 0.05$	Mann-Whitney-U					
132 d [µg kg^{-1}]	Rhizosphere vs. bulk soil habitat	n.d.	n.d.		Mean	<LOD	<LOD	<LOD	<LOD
	Treatment	n.d.	n.d.		S.D.	-	-	-	-
	Habitat x treatment	n.d.	n.d.		$p < 0.001$	-	-	-	-
RES SDZ fraction	n.d.	--->	$p < 0.05$	Mann-Whitney-U					
132 d [µg kg^{-1}]	Rhizosphere vs. bulk soil habitat	n.d.	n.d.		Mean	<LOD	93	<LOD	111
	Treatment	n.d.	n.d.		S.D.	-	29	-	93
	Habitat x treatment	n.d.	n.d.		$p < 0.05$	a	b	a	b
Total PLFA	0.328	--->	$p < 0.05$						
48 d [nmol g^{-1}]	Rhizosphere vs. bulk soil habitat	13.6	0.003	0.53	Mean	18.5	19.1	15.9	13.7
	Treatment	0.6	0.447		S.D.	2.2	3.2	1.3	1.4
	Habitat x treatment	1.6	0.224		$p < 0.05$	b	b	ab	a
Total PLFA	0.048	--->	$p < 0.001$						
132 d [nmol g^{-1}]	Rhizosphere vs. bulk soil habitat	223.5	0.000	0.95	Mean	26.7	23.0	44.1	38.0
	Treatment	20.3	0.001		S.D.	0.3	1.8	2.6	2.9
	Habitat x treatment	1.2	0.291		$p < 0.001$	a	a	b	c

Table S3 continued.

Bacteria:fungi ratio	0.046	--->	$p < 0.001$						
48 d	Rhizosphere vs. bulk soil habitat	3.9	0.071		Mean	4.5	3.2	2.5	2.5
	Treatment	0.8	0.38		S.D.	2.1	1.6	0.1	0.3
	Habitat x treatment	0.9	0.369		$p < 0.001$	a	a	a	a
	0.046	--->	$p < 0.001$						
Bacteria:fungi ratio	Rhizosphere vs. bulk soil habitat	1.5	0.242		Mean				
132 d	Treatment	0.3	0.623		S.D.	0.6	0.9	0.5	1.0
	Habitat x treatment	0.2	0.693		$p < 0.05$	a	a	a	a
$Gram^+$:$Gram^-$ ratio	0.098	--->	$p < 0.001$						
48 d	Rhizosphere vs. bulk soil habitat	3.6	0.082		Mean	1.1	1.0	0.9	0.8
	Treatment	1.4	0.258		S.D.	0.3	0.2	0.0	0.1
	Habitat x treatment	0.0	0.856		$p < 0.001$	a	a	a	a
$Gram^+$:$Gram^-$ ratio	0.074	--->	$p < 0.05$						
132 d	Rhizosphere vs. bulk soil habitat	6.7	0.023	0.36	Mean	1.0	1.0	1.2	1.0
	Treatment	4.2	0.063		S.D.	0.0	0.1	0.2	0.1
	Habitat x treatment	8.2	0.014	0.41	$p < 0.05$	a	a	a	b
PLFA pattern PC1	0.056	--->	$p < 0.05$						
48 d [score value]	Rhizosphere vs. bulk soil habitat	11.9	0.005	0.50	Mean	-0.74	-0.28	-0.75	-0.94
	Treatment	1.8	0.200		S.D.	0.36	0.03	0.09	0.08
	Habitat x treatment	11.6	0.005	0.49	$p < 0.05$	a	b	a	a
PLFA pattern PC2	0.040	--->	$p < 0.001$						
48 d [score value]	Rhizosphere vs. bulk soil habitat	0.0	0.913		Mean	0.34	-0.25	0.04	0.00
	Treatment	2.4	0.148		S.D.	0.57	0.56	0.03	0.03
	Habitat x treatment	1.9	0.196		p < 0.001	a	a	a	a
PLFA pattern PC1	0.255	--->	$p < 0.05$						
132 d [score value]	Rhizosphere vs. bulk soil habitat	342.4	0.000	0.97	Mean	0.19	-0.22	1.60	1.15
	Treatment	32.4	0.000	0.73	S.D.	0.04	0.15	0.17	0.19
	Habitat x treatment	0.1	0.775		$p < 0.05$	b	a	d	c
PLFA pattern PC2	0.057	--->	$p < 0.05$						
132 d [score value]	Rhizosphere vs. bulk soil habitat	1.1	0.305		Mean	-0.10	-0.06	0.20	-0.18
	Treatment	4.4	0.057		S.D.	0.09	0.07	0.24	0.19
	Habitat x treatment	6.6	0.024	0.36	$p < 0.05$	ab	ab	b	a
Pseudomonas PC1	0.127	--->	$p < 0.05$						
DGGE pattern 48 d	Rhizosphere vs. bulk soil habitat	346.2	0.000	0.97	Mean	0.67	0.38	-0.23	-0.50
[score value]	Treatment	34.1	0.000	0.74	S.D.	0.17	0.07	0.05	0.04
	Habitat x treatment	0.0	0.846		$p < 0.05$	a	b	c	d

Table S3 continued.

Pseudomonas PC2	0.077	--->	$p < 0.05$						
DGGE pattern 48 d	Rhizosphere vs. bulk soil habitat	330.6	0.000	0.97	Mean	-0.13	-0.19	0.81	0.52
[score value]	Treatment	15.2	0.002	0.56	S.D.	0.11	0.08	0.02	0.12
	Habitat x treatment	6.2	0.028	0.34	$p < 0.05$	a	a	b	c
Pseudomonas PC1	0.35	--->	$p < 0.05$						
DGGE pattern 132 d	Rhizosphere vs. bulk soil habitat	376.2	0.000	0.97	Mean	0.54	0.55	-0.72	-0.69
[score value]	Treatment	0.1	0.750		S.D.	0.20	0.11	0.10	0.07
	Habitat x treatment	0.0	0.831		$p < 0.05$	a	a	b	b
Pseudomonas PC2	0.087	--->	$p < 0.05$						
DGGE pattern 132 d	Rhizosphere vs. bulk soil habitat	56.8	0.000	0.83	Mean	-0.12	0.05	-0.49	-0.45
[score value]	Treatment	3.4	0.090		S.D.	0.15	0.03	0.02	0.17
	Habitat x treatment	1.3	0.270		$p < 0.05$	a	a	b	b
Betaproteobacteria PC1	0.003	--->	$p < 0.001$						
DGGE pattern 48 d	Rhizosphere vs. bulk soil habitat	5,388.0	0.000	1.00	Mean	-0.76	-0.84	0.75	0.74
[score value]	Treatment	5.1	0.043		S.D.	0.07	0.03	0.03	0.00
	Habitat x treatment	3.0	0.106		$p < 0.001$	a	a	b	b
Betaproteobacteria PC2	0.013	--->	$p < 0.001$						
DGGE pattern 48 d	Rhizosphere vs. bulk soil habitat	46.2	0.000	0.79	Mean	0.31	-0.07	0.51	0.47
[score value]	Treatment	15.1	0.002		S.D.	0.06	0.21	0.05	0.00
	Habitat x treatment	10.0	0.008		$p < 0.001$	a	b	a	a
Betaproteobacteria PC1	0.026	--->	$p < 0.001$						
DGGE pattern 132 d	Rhizosphere vs. bulk soil habitat	4,769.3	0.000	1.00	Mean	-0.86	-0.86	0.98	0.86
[score value]	Treatment	6.2	0.028		S.D.	0.03	0.02	0.02	0.10
	Habitat x treatment	5.1	0.043		$p < 0.001$	a	a	b	b
Betaproteobacteria PC2	0.164	--->	$p < 0.05$						
DGGE pattern 132 d	Rhizosphere vs. bulk soil habitat	165.1	0.000	0.93	Mean	-0.17	-0.16	-0.54	-0.36
[score value]	Treatment	16.4	0.002	0.58	S.D.	0.04	0.06	0.01	0.05
	Habitat x treatment	13.2	0.003	0.52	$p < 0.05$	a	a	b	c

Table S3 continued.

b) Earthworm burrow vs. bulk soil, field experiment

	Levene's test	*F*-value	Test-level	Eta^2		Bulk soil		Burrow soil	
Total SDZ	0.000	--->	$p < 0.001$			**Control**	**SDZ**	**Control**	**SDZ**
[µg kg^{-1}]	Bulk vs. burrow habitat	129.3	0.000	0.93	Mean	<LOD	51.3	<LOD	99.8
	Treatment	1258.1	0.000	0.99	S.D.	-	1.1	-	8.8
	Habitat x treatment	129.3	0.000	0.93	$p < 0.001$	a	b	a	c
Enzyme pattern PC1	0.335	--->	$p < 0.05$						
[score value]	Bulk vs. burrow habitat	10.7	0.007	0.47	Mean	0.66	0.34	-0.67	-0.33
	Treatment	0.0	0.986		S.D.	0.74	0.76	0.47	0.40
	Habitat x treatment	1.2	0.299		$p < 0.05$	a	ab	b	ab
Enzyme pattern PC2	0.494	--->	$p < 0.05$						
[score value]	Bulk vs. burrow habitat	0.6	0.456		Mean	0.01	-0.25	-0.20	0.44
	Treatment	0.3	0.567		S.D.	0.91	0.61	0.41	0.49
	Habitat x treatment	2.0	0.181		$p < 0.05$	a	a	a	a
Pseudomonas PC1	0.062	--->	$p < 0.05$						
DGGE pattern	Bulk vs. burrow habitat	113.3	0.000	0.90	Mean	-0.43	-0.34	1.16	-0.39
[score value]	Treatment	100.5	0.000	0.89	S.D.	0.12	0.26	0.06	0.03
	Habitat x treatment	128.2	0.000	0.91	$p < 0.05$	a	a	b	a
Pseudomonas PC2	0.772	--->	$p < 0.05$						
DGGE pattern	Bulk vs. burrow habitat	47.9	0.000	0.80	Mean	-0.27	-0.36	0.00	0.63
[score value]	Treatment	8.6	0.012	0.42	S.D.	0.24	0.17	0.16	0.16
	Habitat x treatment	15.3	0.002	0.56	$p < 0.05$	a	a	a	b
Betaproteobacteria PC1	0.000	--->	$p < 0.001$						
DGGE pattern	Bulk vs. burrow habitat	704.8	0.000	0.98	Mean	-0.76	-0.85	0.57	1.05
[score value]	Treatment	10.2	0.008		S.D.	0.16	0.02	0.00	0.18
	Habitat x treatment	21.8	0.000	0.65	$p < 0.001$	a	a	b	c
	0.000	--->	$p < 0.001$						
Betaproteobacteria PC2	Bulk vs. burrow habitat	2.8	0.121		Mean	0.20	0.02	-0.63	0.41
DGGE pattern	Treatment	11.0	0.006		S.D.	0.35	0.00	0.00	0.38
[score value]	Habitat x treatment	21.7	0.000	0.64	$p < 0.001$	ab	ab	a	c

Table S3 continued.

