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Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass

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Abstract

Increasingly often soil residual concentrations of pharmaceutical antibiotics are detected, while their ecotoxic relevance is scarcely known. Thus, dose related effects of two antibiotics, sulfapyridine and oxytetracycline, on microorganisms of two different topsoils were investigated. The fumigation-extracted microbial C (E_C) and ergosterol were determined to indicate soil microbial and fungal biomass, respectively. Microbial activity was tested as basal respiration (BR), dehydrogenase activity (DHA), substrate-induced respiration (SIR), and Fe(III) reduction. The BR and DHA were uninfluenced even at antibiotic concentrations of 1000 μg g^{-1}. This revealed that an activation of microbial growth through nutrient substrate addition is required to test possible effects of the bacteriostatic antibiotics. In addition, the effects of both antibiotics were time dependent, showing that short-term tests were not suitable. Clear dose–response relations were determined with SIR when the short-term incubation of 4 h was extended into the growth phase of the microorganisms (24 and 48 h). The Fe(III) reduction test, with a 7-d incubation, was also found to be suitable for toxicity testing of antibiotics in soils. Effective doses inhibiting the microbial activity by 10% (ED_{10}) ranged from total antibiotic concentrations of 0.003–7.35 μg g^{-1}, depending on the antibiotic compound and its soil adsorption. Effective solution concentrations (EC_{10}), calculated from distribution coefficients, ranged from 0.2 to 160 ng g^{-1}. The antibiotics significantly ($p < 0.05$) reduced numbers of soil bacteria, resulting in dose related shifts in the fungal:bacterial ratio, which increased during 14 d, as determined from analysis of ergosterol and E_C. It was concluded that pharmaceutical antibiotics can exert a temporary selective pressure on soil microorganisms even at environmentally relevant concentrations.

Keywords: Dose–response curves; Effective doses; Ergosterol; Fumigation extraction; Pharmaceutical antibiotics

1. Introduction

Pharmaceutical antibiotics are widely used for the medical treatment of microbial infective diseases. Consequently, tons of antibiotics are annually administered to humans and animals, especially to livestock (Thiele-Bruhn, 2003). Most pharmaceutical antibiotics are designed to be quickly excreted from the treated body, either unaltered or as metabolites, some of which are still

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bioactive (e.g. Zuccato et al., 2001). Thus, sewage sludge and manure, used as fertiliser for agricultural land, are often contaminated with antibiotics (Thiele-Bruhn, 2003). Residues of pharmaceutical antibiotics have already been discovered in soils in concentrations of up to 300 ng g^{-1} for tetracyclines and 11 ng g^{-1} for sulfonamides (Hamscher et al., 2002; Höper et al., 2002). In addition, various antibiotics occur naturally in soils because antibiosis is a natural defence mechanism, with numerous soil microorganisms producing antibiotics via their secondary metabolism (Thomashow et al., 1997). However, the concentrations and types of pharmaceutical antibiotics released into soil differ widely from the natural background. Hence, unwanted effects of these antibiotics on soil microorganisms and microbial turnover processes in soils are suspected and require investigation.

To date, a wide range of bioassays exist and have been used to determine effects of different chemicals on soil microorganisms (Hart and Brookes, 1996; Welp and Brümmer, 1999). However, reports on the effects of pharmaceutical antibiotics on soil microorganisms are scarce and inconsistent. For example it has been reported that some antibiotics inhibit microorganisms (Colinas et al., 1994) and dose related effects have been determined (Herron et al., 1998; Pfeiffer et al., 1998). In contrast, a promotion of microbial growth and activity was reported in other studies (Patten et al., 1980; Höper et al., 2002). Soil contamination with the sulfonamide sulfachloropyridazine resulted in small changes in the community-level physiological profile and an increased pollution induced community tolerance against further additions of sulfachloropyridazine (Schmitt et al., 2004). Microbial resistance induced by antibiotics has also been observed (Huysman et al., 1993; Fründ et al., 2000).

