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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.

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.

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

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_3PO_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,