c) Macroaggregate interior vs. surface soil, field experiment

	Levene's test	*F*-value	Test-level	Eta^2		Interior		Sufrace	
Total SDZ	0.002	--->	$p < 0.001$			**Control**	**SDZ**	**Control**	**SDZ**
[µg kg^{-1}]	Surface vs. interior habitat	10.6	0.009		Mean	<LOD	60.9	<LOD	94.4
	Treatment	227.6	0.000	0.96	S.D.	-	14.3	-	15.8
	Habitat x treatment	10.6	0.009		$p < 0.001$	a	b	a	b
Enzyme pattern PC1	0.330	--->	$p < 0.05$						
[score value]	Surface vs. interior habitat	5.5	0.037	0.31	Mean	1.21	-0.36	-0.44	-0.41
	Treatment	4.4	0.057		S.D.	0.36	0.63	1.01	0.78
	Habitat x treatment	4.8	0.049	0.29	$p < 0.05$	a	b	b	b
Enzyme pattern PC2	0.777	--->	$p < 0.05$						
[score value]	Surface vs. interior habitat	0.1	0.803		Mean	-0.06	0.11	0.05	-0.09
	Treatment	0.0	0.929		S.D.	0.28	0.28	0.46	0.34
	Habitat x treatment	0.8	0.393		$p < 0.05$	a	a	a	a
Pseudomonas PC1	0.746	--->	$p < 0.05$						
DGGE pattern	Surface vs. interior habitat	30.7	0.000	0.72	Mean	0.89	-0.42	0.06	-0.53
[score value]	Treatment	124.0	0.000	0.91	S.D.	0.12	0.22	0.13	0.20
	Habitat x treatment	17.4	0.001	0.59	$p < 0.05$	a	c	b	c
Pseudomonas PC2	0.027	--->	$p < 0.001$						
DGGE pattern	Surface vs. interior habitat	58.0	0.000	0.83	Mean	0.17	0.70	-0.57	-0.30
[score value]	Treatment	12.1	0.005		S.D.	0.05	0.27	0.34	0.14
	Habitat x treatment	1.2	0.279		$p < 0.001$	a	ab	b	b
Betaproteobacteria PC1	0.121	--->	$p < 0.05$						
DGGE pattern	Surface vs. interior habitat	0.1	0.735		Mean	-0.77	0.70	-0.46	0.53
[score value]	Treatment	38.8	0.000	0.76	S.D.	0.15	0.19	0.61	0.43
	Habitat x treatment	1.4	0.246		$p < 0.05$	a	b	a	b
	0.833	--->	$p < 0.05$						
Betaproteobacteria PC2	Surface vs. interior habitat	0.7	0.422		Mean	0.03	-0.17	-0.21	0.34
DGGE pattern	Treatment	1.1	0.310		S.D.	0.38	0.40	0.23	0.29
[score value]	Habitat x treatment	5.0	0.054		$p < 0.05$	a	a	a	a

Supplementary figures

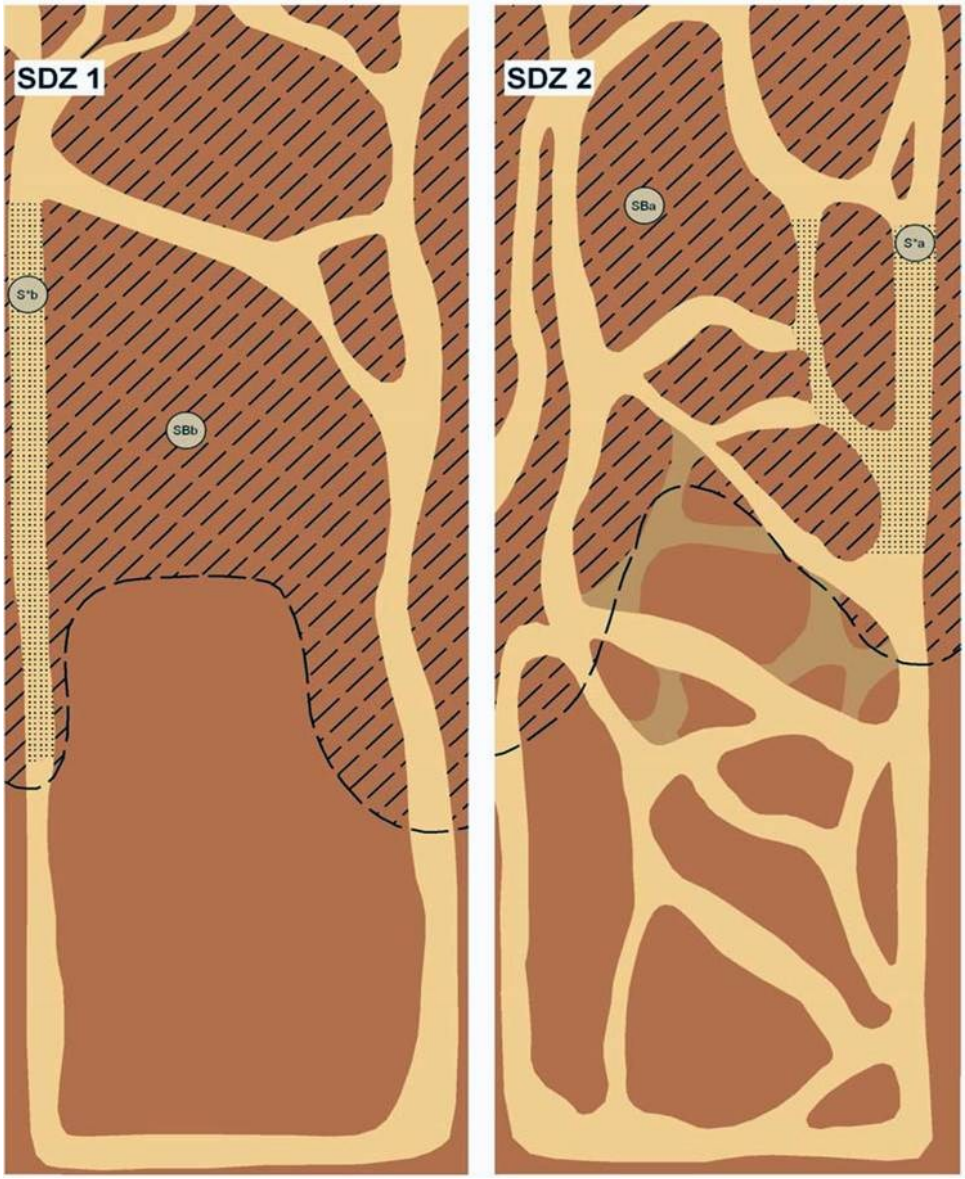


Figure S1. Two selected 2D-terraria with *Lumbricus terrestris* (L.) burrows of the laboratory experiment revealed after 30-d pre-incubation. Earthworms were extracted from soil before manure application. The dashed area marks the infiltration depth of manure into the soil cuvettes. DIRFT samples were obtained from the dotted area, those for microbial analyses from grey circled spots.

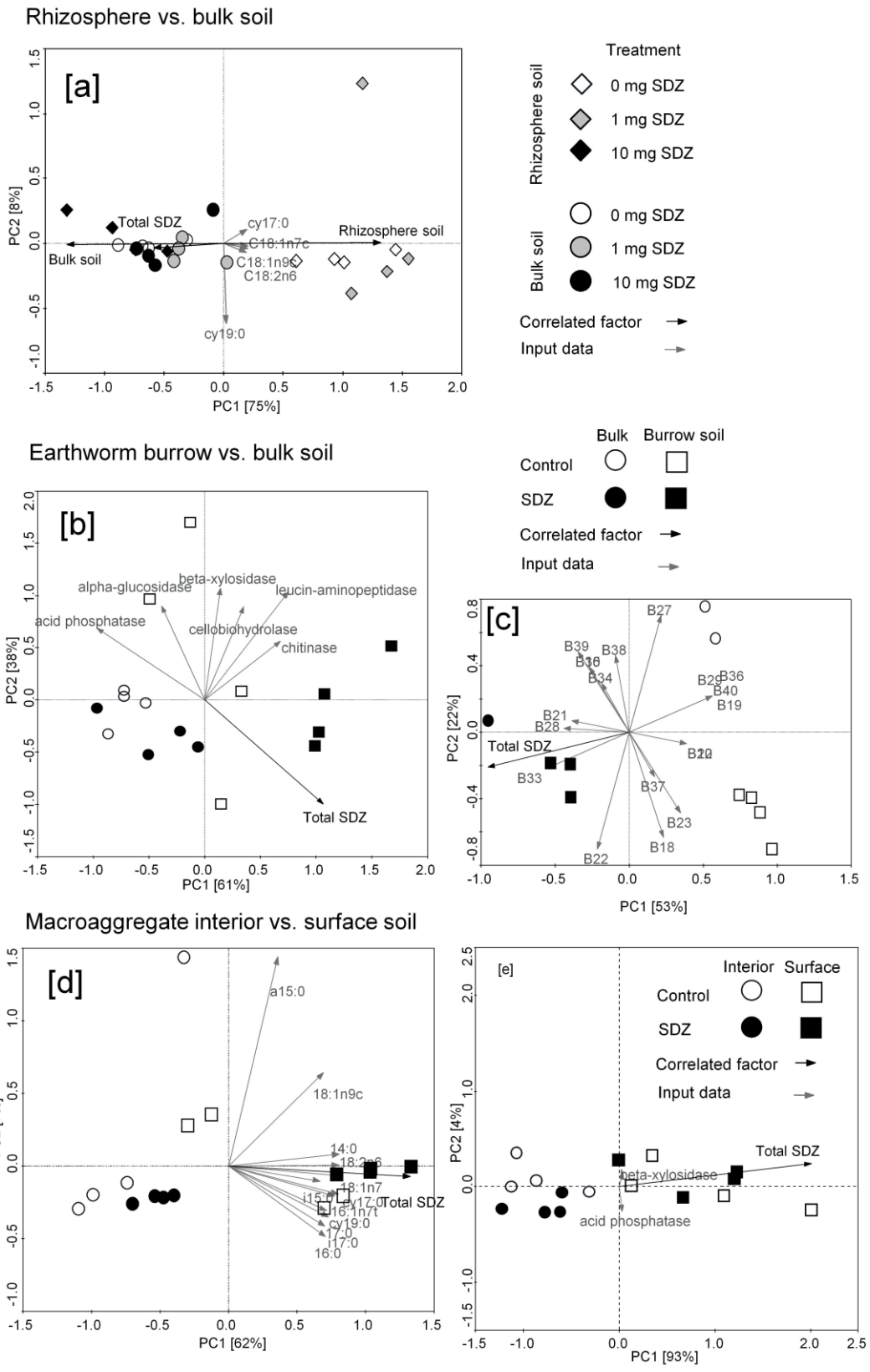


Figure S2.1. Laboratory experiments. [a] Microbial PLFA pattern in rhizosphere vs. bulk soil; [b] soil exoenzyme activities of earthworm burrow vs. bulk soil; [c] Betaproteobacteria 16S rRNA gene fragment DGGE data of earthworm burrow vs. bulk soil; [d] PLFA pattern of soil macroaggregate interior vs. surface; [e] soil exoenzyme activities of soil macroaggregate interior vs. surface.