The overall goal of our study was to investigate the effects of two pharmaceutical antibiotics on the indigenous microorganisms of topsoil samples from two agricultural soils. The selected compounds belong to two antibiotic structural classes that are widely used for the medication of livestock. In particular, our objectives were: (i) to assess the suitability of established soil microbial activity assays—generally used to evaluate the toxicity of chemicals—to indicate effects of pharmaceutical antibiotics; (ii) to determine the concentration and time dependent effects of the two antibiotics on the soil microbial activity and to derive effective doses for microbial inhibition; and (iii) to investigate effects on soil microbial biomass and overall structural composition of the indigenous microbial community of one soil sample.

2. Materials and methods

2.1. Soil samples

Topsoils were sampled from a sandy Eutric Cambisol (sampling depth 2–10 cm) and a sandy loam Albic Luvisol (sampling depth 2–25 cm) under permanent grassland, after the turf (0–2 cm) was removed. The soils were located at the experimental agricultural station of the University of Rostock, Germany, and are typical for the young pleistocene soilscape of northern Germany. Neither soil had received any fertiliser, such as manure, throughout the last decades and, thus, background concentrations from pharmaceutical antibiotics were not detectable. Selected soil characteristics were analysed according to the methods described in Sparks et al. (1996) and are reported in Table 1. The two soil samples differed significantly in their concentration of C from soil organic matter (OC), clay, and pedogenic oxides (Table 1). The field moist soil samples were homogenised and sieved to <2 mm.

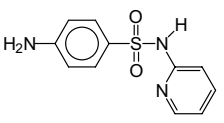
2.2. Chemicals

Two antibiotics, the sulfonamide, sulfapyridine (SPY), and the tetracycline, oxytetracycline hydrochloride (OTC), were selected for the investigations. Their chemical structures and selected physicochemical properties are displayed in Fig. 1. The antibiotics with a purity of >99% were purchased from Sigma (Taufkirchen, Germany). All salts and acids used in this study were of analytical grade and obtained from Merck (Darmstadt, Germany) and all solvents were of gradient grade and obtained from Riedel de Haën (Taufkirchen, Germany).

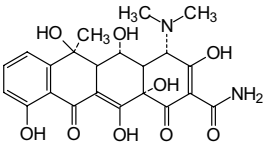
Table 1
Selected properties of the investigated topsoil samples (<2 mm)

Soil type	pH $CaCl_2$	$CaCO_3$ (%)	OC (%)	N_{tot} (%)	Clay (%)	Silt (%)	Sand (%)	CEC_{pot}[a] ($cmol_c\ kg^{-1}$)	Fe[b] ($g\ kg^{-1}$)	Mn[b] ($g\ kg^{-1}$)	Al[b] ($g\ kg^{-1}$)
Luvisol	7.1	1.28	1.56	0.134	9.9	20.4	68.4	13.1	2.11	0.11	0.79
Cambisol	6.6	0.65	0.79	0.075	3.1	15.9	80.9	5.33	1.25	0.05	0.72

[a] Potential cation exchange capacity determined with 0.1 M $BaCl_2$ at pH 8.1.
[b] Active pedogenic oxides extracted with oxalic acid ammonia-oxalate.



Sulfapyridine	$C_{11}H_{11}N_3O_2S$
molecular weight	249.29 g mol^{-1}
water solubility	268 mg l^{-1}
log K_{ow}	0.35
pK_a	2.9 / 8.4



Oxytetracycline	$C_{22}H_{24}N_2O_9$
molecular weight	460.44 g mol^{-1}
water solubility	313 mg l^{-1}
log K_{ow}	-0.90
pK_a	3.3 / 7.3 / 9.1

Fig. 1. Molecular structure and physicochemical data (cited in Thiele-Bruhn (2003)) of the selected pharmaceutical antibiotics; K_{ow} is the octanol/water partition coefficient; pK_a is the dissociation constant.

