The effects
of veterinary antibiotics
on soil microbial communities

Heike Schmitt

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The effects of veterinary antibiotics on soil microbial communities

De effecten van veterinaire antibiotica op bacteriëngemeenschappen in de bodem

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Het gevoel is verstandig, en het denken is gevoelig.

Connie Palmen, De Vriendschap

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CHAPTER 1
GENERAL INTRODUCTION

Clearly if microbial diversity means nothing and cannot be measured the reader will be well advised to turn the page and seek enlightenment and advancement elsewhere.

And yet one cannot help but wonder.

Thomas P. Curtis and William T. Sloan

VETERINARY PHARMACEUTICALS

Pharmaceuticals are designed to interfere with biological systems. Once they reach environmental compartments in significant concentrations, effects can thus also be expected on environmental species (Kümmerer 2001). Compared with pesticides and biocides, the environmental risks of pharmaceuticals have been studied less frequently. Still, during the last few years, human and veterinary drugs received increasingly more attention[1]. Recently, their possibly drastic effects were illustrated by the finding that the pain killer diclofenac led to population declines in vultures due to contamination of carcasses (Oaks et al. 2004).

Veterinary drugs form a very heterogeneous group of chemicals, used for a wide range of purposes in companion and farm animals. In the Netherlands, the number of active ingredients amounts to 200 (Gezondheidsraad 2001). Volume-wise, the anthelmintics and antibiotics are the most important groups, both represented by a range of compound classes (FEDESA 1999). Both are used in animal husbandry: anthelmintics to control worms, and antibiotics to cure bacterial infections.

The main entry route of veterinary drugs into the environment is via soil. Manure of treated farm animals often contains significant amounts of the active ingredients or degradation products, and these can reach agricultural soils with the application of manure as fertiliser. Other minor exposure routes include fish farming (Halling-Sørensen et al. 1998). Reviews of (veterinary) drugs indicate soil effect studies are relatively scarce as compared with aquatic studies (Halling-Sørensen et al. 1998, Thiele-Bruhn 2003, Boxall et al. 2004). Effect-wise, the two most important groups of veterinary pharmaceuticals (Boxall et al. 2003a) are again the anthelmintics and the antibiotics. Anthelmintics have strong effects on insects that are part of the dung pat fauna (Herd 1995). As seen in single species tests and as expected from their mode of action (Backhaus et al. 1997, Holten Lützhøft et al. 1999), antibiotics are highly toxic to environmental bacteria. Further, they have the potential to induce resistance in human pathogens (WHO 2001).

A current prioritisation exercise emphasised the potential environmental risk of many antibiotics for herd treatment (Boxall et al. 2003b). Some ecto-and endoparasitic anthelmintics, coccidiostats, and antifungal compounds were also assigned to the highest risk class. The many data gaps for the latter precluded a full assessment, though, illustrating the paucity of ecotoxicological and analytical data for many veterinary drugs in the terrestrial environment (Lahr 2004).

In Europe, human and veterinary drugs belong to the substances requiring an authorisation procedure (Directives 2001/82/EC and 2001/83/EC (European Community 2001a, b)). An evaluation of their potential environmental risks became a goal of the authorisation of veterinary drugs more than ten years ago (Directive 1990/676/EEC (European Economic Community 1990) and Directive 92/18/EEC (European Economic Community 1992)).

[1] The number of relevant publications in the Current Contents database started to rise exponentially in 2001 from around three to 19 per year, when stringent selection criteria are used ((pharmaceuti* or human drug* or veterinary drug*) and (ecotoxicol* or environmental fate)

However, new guidance documents are only currently being developed and discussed in international bodies[2] (VICH 2000, CSTEE 2001, VICH 2004). Thus, studies addressing the methodology to detect environmental effects sensitively and reliably come in good time.

To summarise, antibiotics are among the most environmentally relevant veterinary drugs. This is due to their high usage and their possible effects on bacteria, one of them being the induction of antibiotic resistance. Therefore, this thesis focuses on the environmental effects of veterinary antibiotics.

VETERINARY ANTIBIOTICS

Compounds and usage

The main antibacterial compound classes in veterinary pharmacotherapy are the tetracyclines and sulfonamides, aminoglycosides, beta-lactams, and macrolides. Their market shares vary per country (Björnerot et al. 1996, Ungemach 2000, Winckler & Grafe 2000, Boxall et al. 2002, Thiele-Bruhn 2003), but in general, the tetracycline and sulfonamide groups are used most intensively. The distribution of the classes in the Netherlands is given in Figure 1. In Europe, antibiotics are most extensively used in the United Kingdom and Spain, with respect to both total amount and dosage per kg animal reared (Ungemach 2000). The total amount of antibiotics used in Europe was approximately 5093 tons per year in 1997, of which about 3494 tons were used therapeutically, and the remainder for growth promotion (European Agency for the Evaluation of Medicinal Products 1999). In the meantime, the use of growth promoters has been phased out in the EU – only monensin sodium, salinomycin sodium, flavophospholipol and avilamycin are currently authorised as growth promotors, and their use will end this year (European Community 2003).

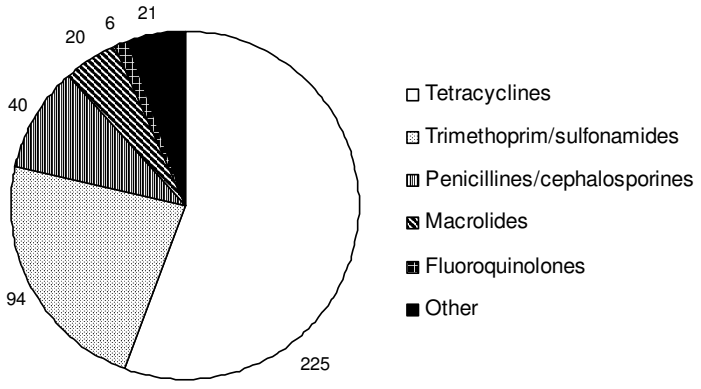


Figure 1: tonnage of veterinary use of antibiotics in the Netherlands in 2002 per compound class (VANTURES 2002).

[2] Interestingly, international harmonization is performed by a group of experts including an identical number of both governmental institutions and industry, the VICH (Montforts & de Knecht 2002).

Occurrence in the Environment

Only recently, trace-level analytical methods for antibiotics in environmental matrices have been applied in extensive monitoring exercises, leading to the detection of antibiotics in surface water and wastewater (Hirsch et al. 1999, Ternes 2001, Heberer 2002, Sacher & Stoks 2003, Schrap et al. 2003). Typical antibiotic concentrations are in the range of some ng/L up to some µg/L, and clarithromycin, (dehydro-)erythromycin, and sulfamethoxazole were most frequently found among the investigated substances. It is difficult to distinguish between human and veterinary sources for aquatic antibiotic residues, though, as many compounds are used in both areas. Few investigations addressed soil contamination with antibiotics (Thiele-Bruhn 2003), and detected tetracycline and sulfonamide residues in soil (Hamscher et al. 2002, De Liguoro et al. 2003, Blackwell et al. 2004b).

Aquatic ecotoxicology of antibiotics

Still, relatively little is known regarding the ecotoxicology of antibiotics, and most data address aquatic endpoints. Toxicity of antibiotics in the classic tests using algae, fish and *Daphnia* is low, as exemplified by the 48 h-EC_{50}[3] for *Daphnia magna* of 221, 340 and 680 mg/L for sulfadiazine, tetracycline and tylosin, respectively (Wollenberger et al. 2000).

As expected from their antimicrobial activity, bacterial test systems react much more sensitive. In tests performed with aquatic cyanobacteria (*Microcystis aeruginosa*), the lowest reported EC_{50} was 3.7 µg/L for amoxillin, and oxytetracycline and sulfadiazine had EC_{50} values of 207 and 135 µg/L, respectively (Holten Lützhøft et al. 1999). For tetracycline and tylosin, EC_{50} values of 90 and 34 µg/L were found (Halling-Sørensen 2000). Toxicity of some antibiotics to bacteria in the order of some µg/L has also been observed for other single strains such as *Pseudomonas putida* (Al-Ahmad et al. 1999, Kümmerer et al. 2000), *Vibrio fischeri* (Backhaus & Grimme 1999), *Vibrio harveyi* (Thomulka & McGee 1993), *Nitrosomonas europae* (Halling-Sørensen 2001), as well as sewage slude consortia (Halling-Sørensen 2001, Halling-Sørensen et al. 2002). In tests with *V. fischeri* and sewage sludge consortia, it occurred that short-time testing can underestimate the toxicity of antibiotics. It was argued that many antibacterial compounds show bacteriostatic effects, and their toxic effects thus only become apparent if the endpoint observed includes cellular growth (Backhaus et al. 1997, Froehner et al. 2000, Kümmerer et al. 2004).

Terrestrial ecotoxicology of antibiotics

Classic test endpoints for soil such as earthworms, enchytraeids and springtails react very insensitive to antibiotics: LC_{50}[4] values often exceed 1000 mg/kg (Baguer et al. 2000). Plants such as *Phaseolus vulgaris*, *Panicum miliaceum*, *Pisum sativum* and *Zea mays* are also only affected at chlortetracycline concentrations beyond 100 mg/L (Batchelder 1982).

[3] EC_{50}: concentration of an agent leading to the inhibition of a test parameter by 50%

[4] LC_{50}: concentration of an agent leading to death of 50% of the investigated test organisms

In forest soil bacteria, Colinas et al. (1994) found that a mixture of oxytetracycline and penicillin would reduce counts of total and active bacteria at a concentration of 10 mg/kg each. In carbon mineralisation tests based on an OECD guideline (2000b), effects have been manifest for all, oxytetracycline, tylosin, and sulfachloropyridazine. A reduction in respiration has been observed at concentrations of 30-73 mg/kg soil dry weight (Vaclavik et al. 2004). The sulfonamide sulfapyridine and oxytetracycline were shown to reduce substrate-induced respiration of soil bacterial communities with an EC_{10}[5] of 50 and 810 µg/kg, respectively (Thiele & Beck 2001), when a test protocol with extended observation times was used. The EC_{50} concentrations were slightly lower as in the abovementioned study.

Antimicrobial effects of oxytetracycline were also observed in multispecies soil systems: the activity of the enzyme phosphatase was decreased at concentrations of 0.01 and 1 mg/kg, and at higher concentrations, basal respiration was also affected (Boleas et al. 2003). Another tetracycline, doxycycline, led to decreases in phosphatase activity at concentrations higher than 1 mg/kg (Fernández et al. 2004).

A thorough investigation on the effects of tylosine at one high concentration (2000 mg/kg) was performed by Westergaard et al. (2001), who noted lasting changes in morphotypes, community structure and the potential physiological profile of the bacterial community. In a comparable study with microcosms amended with both manure and tetracycline, a decrease in bacterial diversity was also found. This was accompanied by a slight reduction in substrate induced respiration, and a shift towards a more fungi-dominated community. However, most of these effects were temporary and only seen at the higher of two concentrations tested (500 mg/kg) (Hund-Rinke et al. 2004), which is in contrast to the effects of oxytetracycline on respiration described above.

To conclude, few studies have so far addressed the effects of antibiotics on the soil microbial community. These have sometimes shown conflicting results, and did not cover a complete dose range.

METHODOLOGY: MICROBIOLOGICAL COMMUNITY ECOTOXICOLOGY

Microbiological ecotoxicology is much less developed than other branches of ecotoxicology, although (or maybe because) the diversity of bacteria exceeds the diversity of the eukaryotes by far (Woese & Fox 1977). Sometimes, effects of chemicals on microorganisms are rather determined to characterise the bioavailable fraction of contaminants (Daunert et al. 2000) than to determine adverse impacts. Remarkably, some bacterial species can survive in the most hostile environments (Vreeland et al. 1998, Madigan 2003) and degrade the most recalcitrant xenobiotics (Quensen et al. 1998). These and their potentially fast growth are possible reasons why they are frequently not seen as a toxicants' target, but rather as a tool for bioremediation (Griffin et al. 2004). However, bacterial consortia react more sensitively than other soil organisms to some toxicants, such as heavy metals (Giller et al. 1998) or some organic pollutants (van Beelen et al. 2003). Further, microorganisms play a central role in many

[5] EC_{10}: concentration of an agent leading to a reduction in a test parameter of 10%

ecosystem processes such as decomposition and nutrient cycling (Bloem & Breure 2003), and they belong to the most important group of soil organisms not only in number (Fitter 2005), but also in weight (Pankhurst et al. 1997). In soil, bacteria grow much more slowly than in liquid cultures (turnover times are in the range of 15-900 days (Harris & Paul 1994, Bååth 1998, Whitman et al. 1998), inhibiting fast regrowth after a disturbance. Some important functional groups ("keystone species") further show a higher sensitivity to environmental disturbances (Torstensson 1996, Anderson 2003), which can be linked to their small functional redundancy. This holds for example true for bacteria involved in nitrification and nitrogen fixation (van Beelen & Doelman 1997).