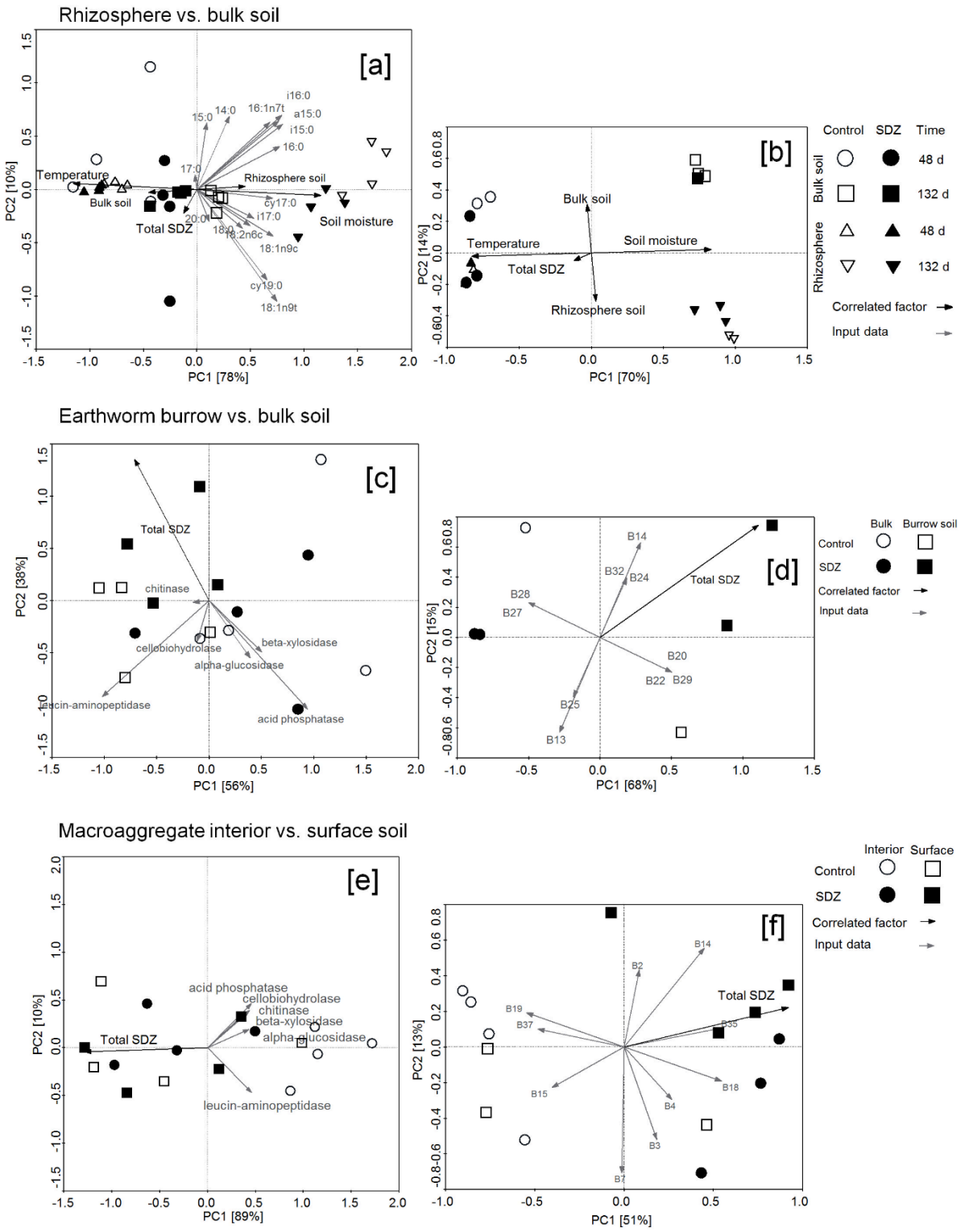


Figure S2.2. Field experiments. [a] Microbial PLFA pattern of rhizosphere vs. bulk soil; [b] Betaproteobacteria 16S rRNA gene fragment DGGE data of rhizosphere vs. bulk soil; [c] soil exoenzyme activities of earthworm burrow vs. bulk soil; [d] Betaproteobacteria 16S rRNA gene fragment DGGE data of earthworm burrow vs. bulk soil; [e] soil exoenzyme activities of soil macroaggregate interior vs. surface; [f] Betaproteobacteria 16S rRNA gene fragment DGGE data of soil macroaggregate interior vs. surface.

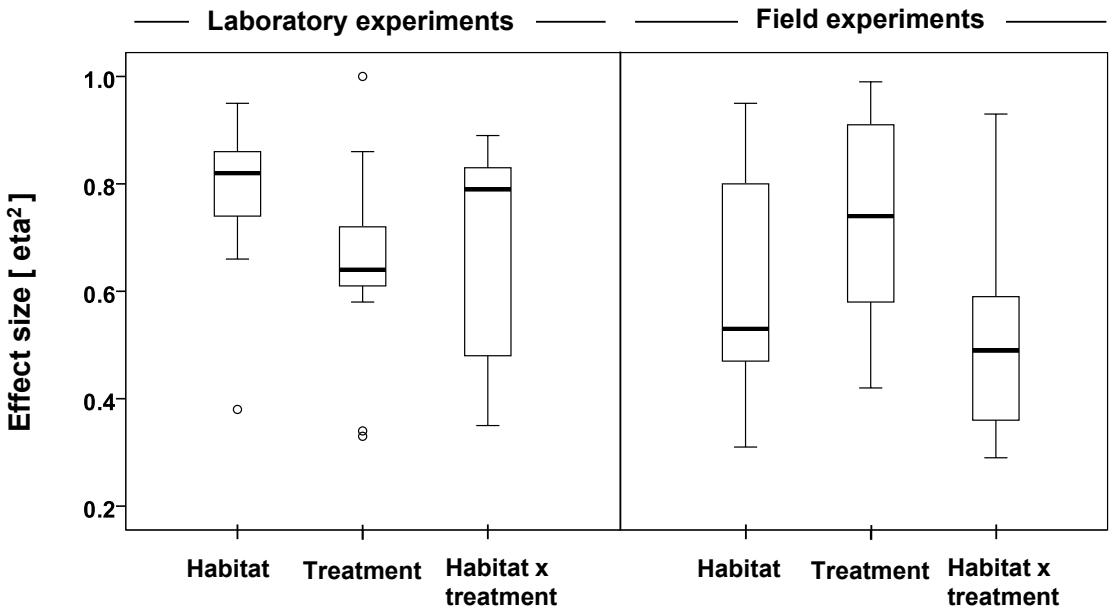


Figure S3. Box plots of effect sizes (eta^2) derived from the significant two-way ANOVA factors habitat, treatment, and habitat x treatment of diverse microbial measures of the laboratory and field experiments. The dark horizontal line within the box marks the median value.

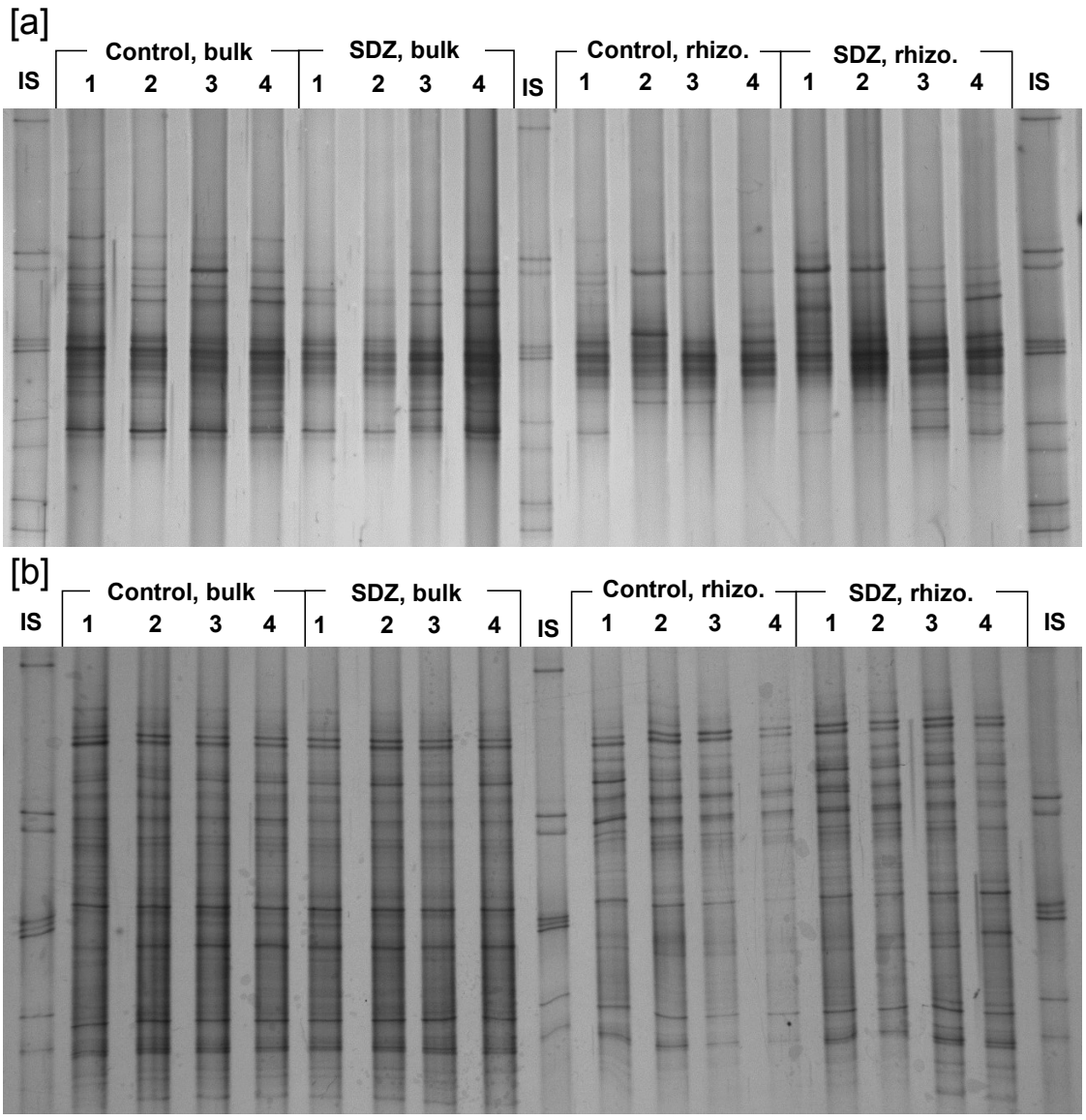


Figure S4: Field experiment: *Pseudomonas* [a] and Betaproteobacteria [b] 16S rRNA gene fragment DGGE of bulk soil (bulk) and rhizosphere soil (rhizo.) of the control- and SDZ-manure treatment at incubation time 132 d.