2.3. Sample preparation and incubation

For microbial activity tests using moist soil, soil moisture was adjusted to 50% of the water holding capacity (WHC) using ultrapure water. For the application of the antibiotics to the soil samples, quartz sand (p.a. grade, Merck, Darmstadt, Germany) was spiked with different volumes of antibiotic standard solution in methanol (100 μg ml^{-1}). After evaporation of the methanol the spiked sand was mixed with the soil samples. To determine dose–response curves from antibiotic effects on microbial activity parameters, the investigation of a broad range of antibiotic concentrations was given preference to the number of repetitions. Thus, duplicate samples were amended with SPY and OTC in six logarithmically increasing concentrations that ranged from 0 to 1000 μg g^{-1}. Additional doses were tested for both or only one of the antibiotics (in parentheses), i.e. 0.02 (SPY), 2.0 (OTC), 3.0 (OTC), 5.0 (OTC), 20.0 (OTC), 50.0, and 500 μg g^{-1}. Samples were incubated in the dark at 20 and 25 °C, respectively, to investigate Fe(III) reduction. Incubation times varied among the different tests and ranged from 4 h to 7 d.

To investigate antibiotic effects on soil microbial and fungal biomass, respectively, soil samples, corresponding to 100 g dry weight, were placed in 500 ml Erlenmeyer flasks stoppered with cellulose plugs to allow gas exchange while minimising water evaporation. Soil moisture was adjusted to 50% of WHC and corrected every two days if necessary. To activate soil microbial growth, samples were amended with an additional 2000 μg C g^{-1} dry soil added as milled maize straw (90% of additional C) and glucose (10% of additional C), and incubated for three days prior to the application of the antibiotics at final concentrations of 0, 10, 100, and 1000 μg g^{-1}. Quadruplicate samples were incubated at 25 °C in the dark. After 0, 1, 3, 7, 10, and 14 days samples were analysed for microbial biomass C by the chloroform fumigation-extraction method and for ergosterol.

2.4. Sample analysis

The following soil microbial assays were used: Soil basal respiration (BR; Isermeyer, 1952); substrate-induced respiration (SIR; Anderson and Domsch, 1978); dehydrogenase activity (DHA; Thalmann, 1968); Fe(III) reduction (Welp and Brümmer, 1985); chloroform fumigation-extraction (CFE; Vance et al., 1987); ergosterol extraction (Gessner and Newell, 1998; modified).

Following the CFE of soil samples, extracted organic C was quantified using non-dispersive infrared spectrometry (LiquiTOC, Elementar Analysensysteme, Hanau, Germany). Calibration was done using potassium biphthalate (Merck, Darmstadt, Germany) as an external standard. Microbial biomass C was calculated as E_C, where E_C represents C extracted from chloroform fumigated samples minus C extracted from non-fumigated samples.

To extract ergosterol, samples were ultrasonicated with 550 J ml^{-1} for 5 min at 22 °C using a Sonics Vibracell 600 (Sonics & Materials Inc., Newton, CT), equipped with a 19 mm sonotrode. This method proved to be more exhaustive than Soxhlet extraction according to Zelles et al. (1987). Recovery rate of ergosterol standard solution added to soil samples was 108%. Ergosterol was analysed by HPLC-UV (HP 1050 system, Hewlett-Packard, Palo Alto, CA). Separation was done with a C18-100-5 reversed phase 250 × 4.6 mm column (Macherey-Nagel, Düren, Germany) as stationary phase. The column was thermostated at 28 °C and methanol (95%) and water (5%) were delivered isocratically at a flow rate of 1.5 ml min^{-1}. Ergosterol was detected at 254 nm with a retention time of 9.3 min and quantified against an external standard (Fluka, Buchs, Switzerland).

The extractable concentrations of the antibiotics, residing after various incubation times in the soil samples were investigated. The tetracycline OTC was extracted according to the modified method of Hamscher et al. (2002) using citric ethylacetate and ultrasonication whereas the sulfonamide SPY was extracted using methanol (Thiele-Bruhn, 2003). After centrifugation at 1700 × g, aliquots of the supernatants were evaporated and redissolved in 1 ml of methanol. Both antibiotics were analysed with the above-mentioned HPLC system and separated at a column temperature of 22 °C with methanol and 0.01 M H_3PO_4 delivered in a gradient program at 1 ml min^{-1}. Retention time was 14.0 min for SPY and 16.7 min for OTC, detected at 254 nm. Quantification was done against external standards.