Bacterial communities should thus be protected from adverse impacts of toxic compounds in environments such as nature reserves or agricultural settings in order to maintain soil health and fertility.

Many areas of ecotoxicology rely heavily on single-species based test systems, such as the triad of algae-daphnia-fish tests in aquatic toxicology. Single species tests also exist for bacteria (Microtox®), but cannot be expected to be representative for highly diverse microbial communities. In regulatory ecotoxicology, carbon and nitrogen mineralisation are the only internationally standardised test systems assessing soil microbial ecotoxicity (OECD/OCDE 2000a, b), however, these are often insensitive (van Beelen & Doelman 1997, Broos et al. 2005), due to functional redundancy for very common processes or due to acclimatisation during the assay. There are relatively few alternative test systems; either based on total biomass, respiration, cellular growth or enzymatic activities (e.g. substrate induced respiration, thymidine or leucine incorporation, or nitrogenase activity). All of these also respond to changes in general soil conditions such as nutrient level, temperature and humidity. In this thesis, the focus is therefore placed on a method, which allows to more firmly establishing the causal relation between a toxicant and its community effects.

Pollution-induced community tolerance (PICT)

Ecotoxicological testing based on the principle of pollution-induced community tolerance (PICT) has been initiated by a Swedish group in the late 80's (Blanck et al. 1988). PICT is based on the notion that a community would respond to the selection pressure exerted by a toxicant by

- changes in community composition towards more tolerant species and individuals,
- changes in physiology towards more tolerant individuals, and
- changes in the prevalence of genes transferring resistance mechanisms.

The testing of PICT is based on a two-step procedure, both of which involve exposure to the same toxicant. In the "PICT selection phase", the community is exposed to the toxic agent, and toxicant-induced succession according to the abovementioned responses renders the community more tolerant. The selection phase can either take place in the field or in microcosms, mimicking environmental exposure and habitat conditions as far as possible. In the second phase, the "PICT detection phase", the community tolerance to the toxicant is determined under controlled conditions in the laboratory. The sensitivity is then compared

between control and exposed communities (Blanck 2002). If the tolerance of the exposed communities exceeds the tolerance of the control communities, the toxicant has exerted adverse effects on some community members (Figure 2).

PICT can specifically be linked to the influence of a single toxicant, because co-founding parameters should influence the community tolerance to that toxicant to a lesser extent. As the community tolerance starts to increase upon disappearance of the most sensitive community members, PICT is inherently sensitive. Further, the PICT signal can be detected with little variation, if established laboratory toxicity assays can be applied for the determination of the community tolerance.

In soil, PICT has mainly been applied to study the effects of heavy metals on microbial communities, both in microcosm and field studies. To the best of our knowledge, no investigations on community tolerance increases due to antibiotic exposure were performed previous to our studies.

Short-term tests for the detection of PICT so far included plate counts on selective media (Díaz-Raviña et al. 1994, Kelly et al. 1999b, Müller et al. 2001a, Rasmussen & Sørensen 2001), thymidine and leucine incorporation (Díaz-Raviña & Bååth 1996a, b, Pennanen et al. 1996, Bååth et al. 1998, Witter et al. 2000, Díaz-Raviña & Bååth 2001), substrate induced respiration (Gong et al. 2000), and mineralisation of ^{14}C acetate (van Beelen et al. 2001). Further, a number of studies (Rutgers et al. 1998, Rutgers & Breure 1999, van Beelen et al. 2004) used the ability of the bacterial community to respire a range of organic substrates contained in multiwell plates, a method which is described in more detail below.

Recent reviews have summarised the PICT concept and have highlighted some requirements for its successful application (Blanck 2002, Boivin et al. 2002). First, a realistic community tolerance determination requires a PICT detection technique representative of the whole community. This is difficult for soil bacteria, as most short-term tests work with soil extracts that only contain parts of the total community. Second, toxicant-induced succession is dependent on the existence of community members with different sensitivities in the first place. The absolute amount of PICT measured thus depends on the range of sensitivities in the community (Molander 1991). PICT can be expected to be successful with compounds with a specific mode of action, as the variation in species sensitivities is larger for these compounds as compared with narcotic toxicants (van Beelen et al. 2001). Time-wise, the selection phase should be long enough to allow for toxicant-induced succession, while the detection phase should be short enough not to shift community tolerance further (Blanck 2002). Last, the specificity of the PICT response can be decreased by co-tolerance or multiple tolerance: Some toxicants share tolerance mechanisms or have comparable mode of actions, such that exposure to one subtance can also increase tolerance to another one.

Substrate utilisation in microwell plates (Biolog)

The degradative capacity of bacteria has long been used to characterise the functional diversity of bacterial strains. Miniaturising the assay to multiwell plates has eased the application of this method. The multiwell plates, commercially available under the name of "Biolog®", contain one organic substrate in each well, together with a tetrazolium violet

redox dye. When bacteria are inoculated into the plate, bacterial growth and respiratory activity change the redox state. Upon oxidation of the substrates, the dye is reduced to the purple-coloured formazan (Biolog 2000). The colour formation can be evaluated by use of a plate reader at the absorption maximum of the formazan dye (590 nm).

Later, this assay was applied to study also bacterial communities (Garland & Mills 1991) by preparing so-called community level physiological profiles (CLPP, see below). To this end, the so-called Ecoplate has been developed, which includes substrates more relevant for environmental samples (Insam 1997) and which contains triplicate substrates per plate.

The main advantages of using Biolog plates are the high degree of automation of the assay and the multiple endpoints studied in one assay. On the other hand, concerns are raised that Biolog plates are biased towards fast-growing heterotrophs (Smalla et al. 1998, Preston-Mafham et al. 2002), and that Gram positive bacteria contribute little to the colouring (Heuer & Smalla 1997). They are thus likely to represent only a limited part of the community, comparable to the culturable bacteria.

Community-level physiological profiles (CLPP)

As mentioned above, the main application of Biolog plates lies in the determination of the community-level physiological profile of a bacterial community, as pioneered by Garland and Mills (1991) and reviewed in Garland (1997) and Mills& Garland (2002). CLPP is based on the pattern of organic substrates that a microbial community can respire.

The CLPP procedure consists of the extraction of bacteria from different environmental habitats. The extracts are then inoculated in the plates, and colour development is monitored with a plate reader over a range of several days. The patterns of different communities can be compared by use of multivariate techniques, and therewith, the relatedness of the communities is determined.

Most often, CLPP analyses are based on Biolog plates. However, recent applications include the testing of whole soil samples instead of bacterial extracts (Campbell et al. 2003). These investigations can be seen as a response to the criticism that the plates provide for a highly selective environment not representative of the whole soil. More concerns touch upon such method parameters as the standardisation of the inoculum density and the data evaluation of the colour readings (Preston-Mafham et al. 2002).

CLPP has frequently been applied for ecological research questions. However, there are also a number of publications addressing toxicants and their effects on microbial communities. The range of substances investigated is wider than for PICT and includes pesticides (Engelen et al. 1998, el Fantroussi et al. 1999), heavy metals (Ellis et al. 1995, Lehman et al. 1997, Kelly & Tate 1998b, a, Kelly et al. 1999a, Ellis et al. 2001, Rasmussen & Sørensen 2001, Barranguet et al. 2003, Lawrence et al. 2004), linear alkyl sulfonates (Vinther et al. 2003), and complex mixtures like oil and diesel (Bundy et al. 2002, 2004) or landfill leachates (Röling et al. 2000).

PICT with Biolog plates

Recently, Biolog plates have also been applied in PICT investigations (Rutgers et al. 1998, Rutgers & Breure 1999, van Beelen et al. 2004, Boivin et al. in press). To this end, bacterial inocula extracted from one community are exposed to a range of toxicant concentrations spiked to the multiwell plates. Reductions in the colouring upon toxicant amendment are used to derive dose-response relationships and determine the community tolerance.

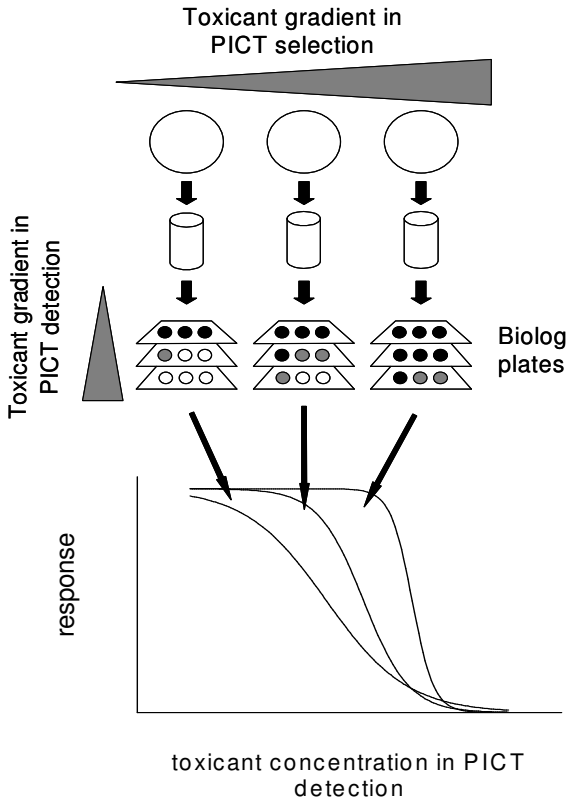


Figure 2– Principle of PICT testing with Biolog plates. In the PICT selection step, soil communities exposed to different concentrations of toxicants show physiological adaptations and/ or replacement of the most sensitive individuals and species, leading to an increase in overall community tolerance. The increase in tolerance is analysed in the PICT detection step, which involves a second toxicant gradient. Bacterial extracts of the communities are inoculated into Biolog plates, which are additionally amended with a dose range of the same toxicant. Colour formation is greatly affected by addition of the toxicant to the Biolog plates in the sensitive control communities, while highly tolerant communities show little colour decrease. Dose-response analysis of the colour formation versus the toxicant concentration in the Biolog plate reveals differences in community tolerance indicative of an exposure in the PICT selection step.

Molecular diversity

The advent of DNA and RNA analysis has opened up new horizons for microbial ecology. After centuries of studying single microbial species in culture, molecular methods revealed that the diversity of microorganisms greatly exceeds what can be comprehended by culturing approaches (Amann et al. 1995).

Several approaches can be taken to study the diversity of microbial communities on a molecular basis. Most of these make use of the characteristics of the genes coding for the small subunit (16S) of bacterial ribosomes (O'Donnell & Görres 1999). Some parts of these ribosomal RNA (rRNA) sequences are highly conserved between species, whereas others are very variable. Thus, polymerase chain reaction (PCR) primers targeting the conserved regions can amplify sequences from a broad range of microbes. Due to the variable sequences, the amplification products are of different nature (Woese & Fox 1977). DNA and RNA can be extracted directly from the environment and the 16S rRNA genes be amplified. To analyse sequence diversity, the PCR products can be characterised by enzymatic restriction reactions (terminal restriction fragment length polymorphism, t-RFLP) or by their melting behaviour (in denaturing gradient gel electrophoresis, DGGE (Muyzer 1999). DGGE analyses are capable of capturing the numerically dominant community members present at frequencies of approximately 0.5% and higher (Gelsomino et al. 1999). Further, the fragments can be cloned and sequenced, in order to yield information on the phylogeny of the bacteria.

These approaches are used in a majority of investigations in microbial ecology (Oren 2004), and there is a wealth of protocols (Akkermans et al. 1995, van Elsas et al. 2005). Only recently have molecular techniques begun to be used in ecotoxicology[6]. Still, 16S based techniques have been applied to study bacterial community changes induced by toxicants, be it without mentioning of ecotoxicology. The investigated compounds include pesticides (Engelen et al. 1998, el Fantroussi et al. 1999, Seghers et al. 2003), other organic pollutants (Siciliano et al. 2000, Brandt et al. 2004), fumigants (Toyota et al. 1999, Ibekwe et al. 2001), complex mixtures (Röling et al. 2000) and, most frequently, heavy metals (Smit et al. 1997, Kandeler et al. 2000, Kozdrój & van Elsas 2001, Müller et al. 2001b, Rasmussen & Sørensen 2001, Gillan 2004, Gremion et al. 2004). One study investigated the effect of high concentrations of the macrolide antibiotic tylosin on the soil microbial diversity (Westergaard et al. 2001). In these investigations, DGGE often turned out to be a sensitive effect measure. Interestingly, many of these publications used both molecular and cultural approaches, such as CLPP analyses.

A number of more recent applications in molecular microbiology allow a more in-depth characterisation of microbial communities, such as clone libraries based on 16S rDNA sequences (Moffet et al. 2003, Schloss & Handelsman 2004), or metagenomic approaches (Handelsman 2004). Their application to ecotoxicological research questions is highly interesting. However, these are laborious, and their use in regulatory ecotoxicological test schemes is still unlikely. These are therefore not the focus of this thesis.

