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

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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 experiments 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.

Keywords Combined stress · Sulfadiazine · Soil moisture · Dissipation · Community structure · Rhizosphere

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Introduction

The usage of antibiotics is a 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).

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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 decline 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).

The soil moisture content influences the microbial activity (Orchard and Cook 1983). The relationship of microbial activity and water availability is parabolic and not continuously increasing (Moyano et al. 2013). Fluctuating water contents, in turn, might stimulate microbial growth due to the release of dissolved organic carbon 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-day 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 ribosomal RNA (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).

Material and methods

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}) of 45.8 % *w/w* (Förster et al. 2009).

Uncontaminated pig manure was produced 1 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 (Supplementary Table S1).

Eighty-eight pots ($9 \times 9 \times 20\ cm^{-3}$) 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 (58:58 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 days 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). Based on pre-experiments, 7-d drying phases were used 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 day) and immediately after manure application (0 day), as well as at the end of each drying (7 and 34 days) and rewetting (20, 27, and 49 days) period. Four replicates were taken per sampling date, split into subsamples, and stored at −20 °C.

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 the moist soil equivalent to 10 g dm. After processing the suspensions in an end-over-end shaker for 24 h and centrifugation at 3000×*g* for 15 min, supernatants were sampled. The remaining pellet was resuspended 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).

Determination of microbial community structure based on phospholipid fatty acids

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×0.4 mm×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, and i-17:0 ($Gram^+$ bacteria); cy17:0, cy19:0, and 18:1ω7c ($Gram^-$ bacteria); and 18:2ω6c and 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 were derived from the ratio of cy17:0+cy19:0 to 18:1ω7c (16:1ω7c was excluded since it was not safely identified).

Determination of bacterial communities based on 16S rRNA gene fingerprinting

Total community DNA was extracted from 0.5-g soil sample, using the FastDNA® Spin Kit for the soil and the Geneclean® Spin Kit for purification (MP Biomedicals, Heidelberg, Germany). The 16S rRNA gene fragments of Betaproteobacteria and Gammaproteobacteria of the genus *Pseudomonas* were amplified using a nested PCR approach. In the first step, the specific primer pairs F311Ps/R1459Ps (Milling et al. 2005) respectively F948β/R1494 (Gomes et al. 2001) were used. In the second step, the universal primer sets F984GC and R1378 (Heuer et al. 1997), which contained the GC clamp, were 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× TAE buffer. The 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.

Data analysis

Mean values±standard deviation (SD; Supplementary 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 (Supplementary Table S2). All results were calculated on

Table 1 Mean values of $PLFA_{tot}$ concentration, bacteria-to-fungi ratio (bac/fungi), Gram-positive-to-Gram-negative bacteria ratio ($Gram^+/Gram^-$), cyclopropyl-to-precursor PLFA ratio (stress), sample scores of the first and second principal components (PC1 and PC2) of the Betaproteobacteria and *Pseudomonas* DGGE data, mild-solvent extractable (MSE) SDZ, residual SDZ (RES) fraction, 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). Soil of the DMR treatments was dried from day 0 until day 7 and from day 27 until day 34

Time		−1 day	0 day	7 days					20 days				27 days				34 days				49 days		
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	2,459 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	2,434	2,350	<LOQ	<LOQ	1,433	1,605	<LOQ	<LOQ	1,907	1,575	<LOQ	<LOQ	1,526 a	1,079 b	<LOQ	<LOQ	1,330	1,144

Significant differences between treatments at incubation time −1 (before manure application), 0 (after manure application), 7, 20, 27, 34, and 49 days are indicated with different letters ($p<0.05$ or $p<0.001$, in case of violated Levene's test, cf. Table 1)

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

oven-dm basis (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 (Supplementary 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). Principal 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 components (PC2) were extracted from the output file to calculate a two-way ANOVA with post hoc tests.

Results

Soil moisture

The two different soil moisture regimes CMR and DMR resulted temporarily in a significantly different moisture status of the soils, as intended (Table 1). The ANOVA indicated that the moisture treatment ($F=110.9$), time ($F=10.1$), as well as the interaction of both factors ($F=79.9$) had a 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 days 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).