2.5. Data analysis

Non-linear sigmoidal equations were fitted to the data from soil microbial activity tests using the best-fit method and the SPSS 11.0 software (SPSS, Chicago, IL), when clear dose–response curves were obtained. The selected equations fitted the data with R^2 ranging from 0.79 to 0.98 and the standard error of the curve fit ranging from 0.1% to 6.2%. From these curves effective doses resulting in 10% and 50% inhibition of microbial activity (ED_{10}, ED_{50}), compared to the unamended control sample, were derived. According to Welp (1999), the dose is the total antibiotic concentration initially added to the samples. To determine the corresponding effective concentrations (EC_{10}, EC_{50}), solution concentrations were calculated from the distribution coefficients (K_d [ml g^{-1}]) of the antibiotics in the investigated soils. The distribution coefficients were determined in batch trials according to the guideline 106 of the Organisation for Economic Co-operation and Development (2000) and are defined as

$$K_d = (x/m)/C$$

where x/m is the amount adsorbed per gram of soil (μg g^{-1}) and C is the solution antibiotic concentration (μg ml^{-1}). Solution concentrations were normalised to the soil-to-solution ratio used in batch experiments (g soil × [ml solution]$^{-1}$), to obtain similar units of EC and ED values.

The significance of the antibiotic effects on soil ergosterol or fumigation-extracted C was evaluated using the least significant difference (LSD) test at a significance level of $p < 0.05$. Concentrations of ergosterol and E_C were not converted to fungal and microbial biomass, respectively, because the ergosterol concentration of fungal species is highly variable, while the conversion factor for E_C is less variable (Djajakirana et al., 1996).

3. Results and discussion

3.1. Antibiotic effects on soil microbial activity

Both SPY and OTC had no effect on BR and DHA. These parameters were not significantly different from the unamended control, regardless of the type and added dose of the antibiotic (Table 2), except for the effect of SPY on DHA, where a total dose of 300 μg g^{-1} corresponded to a 10% decline (ED_{10}) in the loamy Luvisol. In our study, the antibiotics SPY and OTC were added to the soil samples in doses of up to 1000 μg g^{-1}, which exceeded the residual concentrations typically found in the environment (e.g. Hamscher et al., 2002) by several orders of magnitude. This contrasts the fact that most

Table 2
Effective doses (ED; μg g^{-1}) and effective solution concentrations (EC; ng g^{-1}) of the pharmaceutical antibiotics SPY and OTC influencing soil microbial activity of two different topsoil samples as determined using selected soil bioassays and distribution coefficients of the antibiotics (K_d) for the adsorption to these soil materials

Test/duration	Effect[a]	SPY		OTC	
		Cambisol	Luvisol	Cambisol	Luvisol
DHA[b]/16 h	ED_{10}		300	>500[c]	>500[c]
BR[d]/24 h	ED_{10}		>1000[c]		>1000[c]
SIR[e]/4 h	ED_{10}	>1000[c]		>1000[c]	
SIR/1 × 24 h/2 × 24 h	ED_{10}	0.05[f]	1.17[g]	0.81[f]	0.93[g]
	ED_{50}	6.2[f]	11.5[g]	19.1[f]	31.2[g]
	EC_{10}	7.1[f]	56.0[g]	0.50[f]	0.51[g]
	EC_{50}	890[f]	550[g]	11.8[f]	17.1[g]
Fe(III)[h]/7 d	ED_{10}	1.14	0.003	5.50	7.35
	ED_{50}	86.5	6.45	156	9.68
	EC_{10}	160	0.14	3.4	4.0
	EC_{50}	12400	310	96.0	5.3
K_d (l kg^{-1})		7.0	20.9	1625	1825

[a] Effective dose or concentration of 10% or 50% activity inhibition as compared to the unamended control.
[b] Dehydrogenase activity.
[c] No effect observed up to the largest tested concentration.
[d] Base respiration.
[e] Substrate-induced respiration.
[f] Incubation time 1 × 24 h.
[g] Incubation time 2 × 24 h.
[h] Fe(III) reduction.