Diversity of resistance genes

A notorious side effect of the usage of antibiotics is the increase in antibiotic resistant human pathogens, which poses a major risk for public health (World Health Assembly 2005). It is

[6] Between 1988 and 2005, only three microbiological publications are identified by the search engine "web of science" that contain both "16S" and "ecotoxicology" or "ecotoxicological".

known that antibiotics also affect the commensal flora in treated individuals (Sullivan et al. 2001). Thus, the occurrence of antibiotic resistance genes in environmental bacteria might be another effect of the exposure to antibacterial agents (Nwosu 2001, Séveno et al. 2002). This is the more important as resistance mechanisms are thought to originate from environmental bacteria producing antibiotics (Alonso et al. 2001). Apart from the environmental effects of antibiotics, there might thus be human-health related risks of the use of veterinary antibiotics.

Antibiotic resistance is routinely tested in clinical microbiology. Culturing methods are most often used, such as broth dilution or disk diffusion assays (Bergan et al. 1997). Still, the genetic base of antibiotic resistance is well known for many antibiotics (Huovinen et al. 1995, Van Bambeke et al. 2000, Aarts et al. 2001, Chopra 2001). This allows for molecular methods such as PCR to be used for the direct detection of resistance genes in the environment (Smalla & van Elsas 1995).

The effect of antibiotic use in humans and animals on resistance in the environment has not yet been elucidated. A number of PCR-based studies found that the diversity of tetracycline resistance genes in ground water increases under the impact of a pig farm (Chee-Sanford et al. 2001, Aminov et al. 2002), and that the application of manure increases the tetracycline resistance gene diversity in soil (Hund-Rinke et al. 2004). Other investigations also identify resistance genes in certain environments, but do not provide data on the background of resistance in pristine habitats (Schnabel & Jones 1999, Smalla et al. 2000, Tennstedt et al. 2003). Yet other researchers find that the prevalence of streptomycin and gentamycin resistance genes does not or only slightly differ between pristine and polluted habitats (Heuer et al. 2002, van Overbeek et al. 2002).

Diverging conclusions have also been drawn in studies based on the resistance profile of bacterial isolates. On one hand, an influence of antibiotics on resistance has been suggested for ruminal bacteria (Scott et al. 1997) and soil bacteria (Jensen et al. 2001, Jensen et al. 2002, Onan & LaPara 2003, Agersø et al. 2004, Jackson et al. 2004). On the other hand, resistant bacteria have also been identified in clean environments (Lee et al. 1993, Stanton & Humphrey 2003, Guardabassi & Dalsgaard 2004, Jackson et al. 2004).

To conclude, the picture of antibiotic resistance in the environment and its causes and effects is complex. Still, the importance of research elucidating the role of the environment in the ecology of antimicrobial resistance has been highlighted (American Academy of Microbiology 1999).

RESEARCH QUESTIONS AND OUTLINE OF THIS THESIS

The environmental effects of many veterinary drugs are so far insufficiently known. Among the pharmaceuticals, antibiotics should receive special attention, due to their usage patterns and occurrence in the environment, and due to their mode of action and the effects on environmental bacterial species that have been detected so far. At the moment, the risks to humans and the environment associated with the exposure to veterinary antibacterial agents cannot be satisfactorily estimated.

The objective of this thesis is to investigate the effects of veterinary antibiotics on soil microbial communities and to contribute to an evaluation of the relevance of these effects. Specifically, this thesis aims at addressing the nature of changes that occur in microbial communities of agricultural soils upon fertilisation with antibiotic-contaminated manure.

The methods applied in this thesis are chosen with respect to their potential applicability for a regulatory risk assessment of antibiotics. Thus, preference is given to simple, rapid, sensitive and standardisable test systems. Methods directed at different parts of the microbial community are used simultaneously. Toxicant-specific effects are determined, based on the principle of pollution-induced community tolerance (PICT). The community-level physiological profiles (CLPP) are studied as a method reflective of the culturable part of the community. Possible effects on the numerically dominant proportion of the community are investigated by 16S rDNA DGGE, and the diversity of resistance genes is determined in whole-community DNA extracts. Antibiotic community effects are studied in microcosms, and antibiotic resistance is evaluated in both laboratory and field studies.

This thesis starts out with two methodological chapters. Chapter 2 focuses on method optimisation and the significance of CLPP analysis. An inoculum-dilution based method is presented in detail, and its performance is compared with traditional CLPP analyses. Chapter 3 discusses the influence of method parameters on determining PICT effects of antibiotics. The importance of nutrient amendments for the detection of the effects of growth-inhibiting antibiotics is addressed, together with the possibility to use Biolog plates for the study of the growth-inhibiting effects of antibiotics.

In Chapter 4, 5 and 6, these methods are applied to four antibiotics. Chapter 4 addresses the sulfonamide sulfachloropyridazine and its effects as studied with PICT and CLPP. In Chapter 5, two other compound classes, tetracyclines and macrolides, are tested. In addition to PCIT and CLPP, 16S rDNA DGGE was applied to determine the effects of oxytetracycline and tylosin, and conclusions regarding the nature of the community effects are derived. Chapter 6 addresses antibiotic co-tolerance, because the concern has been raised that co-tolerance would limit the applicability of the PICT methodology. Further, the performance of the PICT methodology is studied with respect to response variation.

In Chapter 7, tetracycline and sulfonamide resistance is studied. In detail, the effect of the antibiotics as selective pressure is evaluated in microcosms, and the changes in resistance diversity upon fertilisation with manure is addressed in field studies. Finally, chapter 8 evaluates the methodology applied in this thesis, and discusses the relevance of the effects that have been determined.

Table 1– Physicochemical properties, mode of action and resistance mechanisms of important groups of antibiotics. Physicochemical properties are given for the compounds marked in bold, and substances investigated in this thesis are underlined

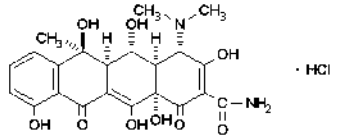
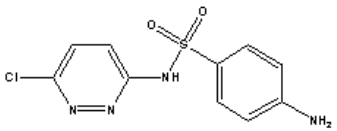
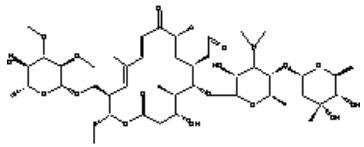
Antibiotic Class	Class members (Boxall et al. 2002)	Physico-chemical properties (Tolls 2001, Blackwell et al. 2004a)	Mode of action	Resistance mechanisms and genes	Structures
Tetracyclines	Tetracycline **<u>Oxytetracycline</u>** Chlortetracycline Doxycycline Minocycline	CAS: 79-57-2 MW: 460.44 log K_{OW}: -1.22 $pK_{a,1}$: 3.27 $pK_{a,2}$: 7.32 $pK_{a,3}$: 9.11	Inhibit protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome (Chopra 2001)	Efflux pumps *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(Z), *tet*(30), *tet*(31),*tet*(K), *tet*(L),*otr*(B),*tet*P(A),*tet*(V) Ribosomal protection proteins *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T), *otr*(A), *tet*P(B) (Chopra 2001)	
Sulfonamides	Sulfamethoxazol **<u>Sulfachloro-pyridazine</u>** Sulfadimidine Sulfadiazine	CAS: 80-32-20 MW: 284.7 log K_{OW}: -0.52 $pK_{a,1}$: 1.76 $pK_{a,2}$: 5.71	Inhibition of dihydropteroase, an enzyme involved in folic acid production (analogy with substrate para-aminobenzoic acid). Folic acid is coenzyme in nucleic acid synthesis (Sköld 2001)	Modifications of dihydropteroate synthase *sul*(I), *sul*(II), *sul*(III), mutations in the dihydropteroate synthase gen *folP* (Sköld 2000, Perreten & Boerlin 2003)	

Table 1 - continued

Antibiotic Class	Class members (Boxall et al. 2002)	Physico-chemical properties (Tolls 2001, Blackwell et al. 2004a)	Mode of action	Resistance mechanisms and genes	Structures
Macrolides	**Tylosin** Erythromycin Clarithromycin	CAS: 1401-69-0 MW: 916.1 log K_{OW}: 3.5 $pK_{a,1}$: 7.1	Inhibition of protein synthesis by binding to bacterial 50S ribosome and inhibiting polypeptide translation (Walsh 2003)	Modifications of 23S rRNA by methylation *erm*(A), *erm*(B), *erm*(C), *erm*(D), *erm*(E), *erm*(F), *erm*(G), *erm*(H), *erm*(I), *erm*(N), *erm*(O), *erm*(Q), *erm*(S),*erm*(T), *erm*(U), *erm*(V), *erm*(W), *erm*(X),*erm*(Y), *erm*(Z) (Roberts et al. 1999)	

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CHAPTER 2

OPTIMISATION OF THE ANALYSIS OF COMMUNITY-LEVEL PHYSIOLOGICAL PROFILES

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ABSTRACT

Patterns of sole carbon source utilization have been applied to characterize bacterial communities from many habitats. The so-called community level physiological profiles (CLPP) are most often based on the inoculation of multiwell plates containing a range of carbon substrates (Biolog plates) with bacterial suspensions. This study addresses some methodological concerns that have been raised and presents possible solutions regarding (1) sampling methodology and sample storage, (2) inoculum density standardisation and data evaluation, and (3) the ecological relevance of CLPP. It turned out that freezing of the bacterial extracts in liquid nitrogen reduced the overall activity of the inocula, but the effect on the CLPP was small compared with the community changes investigated. Storage of the extracts at –70ºC for up to 83 weeks neither affected the activity nor the CLPP, while storage at -20ºC led to greatly changed fingerprints. A data evaluation method based on the inoculation of a dilution series of the bacterial extracts into multiple Biolog plates had higher discriminatory capacity than other CLPP calculation methods frequently applied. Further, this method does not require adjusting the inoculum density of the original community. Last, we have investigated the ecological relevance of CLPP by determining how many bacterial strains are involved in the fingerprint. About 20 to 100 bacterial species were required to respire the whole range of substrates in an ECO plate, a number which is comparable to other fingerprinting methods in microbial ecology. These attributes make the Biolog method, following the inoculum-density independent approach described in this investigation, a promising tool in microbial ecology.

INTRODUCTION

Community-level physiological profiling (CLPP), one of the many methods to characterize microbial communities, is based on the ability of the community to utilize a range of carbon substrates. Since first proposed by Garland and Mills (Garland & Mills 1991), CLPPs have been used frequently to characterise microbial communities of different habitats like sediment, seawater, fresh surface water, oligotrophic groundwater, compost and soil (Garland 1996, Grayston & Campbell 1996, Insam et al. 1996, Papert et al. 2004).. This technique has also shown its value in the field of ecotoxicology, by discriminating between polluted and non-polluted microcosms and field sites (Knight et al. 1997, Lehman et al. 1997, Breure & Rutgers 1998, Engelen et al. 1998, Kelly & Tate 1998, el Fantroussi et al. 1999, Röling et al. 2000, Ellis et al. 2001, Rasmussen & Sørensen 2001, Bloem & Breure 2003, Vinther et al. 2003, Brandt et al. 2004, Bundy et al. 2004, Gremion et al. 2004, Schmitt et al. 2004, Boivin et al. in press). CLPP has already been applied in a large monitoring program of Dutch soils (Bloem & Breure 2003)

Community-Level Physiological Profiling (CLPP) most frequently involves inoculation of bacterial suspensions of environmental samples into Biolog multiwell plates. Biolog plates were initially developed for characterisation of Gram-positive (GP-plates) and Gram-negative (GN-plates) heterotrophic microbial strains (Bochner 1989) and contain different carbon substrates and a redox dye. The oxidation of the substrates leads to intracellular redox changes. Subsequently, the tetrazolium dye is reduced, yielding to formation of a deep violet

formazan, which is spectrophotometrically detected during incubation. Onset and rate of colour formation differs per substrate, which ultimately results in a pattern of colour intensities across the wells. From this pattern, a CLPP can be obtained, which is characteristic for the bacterial community extracted from an environmental sample (Mills & Garland 2002).

The CLPP method is by no means without controversy (Preston-Mafham et al. 2002). In rhizosphere and activated sludge samples, the CLPP was discussed to be the result of a few strains only, and was therefore judged not to be representative of the total community (Smalla et al. 1998). The CLPP potentially assesses the bacteria that can grow in the nutrient-rich, pH neutral, and aqueous environment of the Biolog wells (Garland 1997, van Elsas & Rutgers 2005). The conditions in the Biolog plates are probably as restrictive as in other culturing techniques (Amann et al. 1995), and might thus represent a few percent of the total community. Still, the representation of parts of the total diversity only hampers most methods in molecular ecology. In order to get some insight in the level of complexity captured with the CLPP method, we estimated the number of bacterial strains involved in the utilisation of the Biolog substrates.

