Impact of the antibiotic sulfadiazine and pig manure on the microbial community structure in agricultural soils

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ABSTRACT

Large amounts of veterinary antibiotics enter soil via manure of treated animals. The effects on soil microbial community structure are not well investigated. In particular, the impact of antibiotics in the presence of manure is poorly understood. In this study, two agricultural soils, a sandy Cambisol (KS) and a loamy Luvisol (ML), were spiked with manure and sulfadiazine (SDZ; 0, 10 and $100\,\mu g\,g^{-1}$) and incubated for 1, 4, 32 and 61 days. Untreated controls received only water. The microbial community structure was characterised by investigating phospholipid fatty acids (PLFA) and using PCR–denaturing gradient gel electrophoresis (DGGE) of 16S rDNA. The total concentration of PLFA increased with addition of manure and was reduced by both SDZ concentrations at incubation times >4 days. The SDZ addition decreased the bacteria:fungi ratio. The largest stress level, measured as ratio of PLFA (cyc17:0 + cyc19:0)/(16:1ω7c + 18:1ω7c), was found for the controls, followed by the manure treatments and the SDZ treatments. A discriminant analysis of the PLFA clearly separated treatments and incubation times. Both soils differed in total PLFA concentrations and Gram$^-$:Gram$^+$ ratios, but showed similar changes in PLFA pattern upon soil treatment. Effects of manure and SDZ on the bacterial community structure were also revealed by DGGE analysis. Effects on pseudomonads and β-proteobacteria were less pronounced. While community structure remained altered even after two months, the extractable concentrations of SDZ decreased exponentially and the remaining solution concentrations after 32 days were ≤27% of the spiking concentration. Our results demonstrate that a single addition of SDZ has prolonged effects on the microbial community structure in soils.

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1. Introduction

Antibiotics are used worldwide in livestock farming as growth promoters or to treat infectious diseases. Sulfonamides are the second most important antibiotic class in European countries, with an estimated consumption of $78\,t\,y^{-1}$ in livestock farming and especially pig production (Thiele-Bruhn, 2003). After feeding antibiotics to livestock, about 90% is excreted as parent compound or metabolite (Halling-Sørensen et al., 1998). The antibiotics reach soil either directly when livestock excrete on pastures or indirectly when slurry and manure is spread onto agricultural soils. Sulfonamide residues have been detected in agricultural soils at concentrations of up to $11\,\mu g\,kg^{-1}$ (Höper et al., 2002). Several studies on the fate of sulfonamides in soil suggested that biological effects are likely (Sukul and Spiteller, 2006).

Sulfonamides are broad-band bacteriostatic antibiotics which inhibit dihydropteroate synthesis in the folic acid pathway (O'Neil et al., 2001) reducing the reproduction of bacteria. Therefore soil microbial biomass, structural composition and enzyme activities may be affected. Only a few studies exist investigating the effects of antibiotics on microbial structure and function. Sulfonamide antibiotics were reported to affect general and potential microbial activities and the bacterial community structure (Zielezny et al., 2006) and shifts from bacteria to fungi were observed (Thiele-Bruhn and Beck, 2005). However, effects depended on the addition of glucose as a C and energy source. Therefore the effect of sulfonamides is very likely linked to substrate addition to promote microbial growth. This might be relevant for manure as well, being the main carrier of antibiotics to soil, which aspect needs further investigation.

Manure is used to improve soil nutrient status and fertility in agricultural soil. Moreover, it affects the soil microbial population, mostly stimulating microbial growth and activities. Böhme et al. (2005) and Bossio et al. (1998) showed an increase in microbial C and enzyme activities caused by organic fertiliser. Structural changes in the soil community have been determined by phospholipid fatty acids (PLFA) profiling and were attributed to

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Gram$^-$ bacteria (Böhme et al., 2005). However, these stimulating effects of manure might be reduced by veterinary antibiotics, especially since manure was shown to influence soil physiological properties and the mobility and availability of antibiotics (Kahle and Stamm, 2007; Kreuzig and Höltge, 2005; Thiele-Bruhn and Aust, 2004). Yet the effects of sulfonamides in combination with manure were only scarcely investigated. Schmitt et al. (2005) investigated the effect of a sulfonamide antibiotic on the pollution-induced community tolerance (PICT) of soil. Community tolerance was enhanced with addition of pig slurry. Parallel investigations to the study presented here using the same samples found effects on the bacterial antibiotic resistance and nitrogen turnover processes (Heuer and Smalla, 2007; Kotzerke et al., 2008). Changes in the structural composition of the soil microflora are therefore likely. However, studies are lacking on the effects of sulfonamides in combination with manure on soil microbial community structure.

Consequently, the aim of this study was to examine the effect of manure plus the sulfonamide sulfadiazine (SDZ) on the structural diversity of the soil microbial community using PLFA and PCR–denaturing gradient gel electrophoresis (PCR–DGGE) patterns. We tested the hypotheses that (i) SDZ affects the total amount of microbial biomass in manured soil, (ii) manure plus SDZ affect bacterial community structure, but some groups like pseudomonads, which are believed to be intrinsically resistant to SDZ, are not affected, (iii) the fungal biomass is favoured mainly by an application of the bacteriostatic SDZ, and (iv) stress levels are increased by SDZ. This was achieved by a microcosm experiment with two soils that were differentially treated with manure and SDZ. PLFA profiling and PCR–DGGE analysis were carried out, methods which have already shown to be very sensitive for studying microbial community changes (Ramsey et al., 2006).

2. Material and methods

2.1. Soil samples

Two agricultural field soils, typical for Middle Europe, were sampled for the incubation experiments and characterised by selected soil properties (Table 1).

A periodically manured loamy sand Gleyic Cambisol was sampled at Kaldenkirchen, Germany (KS), and a silt loam Orthic Luvisol at Merzenhausen near Jülich, Germany (ML), which was never fertilised with manure.