Fate of the SDZ parent compound

No SDZ (MES+RES) was discovered in SDZ 0 CMR and DMR soils (<LOQ; Table 1). In SDZ 4 samples, the mild-solvent extractable (MES) SDZ dissipated over time ($p<0.001$) from 2,459 and 775 μg kg^{-1} (7 days) to 97 and 66 μg kg^{-1} (49 days) in CMR and DMR soils, respectively. The MES fraction of the SDZ 4 treatment significantly ($p<0.001$) interacted with the moisture regime (Tables 1 and 2). This was reflected by MES concentrations being significantly larger ($p<0.05$) by a factor of 3.2 (7 days), 1.7 (27 days), and 1.8 (34 days) 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 days) to 1,330 and 1,144 μg kg^{-1} (49 days) under DMR and CMR conditions (Tables 1 and 2). The trend of higher SDZ concentrations in the RES fraction in DMR compared to that in CMR soil was significant ($p<0.05$) at incubation time of 34 days, i.e., SDZ became increasingly sequestered as incubation time proceeded in DMR.

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 days (Tables 1 and 2). At individual incubation times, the SDZ treatment significantly increased ($p<0.05$) the $PLFA_{tot}$ concentrations by a factor of 1.6 (20 days) and 1.5 (27 days) 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 by a factor of 2.3 in SDZ 4/CMR compared to the SDZ 0/CMR soils (Table 1).

Table 2 Two-way ANOVA for the factors moisture regime (CMR/DMR), treatment (SDZ 0/SDZ 4), interaction of moisture regime × 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) axes of the principal component analyses of Betaproteobacteria and *Pseudomonas* DGGE data, mild-solvent extractable (MSE) SDZ, and residual SDZ (RES) fraction (μg kg^{-1}). Shown are the *F* values and the significances

Factors/parameters	$PLFA_{tot}$	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 × treatment	11.3***	0.1	1.3	2.6	1.6	3.8	0.7	1.3	52.0***	2.0

In case of a violated Levene's test ($p<0.05$), only factors with $p<0.001$ are expected as significant

*$p<0.05$; **$p<0.01$; ***$p<0.001$

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 days (Table 1). In contrast, when SDZ was present, we found 1.4- (20, 49 days) and 4.4-fold (27 days) 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 × 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 days.

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 days (Table 1). Similarly, the ratios of Gram-positive-to-Gram-negative bacteria ($Gram^+/Gram^-$) indicated significant shifts ($p<0.05$) toward the $Gram^+$ bacteria from 1.4 (−1 day) to 2.1 and 2.3 (0 day) 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 day, but they were unaffected by the presence of SDZ (Tables 1 and 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 (Tables 1 and 2). The stress ratio was influenced by the different moisture regimes ($p<0.01$). The first drying event at incubation time 7 days 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 days 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 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 ($F=5.4$; $p<0.05$).

DGGE analyses of the 16S rRNA gene

Genotypic shifts within the Betaproteobacteria and *Pseudomonas* community were indicated by band appearance or loss (Supplementary Fig. S1a, b). The accordance of principal component analyses with PC1 and PC2 explained 28 % (Betaproteobacteria) and 34 % (*Pseudomonas*) of the total variance (Fig. 1a, b). ANOVA analyses of the PC2 score values (see "Data analysis" section) 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 were determined within the Betaproteobacteria community profile at incubation times 7 and 27 days (PC2 scores), and 34 days (PC1 scores) of the SDZ 0 treatment and at 7 and 34 days (PC1 scores) and 27 and 34 days (PC2 scores) of the SDZ 4 treatment, respectively (Tables 1 and 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+PC2) and to the treatment ($p<0.05$, PC2). This was reflected by significant shifts in *Pseudomonas* community structure when SDZ was present ($p<0.05$) in CMR soil at incubation time 7 (PC1) and 34 days (PC2) and in remoistened DMR soil at day 20 (PC2; Fig. 1b, Tables 1 and 2). 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 $F=5.3$, $p<0.05$).