In a second part of this investigation, practical issues of CLPP determinations were addressed. Due to practical reasons, it is often desirable not to inoculate the Biolog plate directly after extraction of the bacterial community from the sample. Therefore, the effects of freezing the bacterial suspensions and storing them at two different temperatures were investigated.

A critical experimental parameter for CLPP experiments is the inoculum density, i.e. the amount and activity of the bacteria in the inoculum. The inoculum density is known to affect the substrate utilization response and therewith the CLPP in many ways (Garland 1996). Thus, methods for standardisation of inoculum density are being used (Haack et al. 1995, Garland 1997), such as microscopic cell counting and colony forming units on agar plates. The ratio of an individual carbon source response to the average response in the plate has also been frequently applied as a method to correct for inoculum density (Garland & Mills 1991, Garland 1996, Heuer & Smalla 1997).

The investigation described here discusses an inoculum-density independent approach (van Elsas & Rutgers 2005), consisting of monitoring the colour development of a number of serial dilutions of the same bacterial suspension. We set out to compare the discriminatory power of this approach with other approaches frequently used, such as readings of each well at a certain average colouring, or kinetic parameters of the colour formation curves.

The research described in this paper has three aims. The first is to evaluate a practical and stable CLPP analysis procedure by studying conservation of bacterial samples. The second aim is to study the influence of the inoculum density on data evaluation procedures, in order to identify a robust and sensitive evaluation method. Finally, the third aim is to study the ecological relevance of CLPP by estimating the number of bacterial strains that is involved in one CLPP.

MATERIAL AND METHODS

Sampling and extraction

The following sampling and extraction methods were used for different matrices: soil, sediment and aquatic biofilm.

Soil

For field soils, 25 g of the soil sample, based on dry weight, was mixed with 250 ml sterile bistris buffer (Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane,10 mM, pH 7) in a blender for 1 minute at the highest speed. For microcosm experiments, microbial inocula were extracted from the soil by shaking 20 g of soil for 1 minute in a blender with 200 ml sterile phosphate buffer (100 mM, pH 7). Large soil particles were settled by centrifuging the suspension for 10 minutes at 500 g. The supernatant on top contained the extracted bacteria, and was used as undiluted bacterial suspension.

Sediment

The upper 2 mm of the sediment was sampled transported cool and stored overnight at 4°C in the dark. The bacteria in the sediment sample were extracted by sonicating 1 ml of the sediment sample with 4 ml sterile bistris buffer (10 mM, pH 7), giving 3 pulses of 5 seconds (68 watt), with intervals of 15 seconds. The samples were kept on ice during the sonication procedure to prevent overheating. To settle large sediment particles, the suspensions were centrifuged for 10 minutes at a speed of 500 g.

Biofilm

Aquatic biofilms were grown on glass slides by hanging them in the surface water attached to floating racks for one month (Boivin et al. in press). The biofilm was scraped off the glass plate and harvested with a sterile razor blade, transported cool and stored overnight at 4 °C in the dark. The extraction was done by mixing 7.5 cm² of the biofilm sample in 5 ml sterile bistris buffer (10 mM, pH 7) with a tissue homogeniser (25000 rpm) during 30 seconds and sonicator, giving 1 pulse of 5 seconds (68 watt). The samples were kept on ice during the mixing procedure to prevent overheating. To settle large particles, the suspensions were centrifuged for 10 minutes at a speed of 500 g.

Storage conditions of microbial suspensions

Storage of the bacterial inocula instead of immediate use can significantly ease sample handling and experimental design. In order to investigate sample storage methods, we studied the effect of freezing of the bacterial extracts on the CLPP. Frozen extracts were obtained by quickly freezing portions of 2 ml of bacterial inocula in liquid nitrogen.

The effect of freezing of bacterial inocula was investigated in a set of fourteen soil samples, stemming from two microcosm experiments. One experiment with 8 samples was performed to study the effect of the antibiotic tetracycline on soil microbial communities (Schmitt et al., submitted for publication), three other field soil samples originated from a field monitoring

program of the Netherlands. CLPP profiles were obtained as described below from dilution series of both fresh and frozen bacterial inocula.

The effect of storage time on samples kept at -20°C was determined for a large set of soil samples originating from the Demmerikse Polder (NL) and containing varying levels of metal contamination. The samples were stored for 1 until 43 weeks (Boivin 2005). Biofilm samples were used to investigate storage at –70 °C. The biofilm samples were derived from aquaria treated with a range of copper concentrations (1, 3, 10 µM and a reference) and kept at different temperature levels (10, 14 and 22 ^{0}C) during 2 months (Boivin et al. in press). Further, CLPP was determined twice in time for a set of nineteen samples originating from different matrices: soil, biofilm and sediment.CLPP analyses were based on Eco-plates from Biolog Inc. (Hayward, CA, USA), which consist of three sections of 32 wells containing a freeze-dried mineral medium, a tetrazolium dye and different carbon substrates. When the carbon source is utilised a redox reaction takes place in the bacterial cells and the soluble and colourless tetrazolium dye is transformed into the insoluble purple formazan, which can be measured with a spectrophotometer at a wavelength of 590 nm.

CLPP calculation methods

CLPP based on an inoculum dilution method

First, community level physiological profiles (CLPPs) were made by applying an inoculum-density independent approach (van Elsas & Rutgers 2005). This approach is based on the inoculation of serial bacterial dilutions, and requires a set of plates for one CLPP analysis.

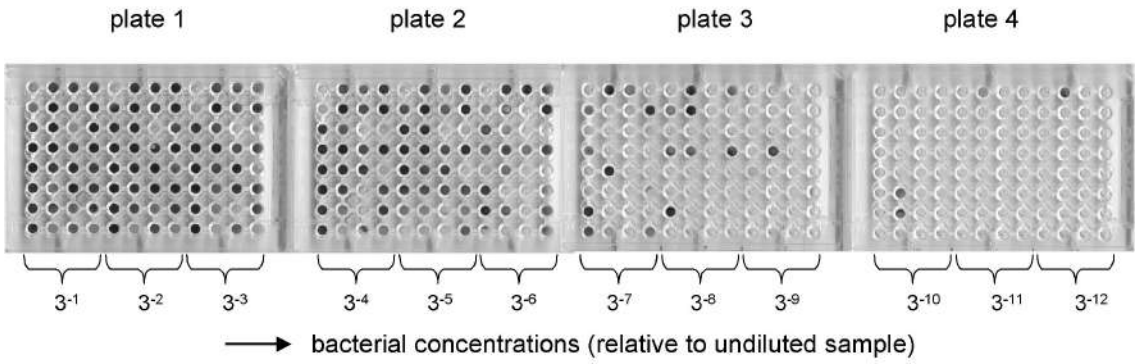

Figure 1. Colour development of several bacterial dilutions after seven days incubation in Eco-plates.

Two vials with inoculum of a specific sample were thawed at room temperature. For each sample a dilution series of twelve dilutions by 3-fold steps was made with a sterile buffer in glass tubes (3^{-1}-3^{-12}). The dilutions aimed at covering a complete range of colour formation (Figure 1). Four Eco-plates were used per sample; in each section one bacterial dilution was inoculated with 100 µl suspension per well. The plates were placed in a microplate stacker and autosampler in a humified chamber (> 85 %) and incubated in the dark at 20 ± 1 °C. The

colour formation in the plates was measured at a wavelength of 590 nm every 8 hours during 7 days, using an auto-sampler and a microplate reader (Expert 96, Asys Hitech, Eugendorf, Austria).

A system for data management was necessary because of the large amount of data obtained from the micro plate reader. For this purpose a software tool was developed using Visual Basic (SimpleDat, produced by W. Roelofs, RIVM, Bilthoven, The Netherlands). This software tool converted the spectrophotometric readings in a quick, simple and ordered way, and calculated the CLPP profile, following the inoculation-density independent approach.

The colour development of each well (WCD) was corrected by subtracting the WCD of the control well not containing any substrate. For each substrate the WCD was plotted against time, and the maximum WCD and the area under the curve (AUC) for each dilution were calculated. The responses were standardised by dividing the observed response by the maximum theoretical response, so the maximal t could be set to 1, which serves a more robust curve fitting. The maximal theoretical response for each well was derived from a collation of previously performed experiments with the same experimental set-up, for soil, sediment, biofilm, and surface water, respectively (Wouterse et al. 2002).

For both the maximum WCD and the AUC (abbreviated as A_s), the bacterial dilution was determined where a reduction of the colouring by 50% occurred.

A_s was plotted against the bacterial dilution (BD), which led to 31 curves, one for each substrate (Figure 2). These sigmoid curves were fitted with the equation for a log logistic distribution (equation 1) (Haanstra et al. 1985).

$$As = \frac{t}{1+10^{h(\log BD_{50}-\log BD)}} \qquad \text{Equation 1}$$

where A_s is either maximum WCD or AUC, t is the maximum of the curve, log BD is the logarithm of the bacterial dilution, log BD_{50} is the inflexion point of the curve and h is the hillslope, i.e. the standardised slope of the curve at the inflexion point.

The same is done for the average response ($A_{average}$) of each bacterial dilution, which was calculated from the 31 individual responses (A_s). $A_{average}$ was plotted as a function of the bacterial dilution and fitted with equation 1, again yielding values for the inflexion point (log $BD_{50average}$) and the hillslope ($h_{average}$) (Figure 2).

For each substrate of the Biolog plate, the log BD_{50s} was used to determine the relative abundance (RA_s) of substrate conversion, which will be used for constructing the fingerprint, by following equation 2.

$$\log RA_S = \log BD_{50average} - \log BD_{50s} \qquad \text{Equation 2}$$

where RA_S is the bacterial dilution necessary to convert 50% of the specific substrate relative to the bacterial dilution needed to convert 50% of all substrates in the Biolog plate.

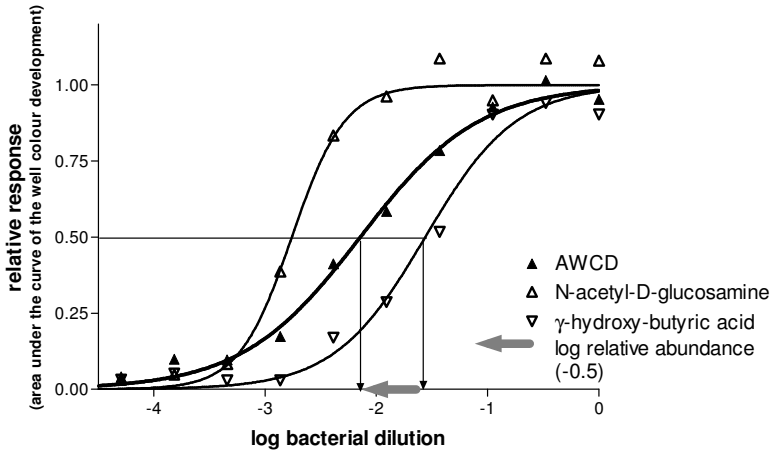

Figure 2. Graphical demonstration of the calculation of the log relative abundance based on the normalised colour development in the Biolog plates.

A positive value for an individual substrate indicates that the potency to convert this substrate is above average in the particular sample and vice versa for a negative value. In cases of unrealistically high and low values for the relative abundance, this value was artificially set to +2 respectively -2. Ultimately, this procedure yields a CLPP of relative abundances for all 31 substrate conversions (Figure 3). As this procedure makes use of the characteristics of substrate utilisation upon dilution, it is independent of the absolute number and activity of bacterial cells in the original inoculum.

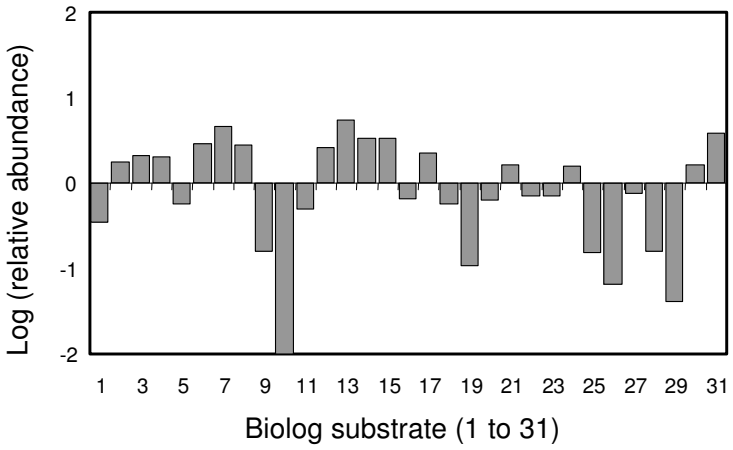

Figure 3. Graphical representation of a community-level physiological profile (CLPP) of one bacterial community inoculated in Biolog Eco-plates.

