The veterinary antibiotic oxytetracycline and Cu influence functional diversity of the soil microbial community

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Oxytetracycline reduces the functional diversity of soil microbial community, and the combination of Cu and oxytetracycline leads to a further reduction.

Abstract

There are increasing concerns over the effects of veterinary antibiotics and heavy metals in agricultural soils. The widely used veterinary antibiotic oxytetracycline (OTC), Cu and their combination on soil microbial community function were assessed with the Biolog method. The microbial community was extracted from the soil and exposed to a 0.85% sodium chloride solution containing OTC (0, 1, 5, 11, 43, 109 and 217 μM), or Cu (0, 10, 20, 100 and 300 μM), or combination of the two pollutants (OTC 0, 5, 11 μM and Cu 0, 20 μM). Functional diversity, evenness, average well color development (AWCD) and substrate utilization decreased significantly with increasing concentrations of OTC or Cu ($p < 0.005$). The critical concentrations were 11 μM for OTC and 20 μM for Cu. The combination of OTC and Cu significantly decreased Shannon's diversity, evenness and utilization of carbohydrates and carboxylic acids compared to individual one of the contaminants. The antibiotic OTC and Cu had significant negative effects on soil microbial community function, particularly when both pollutants were present.

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1. Introduction

Veterinary pharmaceuticals are widely used for the therapy of infectious diseases of animals in intensive farming system (Halling-Sørensen, 2000; Boxall et al., 2003). They are designed to act very effectively at low doses and to be completely excreted from the body after a short time of residence. Only a fraction of the ingested antibiotics is metabolized in the animals, hence a large percentage of the antibiotics are excreted and released into the environment e.g. via manure and sludge used as fertilizer on fields or effluent from aquaculture (Jørgensen and Halling-Sørensen, 2000). Therefore, residual concentrations of pharmaceutical antibiotics can be found in

soils, surface and ground water (Hamscher et al., 2002; Kolpin et al., 2002; Simon, 2005). The fate of antibiotics including sorption and fixation, mobility and transport is well documented (Tolls, 2001; Figueroa et al., 2004; Kulshrestha et al., 2004), whereas the knowledge about their ecotoxicity and the effect of antibiotics on soil microbial functioning is scarce (Thiele-Bruhn, 2003; Boxall et al., 2003).

Under intensive pressure of industrialization in both developing and developed countries, increasing amounts of copper and other heavy metals are entering agricultural fields and contaminate the food-chain. Wastewater irrigation (Luo et al., 2003), compost application, including municipal waste, sewage sludge or their combination (Zheljazzkov and Warman, 2003), and the application of agrochemicals containing heavy metals (Besnard et al., 2001) have been reported to contribute to the input of copper and other heavy metals in agricultural

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Fig. 1. Functional diversity and evenness following addition of OTC. The error bar is the standard error of the means ($n = 3$).

soils. Excess copper in soils is toxic to plants and soil organisms. Plant yield reduction and growth retardation (Moreno et al., 1997) and changes in community structure of soil microorganisms and nematodes (Giller et al., 1998; Ellis et al., 2001) have been reported in metal-contaminated soils. However, conflicting findings with respect to microbial responses to metals may arise from differences in bioavailability (McGrath, 1994). Microorganisms respond mainly to the soluble metal fraction and the proportion of total metals present in this fraction may differ with soil type, environmental conditions and time of measurement relative to metal inputs (Dar and Mishra, 1994).

Soil microorganisms play important roles in many ecosystem processes such as biogeochemical cycling of nutrients, soil structural and hydrological properties and energy flow (Doran and Zeiss, 2000; Lesser et al., 2004; Driver et al., 2004; Šantrůčková et al., 2004). Thus, maintenance of the biological activity in the soil is generally regarded as a key feature of sustainable production to ensure ecosystem functions (Swift, 1994), and soil microbial properties are often used as indicators of soil quality. Microbial community function provides a practical and ecologically relevant measure of microbial diversity (Zak et al., 1994). Community functional diversity indicates its potential activity, i.e. the capability of the community to adapt metabolism and/or composition and size to different conditions. Community level physiological profiles (CLPP) (Garland and Mills, 1991) assessed by BIOLOG Microplate® have been widely used to investigate functional diversity of soil microbial communities (Garland, 1997; Di Giovanni et al., 1999; Mäder et al., 2002; Li et al., 2004). Despite a number of limitations, e.g. focus on bacterial species that are able to respond rapidly to the substrates, changes in community composition during growth (Preston-Mafham et al., 2002), the method can provide insights into the effect of disturbance on microbial communities.