Soil samples were taken and pooled from the ploughed topsoil horizon (A$_p$) in April 2005. Manure used in this study was obtained from mature pigs (60 pigs, 80–100 kg, 6 months old) which had not been treated with antibiotics. Soils were sieved (2 mm) and air-dried for 2 days. Manure was suspended in water or aqueous SDZ solution and thoroughly mixed with soil (320 g manure in 160 ml water added to 8 kg soil) to amend soil with typically used agricultural amounts of manure of 40 mg g^{-1} soil and 0, 10 or 100 µg SDZ g^{-1} soil, corresponding to treatments S0, S10 and S100, respectively. The lower antibiotic concentration of 10 µg g^{-1} is

a normal concentration in livestock husbandry. 100 µg g^{-1} simulates a very intensive therapy and use of undiluted manure. Manure had a slightly acidic pH of 6.3 (Table 1) and manure addition increased pH of the acidic soil KS by 0.3 units and decreased pH of the neutral soil ML by 0.6 units. Due to the high organic C content of 41% in manure (Table 1), the organic C content in manured soils increased at the beginning of the experiment by 0.2% compared to the untreated control soils. After the incubation experiment the organic C content was not significantly different in respective samples with and without manure. The untreated control (U) was only amended with 40 ml water kg^{-1} soil to achieve the same water content. Each treatment was prepared in pots ($n = 4$) with a total sample fresh weight of 2 kg and incubated in the dark at 10 °C. Each treatment was sampled after 1, 4 and 32 days. For selected analyses, incubation time was extended to 61 days. Water content was controlled to keep the soil moisture at 11% and 16% for KS and ML, respectively, corresponding to 50% of the maximum water holding capacity.

2.2. Determination and analysis of antibiotic concentration

The SDZ concentration of the soil samples was determined by a sequential extraction procedure using 0.01 M CaCl$_2$ (soil–solution ratio 1:5) (SDZ$_{CaCl2}$) for the mobile fraction, followed by an extraction step with methanol (soil–solution ratio 1:2.5) (SDZ$_{MeOH}$) representing the total desorbable and hence potentially bioavailable SDZ fraction (Thiele-Bruhn and Aust, 2004). SDZ$_{tot}$ is the sum of both fractions. For each extraction step, samples were sonicated (30 min) and centrifuged (30 min, 1700 g min^{-1}). Supernatants were evaporated and freeze-dried prior to resuspension in 1 ml MeOH. For analysis, a high performance liquid chromatography system (HP 1050, HP, Böblingen, Germany) with a 250 mm × 4.6 mm Nucleosil C18 reversed phase column (Macherey-Nagel, Düren, Germany) and a diode array detector (Agilent G1315B, Agilent, Böblingen, Germany) operated at 254 nm was used. The mobile phase consisted of methanol and 0.01 M H$_3$PO$_4$, with a flow rate of 1 ml min^{-1}. SDZ was quantified using the HP ChemStation software and external standards (limits of detection and quantification of 1 µg L^{-1} and 10 µg L^{-1}, respectively). Spectra were identified if the accordance with the software database was above 90%. The recovery rate was previously described to range from 90% to 103% in soil and soil–manure mixtures (Thiele-Bruhn and Aust, 2004).

2.3. Determination and analysis of phospholipid fatty acids

Lipids were extracted according to Frostegård et al. (1993). Soil samples (20 g fresh weight) were shaken for 2 h with a mixture of citric acid, chloroform and methanol (0.8:1:2, v/v/v) and centrifuged for 30 min at 2500 g min^{-1} (Bligh and Dyer, 1959). The resulting extracts were sequentially fractionated into neutral-, glyco- and phospholipids with chloroform, acetone and methanol using silica gel-filled solid phase extraction cartridges (SPE). The phospholipids (PLFA) were subjected to alkaline methanolysis using 0.2 M methanolic KOH. An internal standard, c19:0 (methylene nonadecanoate, Sigma–Aldrich, Taufkirchen, Germany) was added to quantify the PLFAs. PLFAs were separated by an HP 6890 gas chromatograph equipped with a 30 m × 0.2 mm fused silica capillary column (Optima 5 MS, Macherey-Nagel, Düren, Germany) and detected with mass spectrometry (Hewlett Packard MSD 5973, Palo Alto, CA, USA). Helium was used as carrier gas with a flow rate of 1.5 ml min^{-1}. The initial oven temperature was 60 °C for 1 min, ramped to 150 °C at 10 °C min^{-1} and increased to 320 °C at 5 °C min^{-1}, and held for 25 min. Peaks were identified using a fatty acid methyl ester mixture (FAME 37, Supelco, Taufkirchen,

Table 1
Soil properties of the bulked top soil samples and the used manure

	Manure	KS (sandy Cambisol)	ML (loamy Luvisol)
pH (CaCl$_2$)	6.3	5.5	7.2
Organic C (g 100g^{-1})	41.2	1.0	2.1
Total N (g 100g^{-1})	3.15	0.10	0.11
Clay (g 100g^{-1})		3.6	15.4
Silt (g 100g^{-1})		23.1	78.2
Sand (g 100g^{-1})		73.3	6.4
Max. water capacity (g 100g^{-1})		27	46

Germany) and a bacterial PLFA mixture (CP-Mix, Biotrend, Cologne, Germany) as external standards.

The branched phospholipids i15:0, a15:0, i16:0, i17:0, a17:0 were used as indicators for Gram-positive bacteria (Gram$^+$), while the PLFAs 16:1ω5c, 16:1ω7t, 18:1ω5c, 16:1ω7c, cy17:0, 18:1ω7c and cy19:0 were considered as Gram-negative bacteria (Gram$^-$) markers (Federle, 1986; Frostegård et al., 1993; Greyston et al., 2004). The unsaturated PLFA 18:2ω6 was used as a fungal biomass indicator, while 15:0 and 17:0 PLFAs were considered as general bacterial markers (Federle, 1986). The total amount of PLFA (PLFA$_{tot}$) is the sum of all microbial phospholipids mentioned. Ratios of Gram$^-$:Gram$^+$ PLFA and bacteria:fungi PLFA were used as indicators for changes in the relative abundance of these microbial groups (Bardgett et al., 1996). The stress level (cyc:precursor) for the microbial community was calculated from the ratio of (cyc17:0 + cyc19:0)/(16:1ω7c + 18:1ω7c) (Bossio and Scow, 1998).