CLPP based on kinetic parameters

In order to evaluate the influence of different data treatment methods, CLPP fingerprints were also derived from kinetic parameters based on single plates. This analysis was based on a dataset from soil microcosms exposed to a range of tetracycline concentrations. For two representative bacterial dilutions of each inoculum (3^{-2} and 3^{-4}), the following kinetic parameters of the colour formation curve were determined and used as CLPP fingerprint input: the lag time until onset of colouring, the maximum rate of colouring, and the maximum colouring. Nonlinear curve-fitting based on the Gompertz function was applied to yield the kinetic parameters according to equation 3. The colour readings were fitted with and without natural logarithmic transformation of the absorption readings (Garland et al. 2001).

$$Y=M*\exp(-\exp(u/M*e*(lambda-x)+1)) \qquad \text{Equation 3}$$

with M=maximum colouring, u=maximum colour formation rate, lambda=lag time until onset of colouring, and x= either the absorption, or the ln-transformed absorption (ln (absorption / absorption, t=0)).

For samples with unrealistic curve fit results or missing curve fits, 0 was used as fingerprint for M (if M from curve fit was >6), 168 hours as lambda (if lambda was <0 or > 168), and 0 as U (if U from curve fit was >0.3).

Further, the readings of the individual substrates where the AWCD was closest to 0.5 were used as fingerprint input, both unchanged and after standardization by division by the AWCD (Garland 1996). The number of substrates utilized was determined as the number of substrates yielding an absorption of >0.3 for a point in time where the absorption is exponentially increasing (t=90 hours) and at the end of the whole plate measurement series (t=168 hours).

Next to CLPP, some overall endpoints were analysed and compared. To survey the activity in the Eco-plate of the bacterial community, the average well colour development (AWCD) after 7 days of incubation was used. Additionally, two endpoints were calculated following equation 1, the hillslope (steepness of the curve) and the BD_{50}, the amount of suspension needed for 50% average response (Figure 2). Simultaneously, the number of colony forming units (CFUs) was determined by plating the bacterial suspension of each sample on Tryptone Soy Broth agar (0.3 g/l). The agar plates were incubated in the dark at 25 °C, the colonies were counted after 8 days.

Statistics

Multivariate analyses were applied to analyse the differences in CLPPs using the software package Canoco V.4.0 (CPRO-DLO, Wageningen, The Netherlands). The CLPPs were first analysed with a detrended component analysis (DCA). The length of gradient (based on DCA) was lower than 3 (van Wijngaarden et al. 1995) and therefore the different samples were collated in a principal component analysis (PCA). Significance of correlations with environmental variables and the significance of the axes were tested in a redundancy analysis

(RDA) using a Monte Carlo permutation test. The different variables were tested separately, 9999 permutations were performed and a significance level (p) of 0.05 was chosen.

All parameters were verified for normality using the Kolmogorov Smirnov test (GraphPad Prism, version 4.01). A paired t-test was also used to compare the CLPPs between two treatments on the same set of samples. To this end, the samples were compared both jointly (all joined fingerprints of all samples of one treatment compared to all fingerprints of all samples of the other treatment) and separately.

A paired t-test was used to statistically compare the other endpoints like AWCD, hillslope, BD_{50} and the number of CFUs. If the data were not normally distributed a Wilcoxon paired t-test was used.

Relevance of CLPP: Strains and CLPP predictions

The number of bacterial strains that contribute to the overall plate colouring was determined by a combination of experimental and modelling approaches. In brief, the procedure consisted of isolating single strains from different habitats from a Biolog plate after seven days incubation. The colour formation profile of the isolated strains was determined for one dilution. Then, the colour formation of communities composed of these single strains were simulated using the program Microsoft Excel plug-inn Crystal Ball (version 4.0, 1988-1996 Decisionering, Inc.). In order to isolate different bacterial strains, CLPPs of three samples originating from different habitats were determined according to the method previously described. After six days of incubation in the Eco-plates, samples were taken from wells of the highest and the lowest bacterial dilution in which colour formation was observed. 64 samples per habitat were spread on agar containing ¼ of the concentrations in a standard mineral medium (Evans et al. 1970), 0.3 g Tryptone Soya Broth (TSA)/l and a mixture of 6 different carbon sources also present in the Biolog plate: proline, N-acetyl-D-glucosamine, α-D-lactose, itaconic acid, tween 40 and L-phenylalanine (0.5 g carbon/l each), pH 7. After 7 days of incubation at 20 °C, from each agar plate the most dominant strain was plated on a new agar-plate, with the same recipe, to obtain pure strains. After the second isolation procedure, one bacterial colony was suspended in a 30 ml flask containing the same compounds and concentrations used for the isolation of the strains. The flasks were continuously shaken at 80 rpm at 25 °C and the growth was monitored by means of turbidity measurements at 750 nm using a spectrophotometer. At the end of the exponential growth phase the suspensions were divided over 2 ml Eppendorf vials, rapidly frozen in liquid nitrogen and stored at -70 ºC until analysis. This procedure yielded 122 strains in total.

Carbon substrate profiles for each strain were made by inoculation of thawed bacterial suspensions in Eco-plates, after inoculum standardisation by spectrophotometric measurements at a wavelength of 750 nm (OD_{750nm}). Binary species-level physiological profiles (cut-off value of 0.3 $OD_{590\ nm}$) were determined. To confirm that the binary profiles could be used to predict the colouring of communities composed of several strains, 10 communities were assembled, containing 2 to 6 isolates. The colour formation of these communities was compared with predictions based on the binary physiological profiles of the single strains.

Based on the data described above, the percentage of strains that had a positive response on a particular carbon source was calculated. This was assumed to be the chance that an average strain could utilise that particular carbon source. With these chances as guideline new strains were simulated by Crystal Ball, and the colouring of 250 communities combined from these strains was simulated.

RESULTS

Storage conditions: Influence of freezing of bacterial suspensions on CLPP profiles

The effect of freezing bacterial suspensions on the CLPP was investigated using a set of fourteen soil samples. Community-level physiological profiles (CLPP) were assessed immediately after extraction of the bacterial communities and after rapidly freezing and the bacterial suspension with liquid nitrogen and storage at –70 °C not longer than 2 days. No significant differences ($p = 0.21$) were observed with a Wilcoxon paired t-test between the CLPPs of the frozen and freshly inoculated bacterial suspensions. To maximally reduce the dataset a principal component analysis (PCA) was made (Figure 4). No clear shifts or distortion of the dataset structure after freezing were observed in the PCA. To statistically test if the environmental variables correlated with the CLPP shifts, a Monte Carlo Permutation test was performed in a redundancy analysis (RDA). The freezing of the bacterial suspensions did not significantly ($p = 0.35$) correlate with the CLPP shifts. Also, no significant differences ($p = 0.38$) were observed between the frozen and freshly inoculated bacterial suspensions based on their average well colour development (AWCD) after 7 days incubation in the Biolog plate. However, the number of colony forming units (CFU) significantly ($p = 0.0004$) declined from 7.3 to 6.9 log CFU/g DW representing 60% decrease in absolute numbers (or 5% decrease on a log scale). The increase of the BD_{50} ($p = 0.03$) from a bacterial dilution of $10^{-1.36}$ to $10^{-1.2}$, representing an activity loss of 27%, also pointed to a decrease in bacterial activity. An increasing BD_{50} means that a more concentrated inoculum was needed for 50% average colour formation in the Biolog plate after freezing the bacterial community.

Storage conditions: Storage of frozen bacterial suspensions at –20 °C

The influence of storage of the bacterial suspensions at –20ºC was investigated by comparing the community level fingerprints of community bacterial extracts stored at -20°C for one up to 43 weeks. The extracts originated from a set of 44 soils with a different heavy metal contamination located in the Demmerikse polder, the Netherlands. Figure 5 revealed that the fingerprints clearly changed upon storage for 43 weeks. In addition, a shift in the profiles occurred also between the first two weeks and nine weeks. The CLPP shifts observed in the PCA (Figure 5) were found to correlate significantly ($p = 0.0001$) with the storage time, which was the variable explaining most of the variation in the data.

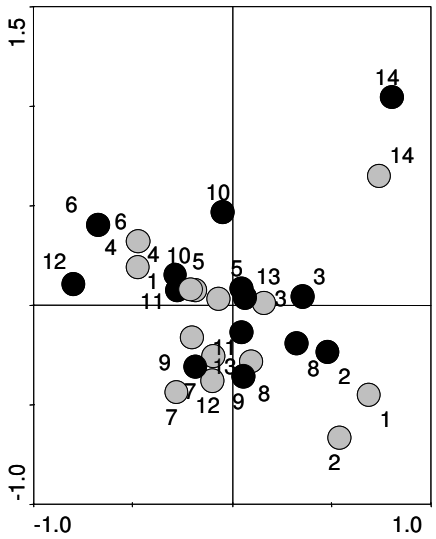

Figure 4. Principal component analysis (PCA) of community-level physiological profiles (CLPP) of fresh and frozen samples. Black circles represent the samples frozen with liquid nitrogen and stored at –70 °C and the grey circles represent fresh samples. First axis 15.7%, second axis 12.8%.

The significance of the other environmental variables changed from 0.024 to 0.35 for the soil lead concentration and from 0.022 to 0.004 for the organic matter content, respectively, between analyses of the samples after 1 week only and all samples. The influence of soil lead content was therefore not detectable in the dataset including the samples stored for up to 43 weeks.

Additionally, a subset of sixteen soil samples stored at –20 °C was replicated after varying periods of time (1 until 43 weeks). Overall, the fingerprints were significantly ($p < 0.0001$) different after storage at -20 °C following the Wilcoxon paired t-test, when all samples were tested simultaneously in a paired t-test. Thus, it was not possible to reproduce the CLPPs of soil samples when stored at -20 ^{o}C. The influence of freezing varied between bacterial communities, as apparent from paired t-tests for each separate sample before and after storage. Furthermore, a multivariate analysis (RDA) showed a significant ($p = 0.0002$) influence of the storage time on the CLPP of the replicates, time being the strongest explanatory variable. Bacterial communities conserved for 43 weeks at –20 °C utilised the substrates L-serine, γ-hydroxy-butyric acid, glycogen, α-ketobutyric acid, phenyl-ethylamine, α-D-lactose and putrescine to a lesser extent than bacterial communities stored at –20 °C for one week. On the other hand, D-galactonic acid γ-lactone was relatively more utilised compared to the bacterial communities stored only for a short period of time at –20 °C.

Moreover, endpoints for overall activity like the AWCD and BD_{50} showed significant differences between the replicates: $p = 0.0001$ and 0.0005, respectively. The average colour development (AWCD) after 7 days incubation in the Biolog plate decreased 33%, from 1.68 to 1.12. As the BD_{50} increased from $10^{-1.79}$ to $10^{-0.68}$, a more concentrated bacterial suspension was needed to obtain 50% colour formation in the Biolog plate, indicating an activity loss of 92% during storage.

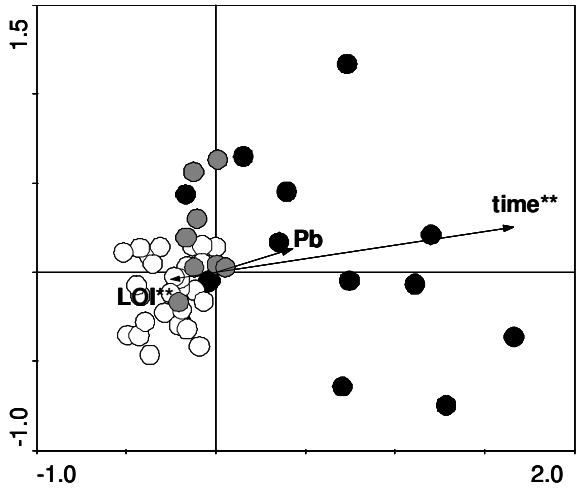

Figure 5. Principal component analysis (PCA) of community-level physiological profiles (CLPP) of bacterial inocula from soil samples of a field with varying metal contamination and the dependence of time of inoculum storage at –20 °C. Open circles: 1-2 weeks. Grey circles: 9 weeks. Black circles: 43 weeks. Variance explained: First axis 23%, second axis 11%. Levels of significance variables. *: $p<0.05$, **: $p<0.01$.

Storage conditions: Storage of frozen bacterial suspensions at –70 °C

To investigate the influence of storage time at –70 °C, CLPPs were calculated for a large set of mesocosm biofilm samples affected by copper. The storage time of the bacterial inocula at -70 °C varied between 2 and 90 weeks.

Figure 6 shows differences between the CLPPs in a PCA. In contrast to samples stored at 20°C, there was no shift in the CLPP with an increased storage time. Changes in CLPP were rather related to the copper treatment, as apparent from a Monte Carlo Permutation test in a redundancy analysis (RDA). The experimental variables such as exposure time ($p = 0.0001$) and copper concentrations ($p = 0.0001$) had a significant correlation with the CLPP shifts observed in the PCA. In contrary, the CLPP shifts observed in the PCA were not significantly correlated with the duration of storage at –70 °C ($p = 0.14$).