Our interests in oxytetracycline (OTC) and its effect on soil microorganisms was provoked by the evidence that it was widely used, its concentration in fresh feces of Simmental calves treated with 60 mg/kg/d of OTC can be very high

(871.7 mg/kg, De Liguoro et al., 2003) and has been shown to reduce nitrification rates and growth of bacteria (Halling-Sørensen, 2001; Halling-Sørensen et al., 2003). OTC is active in vitro against gram-positive and gram-negative bacteria, but has little effect on fungi (Anderson and Domsch, 1993). It inhibits protein synthesis by disrupting amino acid chain elongation at the 30S subunit of ribosomes (Backhaus and Grimme, 1999). Cu concentrations are high in soils in some regions in China because of the input of agricultural chemicals containing Cu and the use of Cu in animal feed as growth promoter. In these regions the use of antibiotics in animal husbandry is also increasing. Thus, soils in these regions can be contaminated by both pollutants, but little is known about their effect on soil microorganisms.

Objectives of the present study were therefore to elucidate the effects of OTC and Cu and their combined effects on soil community level physiological profiles as an indicator for microbial community function.

Fig. 2. AWCD of 95 C sources following addition of OTC. The bar is the standard error of the means ($n = 3$). —◆— 0 μM, —◇— 1 μM, —▲— 5 μM, —△— 11 μM, —●— 43 μM, —○— 109 μM, —×— 217 μM.

Fig. 3. Principal component analysis CLPP following the addition of OTC.

2. Materials and methods

2.1. Soil description and preparation

A paddy soil (0–20 cm) was collected from Jiaying city, Zhejiang province in Southeast China. The soil type is Anthrosols with a silt loam (fine-silty, mesic), developed from river alluvium. After transport to laboratory, the nearly-saturated paddy soil was allowed to dry slowly under sheets of paper at 25 °C until water content reached about two-thirds of water-holding capacity. After sieving to 2 mm, and removal of large pieces of plant material and soil animals, the soil was mixed and stored for 2 weeks at 25 °C.

2.2. Experimental design

OTC ($\geq 89\%$, standard grade) was purchased from China Institute of Veterinary Drug Control, Beijing. All reagents used in the experiments were of analytical grade. All the experiments in this study had three replicates in each treatment.

Experiment 1 (dose–effect of OTC): sterile saline solutions (0.85%, m/v) with seven OTC levels, 0, 1, 5, 11, 43, 109 and 217 μM , which are commonly found in soil.

Experiment 2 (dose–effect of Cu): sterile saline solutions (0.85%, m/v) with five Cu (CuCl_2) levels, 0, 10, 20, 100, 300 μM .

Experiment 3 (combined effect): sterile saline solutions (0.85%, m/v) with 20 μM Cu, 5 μM OTC, 20 μM Cu + 5 μM OTC, 20 μM Cu + 11 μM OTC. Controls contained neither Cu nor OTC.

2.3. Community level physiological profiles determined by Biolog-GN plates

Community level physiological profiles (CLPP) of the soil microbial community was assessed as described previously (Schutter and Dick, 2001; Busse et al., 2001) with a few modifications. Briefly, 10 g (dry weight equivalent) of fresh soil was suspended in 100 ml sterile saline solution (0.85%, m/v) with 5 g of 3 mm glass beads on a rotary shaker at 300 rpm for 10 min at 25 °C. Suspensions were allowed to settle for 10 min before diluting 100-fold. Ten milliliter aliquots of the dilutions were added to 90 ml sterile saline solution (0.85%, m/v) containing different concentrations of the contaminants. After mixing, 150 μl of the suspension was added to each well of a Biolog-GN plate. Absorbance of the wells at 590 nm was read using a Biolog automated BIOLOG Microplate™ Reader (Biolog, Hayward, CA, USA) and the data were collected by Microlog 4.01 software (Biolog, Hayward, CA, USA). The plates were then sealed inside a plastic bag and incubated at 25 °C in the dark, and read every 24 h over 7 d, during which no contamination of control wells (only water) was found. The readings at 72 h incubation were used for subsequent analysis (see below). At this reading time the OD had stabilized and the greatest differences in utilization pattern were observed.