2.4. DNA extraction, PCR and DGGE

DNA from manure and soil samples was directly extracted as previously described (Heuer and Smalla, 2007). Amplification of 16S rDNA gene fragments was done using the primer set U968GC and L1401 (Heuer et al., 1997), and PCR products differing in melting properties were separated in a denaturing gradient gel electrophoresis (DGGE) according to Heuer et al. (2002). A denaturing gradient of 3.2–4.6 M urea plus 16.0–23.2% (v/v) formamide was used for DGGE, which was performed in 1× Tris-acetate–EDTA buffer at 60 °C and a constant voltage of 180 V for 4 h. Acid silver staining was used for detection of DNA in DGGE gels (Riesner et al., 1989). For analysis of pseudomonads or β-proteobacteria, their 16S rDNA genes were specifically amplified prior to PCR–DGGE using primer pairs F311Ps/R1459Ps (Gomes et al., 2001) and F948β/R1494 (Milling et al., 2004), respectively. Gels were analysed using image analysis Bio-1D (Vilber-Lourmat, Marne-la-Vallée, France). Only bands which accounted for more than 1% of the total lane intensity were considered.

2.5. Data analysis

SDZ concentrations were modelled with a two-phase coupled pseudo-first order exponential equation of the type $c_t = c_{0.1}\exp(-k_1\ t) + c_{0.2}\exp(-k_2\ t)$, describing the dissipation kinetics of SDZ at two spiking concentrations (S10 = 10 µg g^{-1}; S100 = 100 µg g^{-1}) (Thiele-Bruhn and Peters, 2007). c_t is the calculated actual SDZ concentration at the incubation time t; $c_{0.1}$ and $c_{0.2}$ are the exponential constants, representing in addition the calculated initial SDZ concentration; k_1 and k_2 are the degradation rate constants.

The Shannon–Wiener diversity index:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

was calculated taking all individual PLFAs to investigate the structural diversity of the microflora. p_i is the proportion of each individual phospholipid of PLFA$_{tot}$. Normal distribution of the data was tested by the Kolmogorov–Smirnoff goodness-of-fit test, and homogeneity of the variances was tested by the Levene's test ($p < 0.05$). Differences between the treatments (factor 1, $F_{treatment}$) and the incubation time (factor 2, F_{time}) of PLFA$_{tot}$, Gram$^-$:Gram$^+$ ratio, bacteria:fungi ratio, stress level and Shannon index were calculated by a two-way ANOVA for each soil separately. For the categorical factors soil (F_{soil}), $F_{treatment}$ and F_{time} a simple three-way ANOVA was used to test differences of every dependent variable (PLFA$_{tot}$, Gram$^-$:Gram$^+$, bacteria:fungi, stress level, Shannon index). A discriminant analysis (DA) was carried out to investigate

the PLFA pattern of all treatments and incubation times separately for both soils. The PLFAs were used as dependent variables, while the independent variables were all combinations of treatment with incubation time. For cluster analysis of DGGE the Dice correlation matrix and the unweighted pair group method using arithmetic averages (UPGMA) were used to calculate similarity.

Significant differences were accepted at $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$. All values are mean values (±SEM) of three to four replicates calculated on an oven-dry weight basis (105 °C, 2 days). Statistics were calculated using Statistica 6.0 (Statsoft, Tulsa, OK, USA).

3. Results

3.1. SDZ concentration

There was a strong decline of extractable SDZ$_{tot}$ within 1 day which levelled out at longer incubation times for all treatments. Accordingly, dissipation kinetics were best described using a two-phase coupled, pseudo-first order model (Fig. 1, Table 2).

Dissipation of SDZ was faster in soil ML compared to soil KS and for the S100 spiking level compared to the S10 level. This can be seen from the larger first degradation constants k_1 (Table 2). Calculated disappearance times of 50% of the spiking level ranged from <1 day to 8.5 days. Correspondingly, extractable SDZ concentrations residing in the soil samples after 32 days were <27% of the initial spiking level.

This clearly showed that the potentially bioavailable fractions of SDZ quickly declined with variation among soils and spiking levels. No SDZ was detected in the treatments without SDZ addition.
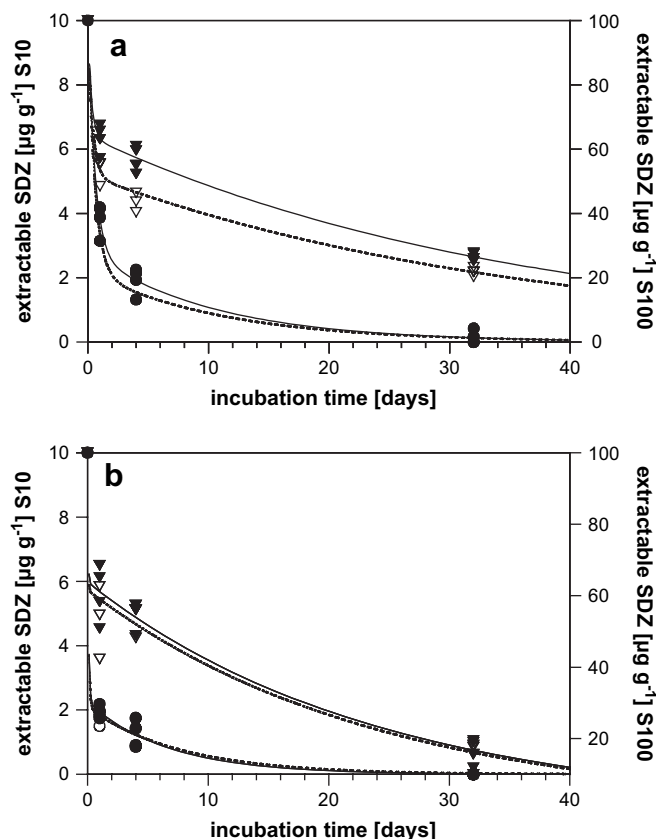

Fig. 1. SDZ concentrations of the SDZ$_{CaCl2}$ and SDZ$_{tot}$ fraction at incubation times 1, 4, 32 days and modelled curve fits of the coupled pseudo-first order equation $c_t = c_{0.1} \times \exp(-k_1\ t) + c_{0.2} \times \exp(-k_2\ t)$ used for the SDZ kinetics (parameters see Table 2); (a) soil KS (Kaldenkirchen), (b) soil ML (Merzenhausen). Spiking concentrations: circle: S10 = 10 µg g^{-1}; triangle: S100 = 100 µg g^{-1}; open symbols: SDZ$_{CaCl2}$ fraction; closed symbols: SDZ$_{tot}$ fraction.