pharmaceutical antibiotics such as SPY and OTC effectively inhibit microorganisms, for which the toxic dose is often several orders of magnitude smaller than for higher organisms (Wollenberger et al., 2000). The BR and DHA results can be explained by several possible causes: (i) a low sensitivity of both methods as it has already been reported for the BR method (Fairbrother et al., 1999); (ii) selective effects of the antibiotics that were not indicated by the microbial activities tested with the two methods; and (iii) shifts in the microbial community structure that compensated for effects on single species. A further cause (iv) could be that, as is typical for numerous antibiotics, SPY and OTC exert a biostatic and not a biotoxic effect, inhibiting bacterial synthesis of nucleic acids and proteins, respectively (Schadewinkel-Scherkl and Scherkl, 1995). Biostasis should not affect non-growing microorganisms. The majority of soil microorganisms are dormant (Jenkinson and Ladd, 1981). These dormant microorganisms maintain respiration by oxidising internal energy sources (Tate, 2000). However, active transport from the exterior into the microbial cell is required before sulfonamides and tetracyclines take effect (Gräfe, 1992). Hence, an activation of soil microbial growth is necessary to obtain clear effects on microbial activity from biostasis.

To stimulate microbial activity and growth, the addition of a nutrient substrate to soil is required. Additionally, the application of antibiotics together with a nutrient substrate is more environmentally relevant because antibiotics mostly reach the soils via manure, sludge and excreta from grazing livestock (Jørgensen and Halling-Sørensen, 2000). The SIR assay involves the addition of glucose as a nutrient substrate. Normally a short incubation time of 4 h is used to register a respiration reaction of the initial microbial population, without invoking microbial growth (Lin and Brookes, 1999). Turnover times of soil bacteria may exceed 2 days (Bååth, 1998). Consequently, the activity of non-growing microorganisms was not influenced within 4 h by the biostatic effects of SPY and OTC even at a concentration of 1000 μg g^{-1} (Table 2). In the case of sulfonamides such as SPY, the biostatic effect can be also delayed for several hours because bacteria possess reserves of growth factors, such as folic acid, whose synthesis is inhibited by sulfonamides (Halling-Sørensen, 2001).

To enable microbial growth in the presence of a nutrient substrate and thus, to allow bacteriostatic effects, the incubation time was extended to 24 h (Fig. 2a; first incubation), into the growth phase of the microbial population. In contrast to the results from the 4-h incubation, strong inhibiting effects of SPY and OTC were determined in the sandy Cambisol. Such a clear dose related inhibition of soil microbial activity was also determined for equivalent doses of OTC and sulfachloropyridazine using the carbon transformation test

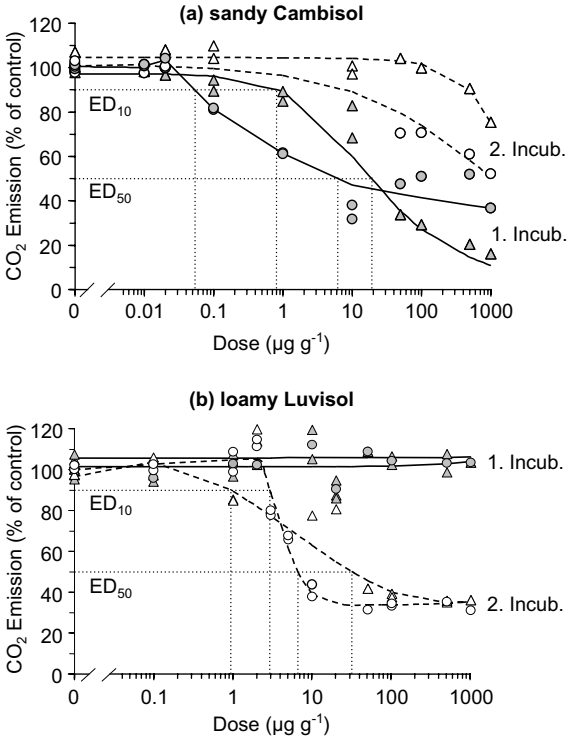


Fig. 2. Dose related effect of sulfapyridine (circles) and oxytetracycline (triangles) on the substrate-induced respiration of topsoil samples of (a) sandy Cambisol and (b) loamy Luvisol. First incubation (solid line, grey symbols) from 0 to 24 h and second incubation (broken line, white symbols) from 24 to 48 h. Incubation at 20 °C and a soil moisture of 50% of the water holding capacity.