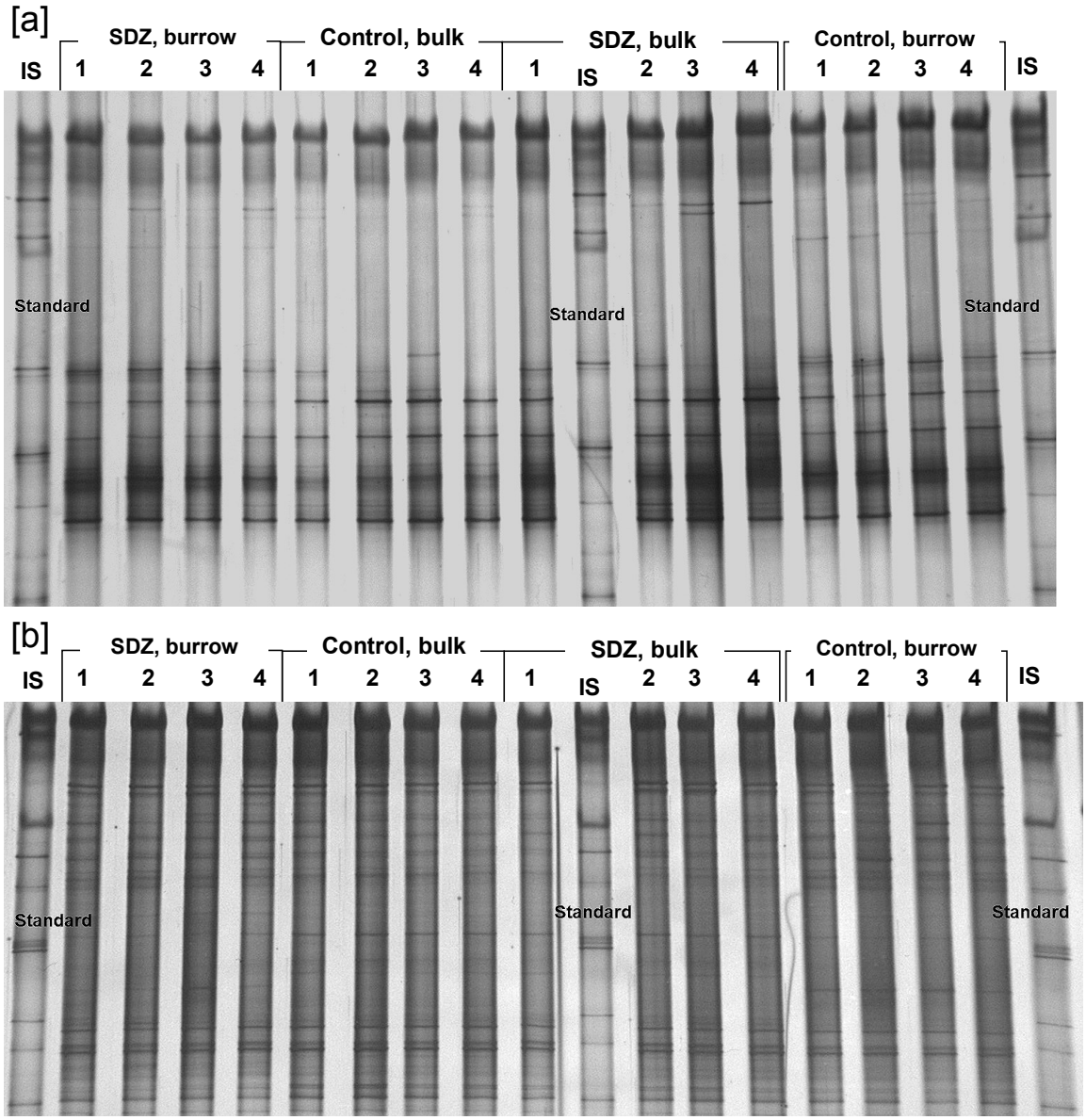


Figure S5. *Pseudomonas* [a] and Betaproteobacteria [b] 16S rRNA gene fragment DGGE of the control and SDZ treatment of earthworm burrows (0-5 mm) and bulk soil (>10 mm/distant bulk soil) from the field experiment.

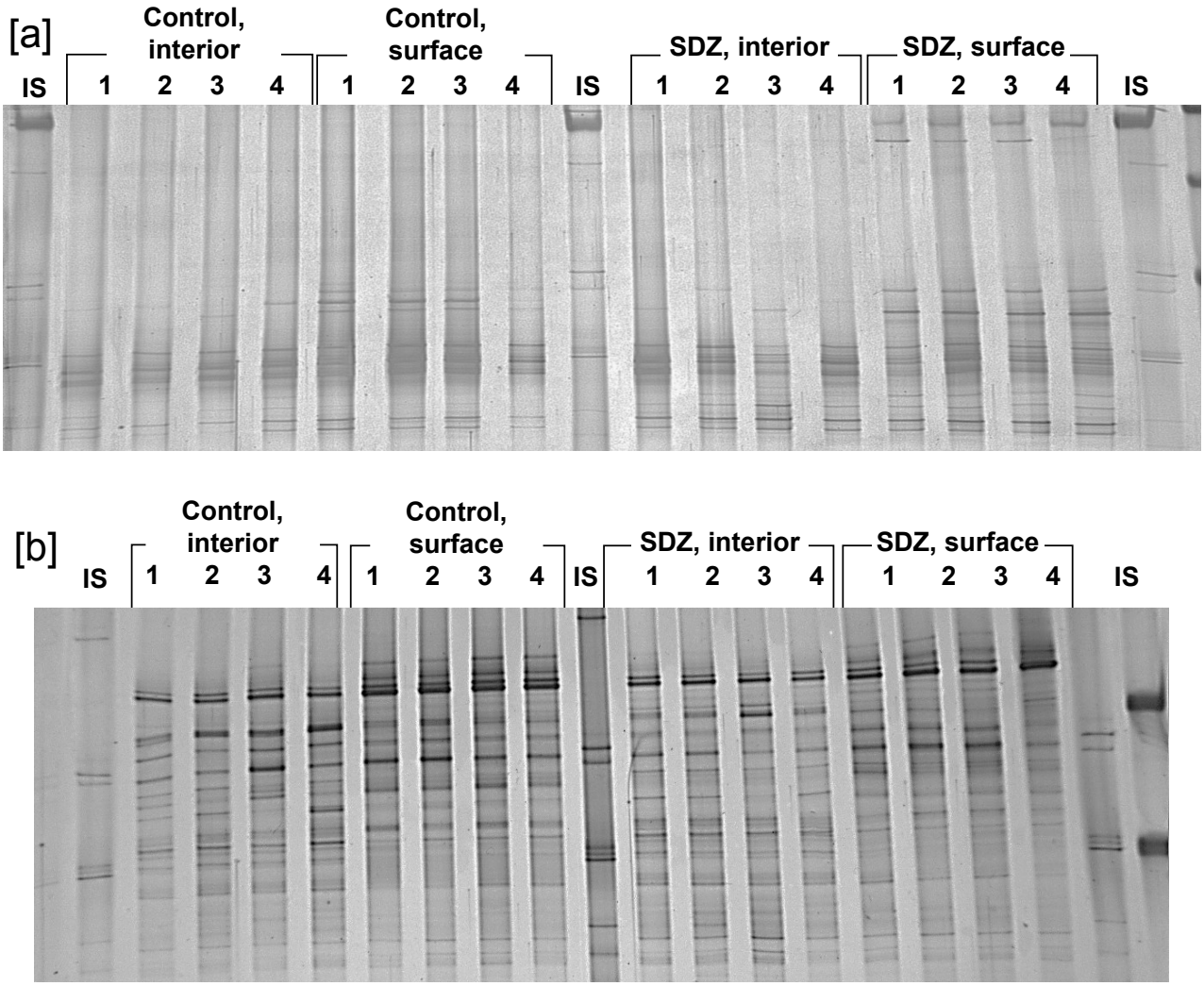


Figure S6. Laboratory experiment: *Pseudomonas* [a] and Betaproteobacteria [b] 16S rRNA gene fragment DGGE of soil from surface and interior fraction of soil macroaggregates of the control and SDZ treatment.

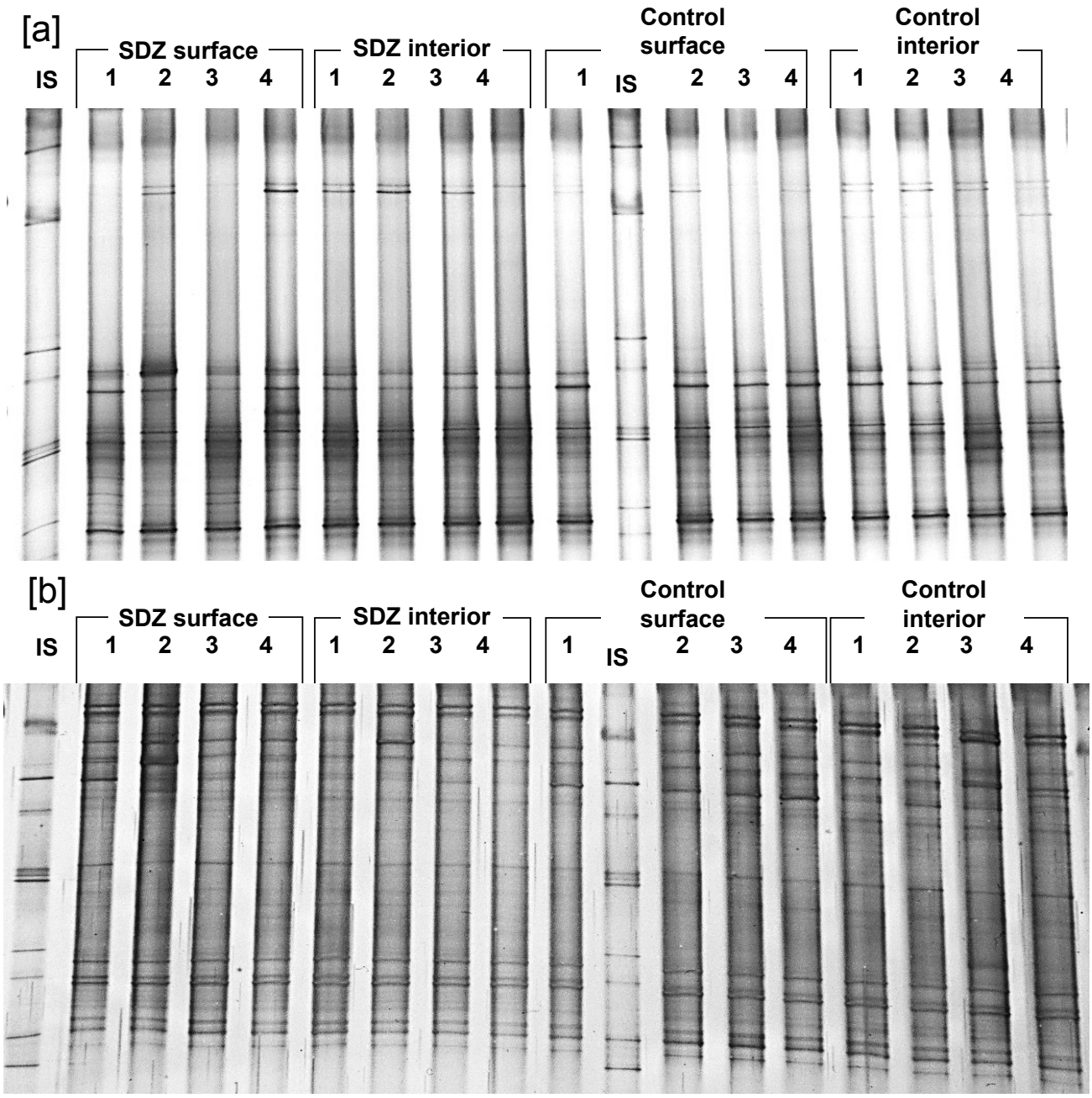


Figure S7. Field experiment: *Pseudomonas* [a] and Betaproteobacteria [b] 16S rRNA gene fragment DGGE of soil from surface and interior fraction of soil macroaggregates of the control and SDZ treatment.

11.3 Supplementary data of Chapter 5

Effects of SDZ-spiked manure under different soil moisture regimes

Table S1. Mean values with standard deviation (± sd) of different parameters (n ≥ 8) calculated on fresh (fm; $kg^{-1} \approx l^{-1}$) and dry mass (dm) basis of the pig manure used in the climate chamber experiment.