Table 2
Parameters of the coupled pseudo-first order equation $c_t = c_{0.1} \times \exp(-k_1\ t) + c_{0.2} \times \exp(-k_2\ t)$ used for the SDZ kinetics

	S10		S100	
	SDZ_{CaCl2}	SDZ_{tot}	SDZ_{CaCl2}	SDZ_{tot}
Sandy Cambisol—KS				
$c_{0.1}$ ($\mu g\,g^{-1}$)	7.79	7.19	47.92	35.93
k_1 (d^{-1})	1.73	1.71	2.72	3.47
$c_{0.2}$ ($\mu g\,g^{-1}$)	2.21	2.81	52.08	64.07
k_2 (d^{-1})	0.09	0.10	0.03	0.03
R^2	0.97	0.99	0.97	0.98
p	<0.05	<0.05	<0.05	<0.05
DT50 (d)	<1	<1	1.7	8.5
C_{32} ($\mu g\,g^{-1}$)	0.15	0.15	21.81	26.64
Loamy Luvisol—ML				
$c_{0.1}$ ($\mu g\,g^{-1}$)	7.93	7.75	38.13	35.81
k_1 (d^{-1})	11.69	12.28	22.91	19.87
$c_{0.2}$ ($\mu g\,g^{-1}$)	2.07	2.25	61.86	64.19
k_2 (d^{-1})	0.13	0.15	0.04	0.04
R^2	0.99	0.99	0.93	0.96
p	<0.05	<0.05	<0.05	<0.05
DT50 (d)	<1	<1	4.8	5.6
C_{32} ($\mu g\,g^{-1}$)	<LOD[a]	<LOD	15.38	16.52

Spiking concentrations: S10 = 10 $\mu g\,g^{-1}$; S100 = 100 $\mu g\,g^{-1}$. DT50: Calculated disappearance times of 50% of the spiking level, C_{32} ($\mu g\,g^{-1}$): measured concentrations after 32 days.

[a] <LOD: below the limit of detection.

3.2. PLFA concentration

The $PLFA_{tot}$ concentrations over incubation time of soil KS ranged from 195 to 372 nmol PLFA g^{-1} and were significantly ($F_{soil} = 92.2$, $p < 0.001$) smaller than for soil ML (212–589 nmol PLFA g^{-1}) (Fig. 2, Table 3).

$PLFA_{tot}$ was significantly larger in manure treatments (S0) versus untreated controls for both soils during the entire incubation period ($F_{treatment} = 30.0$, $p < 0.001$). Adding SDZ (S10, S100) tended to decrease the effect of manure on $PLFA_{tot}$ at all incubation days (1, 4, 32) in both soils, except day 1 in soil ML. This decrease was significant at day 32 for both soils and even led to a significant reduction of $PLFA_{tot}$ concentrations in the SDZ treated samples down to the level of the controls in soil KS at day 32. For all incubation times and both soils, no significant difference in $PLFA_{tot}$ was determined among the two different SDZ spiking concentrations.

The effects of the different treatments on four parameters that were obtained from the PLFA analysis in soil KS are shown in Fig. 3a–d.

The ratio of Gram⁻:Gram⁺ bacterial PLFA markers of all treatments in soil KS varied significantly with the incubation time ($F_{time} = 10.0$, $p < 0.001$, Table 3). The ratio of Gram⁻:Gram⁺ ($F_{treatment} = 3.7$, $p < 0.05$) tended to slightly increase in the manure treatments (S0, S10, S100) compared to the control U (Fig. 3a, Table 3). The control had ratios between 0.8 and 1.1 at all incubation times (1, 4, 32 days), the manure treatments (with or without SDZ) had larger Gram⁻:Gram⁺ ratios between 0.8 and 1.8 for all treatments. For all incubation times no additional effect of SDZ on the ratio of Gram⁻:Gram⁺ was determined compared to the treatments with manure alone. For soil ML, the ratio of Gram⁻:Gram⁺ bacteria ($F_{time} = 14.9$, $p < 0.001$) showed similar trends as for soil KS (Table 3), with higher amounts of Gram⁻ bacteria (Fig. S1a, supplementary data), leading to Gram⁻:Gram⁺ ratios up to 2.6.

The ratio of bacteria:fungi PLFA markers in soil KS was significantly decreased for all incubation times in manure treatments (S0, S10, S100) compared to the control U ($F_{treatment} = 93.6$, $p < 0.001$; Table 3) (Fig. 3b). Moreover, the bacteria:fungi PLFA ratio was in some cases decreased in the SDZ-treated samples (S10, S100) compared to the manure treatment without SDZ (S0). At day 1 fungi were stimulated, at the other incubation times this was an effect of the reduced bacterial biomass. Similar trends were obtained for the bacteria:fungi PLFA ratio in soil ML ($F_{treatment} = 25.5$, $p < 0.001$, Table 3), (Fig. S1b, supplementary data).

The stress level of microbes in soil KS is shown in Fig. 3c and tended to be lower in manure treatments versus control samples independent of the incubation time ($F_{time} = 2.3$, $p > 0.05$) (Table 3). The S10 amendment tended to further reduce the stress level at all incubation times ($F_{treatmen\ t} = 13.9$, $p < 0.001$), while it was similar between S0 and S100. The stress level of the control in soil ML was lower than in soil KS and no significant differences between the control and treatments were determined ($F_{treatment} = 1.3$, $p > 0.05$; Table 3), except day 4 (Fig. S1c, supplementary data).