(Vaclavik et al., 2004). Similarly, the importance of incubation time has been reported for the toxicity of antibiotics to aqueous bacteria, using a bioluminescence test with *Vibrio fischeri* (Backhaus et al., 1997). These results show that short-term tests should not be used to assess compounds affecting metabolic pathways conducive to long-term processes like growth and reproduction (Froehner et al., 2000; Halling-Sørensen, 2001).

While strong antibiotic effects were obtained for the sandy Cambisol, no response to antibiotic addition was observed within 24 h for the loamy Luvisol (Fig. 2b; first incubation). However, after the glucose addition was repeated and the soil sample was incubated for another 24-h period, a significant effect on the SIR was obtained from SPY and OTC (Fig. 2b; second incubation). In contrast, the second 24-h incubation of the sandy Cambisol exhibited smaller effects of the antibiotics as compared to the first 24-h incubation (Fig. 2a). This was accompanied by a slight increase in the total CO_2 evolution from the unamended control sample from the first to the second incubation phase (44 to 57 mg

$CO_2 \times$ [100 g soil 24 h]$^{-1}$) indicating continuous microbial activity. Hence, the recovery of soil respiration during the second 24-h incubation of the sandy Cambisol is attributed to a decrease in the bioavailable antibiotic fraction or an increasing adaptation and resistance of the microorganisms. Such an adaptation and development of resistance in soil microorganisms has already been reported for antibiotic-spiked soil samples (Fründ et al., 2000; Ingerslev and Halling-Sørensen, 2000; Halling-Sørensen et al., 2003; Schmitt et al., 2004). Once a resistance gene is present on a plasmid, the gene can be spread to other bacteria very quickly, especially when selection pressure from antibiotic is high (Gräfe, 1992). In soil, a decrease in the bioavailability of sulfonamides and tetracyclines is mostly due to sorption and fixation processes, while biodegradation is of minor importance (Halling-Sørensen, 2000; Kreuzig et al., 2003).

Adsorption coefficients (K_d) of SPY in the loamy Luvisol were three times larger compared to those in the sandy Cambisol (Table 2). The concentrations of soil organic matter, pedogenic oxides and clay minerals were also about two to three times larger in the loamy Luvisol compared to those in the sandy Cambisol (Table 1). These soil components were identified as the preferred sorption sites for sulfonamide and tetracycline antibiotics (Figueroa et al., 2004; Thiele-Bruhn et al., 2004). Similarly, ED_{50} values of SPY were about two times larger in the loamy Luvisol compared to those in the sandy Cambisol. The adsorption and bioactivity of SPY were likely influenced by the soil sorptive properties, as it was reported for the microbial toxicity of various other pollutants (Welp and Brümmer, 1999). Correspondingly, the effects of the antibiotics neomycin and thiostrepton on soil organisms are fundamentally influenced by their sorption and bioavailability, and by the availability of nutrients (Herron et al., 1998). A similar but much less pronounced relationship between ED_{50} values and sorption coefficients was found for OTC, which was considerably stronger adsorbed in both soil samples (Table 2). In general, the results indicated that for both antibiotics, a strong adsorption led to a smaller and delayed antibiotic effect, while a smaller adsorption and corresponding larger bioavailability of SPY and OTC resulted in a stronger but more rapidly declining effect on microorganisms. The ED_{10} values were in the order of 1 μg g^{-1} (Table 2) and, for SPY in the sandy Cambisol, the ED_{10} was below the trigger value of 100 ng g^{-1} established by the European Medicines Agency (1997) for the environmental assessment of medicinal products. This was even more valid for most of the soil-to-solution ratio normalised EC values that were calculated from the adsorption coefficients (K_d) of the antibiotics in the two soils. The EC ranged from 0.2 to 160 ng g^{-1} (Table 2). This shows that small concentrations of mobile and bioavailable antibiotics might cause significant effects on soil microorganisms.