The influence of storage of inocula at -70 °C was also tested using another set of nineteen samples, whose CLPP determination was replicated after different storage times. These samples originated from different matrices: soil, biofilm and sediment. The bacterial communities were extracted from the matrices and stored at –70 °C during varying periods of time (2 until 84 weeks). The fingerprints of replicates made at different time points were not significantly different from each other ($p = 0.12$) following the Wilcoxon paired t-test. Multivariate analysis (RDA) showed no significant differences between the CLPPs of the replicates ($p = 0.32$). Other endpoints like the AWCD, BD_{50} and CFU also showed no significant differences between the replicates (Table 1).

Table 1. Overview of activity of bacterial inocula influenced by different extraction and storage parameters. Fresh / Frozen: Influence of freezing of inocula in liquid nitrogen, -70°C: before / after storage at -70°C for up to 84 weeks, -20°C: before / after storage of bacterial inocula at -20 °C for up to 43 weeks. Asterisks represent the level of significance. ns: not significant p>0.05; * : significant p<0.05; **: significant p<0.01.

	fresh	frozen	n	-70 °C			-20 °C		
				before	after	n	before	after	n
AWCD	1.58	1.56 ^{ns}	14	1.29	1.34 ^{ns}	19	1.68	1.12 **	16
log CFU	7.3	6.9 **	14	6.55	6.61 ^{ns}	19	-	-	16
BD_{50}	$10^{-1.36}$	$10^{-1.22}$ *	14	$10^{-0.66}$	$10^{-0.65}$ ns	19	$10^{-1.79}$	$10^{-0.68}$ **	16

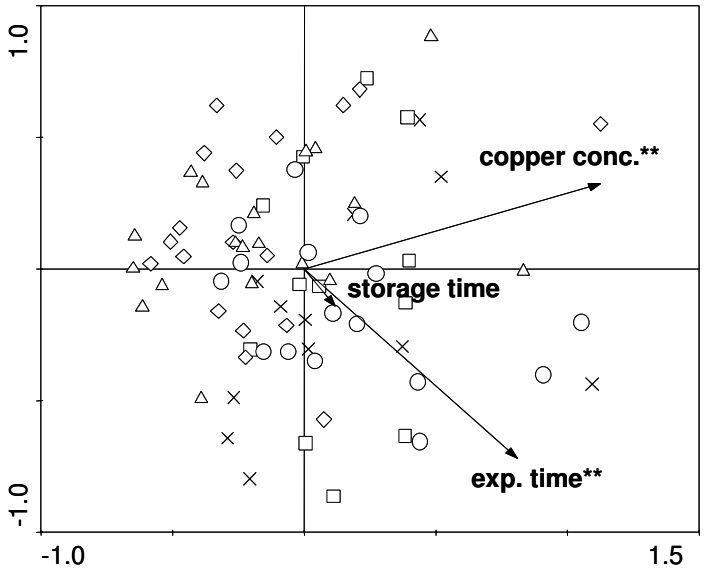

Figure 6. Principal component analysis (PCA) of community-level physiological profiles (CLPP) of biofilm samples exposed to copper and stored, after extraction, at –70 °C. The symbols represent the storage time at –70 °C. Circles: 2 weeks. Triangle: 3-6 weeks. Diamond: 20-30 weeks. Square: 60-70 weeks. Cross: 80-90 weeks storage. First axis 15.2%, second axis 14.3%. Levels of significance variables. *: p<0.05, **: p<0.01.

CLPP calculation methods

Different parameters characterising colour formation in Eco-plates were analysed for their resolving power in CLPP analyses. Among them were a range of kinetic parameters of the colour formation curve, readings at a set AWCD of 0.5, and the method based on an inoculum dilution series (van Elsas & Rutgers 2005). The results are shown in table 2.

Table 2. Discrimatory power of different data evaluation methods for CLPP analyses. The explained variance of the 1st and 2nd axis of PCA analyses and the significance of the correlation with an environmental variable of CLPPs based on kinetic parameters of the colour formation curves (maximum, lag phase and rate as obtained from sigmoidal curve-fitting of ln-transformed absorption over time), on readings at an AWCD of 0.5 both raw readings and readings normalized by the AWCD ($AWCD_n$), and on a inoculum dilution method. The environmental variable is the logarithm of the soil concentration of antibiotics. Asterisks represent the level of significance. ns: not significant $p>0.05$; * : significant $p<0.05$; **: significant $p<0.01$.

Sample	parameter	dilution	Variance explained [%]		Significance environmental variable
			PCA axis 1	PCA axis 2	
tylosin					
	Maximum	2	32.7	26.3	0.4430
	Lag phase	2	30.6	27.2	0.4423
	Rate	2	57.5	17.7	0.0036 **
	Maximum	4	39.4	23	0.1110
	Lag phase	4	31.8	21.9	0.1501
	Rate	4	50.3	17.5	0.2517
	0.5 AWCD	2	53.6	19.9	0.0014 **
	0.5 AWCD	4	44.5	17.9	0.0068 **
	0.5 $AWCD_n$	2	54.4	16.4	0.0003 **
	0.5 $AWCD_n$	4	44.3	19.4	0.0048 **
	AUC, dilution based		46.4	17.3	0.0009 **
	Maximum, dilution based		63.3	11.5	0.0006 **
tetracycline	Maximum	2	39.8	21.3	0.0840
	Lag phase	2	47.5	24.2	0.2395
	rate	2	40.4	20	0.1693
	Maximum	4	30.4	26.8	0.0314 *
	Lag phase	4	42.2	19.8	0.3166
	rate	4	29.1	23.3	0.3941
	0.5 AWCD	2	38.1	32.4	0.3583
	0.5 AWCD	4	35.5	29.8	0.0009 **
	0.5 $AWCD_n$	2	53	23.4	0.2504
	0.5 $AWCD_n$	4	36.1	27.3	0.0004 **
	AUC, dilution based		40.6	22.7	0.1065
	Maximum, dilution based		47.8	18.8	0.0827

When the significance of the influence of the environmental variable is taken as measure for the applicability, both the dilution-based methods and methods based on readings at an AWCD of 0.5 compare favourably. The performance of the kinetic models was limited by the curve-fitting step, as data loss occurred when curve fits did not convert. This occurred frequently when raw absorbance was fitted to a sigmoid growth curve, as the colouring of many wells had not reached the plateau phase. In addition, there were a significant number of wells without colour formation in the higher dilution, such that the total amount of substrates with a failed curve fit or no colouring amounted to 31% (tetracycline data set). Data transformation before curve-fitting as suggested by (Garland et al. 2001) led in many cases to misleading fits, as colour formation curves with low absolute colouring were still fitted. For the tetracycline dataset, the kinetic parameters correctly identified the effect of the highest treatment, which was also apparent from a generally lower activity of that sample (as seen from the AWCD). For the tylosine dataset, the reduction of activity in the highest treatment was smaller, and the kinetic parameters less clearly discriminated the treatments than the inoculum-dilution method and the readings at an AWCD of 0.5.

For both the tetracycline and the tylosin dataset, the correlation of the readings at an average absorption of 0.5 gave highly significant correlations with the environmental stress, rather independent from whether the readings were corrected for the average AWCD or not. Still, this did not hold true for both dilutions tested – for tylosin, the higher dilution more significantly depicted the environmental stress, while for tetracycline, it was the lower dilution. The choice of the dilution of the original inocula therefore determined the outcome of the fingerprint experiment, as illustrated in figure 7, where two PCAs are shown for readings at an AWCD of 0.5 for both the higher and the lower dilution. Thus, normalisation by correcting the readings for the average colouring did not remove the influence of inoculum density on the fingerprints. The methods based on the inoculation of a dilution series, in contrast, make use of the changes in substrate utilisation upon inoculum dilution, and characterise the community by the relation between outdilution of a substrates' utilization and outdilution of the total community. This method does thus not require the choice of one inoculum density, when compared with methods based on readings at a set AWCD.

Relevance of CLPP: Strains and Communities

A combination of experiments and modelling approaches was used to investigate the number of bacterial strains involved in the colour formation in a Biolog plate. In brief, the profiles of a number of environmental isolates were determined. Using these profiles, the substrate utilization was simulated for communities with varying diversity.

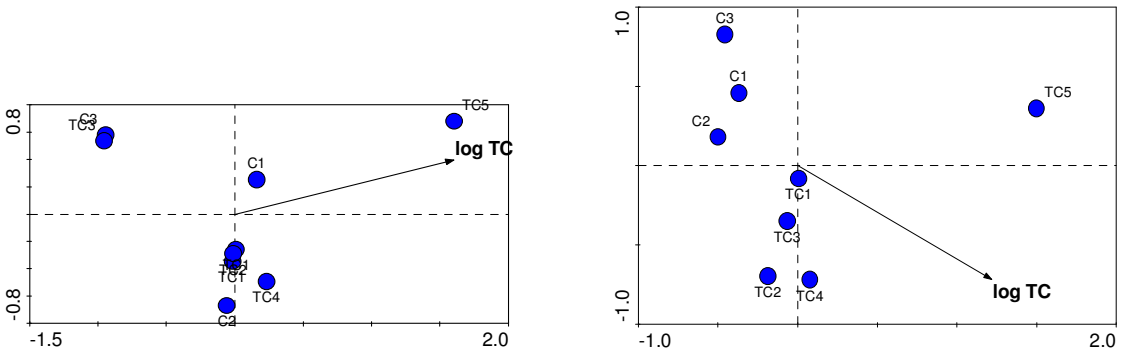

Figure 7. Influence of inoculum dilution on fingerprints based on readings at a set average colouring as seen from principal component analyses. Left: inocula of tetracycline-exposed soils (C1-3: controls, T1-T5: treatments with 0.1, 1, 10,. 100 and 1000 mg/kg tetracycline) tested at a dilution of 3^{-2}, right: the same inocula tested at a dilution of 3^{-4}. Readings for each well were taken when the AWCD was 0.5, and were divided by the AWCD. For the sample TC1, the two readings around 0.5 were taken as independent samples.

A set of 122 isolates was obtained from Biolog plates inoculated with communities from different habitats. The pure isolates produced 93 different profiles. The chance that a certain carbon source was utilised by these isolates differed markedly per substrate, with one substrate (α-cyclodextrine) only being utilised by one strain out of the 122 tested. Four other substrates were used by less than 10% of the strains (L-threonine, glycogen, D-L-α-glycerol phosphate, 2-hydroxybenzoate and α-keto-butyric acid). Nearly half of the substrates (15) were utilized by less than 25% of the isolates. In contrast, only 8 substrates were used by >75% of the isolates, among them L-arginine, pyruvic acid methyl ester and L-asparagine.

In order to test whether patterns of substrate utilisation of single isolate can be applied for predicting community patterns, the utilization of substrates by communities with 2 to 6 members assembled from the investigated strains was tested. After 7 days incubation, the profile could be predicted with 88% accuracy, based on the binary profiles of the single isolates. Information on single strains could thus be used to evaluate the substrate utilization patterns of the communities.

In a following step, the number of substrates utilised was simulated in dependence of the number of strains in the community. To this end, model bacterial communities were constructed, which were based on the profiles of the single isolates. Simulations demonstrated that the number of carbon sources that were utilised on average by a community of 20 strains was 27 substrates out of 31. A community of 50 strains utilised on average 30 carbon sources out of 31 (Figure 8). Soil communities in general utilised all carbon sources available if the inoculum density was high enough. The chance that a whole plate was coloured (31 carbon sources utilised) by 10 strains was very low (< 0.1%). 60 strains resulted in a chance of 50% of a whole coloured plate, and 100 strains resulted in a chance of 80% of a wholly coloured plate.

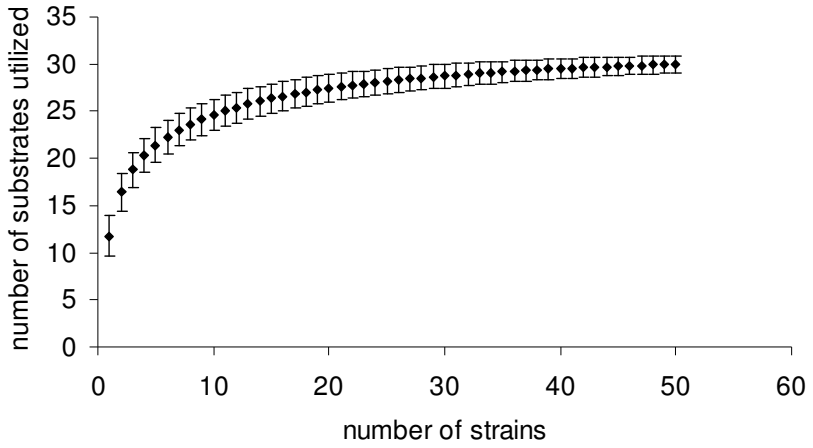

Figure 8. The predicted number of utilised carbon sources per community size.