The Shannon–Wiener index ranged between 2.2 and 2.5 for soil KS for all incubation times (Fig. 3d), with no significant effect of the treatments ($F_{treatment} = 1.0$, $p > 0.05$) or incubation time ($F_{time} = 0.7$, $p > 0.05$, Table 3). In soil ML manure did significantly affect the Shannon–Wiener diversity index at days 1 and 4 ($F_{treatment} = 6.8$, $p < 0.01$; $F_{time} = 7.1$, $p < 0.001$; Table 3). No effect was obtained for the incubation time of 32 days (Fig. S1d, supplementary data).

The inherent differences among the two soils were identified by a three-way ANOVA for $PLFA_{tot}$, Gram⁻:Gram⁺ and bacteria:fungi ratios ($p < 0.001$; $PLFA_{tot}$: $F = 92.2$; Gram⁻:Gram⁺: $F = 31.1$; bacteria:fungi: $F = 59.3$). Soil treatment and soil incubation time
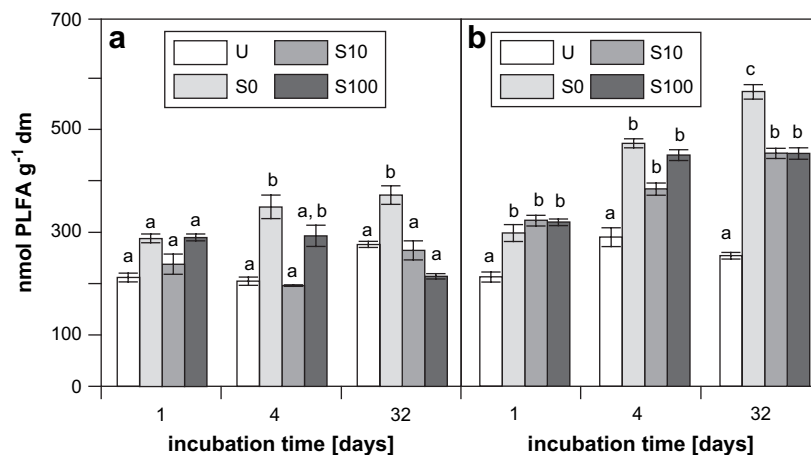
Fig. 2. $PLFA_{tot}$ depending on the sample treatment (U: control soil; S0: manure treatment; S10: manure + 10 μg SDZ g^{-1}; S100: manure + 100 SDZ $\mu g\,g^{-1}$) and incubation time (1, 4, 32 days); (a) soil KS (Kaldenkirchen) and (b) soil ML (Merzenhausen). Different letters indicate significant differences among treatments at a given incubation time and soil (one-way ANOVA, followed by the least significant difference (LSD) test).

Table 3
Multivariate analysis of variance by two-way and three-way ANOVA of the sum of all PLFAs, ratios of Gram⁻:Gram⁺ and bacteria:fungi PLFA, stress level and Shannon index

Factor	PLFA$_{tot}$		bac:fungi		Gram⁻:Gram⁺		Stress		Shannon	
	KS	ML	KS	ML	KS	ML	KS	ML	KS	ML
Two-way										
Treatment (treat)	8.3***	31.7***	93.6***	25.5***	3.7*	0.7	13.9***	1.3	1.0	6.8**
Time	1.0	34.0***	5.5**	0.8	10.0***	14.9***	2.3	1.1	0.7	7.1**
Treat × time	2.7*	3.9**	0.5	0.3	1.8	1.2	0.3	1.6	1.2	5.1**
Three-way										
Soil		92.2***		59.3***		31.1***		8.1**		0.0
Treatment (treat)		30.0***		56.6***		3.4*		13.5***		3.1*
Time		20.3***		6.2**		20.9***		2.6		2.2
Soil × treat		7.1***		5.4**		3.1*		6.3***		0.8
Soil × time		10.9***		11.2***		1.4		1.0		1.3
Treat × time		4.2**		3.6**		1.0		0.4		2.4*
Soil × treat × time		2.2		1.3		1.3		0.8		1.3

The categorical factors are soil (KS, ML), treatment (U, S0, S10, S100), incubation time (1, 4, 32 days). Presented are the F-values with the level of significance.

interactions were significant, but rather small compared with the single factors (Table 3). The stress parameter and the Shannon–Wiener index were most influenced by the treatment factor (stress: $F = 13.5$, $p < 0.001$, Shannon: $F = 3.1$, $p < 0.05$).

The results of a discriminant analysis of the PLFA patterns are shown in Fig. 4. Different community structures were identified at the different soil treatments and incubation times. The Gram⁻ fatty acid markers ($p < 0.05$) contributed most to the separation of the groups. The PLFA pattern of the control samples of soil KS were significantly separated from all other treatments (Fig. 4a), while the differences among the three incubation times were not significant.