Parameters	Unit		Results				
			mean	sd		mean	sd
Dry mass	%	fm	10.8	0.9	-	-	-
Ash content	%	dm	44.6	8.9	-	-	-
pH ($CaCl_2$)	-	-	7.7	0.1	-	-	-
Conductivity	µS cm^{-1}	-	42.2	0.9	-	-	-
TC	g kg^{-1}	fm	36.5	4.3	dm	339.2	40.3
DOC	g kg^{-1}	fm	4.5	0.7	dm	41.9	6.4
TN	g kg^{-1}	fm	6.4	0.4	dm	59.2	3.5
Organic N	g kg^{-1}	fm	2.0	0.4	dm	19.0	3.7
NH_4-N	g kg^{-1}	fm	4.3	0.2	dm	40.2	1.6
NO_3-N	mg kg^{-1}	fm	n.d.	n.d.	dm	n.d.	n.d.

n.d.: not detectable

Table S2. Mean values ± standard deviation (sd) of $PLFA_{tot}$ [nmol g^{-1} dm], bacteria-to-fungi ratio (bac:fungi), Gram-positive-to-Gram-negative bacteria ratio ($Gram^+$:$Gram^-$) and cyclopropyl-to-precursor PLFA ratio (stress), mild solvent extractable SDZ (MES), residual SDZ (RES) fraction [µg kg^{-1}], and soil water content [$\%WHC_{max}$] in SDZ uncontaminated (SDZ 0) and SDZ contaminated (SDZ 4) soil samples, influenced by the dynamic moisture regime (drying, ↓DMR; rewetting, ↑DMR) or control moisture regime (CMR). Significant differences between treatments and all incubation times -1 (before manure application), 0 (after manure application), 7, 20, 27, 34, and 49 d, and separately for the overall average, are indicate with different letters ($p < 0.05$ or $p < 0.001$, in case of violated Levene's-Test, cf. Tab. 1). Significances of SDZ MES and RES fraction among treatments of individual incubation times tested at $p < 0.05$ by Mann-U test (*).

Moisture regime / time	-1 d		0 d				7 d				20 d				27 d				34 d				49 d				SDZ 0		SDZ 4	
Dynamic moisture regime	-		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		Average			
$\bar{x}$ (±sd) of replicates (n)	4	sd	4	sd	4	sd	3	sd	3	sd	4	sd	3	sd	4	sd	3	sd	4	sd	4	sd	4	sd	4	sd	19	sd	17	sd
Soil water [$\%WHC_{max}$]	9^{a}	1	66^{gh}	8	71^{h}	7	12^{a}	3	11^{a}	2	31^{bcd}	2	37^{cd}	2	33^{bcd}	2	38^{de}	4	8^{a}	2	9^{a}	1	31^{bcd}	2	28^{bcd}	5	24^{a}	11	24^{a}	13
$PLFA_{tot}$ [nmol g^{-1}]	34.3^{b}	0.5	107.4^{ij}	6.4	95.5^{ij}	14.8	98.7^{ij}	6.5	75.0^{fgh}	8.9	63.9^{def}	1.5	48.0^{bcd}	14.5	64.6^{def}	13.6	19.1^{a}	0.7	42.3^{bc}	3.6	23.0^{a}	3.6	43.2^{bc}	6.1	31.5^{a}	4.3	60.6^{a}	21.0	37.9^{b}	21.3
Bac:fungi ratio	2.7^{bcd}	0.8	2.7^{bcd}	0.2	2.6^{bcd}	0.5	2.3^{bcd}	0.2	2.8^{cd}	0.3	2.1^{abc}	0.6	2.0^{abc}	0.2	1.5^{a}	0.2	1.5^{a}	0.2	2.1^{abc}	0.6	1.5^{a}	0.2	2.2^{abc}	0.3	2.3^{bcd}	0.1	2.0^{a}	0.5	2.0^{a}	0.5
$Gram^+$:$Gram^-$ ratio	1.4^{abd}	0.2	2.1^{e}	0.2	2.3^{e}	0.2	1.7^{de}	0.2	1.7^{d}	0.1	1.4^{bcd}	0.4	1.3^{bcd}	0.4	1.0^{abc}	0.1	1.2^{abc}	0.1	0.9^{a}	0.1	1.0^{abc}	0.1	1.0^{abc}	0.0	1.0^{abc}	0.1	1.1^{a}	0.3	1.2^{a}	0.3
PLFA stress ratio	-	-	-	-	-	-	4.2^{bcd}	0.4	3.8^{abcd}	0.3	4.6^{cd}	0.0	3.9^{abcd}	0.5	4.1^{bcd}	0.7	4.5^{cd}	0.4	3.4^{abcd}	0.2	2.0^{a}	0.3	3.6^{abcd}	0.5	2.8^{abc}	0.1	3.9^{ab}	0.6	3.3^{a}	1.0
SDZ (MES) [µg kg^{-1}]	-	-	-	-	-	-	<LOQ		2,459*	263	<LOQ	-	124	20	<LOQ	-	107*	15	<LOQ	-	106*	6	<LOQ	-	97	15	-	-	-	-
SDZ (RES) [µg kg^{-1}]	-	-	-	-	-	-	<LOQ		2,434	29	<LOQ	-	1,433	682	<LOQ	-	1,907	475	<LOQ	-	1,526*	188	<LOQ	-	1,330	171	-	-	-	-

Control moisture regime	-		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		SDZ 0		SDZ 4		Average			
$\bar{x}$ (±sd) of replicates (n)	4	sd	4	sd	4	sd	4	sd	4	sd	3	sd	4	sd	4	sd	4	sd	3	sd	3	sd	4	sd	4	sd	18	sd	19	sd
Soil water [$\%WHC_{max}$]	9^{a}	1	66^{gh}	8	71^{h}	7	58^{g}	2	58^{g}	2	30^{bcd}	5	27^{ab}	3	27^{ab}	8	29^{ab}	3	45^{b}	4	49^{ab}	5	28^{bcd}	4	27^{bcd}	8	38^{b}	14	37^{b}	14
$PLFA_{tot}$ [nmol g^{-1}]	34.3^{b}	0.5	107.4^{ij}	6.4	95.5^{ij}	14.8	105.6^{j}	7.4	90.8^{hij}	9.4	43.0^{bc}	3.4	67.3^{efg}	3.6	56.0^{cde}	6.7	83.3^{ghi}	9.7	46.7^{bc}	2.5	20.0^{a}	2.3	40.6^{bc}	2.1	44.0^{bc}	3.5	59.9^{a}	26.2	63.2^{a}	26.3
Bac:fungi ratio	2.7^{bcd}	0.8	2.7^{bcd}	0.2	2.6^{bcd}	0.5	2.6^{bcd}	0.4	3.1^{d}	0.4	1.8^{ab}	0.4	1.9^{abc}	0.3	1.8^{ab}	0.3	1.4^{a}	0.1	1.9^{abc}	0.6	1.6^{a}	0.2	2.2^{abc}	0.1	1.7^{ab}	0.5	2.1^{a}	0.5	2.0^{a}	0.7
$Gram^+$:$Gram^-$ ratio	1.4^{abd}	0.2	2.1^{e}	0.2	2.3^{e}	0.2	1.6^{d}	0.2	1.7^{de}	0.2	1.4^{cd}	0.2	1.1^{abc}	0.1	1.0^{abc}	0.1	1.0^{abc}	0.1	0.9^{a}	0.0	0.9^{ab}	0.1	1.0^{abc}	0.0	1.0^{abc}	0.1	1.2^{a}	0.3	1.1^{a}	0.3
PLFA stress ratio	-	-	-	-	-	-	5.3^{de}	0.2	4.8^{cde}	0.5	4.3^{cd}	0.2	4.7^{cde}	0.4	4.3^{cd}	0.8	6.6^{e}	2.1	3.9^{abcd}	0.6	2.0^{ab}	0.4	3.2^{abcd}	0.1	3.5^{abcd}	0.3	4.3^{b}	1.7	4.3^{b}	0.8
SDZ (MES) [µg kg^{-1}]	-	-	-	-	-	-	<LOQ	-	775*	301	<LOQ	-	112	29	<LOQ	-	62*	14	<LOQ	-	58*	9	<LOQ	-	66	16	-	-	-	-
SDZ (RES) [µg kg^{-1}]	-	-	-	-	-	-	<LOQ	-	2,350	225	<LOQ	-	1,605	136	<LOQ	-	1,575	238	<LOQ	-	1,079*	90	<LOQ	-	1,144	468	-	-	-	-

LOQ: limits of quantitation: 1.25 µg kg^{-1} for SDZ in the $CaCl_2$-extracts and 2.5 µg kg^{-1} in the residual fraction

Figure S1a. Betaproteobacteria DGGE of incubation times (t) -1, 0, 7, 20, 27, 34, and 49 d with SDZ 0 (control), SDZ 4 (SDZ), control moisture regime (CM) and dynamic moisture regime (DR).

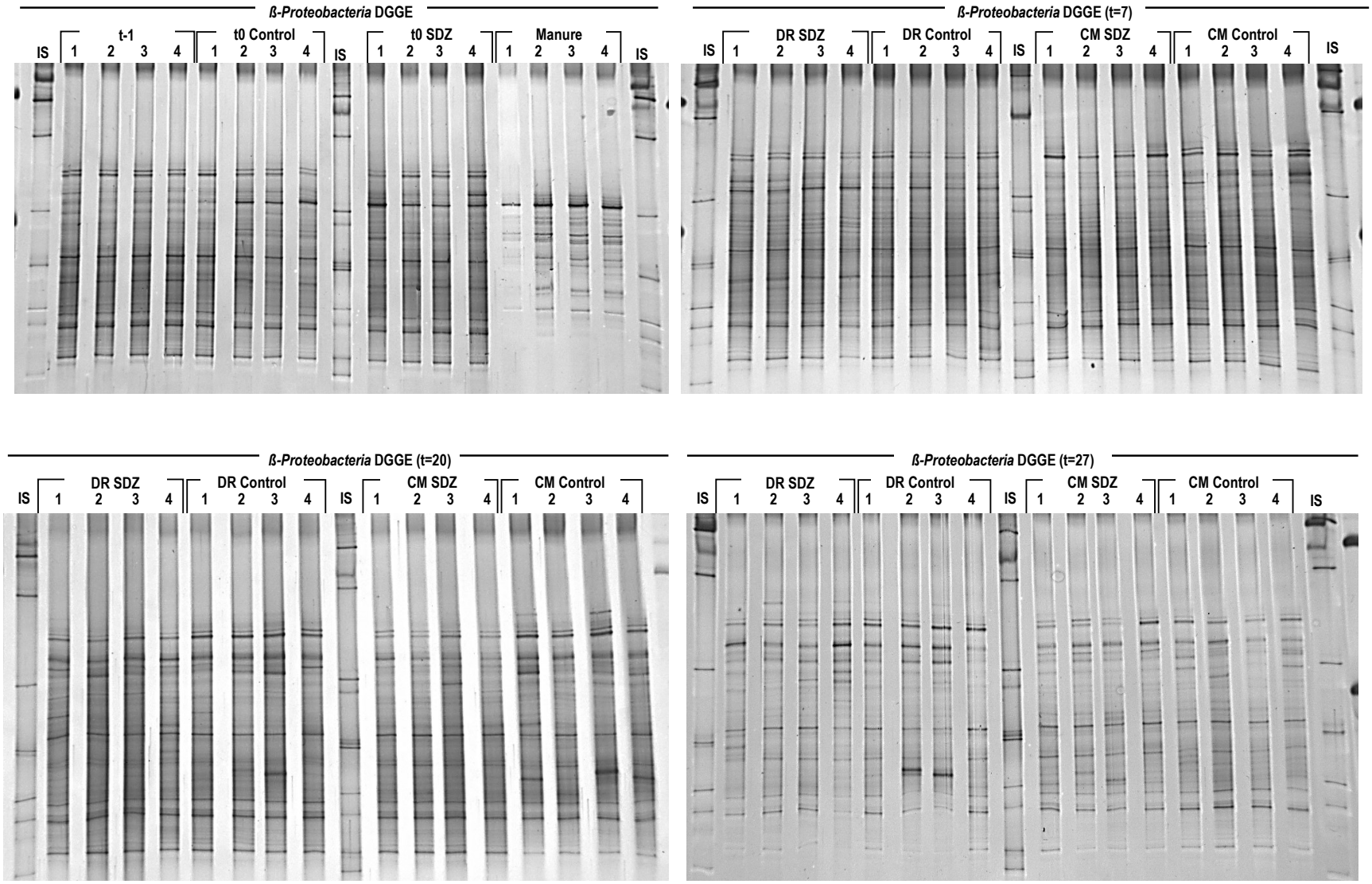


Figure S1a continued.