DISCUSSION

Because of the high biomass and diversity of microbial communities, no single method in microbial ecology can characterise a microbial community completely (Dykhuizen 1998, Westergaard et al. 2001, Wellington et al. 2003, Garbeva et al. 2004). Still, a number of methods for the study of bacterial communities exists, such as methods based on 16S rRNA sequences (Kent & Triplett 2002) or, more recently, on metagenomic analyses (Torsvik & Ovreas 2002, Wellington et al. 2003, Handelsman 2004). Whereas fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) can detect species present in the community at proportions of approximately 0.5% and higher (Gelsomino et al. 1999), techniques that capture a larger proportion of the community can be laborious (Leser et al. 2002). Community-level physiological profiles (CLPP) using Biolog plates are another method in microbial ecology, characterising the part of the community capable of proliferating in the well of a Biolog plate (Mills & Garland 2002), and enabling a quick and easy community analysis. However, a range of technical problems has been highlighted, such as the need for standardisation of the inoculum density and the possible bias introduced by different methods of data treatment (Preston-Mafham et al. 2002). In this paper, we discuss the performance of alternative procedures that overcome the problems raised above.

It is not always possible or desirable to perform the analysis immediately after sampling. First of all, the possibility to store frozen bacterial extracts frozen has therefore been evaluated. A procedure based on rapid freezing of small amounts of bacterial suspensions in liquid nitrogen led to some activity losses (such as reductions in absolute colouring and bacterial counts). It can be expected that bacteria would suffer from hyperosmotic stress upon freezing (Muldrew & McGann 1990), which results in loss of bacteria and therewith activity. However, the distortion of the CLPP of communities after freezing was minor in comparison with the difference between samples under environmental stress. Apparently, the freezing process does not differentially affect specific groups in the community, but the potential physiological activity of the community was correctly captured in the remaining bacteria.

Furthermore, subsequent storage of the inocula was investigated, in order to ease the practical examination. Storage of the microbial communities at -70 ^{0}C resulted in good conservation of microbial communities until 90 weeks after sampling, both with regard to the overall activity and to the fingerprint. In contrast, storage at -20 ^{0}C greatly influenced both the activity and the fingerprints of the samples and is thus not recommended for storage of bacterial inocula.

Inoculum standardisation is one of the problems discussed by many investigators. The influence of the inoculum density on the colour formation requires the inoculum density to be standardised. However, colour development in the Biolog plate is neither linearly related to the absolute cell density, nor to the density of active cells (Garland & Lehman 1999).

Furthermore, the response in the Biolog plates per standardized unit of inoculum is often dependent on environmental parameters of the original sample, such as soil pH or organic matter (Garland 1997). Finally, the advantage of the CLPP approach as a quick and simple assay becomes limited by a time-consuming density determination step (Mills & Garland 2002). An inoculum-density independent method using several bacterial dilutions was consequently developed. This method consumes more than one plate per sample, but was found reproducible and discriminative between microbial communities in a Dutch soil quality monitoring program (Schouten et al. 2000, Bloem & Breure 2003). In essence, using a complete range of bacterial concentrations, the relation between colour formation and dilution of the original inoculum is obtained for each well in the Biolog plate. This enables calculations of the relative amount of the colour forming capacity, which is independent from the initial amount of bacteria. Information of consistently low colouring substrates is thus also captured, as well as of highly colouring ones.

When comparing the performance of other CLPP calculation methods frequently applied, it occurred that kinetic parameters of the colour formation curves showed little discriminatory power, due to loss of data upon failure of curve fitting. A relatively bad performance of the kinetic parameters has also been noted previously (Garland et al. 2001). In our analyses, the discriminative power further varied substantially between two dilutions tested for the kinetic parameters, in accordance with findings of Haack (1995). Methods based on readings of all wells at a set average colour (AWCD=0.5) showed high discriminatory power, but were strongly dependent on the dilution chosen for inoculation. Unfortunately, the dilutions providing higher discriminative power were not identical for two datasets. The finding that CLPP analyses based on readings at a set AWCD value varied with the inoculum dilution is in contrast to the assumption that this parameter is less hampered from inoculum divergences (Garland et al. 2001). Overall, the inoculum-dilution method was the most robust in performance and showed a high discrimatory power, also for inocula not differing in overall activity. Further, no determination of the inoculum density is necessary for this approach, as data analysis is based on the changes in profile upon inoculum dilution. The only prerequisite is that the highest dilutions should colour the magnitude of the wells (M. Wouterse, personal communication).

With the CLPP method, only culturable cells can be captured, since the method is based on growth of community members. The culturable part of the community is considered to be small compared with the total diversity (Torsvik & Ovreas 2002). Estimates range from a few

percent (Faegri et al. 1977, Bakken 1985, Hopkins et al. 1991), to less than one percent (Dykhuizen 1998). This is seen as a general disadvantage of the CLPP method in comparison with molecular methods yielding information on the numerically dominant community members. It was demonstrated in this study that 20 to over 100 bacterial strains were involved in a CLPP analysis using the inoculum-density independent procedure. Many substrates could only be utilized by a minority of the isolates tested, which is in accordance with previous findings for Atlantic sediments (Fredrickson et al. 1991) The absolute amount of strains detected in a CLPP analysis is thus not smaller than detected with other profiling techniques used in microbial ecology, like denaturing gradient gel electrophoresis and PLFA (van Elsas & Rutgers 2005). CLPP analyses thus rather address a different part of the bacterial community than molecular analyses and therefore have their own potential (el Fantroussi et al. 1999, Westergaard et al. 2001, Dunfield & Germida 2003, Ellis et al. 2003, Girvan et al. 2003).

In this context, it is interesting to note that the metabolic profile of soil microbial communities has also been tested in situ. The pattern of substrate utilization in soil samples could, for example, discriminate between soils under different management (Degens & Harris 1997) and under the impact of heavy metals and hydrocarbons (Shi et al. in press), and this method was seen as representative of community function. Still, responses obtained with Biolog plates and with a miniaturized soil microplate method were partly correlated, while CLPP (measured at one dilution) was hampered by smaller discriminatory power (Campbell et al. 2003). The coherence between the inoculum-dilution based method and in situ metabolic activities has yet to be studied. If there was a general concordance between both methods, CLPP could be used as a surrogate for a functional test, although not being a true functional assay in itself.

CONCLUSIONS

The CLPP analysis based on the inoculum-density independent method was found to be reproducible and discriminative between bacterial communities. Bacterial suspensions can be quickly frozen and stored for up to 20 months at -70 ^{0}C without large changes in the CLPP, offering practical advantages. The inoculum-density independent method had a higher discriminatory power than other methods of data analysis. About 20 to 100 bacterial species were found to be responsible for a community-level physiological profile. These attributes make this CLPP analysis, following the inoculum-density independent approach, a promising tool in microbial ecology

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CHAPTER 3

EFFECTS OF ANTIBIOTICS ON SOIL MICROORGANISMS: TIME AND NUTRIENTS INFLUENCE POLLUTION-INDUCED COMMUNITY TOLERANCE

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ABSTRACT

A method for the detection of the effects of antibiotics on soil microbial communities was optimised in the present study. We investigated the influence of measurement time and nutrient status on the pollution-induced community tolerance (PICT), using the sulfonamide sulfachloropyridazine (SCP) as model compound. The tolerance development in soil microcosms that were exposed to SCP under different conditions was compared with the background tolerance in SCP-unexposed microcosms. The tolerance of bacterial extracts from the soil microcosms was determined in Biolog® multiwell plates as the SCP sensitivity of a range of physiological processes. The background tolerance was not affected by soil nutrient amendments, but an influence of the inoculum density in the microtiter plates was observed. Still, the variance of the background tolerance was low, which is in favour of the usage of community tolerance measurements for a selective detection of a toxicant impact. In line with the assumptions of the PICT concept, soil amendment with SCP led to an increase in community tolerance. This tolerance development was enhanced upon additional soil amendment with fresh pig slurry, and less by alfalfa meal addition. Tolerance increases were observed after a soil exposure to SCP of only 7 days, possibly because nutrient input facilitates the fast adaptation of the soil microbial community. However, a further increase in exposure time led to variable changes in the observed tolerance. Prolonged tolerance detection in the microtiter plates (11 days) enabled a clearer differentiation between different soil treatments, as it better resolved the EC_{50} values of processes with a high tolerance to bacteriostatic antimicrobial compounds. For the detection of antibiotic effects on soil microbial communities, it is therefore recommended to use nutrient amendments (possibly fresh pig slurry), to standardize the soil exposure time, and to extend the period of Biolog plate measurement beyond 7 days.

INTRODUCTION

The concept of pollution-induced community tolerance (PICT) has been applied successfully to establish cause-effect relationships between a toxicant and the structure of terrestrial microbial communities (Díaz-Raviña et al. 1994, Díaz-Raviña & Bååth 1996, Bååth et al. 1998, Siciliano et al. 2000, Gong et al. 2002). In PICT investigations, community changes brought about by toxicant-induced succession are quantified by the increase in tolerance of the total community to this substance (Blanck et al. 1988). This increase in tolerance is thought to reflect three possible toxicant effects: the disappearance of sensitive species through direct intoxication and the proliferation of more tolerant species, physiological changes that render the organisms less sensitive, and genetic changes such as acquiring mobile genetic material encoding for more resistance. Pollution-induced community tolerance as a tool in community ecotoxicology has been claimed to have a high specificity and sensitivity for the detection of substance effects (Blanck et al. 1988), reviews in (Blanck 2002, Boivin et al. 2002).

Among the bacterial short-term tests used for the detection of changes in community tolerance, the utilization of a range of carbon substrates in microtiter plates has gained attention as a highly automated test system (Rutgers et al. 1998, Rutgers & Breure 1999). This

system is based on the inoculation of microtiter plates containing one individual carbon substrate per well (Biolog® plates) with bacterial suspensions. Colorimetric measurements of the bacterial carbon source oxidation over a time course of several days reveal the sensitivity of the community to an additional amendment of the multiwell plates with antibiotics or other toxicants (Figure 1).

Biolog plates are also frequently used to generate community-level physiological profiles (CLPP). These can be used to discriminate bacterial communities by the differences in utilization of the single carbon sources (Garland & Mills 1991, Garland 1997). Although only a limited proportion of the total community responds in the growth-dependent Biolog assays (Konopka et al. 1998, Smalla et al. 1998), CLPP has successfully been applied to detect toxicant effects on soil communities (e.g. (Lehman et al. 1997, Engelen et al. 1998, Müller et al. 2001)).

A group of substances that has recently been discussed regarding their environmental effects are pharmaceuticals. Although investigations of antibiotic drugs in bacterial test systems have highlighted that effects on single environmental bacterial strains such as *Microcystis aeruginosa* (Halling-Sørensen 2000), *Vibrio fischeri* (Backhaus & Grimme 1999), and *Aeromonas salmonicida* (Pursell et al. 1995) might occur, their effects on soil microbial communities have scarcely been investigated (Warman & Thomas 1981), (Colinas et al. 1994, Thiele & Beck 2001, Westergaard et al. 2001). Antimicrobials reach agricultural soils through manure of treated farm animals. Tolerance investigations can in this context be used for the evaluation of antibiotic effects in laboratory microcosms and, potentially, in field situations.

Results from a study of the effects of the sulfonamide sulfachloropyridazine on soil microbial communities (Schmitt et al. 2004) show that substrate utilization tests in microtiter plates can indeed be applied for PICT investigations of antibiotics. However, in order to advance future experiments, there is a need for a more detailed investigation into the method parameters. Firstly, it has been speculated that bacteriostatic (growth-limiting) effects of antibiotics might not become apparent under test situations that do not allow for bacterial growth (Backhaus et al. 1997, Thiele-Bruhn 2003). Thus, nutrient amendment of possibly nutrient-limited test soils is hypothesized to be of critical importance to induce toxicant-induced succession. For the PICT detection, in contrast, it has been questioned whether microtiter plate testing might be applicable as short-time test for community tolerance, because of possible changes in community composition due to its duration in the range of days (Blanck 2002).

This investigation is thus directed at optimising laboratory PICT investigations with Biolog plates for the evaluation of antibiotic effects, in relation to the type and amount of nutrient addition to the soils, and the duration of tolerance development and tolerance detection. Additionally, it has been tested whether the potential physiological diversity as studied by CLPP is equally suited to detect antibiotic impacts. The relevance of optimising the testing protocol is twofold: first, tolerance testing can be used as a sensitive ecotoxicological test system for the evaluation of possible substance effects (Blanck 2002), and second, studies of antibiotic effects in the field are only meaningful after investigations of the applicability of the PICT methodology.