In soil ML all control samples differed significantly from all other treatments except for the control samples at day 32 and the S10 treatment at day 4. In contrast to soil KS, differences among the incubation times of control samples were significant, except differences between days 4 and 32. For each of the treatments S0, S10, S100 of both soils PLFA patterns were significantly different between incubation times. The samples with manure alone (S0) showed significant different PLFA patterns than samples with additional SDZ (S10, S100) for the same incubation time and between different incubation times, except S0 at day 4 and S10 at day 1. Moreover, most manure-treated samples (S0) differed significantly
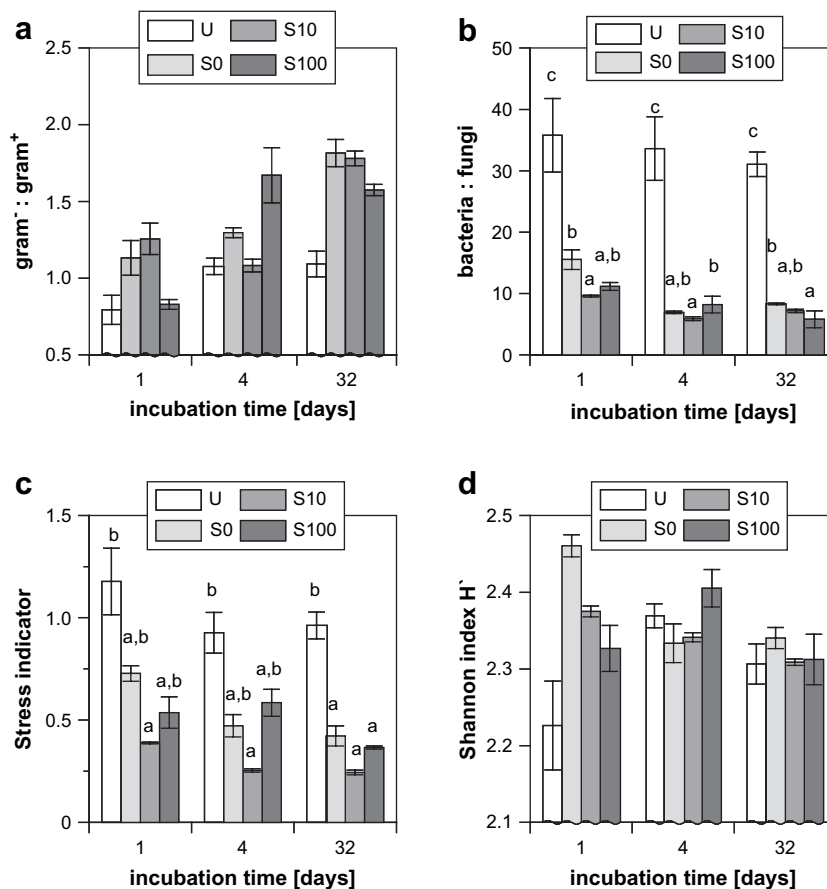
Fig. 3. (a) Gram⁻:Gram⁺ ratio and (b) fungi:bacteria ratio, (c) stress level and (d) Shannon–Wiener Index of soil KS depending on the sample treatment (U, S0, S10, S100) and incubation time (1, 4, 32 days). Different letters indicate significant differences among treatments at a given incubation time (one-way ANOVA, followed by the least significant difference (LSD) test). All bars without letters are not significantly different and get the label "a". Abbreviations: see Fig. 2.
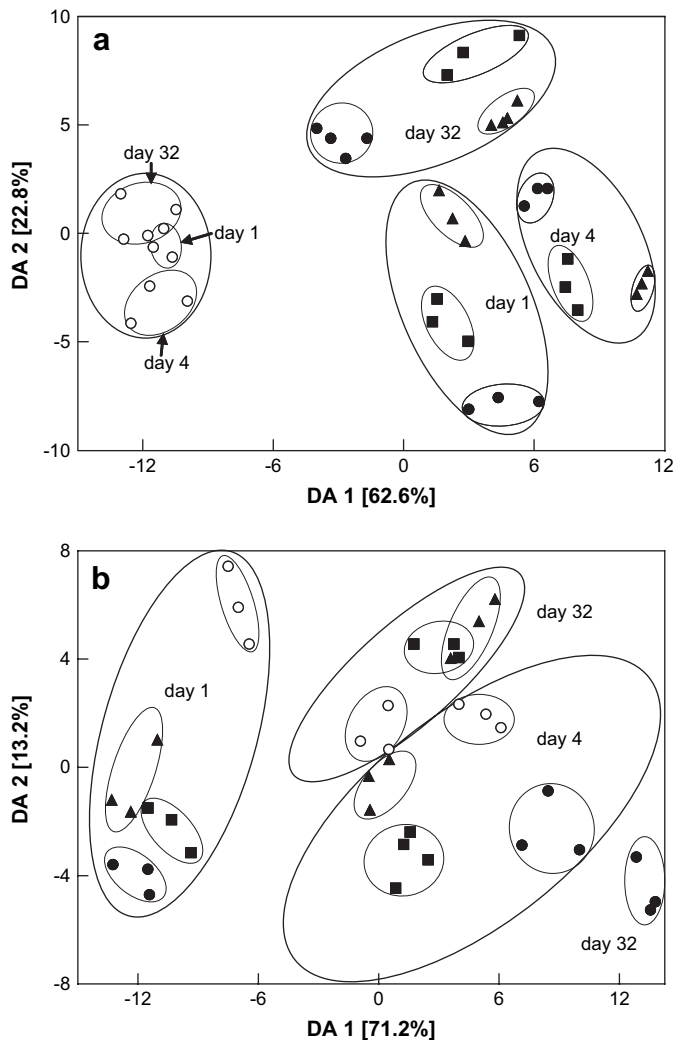
Fig. 4. Discriminant analysis of fatty acids (dependent factors) of all treatments (U, S0, S10, S100) and incubation times (1, 4, 32 days); (a) soil KS, (b) soil ML. All treatments and incubation times were defined as separate cases. Open circle: U; filled circle: S0; triangle: S10; square: S100. Big ellipses grouped different incubation times. Abbreviations: see Fig. 2.

from the manure plus SDZ treatments (S10, S100) within a given incubation time, except day 1 in soil ML and day 4 in soil KS. In contrast, the samples with different SDZ spiking concentrations were not different to each other within the same incubation time.

3.3. DGGE

For both soils, DGGE patterns of 16S rDNA genes of bacteria at day 32, β-proteobacteria at day 61 and DGGE patterns of pseudomonads at days 32 and 61 were investigated. All replicates ($n = 4$) of each treatment were analysed. Banding patterns were nearly identical among replicates, though total band intensity of lanes varied strongly. All gels are presented in Fig. S2a–g (supplementary data).

Pure manure had a very different banding pattern than soil treated with manure (Fig. S2, supplementary data). Most changes in banding pattern were observed in bacterial profiles (up to 8 bands), followed by pseudomonads (up to 5 bands) and β-proteobacteria (up to 3 bands).

Manure treatment increased band intensity, while manure plus SDZ had a more differentiated effect on bacterial populations: it either increased or decreased band intensities (Fig. S2, supplementary

data). This pattern was found in all bacterial groups, with SDZ enhancing or reducing DGGE bands. A cluster analysis was performed for the band profiles (normalised intensity and band pattern) for all treatments (Fig. 5).