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 days (Table 1), simulating a typical drought situation (Landesman and Dighton 2011). The consecutive rewetting of DMR soils completed each of the two drying-rewetting cycles (Table 1); such cycles are known to alter the soil microbial status (Xiang et al. 2008).

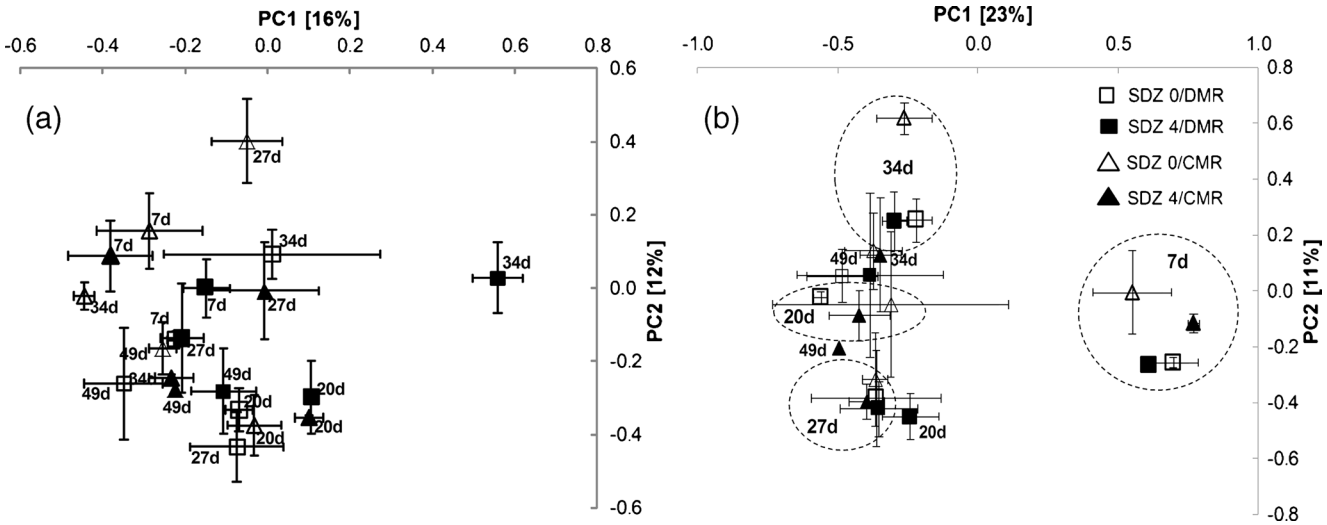

Fig. 1 Community shifts displayed by principal component analysis (PCA) of **a** Betaproteobacteria and **b** *Pseudomonas* 16S rRNA gene fragment DGGE data of the SDZ-uncontaminated (SDZ 0) and SDZ-contaminated (SDZ 4) soil samples, influenced by the dynamic (DMR) or control moisture regime (CMR). Displayed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

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 (Tables 1 and 2), which similarly were reported after application of manure with and without SDZ to unplanted and planted bulk soils (Hammesfahr et al. 2008, 2011a; Reichel et al. 2013). Compared to this, the responses of the measured microbial parameters to the moisture regime were lower or even absent (Fig. 1, Tables 1 and 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 to 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 preadapted 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, and also the low responsiveness of the genotypic community structure and total microbial biomass in SDZ-uncontaminated DMR soil (Fig. 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, Fig. 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, SDZ also dissipated faster in these soils.

The PLFA-derived stress ratio was lowered in SDZ-uncontaminated DMR soils due to the dynamic moisture

changes (Tables 1 and 2). The stress ratio was introduced to monitor the level of bacterial starvation in soil (Bossio et al. 1998), and here, it probably 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 (Tables 1 and 2). The latter finding is in line with the 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 of SDZ effects in soil.

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 might reflect 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.

Overall, 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).

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