2.4. Statistical analysis

Well absorbance values were adjusted by subtracting the absorbance of the control well (water only) before data analyses, and substrates with an OD < 0 were excluded from further analysis. Average well color development (AWCD), calculated as the average optical density across all wells per plate, was used as an indicator of general microbial activity. CLPP diversity was calculated as the Shannon index, where $H' = -\sum p_i \ln p_i$, and p_i was calculated by subtracting the control from each substrate absorbance and then dividing this value by the total color change recorded for all 95 substrates, and evenness was calculated as $E = H' / \ln(\text{richness})$, richness referred to the number of substrates utilized (Zak et al., 1994). The substrates were divided into six substrate categories, according to their chemical nature: carbohydrates, carboxylic acids, amines and amides, amino acids, polymers, and miscellaneous (Preston-Mafham et al., 2002), and average absorbance of each category was calculated. AWCD, substrate utilization of each substrate category, Shannon diversity and evenness were compared by one-way ANOVA and the data from interaction between OTC and Cu by two-way ANOVA with the SPSS 10.0 for Windows. CLPP were analyzed by principal component analysis (PCA) with the SPSS 10.0 for Windows.

3. Results

3.1. Effects of OTC on functional diversity

In the OTC dose–effect experiment (Experiment 1), Shannon diversity decreased significantly as the OTC concentration increased up to 43 μM and remained at this low level at higher OTC concentrations (Fig. 1). Shannon evenness decreased up to 109 μM and increased slightly but not significantly at

Table 1
Biolog substrate utilization potential (optical density, OD) of microbial communities following the addition of OTC

Treatment	Amino acids	Carbohydrates	Carboxylic acids	Polymers	Amines	Miscellaneous
Control	1.45 (0.06)a	2.02 (0.02)a	1.23 (0.02)a	1.44 (0.13)a	0.44 (0.03)a	1.07 (0.04)a
1 μM	0.88 (0.04)b	1.47 (0.07)b	1.01 (0.06)b	0.49 (0.04)b	0.25 (0.03)b	0.78 (0.08)b
5 μM	0.61 (0.00)c	0.71 (0.03)c	0.75 (0.04)c	0.45 (0.04)b	0.23 (0.06)b	0.37 (0.02)c
11 μM	0.35 (0.04)d	0.20 (0.01)d	0.34 (0.02)d	0.28 (0.09)bc	0.05 (0.02)c	0.14 (0.03)d
43 μM	0.19 (0.08)e	0.13 (0.05)de	0.14 (0.05)e	0.04 (0.03)c	0.03 (0.03)c	0.13 (0.05)d
109 μM	0.02 (0.01)f	0.04 (0.00)ef	0.05 (0.02)ef	0.02 (0.00)c	0.03 (0.02)c	0.09 (0.04)d
217 μM	0.01 (0.00)f	0.01 (0.00)f	0.01 (0.00)f	0.01 (0.00)c	0.01 (0.00)c	0.04 (0.01)d

Means in the same column followed by the same letter are not significantly differed at $p < 0.05$, standard error was in bracket ($n = 3$).

Fig. 4. Functional diversity and evenness of soil microbial community following the addition of Cu. The bars are the standard errors of the means ($n = 3$).

217 µM. OTC at 11 µM inhibited Shannon diversity by 20%. Average well color development (AWCD) was used as an indicator of microbial activity in soil (Garland and Mills, 1991). OTC had significant negative effect on AWCD (Fig. 2). AWCD of the control and the treatment of 1 µM OTC increased rapidly after 24 h and reached the maximum within 96-h incubation. Increasing OTC concentrations delayed the onset of AWCD increase, reduced the rate of AWCD increase and decreased the maximum AWCD. Thus, the critical dose for AWCD was 1 µM. The two-dimensional PCA of the CLPP explained 75.4% of the total variance with the first principal component having the greater power of separation (67.9%) (Fig. 3). Therefore differences along PC1 are more important than along PC2. There are two distinct groups: the controls and 1 µM OTC on the one hand

and all higher OTC concentrations on the other. This separation can be explained by the higher number of substrates and the higher utilization of all substrates in the controls and in the treatment with 1 µM OTC. Utilization of 83% of all substrates was highly correlated to the first axis (Pearson coefficient > 0.7 ; $p < 0.05$, data not shown).