Results similar to those found for the long-term SIR were obtained from the Fe(III) reduction test (Table 2). This microbial activity test combines the addition of glucose as a nutrient substrate and a long incubation time of 7 d. The microbial Fe(III) reduction, used as test parameter, requires the activity of aerobic and facultative anaerobic soil bacteria such as *Clostridium* spp. and *Bacillus polymyxa* (Zelles et al., 1986). Thus, the suppression of various species cannot be easily compensated for by other soil organisms. Accordingly, this bioassay was sensitive to antibiotic addition. For the loamy Luvisol the effective doses derived from the sigmoidal curves fitted to the data for microbial Fe(III) reduction (not shown) were mostly smaller compared to those obtained from the long-term SIR method, while for the sandy Cambisol the opposite occurred (Table 2). As found for the sandy Cambisol in the 48-h SIR test, this result was probably due to an already declining effectiveness of the antibiotics, or to an increasing number of antibiotic insensitive microorganisms during the 7-d incubation. In contrast, it is unlikely that the declining antibiotic effect was due to a shortage in microbially reducible Fe(III). The maximum amount of Fe released from a control sample corresponded to <0.1% of the Fe concentration in active Fe(hydr)oxides such as ferrihydrite (see Table 1).

Similar clear dose response curves obtained with the Fe(III) reduction test were reported for organic pollutants such as pesticides, and chlorinated and aromatic solvents by Zelles et al. (1986) and Welp and Brümmer (1999), who suggested that the Fe(III) reduction test is a suitable routine method to test dose related toxic effects of organic pollutants on soil microorganisms. We assume that this holds true for pharmaceutical antibiotics as well.

3.2. Effects of antibiotics on soil microbial biomass and the microbial community

The determination of the microbial biomass C (E_C) and ergosterol as indicators of the total microbial and fungal biomass, respectively, revealed different effects of SPY and OTC in the sandy Cambisol (Fig. 3a and b). After the addition of a nutrient substrate, the ergosterol concentration in the control sample increased during a 14-d incubation on average from 0.47 to 0.96 μg g^{-1}, while the E_C declined from 1190 to 450 μg g^{-1}. According to the findings of Hart and Brookes (1996), the addition of straw and glucose led especially to the growth of fungi. It has to be stated again that in this study, due to the high variability of the fungal ergosterol concentration, ergosterol was used as an indicator but not a measure of fungal biomass. Depending on the spiking concentration, SPY led to a reduction of the E_C but did not affect the ergosterol concentration (Fig. 3a). In contrast, in the presence of OTC the ergosterol concen-

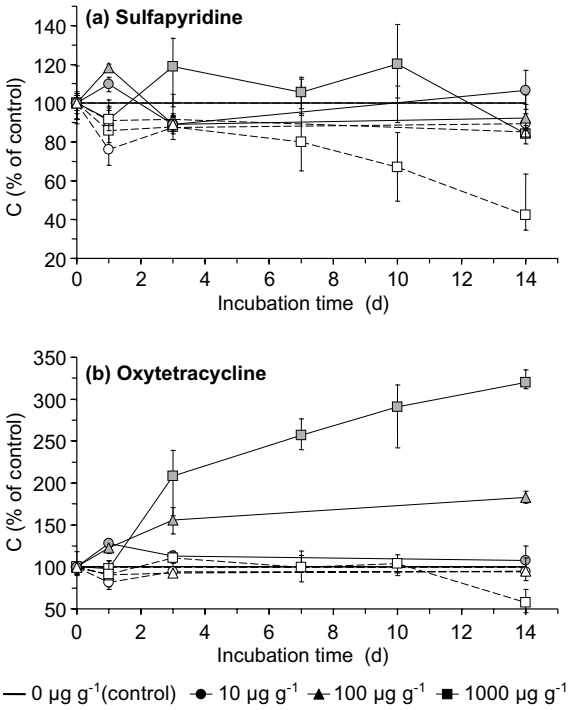


Fig. 3. Time and dose related effects of (a) sulfapyridine and (b) oxytetracycline on the E_C values (white symbols, broken line) and ergosterol concentration (grey symbols, solid line) of a sandy Cambisol. Concentrations in relation to the unamended control (100%, thick line). Incubation at 25 °C and a soil moisture of 50% of the water holding capacity. Error bars that are not shown are smaller than the symbols. E_C is the organic C extracted from the fumigated soil sample minus the organic C from the non-fumigated soil sample.

tration strongly increased during the incubation time (Fig. 3b), whereas the E_C did not significantly differ between amended samples and the unamended control.