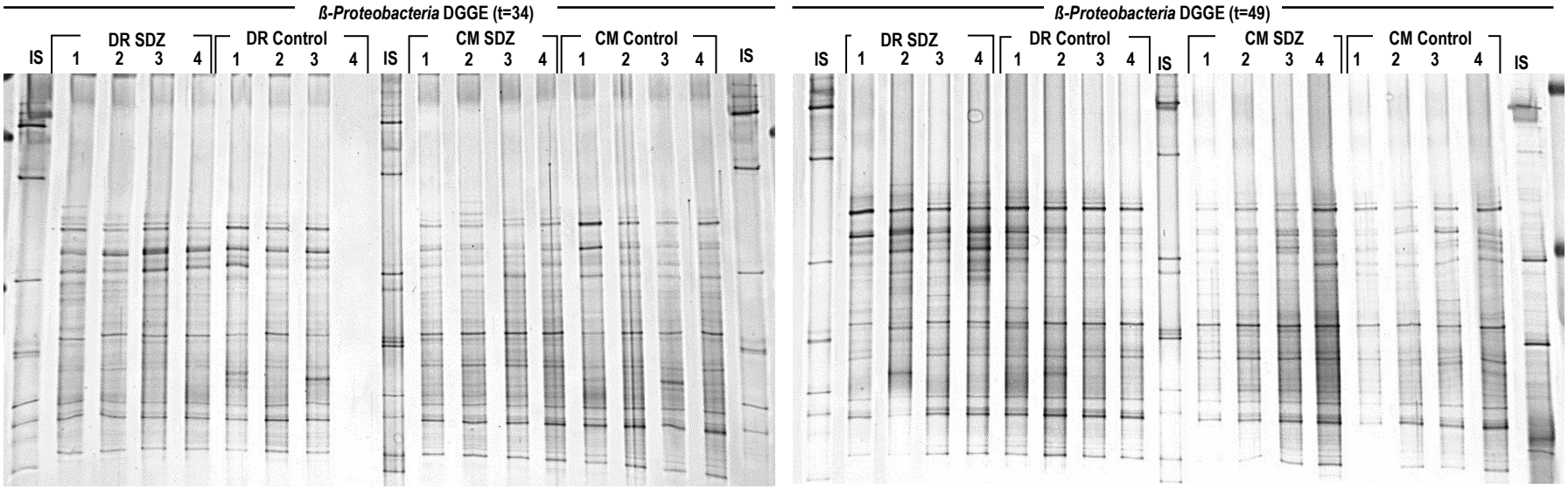


Figure S1b. *Pseudomonas* DGGE of incubation times (t) -1, 0, 7, 20, 27, 34, and 49 d with SDZ 0 (control), SDZ 4 (SDZ), control moisture regime (CM) and dynamic moisture regime (DR).

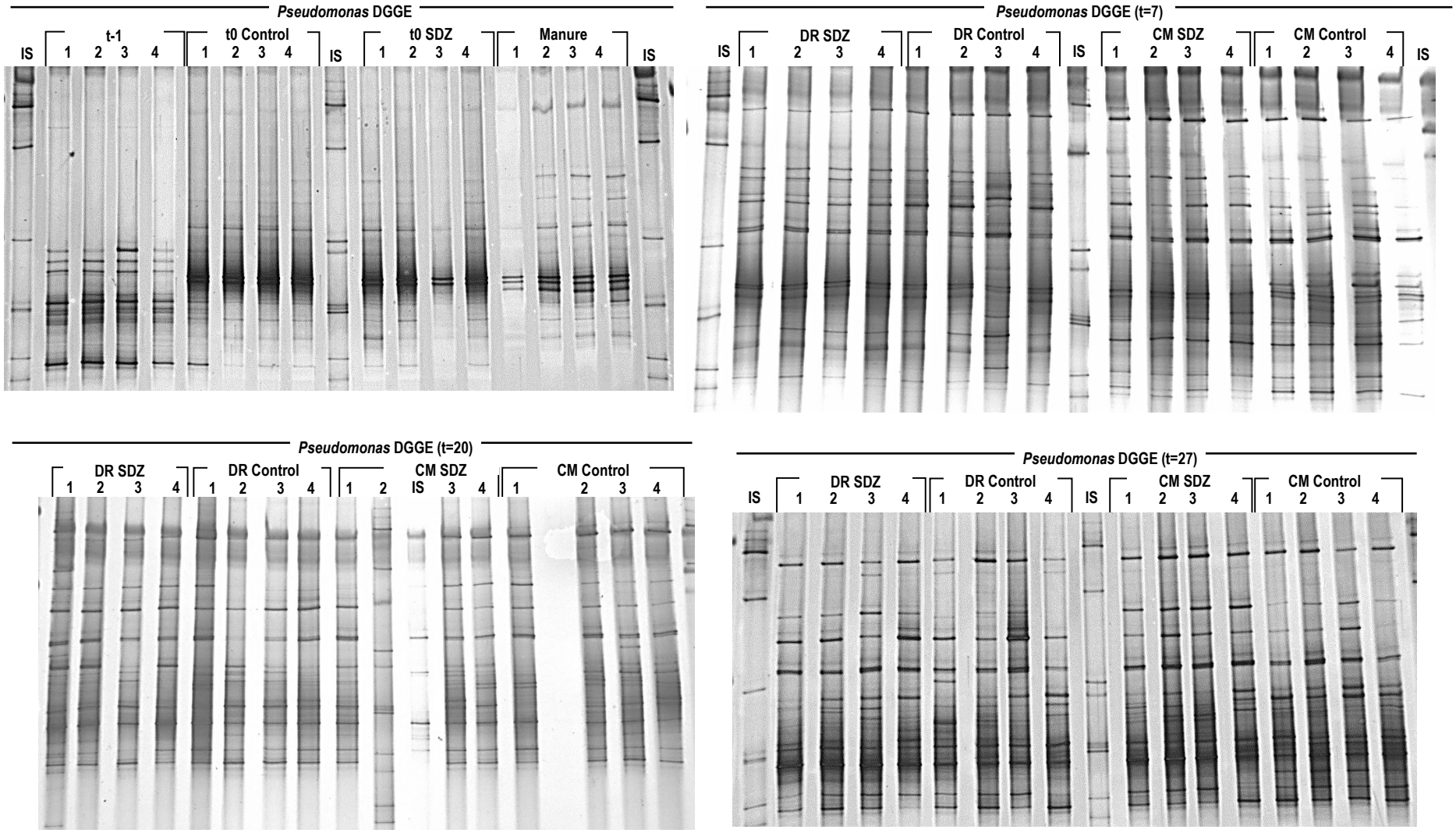
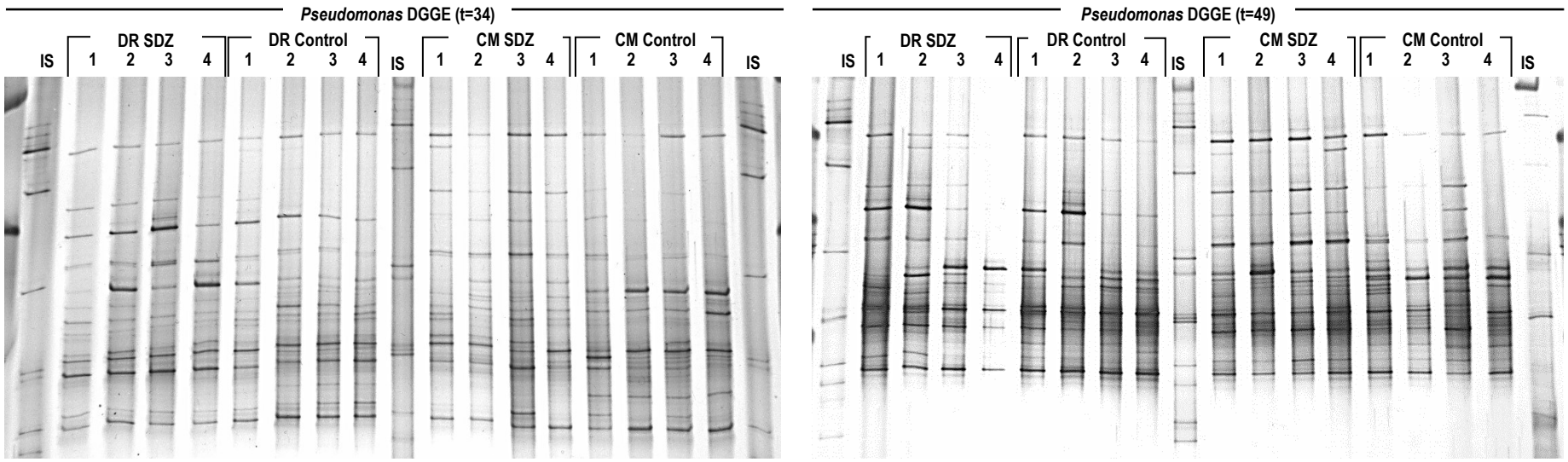


Figure S1b continued.



11.4 Supplementary data of Chapter 6

SDZ effects on *Salix fragilis* L. and *Zea maize* L. plant roots

Table S1. Gradient program for separation and detection of sulfadiazine and its metabolites from background contaminants on a Sunfire C18 HPLC column.

Time (min)		% Solvent A	% Solvent B
0-2		70	30
2-4	linear	0	100
4-8		0	100
8-8.2	linear	70	30
8.2-9		70	30

Table S2. Compound dependent parameters for the sensitive determination of the antibiotic sulfadiazine and its two metabolites in extracts of plant tissues and soil.