Microbial substrate utilization for the six substrate categories (Preston-Mafham et al., 2002), in Biolog plates, decreased to a different extent (Table 1). Carbohydrates were the most strongly utilized, and amines least. Addition of 1 µM OTC suppressed utilization of most substrate groups by about 50% but polymer utilization was decreased by more than 65%, suggesting that the soil microbial community was sensitive to OTC, especially microbes degrading polymers. Utilization of all substrate groups decreased with increasing OTC

Fig. 5. AWCD of 95 C sources after addition of Cu. The bar is the standard error of the means ($n = 3$). —◆— 0 µM, —◇— 10 µM, —▲— 20 µM, —△— 100 µM, —○— 300 µM.

Fig. 6. Principal component analysis of CLPP following the addition of Cu.

Table 2

Biolog substrate utilization potential (OD) of microbial communities following the addition of Cu

Treatment	Amino acids	Carbohydrates	Carboxylic acids	Polymers	Amines	Miscellaneous
Control	0.94 (0.09)a	1.67 (0.07)a	1.05 (0.02)a	1.05 (0.08)a	0.39 (0.07)a	0.85 (0.06)b
10 μ M	0.84 (0.09)ab	1.47 (0.08)b	0.79 (0.04)b	0.80 (0.08)b	0.27 (0.08)a	0.96 (0.03)a
20 μ M	0.71 (0.02)b	0.56 (0.05)c	0.29 (0.02)c	0.22 (0.03)c	0.02 (0.00)b	0.39 (0.03)cd
100 μ M	0.43 (0.07)c	0.02 (0.01)d	0.06 (0.01)d	0.01 (0.00)d	0.02 (0.00)b	0.09 (0.02)d
300 μ M	0.12 (0.01)d	0.01 (0.01)d	0.02 (0.00)d	0.00 (0.00)d	0.01 (0.00)b	0.02 (0.00)d

Means in the same column followed by the same letter are not significantly differed at $p < 0.05$ ($n = 3$).

concentrations. Utilization of each substrate category was inhibited to almost zero at 43 μ M OTC and the critical dose was 11 μ M.

3.2. Effect of Cu on functional diversity

Addition of Cu up to 100 μ M strongly reduced functional diversity and evenness of microbial community (Experiment 2) (Fig. 4), which then remained at a low level up to 300 μ M Cu. The concentration of 20 μ M inhibited AWCD by over 60% and inhibited Shannon diversity by 7.3%. Increasing Cu concentrations delayed the onset of AWCD increase, reduced the rate of AWCD increase and decreased the maximal AWCD (Fig. 5). The PCA plot of the CLPP explained 69.3% of the total variance with 59.8% being explained by PC1 (Fig. 6). The groups were not quite as well as separated for OTC (Fig. 3). The CLPP of the treatments 100 and 300 μ M Cu differed from that at lower Cu concentrations, which were similar to the control. Utilization of 62% of all substrates was positively related to PC1 at $p < 0.05$ (Pearson coefficient > 0.5). Cu significantly decreased the utilization potential of six substrate categories (Table 2). At Cu concentrations ≥ 100 μ M utilization of most substrate groups was almost completely inhibited. The only exception was the utilization of amines. Amine utilization was already very low at 20 μ M Cu. The critical Cu concentration for utilization of the different substrate groups was

20 μ M, which was similar as for Shannon diversity, evenness and AWCD.

3.3. Effects of OTC and Cu on functional diversity

To study the combined effect of OTC and Cu on CLPP (Experiment 3), we used the critical doses of OTC and Cu found in the first two experiments. The combination of 5 μ M OTC and 20 μ M Cu decreased significantly Shannon diversity and evenness compared to the separate application of two pollutants (Fig. 7), indicating an interactive effect on functional diversity. The combination of 11 μ M OTC and 20 μ M Cu showed no decrease in Shannon diversity and evenness, compared to the same concentrations of the single pollutants. The decrease of Shannon diversity and evenness with 11 μ M OTC and 20 μ M Cu was greater than with 5 μ M OTC and 20 μ M Cu. AWCDs for the two combinations were significantly reduced compared to those of the contaminants applied singly (Fig. 8). For each substrate group, the combination of OTC and Cu had a significantly stronger negative effect on the utilization potentials of carboxylic acids and carbohydrates than those of OTC or Cu alone (Fig. 9 and Table 3), whereas there were no significantly lower utilization potentials in the combined treatments for other substrate groups (data not shown). The PCA plot which explained 43.8% of the total variance indicated that the profiles of microbial communities amended with OTC, Cu separately or in combination differed from the control (Fig. 10).