The effects of SPY on E_C and of OTC on ergosterol are significant at $p < 0.05$ for all tested concentrations and increased with increasing antibiotic concentration (Fig. 3a and b). These results indicate that soil fungi increasingly dominated the microbial biomass, while the number of soil bacteria was reduced. Similarly, Patten et al. (1980) reported that in OTC containing excrements from cattle, the number of fungi increased and Colinas et al. (1994) found that a combination of 10 µg g^{-1} OTC and penicillin added to soil led to a reduction in the soil bacterial biomass. However, in the latter study, the numbers of active fungal hyphae were also reduced. Recently, OTC was proposed as a bacterial inhibitor (Bailey et al., 2003). In our study, both pharmaceutical antibiotics exerted a selective pressure on the soil microbial community.

The shift from soil bacteria to soil fungi was also significant at $p < 0.05$ over the whole 14-d incubation period. It increased with time compared to the unamended control sample (Fig. 3a and b), although the detectable concentrations of SPY and OTC strongly declined with time (Fig. 4), following coupled first order kinetics (data not shown). The decline in the extractability of the antibiotics depended on the initial concentration, and it was stronger at the smaller spiking level. This is probably due to the nonlinear adsorption of the tested antibiotics to soil (Thiele-Bruhn, 2003; Thiele-Bruhn et al., 2004). In the sample spiked with 10 µg g^{-1} non-detectable concentrations of SPY were reached within 14 d. However, effects on soil microorganisms were still evident (Fig. 3a). This suggested that structural changes of the soil microbial community, once initiated, might proceed for a longer time of several weeks. Westergaard et al. (2001) reported that changes in soil microbial community structure following a tylosin treatment were evident throughout a 2-month experiment. Furthermore, deviant effects from metabolites as products of the antibiotics (bio-)degradation cannot be excluded. Such different efficacy was shown for degradation products of tetracyclines (Halling-Sørensen et al., 2002).

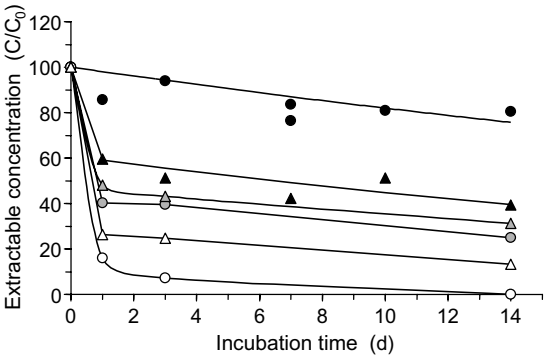


Fig. 4. Time related extractable portions of sulfapyridine (circles) and oxytetracycline (triangles) at different initial spiking levels (C_0 : 10 µg g^{-1} white symbols; 100 µg g^{-1} grey symbols; 1000 µg g^{-1} black symbols) in topsoil of a sandy Cambisol.

By relating our data to a field situation, it is reasonable to hypothesise that, although extractable concentrations of antibiotics in field soils are generally small (e.g. Hamscher et al., 2002), initial concentrations immediately after addition can be high enough to affect soil microorganisms. Moreover, an increase in the effective concentration due to bioaccumulation should also be taken into account (Dojmi di Delupis et al., 1992).

4. Conclusions

Residual concentrations of pharmaceutical antibiotics exert a temporary selective pressure on soil

microorganisms, which is clearly influenced by soil adsorption. To test time and concentration related effects, the fact that numerous antibiotics are biostatic and not biotoxic must be considered. Thus, long-term tests with a nutrient addition to stimulate microbial growth, such as the Fe(III) reduction test are suitable routine methods to test dose related effects of pharmaceutical antibiotics on soil microorganisms. The different effects of antibiotics on the soil microbial and fungal biomass show that further experiments are required, investigating the biodiversity of pharmaceutical affected soil microorganisms.

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