In most cases the unamended control soils had the least similarity to the other treated samples. Most manure-treated samples had smaller distances among each other, with the samples additionally treated with SDZ having the highest similarity to each other. This was true for bacteria in soil KS, and for pseudomonads in soil KS at 32 days and in soil ML at 32 and 61 days.

4. Discussion

4.1. SDZ concentration

The extractable concentration of SDZ rapidly decreased over the incubation time of 32 days. Sulfonamides have a large potential for rapid adsorption to the soil matrix and manure constituents (Kahle and Stamm, 2007; Thiele-Bruhn et al., 2004). The extractable SDZ concentration was larger in soil KS than ML for all incubation times. These differences may reflect the lower organic carbon and clay content of soil KS versus ML. Correspondingly, the sorption coefficients were larger in the clay compared to the sand fraction of soils for several antibiotic compounds (Thiele-Bruhn et al., 2004). Similarly, model studies with sulfonamides and clay minerals and organic substances revealed the importance of these soil properties for the sorption of antibiotics (Gao and Pedersen, 2005; Kahle and Stamm, 2007). The DT50 values were much lower for the S10 treatments, being less than 1 day in soil KS and ML, than for the S100 treatments (6–9 days). Hence, our results indicate that the kinetics largely depend on the applied initial SDZ concentrations. The sorption process of antibiotics is nonlinear for high concentrations, leading to higher extractable amounts (Thiele-Bruhn et al., 2004). The DT50 and resulting residual SDZ concentrations agree with findings from Hamscher et al. (2005) and Kreuzig and Höltge (2005).

The strong absorption of SDZ was mostly due to an immobilisation in soil. In contrast, transformation pathways such as incorporation in microbial biomass or mineralisation are negligible and contribute to less than 2% of the initially spiked SDZ (Kreuzig and Höltge, 2005; our own unpublished data). Major transformation products are the non-bioactive conjugate acetyl-SDZ and the metabolite hydroxyl-SDZ that exhibits strongly reduced antibiotic potential. Hence delayed antimicrobial effects due to the formation of antibiotic active metabolites were not likely. However, a detailed analytical investigation of metabolic pathways was beyond the scope of this study. Chemical analyses confirmed that stronger effects of SDZ in soil KS versus soil ML and by the S100 treatment versus the S10 treatment might be attributed to the larger bioavailable fraction of SDZ. In contrast, the bioavailable fraction decreased over time, while effects on the community structure increased.

4.2. Impacts on the microbial biomass

The total amount of phospholipids is a good indicator for the living microbial biomass and correlates closely with microbial biomass C (Bååth and Anderson, 2003). PLFA$_{tot}$ concentrations differed between the two soils, with maximum values of 370 nmol PLFA g^{-1} in KS and 590 nmol PLFA g^{-1} in ML that reflect soil properties. The larger sand content and lower pH of soil KS compared to soil ML may indicate a lower nutrient status and substrate quality of the soil organic matter, confirmed by the higher C/N ratio in soil KS. In both soils, adding manure increased the PLFA$_{tot}$ to a maximum value of 150% (KS) and 180% (ML) compared to the control samples without manure. These results are in line with
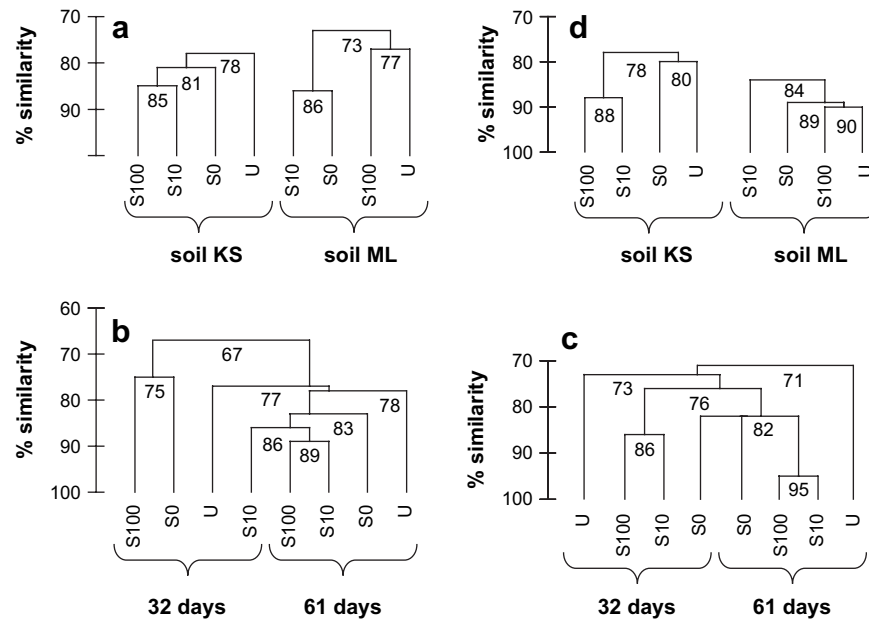
Fig. 5. Cluster analysis of DGGE patterns of (a) bacteria soil KS and ML, day 32, (b) pseudomonads soil KS, day 32 and 61, (c) pseudomonads soil ML, day 32 and 61, (d) β-proteobacteria soil KS and ML, day 61, depending on the sample treatment (U, S0, S10, S100). Cluster was generated using the Dice similarity matrix and UPGMA.

findings from Hund-Rinke et al. (2004) and Böhme et al. (2005) using pig slurry and farmyard manure, respectively. The SDZ treatments (S10/S100) reduced the PLFA$_{tot}$ to a maximum of 75% (KS) and 85% (ML) compared with the manure treatment. Hence, SDZ reduced the stimulating effect of manure on the total PLFA amount. This result confirmed that SDZ had a pronounced effect on the microbial community composition, even though the extracted concentrations diminished rapidly and quickly equalled the maximum concentration of 11 µg kg^{-1} found in agricultural soils (Höper et al., 2002).