Fig. 7. Functional diversity and evenness of soil microbial community following the addition of OTC and Cu singly or in combination. The bars are the standard errors of the means ($n = 3$). CK: 0 μ M, T1: 20 μ M Cu, T2: 5 μ M OTC, T3: 11 μ M OTC, T4: 20 μ M Cu + 5 μ M OTC, T5: 20 μ M Cu + 11 μ M OTC.

Fig. 8. AWCD of 95 C sources following the addition of OTC and Cu singly or in combination. The bars are the standard errors of the means ($n=3$). —◆—0 μ M, —◇—20 μ M Cu, —▲—5 μ M OTC, —△—11 μ M OTC, —●—20 μ M Cu + 5 μ M OTC, —○—20 μ M Cu + 11 μ M OTC.

4. Discussion

Residual concentrations of pharmaceuticals are found throughout the environment, especially in soil, river water and drinking water (Kümmerer, 2001). A nationwide survey of pharmaceuticals in the USA found that the maximum concentration of oxytetracycline was 0.34 μ g/kg in stream water (Kolpin et al., 2002), and the concentration of loosely bound oxytetracycline [extracted by 1 M MgCl_2 (pH8.0)] ranged from 0.6 to 3.3 mg/kg in riverine sediments (Simon, 2005). Previous studies have demonstrated that antibiotics in soils can contribute significantly to the contamination of water bodies and sediments (Kay et al., 2005). Veterinary antibiotics enter the soil mainly through the application of animal manure (Loke et al., 2002; De Liguoro et al., 2003; Hamscher et al., 2002). Animal manures can also be a source of Cu since Cu is a common supplement in animal feed (Ópezalonso et al., 2000; Hostetler et al., 2003).

Due to the widespread contamination of soils with antibiotics, it is essential to evaluate the potential risk of these compounds in the environment. In the present study, soil microbial community was extracted from soil, and exposed directly to OTC or Cu. This method has been widely used to examine the impacts agrochemicals on soil microbial communities (Busse et al., 2001). The present study showed that CLPP of soil microbial communities is sensitive to OTC and Cu, however, the Biolog method has a number of limitations. It relies on the growth (and/or substrate utilization) of microorganisms, thus only a small percentage of the total community is assessed and these may not be relevant in the ecosystem. Fast-growing species dominate the substrate utilization patterns and it has been shown that the community structure changes during the incubation period (Smalla et al., 1998). Thus, the CLPP does not necessarily reflect the original microbial community (Smalla et al., 1998). As a consequence, the color development observed cannot be interpreted in terms of the number of utilizers or the metabolic potential of the original microbial community (Haack et al., 1995). Other potential drawbacks of the Biolog method include the fact that the C sources tested are not necessarily those found in soil and they are present in high concentrations (Campbell et al., 1997). Despite these criticisms, Biolog has been used to assess the impacts of various environmental factors on microbial communities (Gremion et al., 2004; Mäder et al., 2002). CLPP are likely to be very sensitive to environmental factors because they reflect the response of the fast-growing bacteria more sensitive than the majority of soil microorganisms. Effects of environmental factors on CLPP can therefore be regarded as early warning signals.

In the present study, the Biolog method showed that pollutants such as OTC and Cu affected microbial communities, and it was demonstrated that the direct exposure of soil microbial communities to pollutants is a rapid way in predicting its toxicity to soil microbes using the Biolog method. The results of the present study showed that the addition of OTC decreased the functional diversity of soil microbial community

Fig. 9. Utilization potential of carboxylic acids and carbohydrates following addition of OTC and Cu singly or in combination. The bars are the standard errors of the means ($n=3$). CK: 0 μ M, T1: 20 μ M Cu, T2: 5 μ M OTC, T3: 11 μ M OTC, T4: 20 μ M Cu + 5 μ M OTC, T5: 20 μ M Cu + 11 μ M OTC.