Antibiotic/ Metabolite	Precursor Ion	Source parameters		Fragment Ion	Mass analyzer parameters		
		DP	EP		CEP	CE	CXP
	(*m/z*)	(V)		(*m/z*)		(V)	
SDZ	251.15	26	4	65.1	22	59	2
				92.0	22	43	4
				108	13	35	8
N-Acetyl-SDZ	293.20	70	10	65.1	38	29	6
			10	134	14	10	3
			10	198	14	10	3
4-OH-SDZ	267.21	41	9.5	156	10	21	4
				201	10	25	4
				173	10	23	4
SDM	279.01	35	36	186	16	23	4
			5	204	16	23	4
				124	16	35	4
				156	16	25	4

DP: Declustering potential; EP: Entrance potential; CEP: Collision cell entrance potential; CE: Collision energy; CXP: Collision cell exit potential

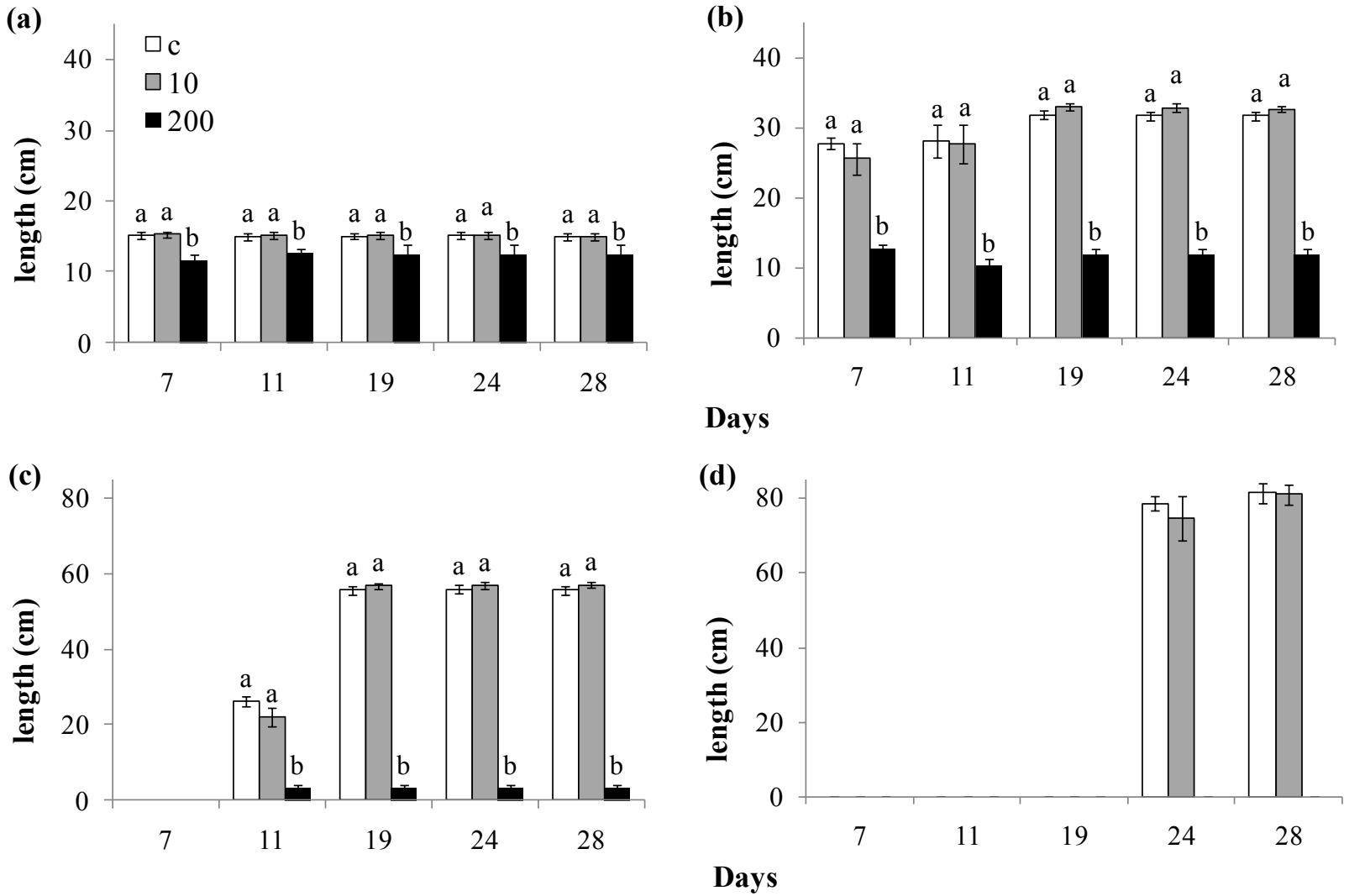


Fig. S1. Length and development of the second (a), third (b), fourth (c) and fifth (d) leaf of maize plants exposed to 0 (control), 10 and 200 mg SDZ kg^{-1} soil. Values denote mean ± SE; HSD post-hoc test ($p < 0.05$). Letters, when present, indicate significant difference among SDZ treatments ($p < 0.05$).

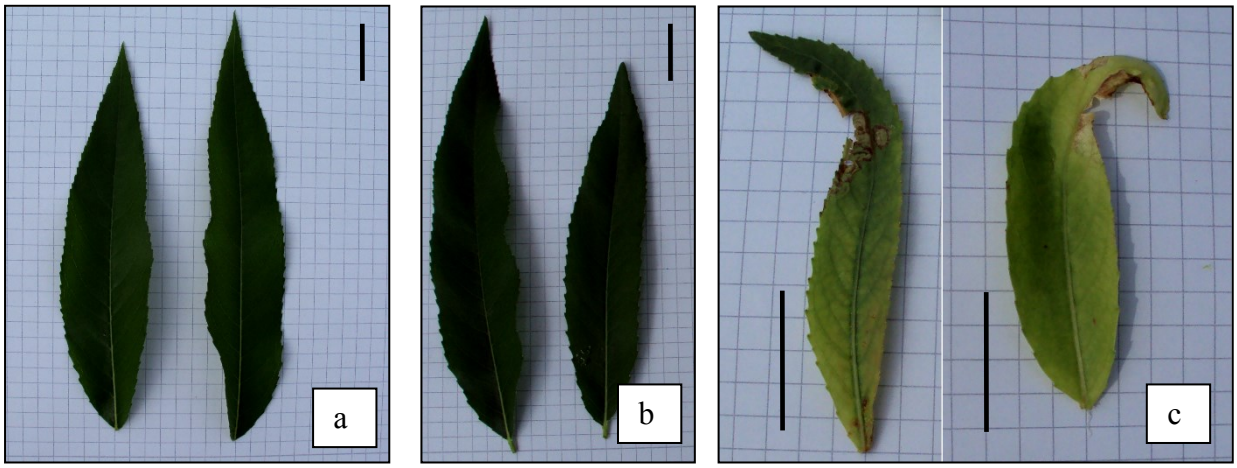


Fig. S2. Adult leaves from *Salix fragilis* L. plants exposed to soil concentrations of 0 (a), 10 (b) and 200 (c) mg SDZ kg^{-1} soil. Black lines correspond to 2 cm.

11.5 Supplementary data of Chapter 7

SDZ effects on plant roots and microbes at different distance to roots

Table S1. Mean values and standard deviation (±S.D.) of exoenzyme activities in bulk soil and rhizosphere soil of willow and maize plants. Data are expressed as nmol MUB (AMC) g^{-1} h^{-1}. SDZ0, 10 and 200 correspond to soil spiking concentrations of 0 (control), 10 and 200 mg SDZ kg^{-1}. Different lowercase letters indicate significant differences within the willow and maize group among both plants by capital letters at significance of $p < 0.05$.

Exoenzyme	**Bulk soil**			**Rhizosphere soil**		
	SDZ0	**SDZ10**	**SDZ200**	**SDZ0**	**SDZ10**	**SDZ200**
				Willow		
Leucin-aminopeptidase	1327.0 ± 61.4 **a**	1336.3 ± 45.1 **a**	883.8 ± 55.0 **b**	1303.9 ± 78.8	1352.6 ± 10.5	1101.6 ± 106.1
Acid phosphatase	1455.5 ± 55.8 **aB**	1468.2 ± 39.2 **aB**	1063.8 ± 38.3 **bB**	1372.8 ± 54.6 **abB**	1501.8 ± 13.8 **aB**	1236.3 ± 83.4 **bB**
ß-cellobiohydrolase	194.9 ± 11.6 **a**	201.2 ± 7.8 **a**	146.5 ± 8.9 **b**	199.8 ± 5.3 **ab**	220.9 ± 11.2 **a**	173.7 ± 13.8 **b**
α-glucosidase	226.4 ±14.4 **a**	238.3 ± 5.9 **a**	164.1 ± 14.2 **b**	217.4 ± 4.0	248.9 ±13.2	202.7 ± 23.7
Chitinase	283.7 ± 13.6 **a**	289.1 ± 13.6 **a**	219.2 ± 13.2 **b**	270.7 ± 8.7 **ab**	301.2 ± 4.9 **a**	242.9 ± 11.9 **b**
ß-xylosidase	178.8 ± 11.7 **a**	187.9 ± 6.9 **a**	129.5 ± 7.8 **b**	177.5 ± 2.1 **ab**	190.3 ± 2.4 **a**	150.8 ± 11.1 **b**
				Maize		
Leucin-aminopeptidase	1484.6 ± 17.2 **a**	1448.6 ± 33.2 **a**	1123.2 ± 69.3 **b**	1415.4 ± 56.1	1506.7 ± 108.4	1260.6 ± 30.0
Acid phosphatase	2021.9 ± 11.2 **aA**	1985.9 ± 37.5 **aA**	1641.5 ± 69.9 **bA**	2004.5 ± 54.1 **abA**	2082.0 ± 119.8 **aA**	1769.3 ± 51.4 **bA**
ß-cellobiohydrolase	203.0 ± 1.6 **a**	198.2 ± 6.1 **a**	162.9 ± 6.9 **b**	195.6 ± 5.6 **ab**	211.0 ± 12.4 **a**	173.3 ±3.2 **b**
α-glucosidase	243.8 ± 11.2	257.6 ± 27.1	195.5 ± 11.2	227.2 ± 8.3	263.9 ± 28.0	219.5 ± 18.6
Chitinase	305.0 ± 0.6 **a**	294.4 ± 7.8 **a**	244.1 ± 6.5 **b**	293.0 ± 10.6 **ab**	302.9 ± 14.6 **a**	258.3 ± 6.3 **b**
ß-xylosidase	180.0 ± 1.0 **a**	182.3 ± 7.5 **a**	143.6 ± 4.6 **b**	178.7 ± 8.7 **ab**	183.1 ± 9.9 **a**	155.8 ± 2.9 **b**

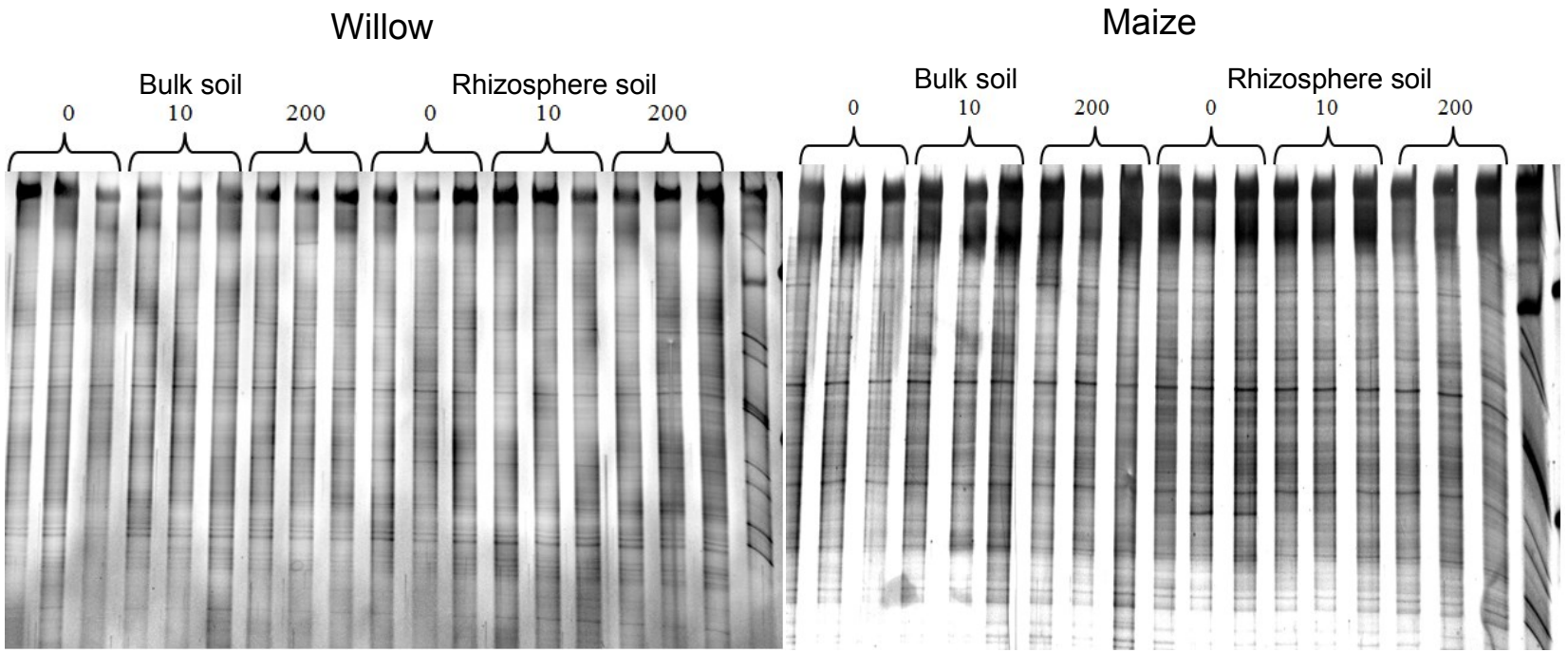


Figure S1. Total bacteria DGGE of bulk soil and rhizosphere soil of SDZ 0, 10 and 200 mg kg^{-1} soil treatments of willow and maize.