A reduction of PLFA$_{tot}$ was also reported by Hund-Rinke et al. (2004) using tetracycline. However, this was only observed for an incubation time of 8 weeks and a high concentration of 500 µg g^{-1} tetracycline. The different effects of the antibiotics may be due to larger adsorption coefficients of tetracyclines, which resulted in higher bioavailable concentrations of SDZ.

4.3. Shifts in microbial community structure

The ratio of Gram$^-$:Gram$^+$ and bacteria:fungi fatty acids was used to study changes in microbial community structure for different treatments. All manure treated samples (with or without SDZ) had larger Gram$^-$:Gram$^+$ ratios of up to 2.6 compared to the control treatments. Similarly, ratios larger than 1 have been reported for cultivated soils with large C content or soils being treated with organic inputs (Bossio et al., 1998; Peacock et al., 2001). In our study, SDZ did not significantly affect Gram$^-$:Gram$^+$ ratios. As SDZ is a broadband antibiotic, there is no a priori expectation that this ratio will be affected. In contrast, Hund-Rinke et al. (2004) found that tetracycline affects the ratio after 8-week incubations. Tetracycline-resistant bacteria are Gram$^-$ and better resist large antibiotic concentrations (Schabel and Jones, 1999). The total incubation time may additionally affect the microorganism structure as substrate quality of the organic matter in soil changes due to degradation and aging, as it was the case in manured samples.

Adding manure caused shifts towards fungi. The calculated bacteria:fungi ratios were reduced to maximally 70% in KS/S0 and to 57% in ML/S0 compared with the control soil. Additional to a reduction in bacterial biomass, SDZ (S10, S100) stimulated fungi

at the beginning of incubation compared to S0 treatments, which is in line with findings of Thiele-Bruhn and Beck (2005), who used sulfapyridine.

In soil KS manure and SDZ affected the stress level of the microflora. The largest cyc:precursor ratio was in the control (U), followed by S0, S100 and S10. Our results showed the largest stress level in the control soil indicating a suboptimal nutritional status compared to all manure treatments (with or without SDZ). Similarly, Bossio et al. (1998) and Bossio and Scow (1998) reported a decrease of cyclopropyl fatty acids under organic fertiliser treatments or straw addition. No information is available on stress levels in environmental samples treated with antibiotics. The effect of SDZ on the stress level in soil KS was inconstant, with a stress reduction in samples S10 and an increase in samples S100. The results from the S100 treatments suggest that a critical concentration of SDZ was reached and impacted the remaining microbial community. Soil ML showed no significant differences between stress indicators in the control and manure treatments. This result together with larger PLFA$_{tot}$ indicate that this soil with better nutrient status, larger organic C, pH and clay content favours microbial functioning better than soil KS, reducing the stress level.

4.4. Effects on the microbial population

The discriminant analysis showed significant differences in the PLFA pattern between control and manure treatments (with and without SDZ). The control soils were mostly unaffected during the entire incubation. Consequently, shifts in treated samples were due to manure and SDZ addition, respectively. Several studies reported a larger influence of incubation time versus treatment (Böhme et al., 2005; Bundy et al., 2004; Hund-Rinke et al., 2004). In contrast to these studies, we found that different treatments were more important than incubation time. The manure treatments differed clearly from the control samples. The effect of treatments was mirrored by the ANOVA results, indicating that incubation time was less important than treatment. An initial SDZ concentration of 10 µg g^{-1} that declined to <3.3 µg g^{-1} within 1 day already caused shifts in the microbial community. These results are in contrast to Kotzerke et al. (2008), using the same experimental design.

Kotzerke et al. (2008) found that effects on the microbial activity and N-transformation processes were only visible for up to 4 days of incubation. Discrepancies between soil community structure and functioning were reported earlier (Coleman and Whitman, 2005). In our study it showed that antibiotic effects on microbial activities may be masked by functional redundancy sustained by a structurally changed microbial community.

4.5. Impacts on the DNA level

The DGGE patterns showed effects of manure and of SDZ on soil bacterial community structures 32 and 61 days after treatment. Effects were less pronounced for pseudomonads and β-proteobacteria. The abundant strains of these groups may be intrinsically resistant to sulfonamides, and thus be not or only indirectly affected by SDZ. This indirect impact may be due to interactions with sensitive bacteria, e.g. less competition for limited resources, or a reduced antagonistic activity. Comparing the DGGE pattern of pure manure with those of the soil samples showed that manure populations did not become major components of the soil community but manure rather indirectly influenced soil microbial community structure, e.g. by nutrients. This confirms the findings of Cools et al. (2001), who found manure-derived bacteria in soils treated with manure only at waterlogged conditions. Additional bands in the SDZ treatments may indicate resistant bacterial populations, as expected for different environmental pollutants (Demanou et al., 2006; Zielezny et al., 2006) and SDZ (Heuer and Smalla, 2007). Overall changes of DGGE banding patterns were illustrated by the cluster analysis. The similarity level was highest for the two SDZ concentrations for most clusters, indicating that low SDZ concentrations altered the bacterial population. In most clusters, controls had the lowest similarity to the manure treatments (S0, S10, S100), confirming the results from PLFA measurements. Some changes in the composition of micro-organisms community in all treatments occurred due to the long incubation period. Changes in community structure therefore also occurred on the genotypic level.

4.6. Conclusion

A single application of SDZ in combination with manure led to a shift in the soil community structure. The effect increased with the initial spiking concentration but gradually differed between soil types. Hence knowledge about the influencing soil physiochemical properties and the indigenous soil community status is needed to assess effects from veterinary antibiotics. Parallel studies revealed that the community shift is not necessarily mirrored by an altered soil functioning but masked by species redundancy. Furthermore, SDZ exhibited a delayed and prolonged effect on the microbial community structure that increased over time periods of months. This is in contrast to the strong dissipation kinetics of bioavailable SDZ in soil leading on the time scale to apparently concentration independent antibiotic action. Furthermore, repeated applications of antibiotics and manure as a normal agricultural practice are likely to influence adaptive mechanisms or may intensifying effects on the soil microbial community structure. These findings stress that there is a high need for further investigations to increase our understanding of the interactions of antibiotics and the structure and function of the soil microbial biomass.

Acknowledgements

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.soilbio.2008.01.010.

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