Table 3

Results of two-way ANOVA on functional diversity, evenness, AWCD and substrate utilization in experiment with OTC and Cu singly or in combination

	Shannon diversity	Shannon evenness	AWCD	Amino acid	Carbohydrate	Carboxylic acid	Polymer	Amine	Miscellaneous
<i>F</i> -value									
Cu (C)	26.89	5.14	273.04	48.31	23.88	31.34	6.85	6.37	27.8
OTC (O)	7.36	2.39	45.76	0.69	13.35	7.42	2.65	1.48	3.42
C × O	14.07	6.44	37.09	0.99	7.55	7.14	1.71	1.39	1.16
<i>P</i> -value									
Cu (C)	0.000	0.043	0.000	0.000	0.000	0.000	0.023	0.027	0.000
OTC (O)	0.008	0.134	0.000	0.527	0.001	0.008	0.111	0.267	0.067
C × O	0.001	0.013	0.000	0.399	0.008	0.009	0.221	0.287	0.347

by 63% at 43 μM and decreased functional evenness by 41% at 109 μM . The results suggest that functional diversity was more sensitive than evenness to OTC hence that capacity of soil microbial community to utilize a wide range of substrates is very sensitive to OTC. OTC has been used to reduce or kill soil microbes to experimentally examine soil function (Bailey et al., 2002). However, our results indicate that the sensitivity of microbial species to OTC varies because the utilization of some substrate groups was affected to a greater extent than that of others. The utilization potential of carbohydrate and polymer decreased sharply with the increase in OTC concentration (Table 1). Thus, the fast-growing bacteria degrading carbohydrates and polymers were sensitive to OTC. In the Cu experiment, the addition of Cu decreased functional diversity by 35% at 100 μM and functional evenness by 30% at 100 μM , indicating that functional diversity and evenness were more or less similarly sensitive to Cu. Cu decreased the utilization potential of three substrate groups (carboxylic acids, polymers and amines) more strongly than that of the other groups. This suggests that fast-growing bacterial species

degrading carboxylic acids, polymers and amines are particularly sensitive to Cu.

Due to the co-occurrence of OTC and Cu in animal manure, the impacts of the combination of OTC and Cu on soil microbial communities need to be evaluated. Results from the current study demonstrated that the addition of OTC and Cu to the growth medium, either singly or in combination, decreased CLPP diversity, evenness and AWCD. The critical concentrations were 11 μM for OTC and 20 μM for Cu. When applied together, the negative effect on diversity, evenness and AWCD was more pronounced indicating a synergistic effect of the two pollutants on soil microbes. Bacteria degrading polymers and carbohydrates were sensitive to OTC and bacteria degrading carboxylic acids, polymers and amines were sensitive to Cu. Therefore, both pollutants affected bacteria degrading polymers, but there are also effects that are specific to each pollutant. One may argue that OTC could chelate Cu, and this chelation could reduce Cu toxicity. However, in the present study toxicity (effects on AWCD and utilization potential of substrate categories) on microbial community in the treatments amended with the combination of OTC and Cu was not reduced compared with the treatments amended with single addition (Figs. 8 and 9), indicating that Cu chelation by OTC may be insignificant.

AWCD and utilization potential of substrate categories in the control was lower in the combination experiment than in Experiments 1 and 2. The combination experiment was conducted 1 week after Experiments 1 and 2. Although the tested soil was kept at 25 °C and about 60% field capacity during that time, it appears that microbial activity declined, possibly due to the exhaustion of substrates in the soil. It cannot be ruled out that the composition of the microbial community and their sensitivity to OTC and Cu also changed during storage. AWCD and utilization potential were lower but the relative sensitivity was not changed.

In conclusion, the present study showed that OTC and excessive Cu concentrations in soils have negative impacts on the functional diversity of soil microbial communities. This suggested that important microbial functions such as the decomposition of residues may be reduced in soils that receive animal manure. Our in vitro experiments should be followed by further studies on the effect of OTC and Cu on community structure and function in soils, both under lab and field conditions.

Fig. 10. Principal component analysis CLPP following complex addition of OTC and Cu singly or in combination.