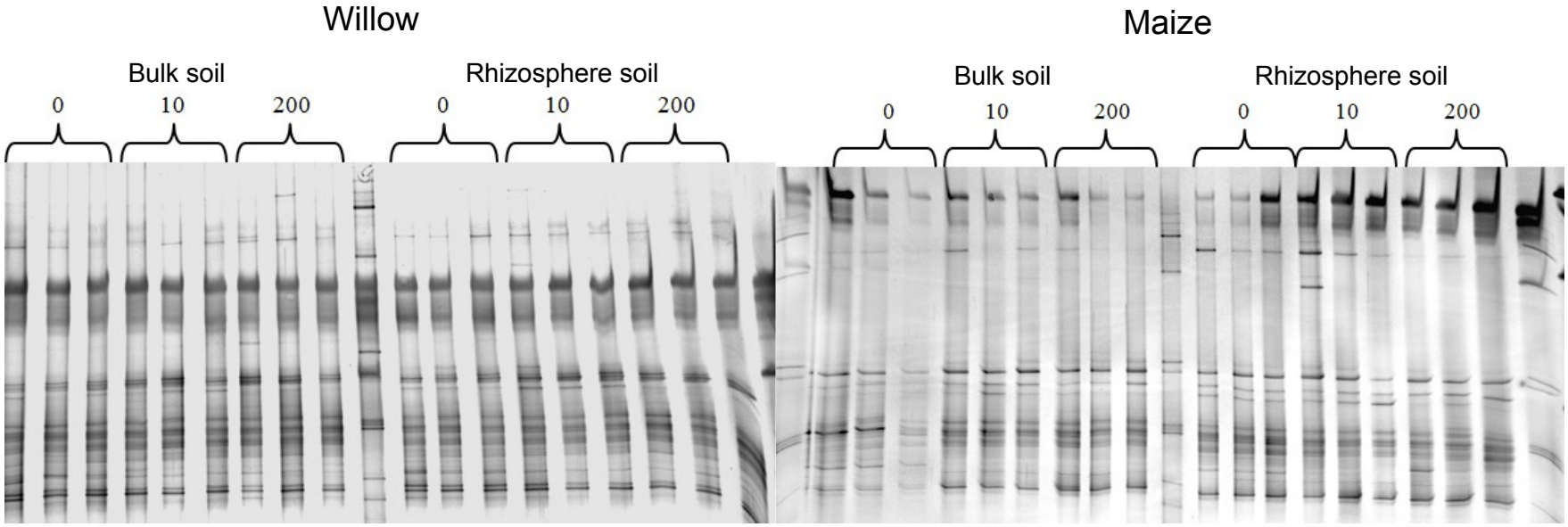


Figure S2. *Pseudomonas* DGGE of bulk soil and rhizosphere soil of SDZ 0, 10 and 200 mg kg^{-1} soil treatments of willow and maize.

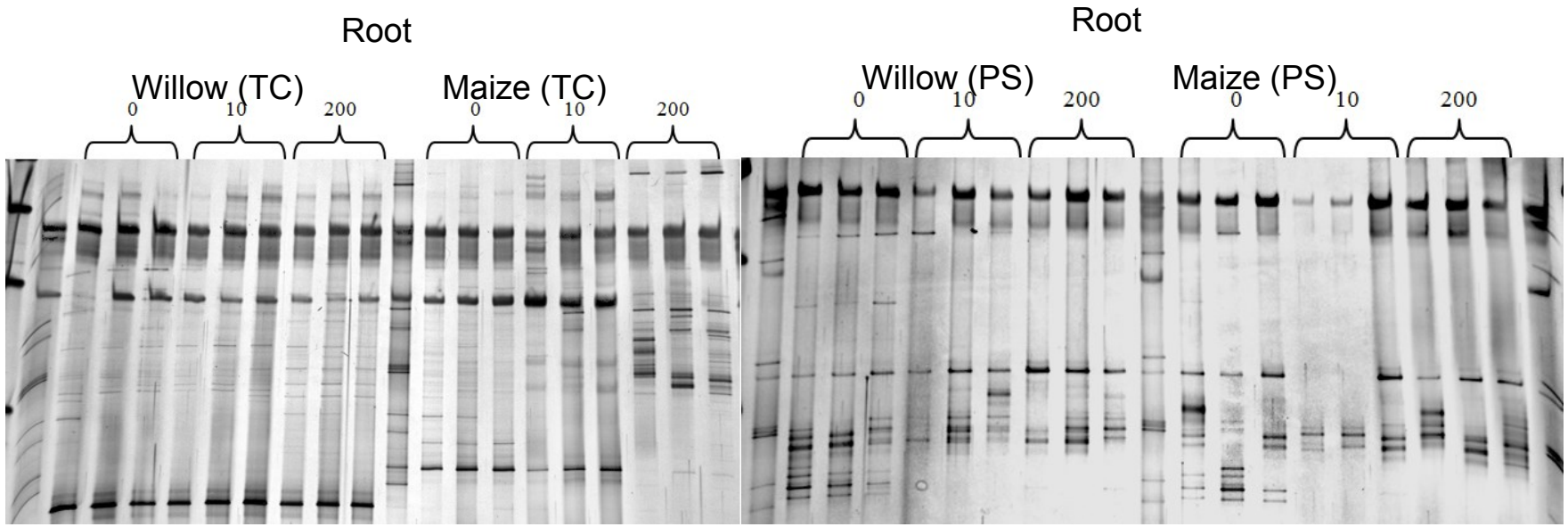


Figure S3. Total bacteria (TC) and *Pseudomonas* (PS) DGGE of root samples of the SDZ 0, 10 and 200 mg kg^{-1} treatments of willow and maize.

A. Selbstständigkeitserklärung

Hiermit erkläre ich, dass die Arbeit selbstständig und ohne fremde Hilfe verfasst und keine anderen als die genannten Quellen und Hilfsmittel genutzt, sowie wörtlich und inhaltlich entnommene Stellen gekennzeichnet wurden.

Trier, den 21. April 2014

B. Danksagung

Eine Danksagung ist nicht leicht, da Worte den vielen glücklichen Fügungen des Lebens und den daran beteiligten Personen ebenso wenig gerecht werden können, wie diese Arbeit der faszinierenden Komplexität des Bodens und seiner Lebensgemeinschaft. Mit den Worten von Max Planck „*Die eigentliche Befriedigung des Forschers ist nicht eine Wahrheit zu besitzen, sondern im fortschreitend, erfolgreichen Suchen nach der Wahrheit“*.

Für die nötige Genetik, Erziehung und Faszination an der Natur möchte ich meinen Eltern Sieglinde und Klaus-Jürgen Reichel, aber auch meinen Großeltern Franziska und Franz Goldschald aufrichtig danken. Meiner Umweltwissenschaftlerin Christiane Giudici möchte ich für die Freude und Liebe in meinem Leben danken.

Meine wissenschaftliche Karriere und Erziehung verdanke ich den Dozenten meiner *Alma Mater* in Trier. Insbesondere danke ich meinem Mentor während der Diplomarbeit Prof. Dr. Christoph Emmerling und meinem Doktorvater und Lehrstuhlinhaber Prof. Dr. Sören Thiele-Bruhn für das langjährige Vertrauen, die Herausforderungen, aber auch die Zeit und den Raum für meine persönliche und wissenschaftliche Entwicklung.

Auch möchte ich allen Kollegen aus Trier und der DFG-Forschergruppe FOR566, den Diplomanden, Hilfswissenschaftlern und technischen Assistenten Elvira Sieberger und Petra Ziegler für die Unterstützung meiner Arbeit danken.

In diesem Sinne wünsche ich allen Lesern und meiner *Alma Mater*:

„*vivat, crescat, floreat ad multos annos*!“

C. Curriculum vitae

Name	Reichel, Rüdiger
Date / place of birth	27th August 1981 / Nürtingen, Baden-Württemberg, Germany
Education	
November 2013	Achieved the permission to perform bachelor exams of studies within the Faculty VI of Regional and Environmental Sciences, University of Trier, Germany
	Project approval by the research fund 2013, University of Trier: *„Seasonal variability of soil microorganisms and its impact on the susceptibility to toxic pollutants*".
	Certificate of gas chromatography - mass spectrometry "H4040A: introduction and training in the GC/MS" by Agilent Technologies, Germany
	Certificate of high performance liquid chromatography - tandem mass spectrometry (HPLC/MS^2) "Basic Training API 3200 Small Molecule" by AB Sciex Technologies, Germany
since 2013	Teacher of practical course for Univ.-Prof. Dr. Sören Thiele-Bruhn at the Soil Science department, University of Trier: Advanced Methods in Soil Science, Chemical Processes in the Environment, Introduction to Field Soil Science
since 2012	Research assistant and co-supervisor of the laboratory of Univ.-Prof. Dr. Sören Thiele-Bruhn, head of the Soil Science department of the University of Trier
2011 - 2012	Scientific assistant and PhD student of the Soil Science department at the University of Trier within the 3rd project phase of the DFG research unit 566
2008 - 2011	Scientific assistant of the Soil Science department at the University of Trier within the 2nd project phase of the DFG research unit 566: "Veterinary Medicines in Soils: Basic Research for Risk Analysis" and PhD student with the topic: "*Soil microbial community responses to antibiotic pharmaceuticals: influence of different soil habitats and moisture regimes*"
2008	Diploma thesis: *Effects of synthetic Cry1Ab protein on fermentation processes in a laboratory biogas plant* (supervisor: Prof. Dr. C. Emmerling)
2006 - 2008	Student assistant in the Soil Science and Geobotany department
2006	Student assistant of the FÖA Landschaftsplanung GmbH within the research and development project "*Effects of freeways and train paths on populations of European bats*"
2003 - 2008	Studies in biogeography with focus on soil science, ecotoxicology, geobotany, and zoology at the University of Trier