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References

- Anderson, J.P.E., Domsch, K.H., 1993. Quantification of bacterial and fungal contributions to soil respiration. *Archives of Microbiology* 93, 113–127.
- Backhaus, T., Grimme, L.H., 1999. The toxicity of antibiotic agents to the luminescent bacterium *Vibrio fischeri*. *Chemosphere* 38, 2291–2301.
- Bailey, V.L., Smith Jr., J.L., Bolton, H., 2002. Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. *Soil Biology and Biochemistry* 34, 997–1007.
- Besnard, E., Chenu, C., Robert, M., 2001. Influence of organic amendments on copper distribution among particle-size and density fractions in Champagne vineyard soils. *Environmental Pollution* 112, 329–337.
- Boxall, A.B.A., Koipin, D.W., Tolls, J., 2003. Are veterinary medicines causing environmental risks? *Environmental Science and Technology* 37, 265A–304A.
- Busse, M.D., Ratcliff, A.W., Shestak, C.J., Powers, R.F., 2001. Glyphosate toxicity and the effects of long-term vegetation control on soil microbial communities. *Soil Biology and Biochemistry* 33, 1777–1789.
- Campbell, C.D., Grayston, S., Hirst, D.J., 1997. Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. *Journal of Microbiological Methods* 30, 33–41.
- Dar, G.H., Mishra, M.M., 1994. Influence of cadmium on carbon and nitrogen mineralization in sewage-sludge amended soils. *Environmental Pollution* 84, 285–290.
- De Liguoro, M., Cibir, V., Capolongo, F., Halling-Sørensen, B., Montesissa, C., 2003. Use of oxytetracycline and tylosin in intensive calf farming: evaluation of transfer to manure and soil. *Chemosphere* 52, 203–212.
- Di Giovanni, G.D., Watrud, L.S., Seidler, R.J., Widmer, F., 1999. Comparison of parental and transgenic alfalfa rhizosphere bacterial communities using Biolog GN metabolic fingerprinting and eubacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR). *Microbial Ecology* 37, 129–139.
- Doran, J.W., Zeiss, M.R., 2000. Soil health and sustainability: managing the biotic component of soil quality. *Applied Soil Ecology* 15, 3–11.
- Driver, J.D., Holeben, W.E., Rillig, M.C., 2004. Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry* 37, 101–106.
- Ellis, R.J., Neish, B., Trett, M.W., Best, J.G., Weightman, A.J., Morgan, P., Fry, J.C., 2001. Comparison of microbial and meiofaunal community analyses for determining impact of heavy metal contamination. *Journal of Microbiological Methods* 45, 171–185.
- Figuerola, R., Leonard, A., Mackay, A., 2004. Modeling tetracycline antibiotic sorption to clays. *Environmental Science and Technology* 38, 476–483.
- Garland, J.L., 1997. Analysis and interpretation of community-level physiological profiles in microbial ecology. *FEMS Microbial Ecology* 24, 289–300.
- Garland, J.L., Mills, A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole carbon source utilization. *Applied and Environmental Microbiology* 57, 2351–2359.
- Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biology and Biochemistry* 30, 1389–1414.
- Gremion, F., Chatzinotas, A., Kaufmann, K., Von Sigler, W., Harms, H., 2004. Impacts of heavy metal contamination and phytoremediation on a microbial community during a twelve-month microcosm experiment. *FEMS Microbial Ecology* 48, 273–283.
- Haack, S.K., Garchow, H., Klug, M.J., Forney, L.J., 1995. Analysis of factors affecting the accuracy, reproductivity and interpretation of microbial community carbon source utilization patterns. *Applied and Environmental Microbiology* 61, 1458–1468.
- Halling-Sørensen, B., 2000. Algal toxicity of antibacterial agents used in intensive farming. *Chemosphere* 40, 731–739.
- Halling-Sørensen, B., 2001. Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. *Archives of Environmental Contamination and Toxicology* 40, 451–460.
- Halling-Sørensen, B., Sengeløv, G., Ingerslev, F., Jensen, L.B., 2003. Reduced antimicrobial potentials of oxytetracycline, tylosin, sulfadiazine, streptomycin, ciprofloxacin, and olaquinox due to environmental processes. *Archives of Environmental Contamination and Toxicology* 44, 7–16.
- Hamscher, G., Sczesny, S., Höper, H., Nau, H., 2002. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Analytical Chemistry* 74, 1509–1518.
- Hostetler, C.E., Kincaid, R.L., Mirando, M.A., 2003. The role of essential trace elements in embryonic and fetal development in livestock. *The Veterinary Journal* 166, 125–139.
- Jørgensen, S.E., Halling-Sørensen, B., 2000. Drugs in the environment. *Chemosphere* 40, 691–699.
- Kay, P., Blackwell, P.A., Boxall, A.B.A., 2005. Transport of veterinary antibiotics in overland flow following the application of slurry to arable land. *Chemosphere* 99, 951–959.
- Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. *Environmental Science and Technology* 36, 1202–1211.
- Kulshrestha, P., Giese, R.F., Aga, D., 2004. Investigating the molecular interactions of oxytetracycline in clay and organic matter: insights on factors affecting its mobility in soil. *Environmental Science and Technology* 38, 4097–4105.
- Kümmerer, K., 2001. *Pharmaceuticals in the Environment: Source, Fate, Effects and Risks*, first ed. Springer-Verlag, Berlin.
- Lesser, M.P., Mazel, C.Z., Gorbunov, M.Y., Falkowski, P.G., 2004. Discovery of symbiotic nitrogen-fixing cyanobacteria in corals. *Science* 305, 997–1000.
- Li, Q.C., Allen, H.L., Willum, A., 2004. Microbial biomass and bacterial functional diversity in forest soils: effects of organic matter removal, compaction, and vegetation control. *Soil Biology and Biochemistry* 36, 571–579.
- Loke, M.L., Tjørnelund, J., Halling-Sørensen, B., 2002. Determination of the distribution coefficient ($\log K_d$) of oxytetracycline, tylosin A, olaquinox and metronidazole in manure. *Chemosphere* 48, 351–361.
- Luo, Y., Jiang, X., Wu, L., Song, J., Wu, S., Lu, R., Christie, P., 2003. Accumulation and chemical fractionation of Cu in a paddy soil irrigated with Cu-rich wastewater. *Geoderma* 115, 113–120.
- Mäder, P., Fließbach, A., Dubois, D., Gunst, L., Fried, P., Niggli, U., 2002. Soil fertility and biodiversity in organic farming. *Science* 296, 1694–1697.
- McGrath, S.P., 1994. Effect of heavy metals from sewage-sludge on soil microbes in agriculture ecosystems. In: Ross, S.M. (Ed.), *Toxic Metals in Soil-Plant Systems*. Wiley, Chichester, pp. 247–273.
- Moreno, J.L., Garcia, C., Hernandez, T., Ayuso, M., 1997. Application of composted sewage sludges contaminated with heavy metals to an agricultural soil. *Soil Science and Plant Nutrition* 43, 565–573.
- Ópezalonso, M.L., Benedito, J.L., Castillo, C., Hernández, J., Shore, R.F., 2000. The effect of pig farming on copper and zinc accumulation in cattle in Galicia (North-Western Spain). *The Veterinary Journal* 160, 259–266.
- Preston-Mafham, J., Boddy, L., Randerson, P.F., 2002. Analysis of microbial community functional diversity using sole-carbon-source utilization profiles – a critique. *FEMS Microbial Ecology* 42, 1–14.
- Šantrůčková, H., Vrba, J., Pícek, T., Kopáček, J., 2004. Soil biochemical activity and phosphorus transformations and losses from acidified forest soils. *Soil Biology and Biochemistry* 36, 1569–1576.
- Schutter, M., Dick, R., 2001. Shifts in substrate utilization potential and structure of soil microbial communities in response to carbon substrates. *Soil Biology and Biochemistry* 33, 1481–1491.
- Simon, N.S., 2005. Loosely bound oxytetracycline in riverine sediments from two tributaries of the Chesapeake bay. *Environmental Science and Technology* 39, 3480–3487.

- Smalla, K., Wschendorf, U., Heuer, H., Liu, W., Forney, L., 1998. Analysis of Biolog GN substrate utilization patterns by microbial communities. *Applied and Environmental Microbiology* 64, 1220–1225.
- Swift, M.J., 1994. Maintaining the biological status of soil: a key to sustainable land management. In: Greenland, D.J., Szabolcs, I. (Eds.), *Soil Resilience and Sustainable Land Use*. CAB International, Wallingford, pp. 33–39.
- Thiele-Bruhn, S., 2003. Pharmaceutical antibiotic compounds in soils — a review. *Journal of Plant Nutrition and Soil Science* 166, 145–167.
- Tolls, J., 2001. Sorption of veterinary pharmaceuticals in soils: a review. *Environmental Science and Technology* 35, 3397–3406.
- Zak, J.C., Willig, M.R., Moorhead, D.L., Wildman, H.G., 1994. Functional diversity of microbial communities: a quantitative approach. *Soil Biology and Biochemistry* 26, 1101–1108.
- Zheljaskov, V.D., Warman, P.R., 2003. Application of high Cu compost to Swiss chard and basil. *The Science of the Total Environment* 302, 13–26.