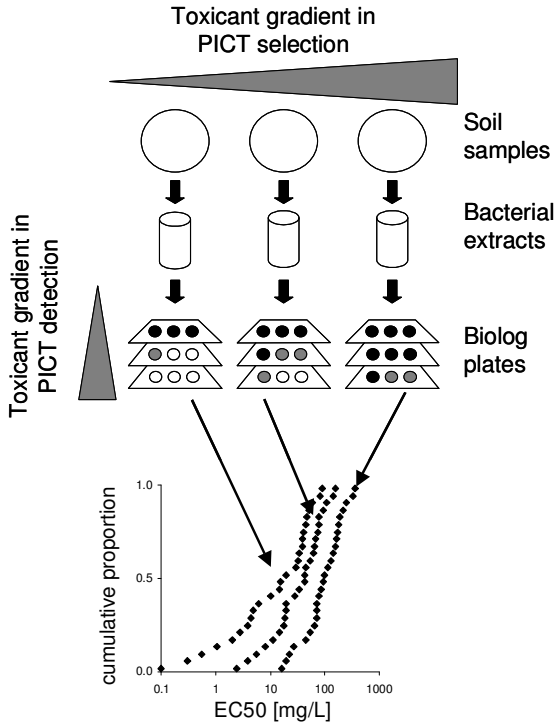

Figure 1. Test scheme for laboratory PICT investigations with Biolog plates. Soil samples are exposed to a gradient of an antibiotic (or other toxicant) in the PICT selection step, resulting in shifts in the community composition. Bacterial inocula extracted from each soil are exposed to a second antibiotic gradient in Biolog plates in the PICT detection step. PICT assumes that bacterial extracts from the soils exposed to the highest toxicant concentrations show the highest tolerance towards the second toxicant addition (more colouring wells in the Biolog plates). Sensitivity distributions show the increased tolerance of the highly exposed samples by a shift to the right.

MATERIALS AND METHODS

Soil and pig slurry sampling

The loamy sandy soil used for PICT analyses originated from an experimental agricultural site (Garbeva et al. 2003). The site received organic fertilisers only twice during the last 20 years: in the course of another field experiment, NPK fertilizer containing heat-treated cow and chicken manure had been applied in May in the two years preceding the soil sampling. Several hundred soil cores from the top 10 cm were sampled with an auger in March and May. The cores were combined and sieved (4 mm) and stored at 10ºC at field humidity in the dark for up to one week.

Pig slurry samples were obtained from the Utrecht University pig farm. The sows had no record of sulfonamide treatment during the preceding months. Other antibiotics had only incidentally been given to individual sows. Aged pig slurry was sampled from a slurry pit that

was located below the pig stable at ambient temperatures and that was regularly amended with fresh pig slurry upon cleaning of the stable. After mixing the slurry by moving the sample beaker up and down, a mixed sample was taken and stored at 4ºC overnight. For the preparation of a fresh pig slurry sample, fresh pig slurry and urine was sampled separately from one individual sow, and stored at 4ºC overnight. Fifteen g of fresh pig slurry were then vortexed with 150 ml urine, resulting in an N and C content of 2.92 and 32 g kg^{-1} ww, respectively, at a dry weight content of 84 g kg^{-1}. The slurry from the slurry pit had a dry weight content of 34 g kg^{-1}, contained 1.91 and 9.58 g N and C per kg ww, respectively, and was used without further treatment. Whereas liquid slurries were used in the PICT experiment, the elemental composition of the slurries was determined with an elemental analyser (EA 1108, Fisons Instrument) in oven-dried and pulverized slurry. In order to avoid ammonium loss, the pig slurry had been acidified before oven-drying (110 ºC overnight).

Soil exposure (PICT selection step)

Subsamples of soil (300 g wet weight) received alfalfa meal in concentrations of 0%, 0.05%, 0.5% and 5% on a dry-weight basis, equivalent to an input of 0, 11.5, 115, and 1150 mg N kg^{-1} dw, respectively, or 20.3, 203 and 2032 kg N ha^{-1} (see table 1) at a mixing depth of 20 cm. An amendment of 0.5% alfalfa is used in N and C mineralization studies according to OECD (Organization for Economic Co-operation and Development) guidelines (OECD/OCDE 2000a, b) and roughly equals agricultural carbon application rates. Sulfachloropyridazine, 4-Amino-N-(6-chloro-3-pyridazinyl)benzenesulfonamide (CAS-No. 80-32-0) was used as the antibiotic compound. SCP for all soil exposure was provided by Novartis (as Na-SCP) and was more than 98% pure. Per alfalfa addition, one subsample served as a control without SCP addition, and a second sample was amended with SCP (100 mg kg^{-1} dw), a concentration that yielded a clear increase in community tolerance in a previous study (Schmitt et al. 2004).

Soil samples were also amended with different amounts and types of manure. Aged pig slurry from the slurry pit was added at 98 ml kg^{-1} soil dw, equivalent to a nitrogen and carbon input of 331 and 1660 kg ha^{-1} at a mixing depth of 20 cm (or 0.19 and 0.96 g N and C kg^{-1} dw, respectively). Fresh slurry was added at 39 and 98 ml kg^{-1} dw, equivalent to an input of 202 and 506 kg N ha^{-1} (for carbon input, see table 1). The lower treatment rate was used in order to reflect normal agricultural nitrogen application rates, the higher treatment rates were chosen to provide strong growth stimulation. One soil sample received water instead of slurry. All samples were adjusted to the same humidity (15%). Per water / pig slurry amendment, one subsample received SCP (115 mg kg^{-1} dw), and a second sample served as control without SCP addition. The SCP addition was slightly higher in this experiment. However, the difference in PICT effect to be expected from previous experiments with a wide range of SCP concentrations is small (community tolerance, EC_{50}, predicted as 18 and 19 mg l^{-1} for an SCP addition of 100 and 115 mg kg^{-1} dw, respectively), so that comparisons can still be drawn with respect to the question whether PICT occurs upon SCP exposure or not.

Soil samples were kept at 25ºC in the dark for up to 15 days in covered, but unsealed plastic trays to allow for sufficient oxygen supply. The humidity was kept at 15% (w/w) by regular addition of deionised water. Microbial extracts were prepared from these soil samples after 8 days (alfalfa) and 7 days (pig slurry). For technical reasons, the exposure times were not

identical (7 and 8 days), however, it is expected that the rate of restructuring of the community decreases after the first days, such that conclusions on the strength of PICT between the two treatments can still be drawn. A second extract was prepared for the alfalfa treatments of 0%, 0.05% and 0.5% after 15 days.

For all bacterial extracts, twenty g soil (wet weight) was mixed with 200 ml 100 mM sodium phosphate buffer (pH 7) for one minute in a blender and subsequently centrifuged at 300 g for 10 min. Portions of the supernatant (2 ml) were frozen in liquid nitrogen and stored at –80ºC until testing. Freezing of samples was necessary in order to be able manipulate the density of bacterial inocula in the PICT experiments, and previous investigations in our laboratory had shown that freezing did not significantly affect CLPP fingerprints (M. Boivin and M. Wouterse, personal communication).

Community level physiological profiling (CLPP)

For the determination of the bacterial metabolic activity, prefilled 96-well microtiter plates ECO microplates (Biolog, Hayward, CA, USA) were used. These contain 31 different organic substrates such as sugars or amino acids, as well as a dried mineral salts medium and a tetrazolium redox dye which enables monitoring the utilization of the organic substrates by the bacterial community (Garland & Mills 1991, Insam 1997). Briefly, the procedure consisted of microtiter plate inoculation, determination of colour development during an incubation period, and evaluation of the contribution of each organic substrate to the total colouring with correction for inoculum density. We extended common procedures for CLPP determination (reviewed in (Mills & Garland 2002) by evaluating serial dilutions of each community (van Elsas & Rutgers 2005). This approach enabled the comparison of samples with different inoculum densities, and represents an alternative to other methods of inoculum density standardisation (Garland & Mills 1991, Garland 1997, Konopka et al. 1998).

The plates were inoculated with 100 µl per well of serial 3-fold dilutions of the original microbial extracts in 10 mM phosphate buffer (pH 7), containing 9 g l^{-1} sodium chloride.

One Biolog plate was inoculated with three serial dilutions, such that each serial dilution was tested on 31 substrates and a water control well. The absorbance at 590 nm was determined once or twice daily until day 6 (144 h). Per soil treatment – substrate combination, data consisted of the colour development over time for 12 serial inoculum densities. Per inoculum dilution, the colour development curves for each of the substrates (well colour development, WCD) as well as the averaged absorption over all substrates (average WCD, AWCD) were integrated over time to give the area under the curve (AUC, dimension: $Absorbance_{590\ nm}$*h) (Guckert et al. 1996). The fingerprint of the metabolic activities in each soil sample (CLPP) was calculated as the relative contribution of each organic substrate to the average colouring (relative abundance). In order to correct for inoculum density, this contribution was calculated for the inoculum dilution yielding a half-maximum activity. To this end, the AUC values for both single substrates and the AWCD were fitted against the inoculum dilution using a log-logistic function (GraphPad Prism version 3.00, GraphPad Software, San Diego, California, USA), with one curve parameter being the dilution at which colouring reached 50% of the maximum colouring (log WCD_{50}). Curve fitting was performed with a fixed top of 1, after

standardizing the AUC values to a maximum of 1 by dividing them by maximum colouring values obtained in a range of field experiments in the Netherlands (Wouterse et al. 2002). The relative contribution of a substance was determined as the $AWCD_{50}$ for the AWCD, divided by the corresponding value for this substance, the WCD_{50}, as illustrated in Figure 6 (left).

Fingerprint=log ($AWCD_{50}$ / WCD_{50})

If substrates had their WCD_{50} at dilutions more than a factor 100 different from the $AWCD_{50}$, their log WCD_{50} were set to 2 or –2, respectively. The relative contributions of all 31 substrates were then analysed using principal component analysis (without further scaling) and redundancy analysis with Monte Carlo permutations.

Tolerance determination (PICT detection step)

The tolerance determination was also based on the metabolic activity in Biolog plates. The plates were amended with SCP to yield nominal concentrations of 0, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 mg l^{-1}. Only one inoculum density of the microbial extract was used per soil treatment, chosen as the dilution which resulted in sufficient colouring (average absorption on day 7 equalling approximately 1.5 in the control soil sample) in the CLPP experiments.

Each plate was inoculated with 100 µl per well of one SCP / inoculum combination, leading to triplicate measurements of each combination of soil sample extract and SCP amendment for each of the 31 Biolog substrates. Colouring was determined one to three times daily for a period of up to 11 days (260 h).

PICT from single metabolic processes: physiological process sensitivity distributions

As a measure of the tolerance, effective concentrations of SCP were determined that reduced the colour formation to 50% of the maximum colouring (EC_{50} values). The colour formation was calculated as the integral of absorption over time per Biolog plate and substrate, yielding AUCs of well colour development. All samples were evaluated for an exposure time of 11 days (260 hours), and additional evaluations were performed for some samples after 6 days (144 hours). For each soil sample, response data was thus obtained for 31 substrates (in triplicate), measured at 10 different Biolog plate SCP concentrations.

Log EC_{50} values and log EC_{10} values (referring to 10% reduction in colour formation) were calculated using log-logistic dose-response curves of a substrates' AUC versus analytically verified SCP concentrations (see below) using GraphPad Prism according to formula 1 (van Beelen et al. 1991).

$y=1/(1+10^{((\log(c)-\log EC_{50})*slope)}$ (1)

$slope=\log(9)/(\log EC_{50}-\log EC_{10})$ (2)

with y=area under the curve (AUC) of the absorption at 590 nm over time, c= concentration of antibiotic in the plate in mg l^{-1}.

Log EC_{50} values with low r^2 values (<0.4) or high standard deviation of log EC_{50} (>1) in the curve fit were excluded. In order to combine information on the individual metabolic processes for one soil sample into one graph, the EC_{50} values were shown as a physiological process sensitivity distribution (ppSD): The log EC_{50} values were ranked and plotted in a cumulative distribution function using plotting positions of (i-0.5)/n.

PICT as seen from the averaged well colour development

In order to integrate data from all substrates into one parameter, the well colour development of all substrates has been averaged. As substrates with different absolute colouring have a different weight upon normal averaging, and as some substrates are not influenced by antibiotic addition to Biolog plates, the average was calculated only from the responding, normalized substrates. To this end, the responding substrates were defined as those with a robust log-logistic dose-response curve of their AUC when plotted against plate concentration (criteria as above). Each responding substrate was normalized by dividing the AUC in the Biolog plates exposed to SCP by the maximum AUC for this substrate (AUC in the control plate without SCP addition). Per plate (thus, per SCP concentration), all AUCs of all responding substrates were averaged to yield average AUCs of rWCDs (responding well colour development), ranging from 0 to 1. These were fitted against the plate SCP concentrations using a log-logistic dose-response curve as above.

Analysis of SCP concentrations in soil and Biolog plates

Soil SCP concentrations were not analysed because previous experiments showed that analytically determined concentrations match well with nominal soil concentrations and that 100 mg kg^{-1} SCP diminishes with a first-order half life of about 8.5 days under the conditions used (Schmitt et al. 2004).

Concentrations of SCP in Biolog plate inocula were verified with HPLC or LC-MS/MS. The HPLC analyses were performed using a Supelco discovery C_{18} column (15 cm * 4.6 mm, 5 µm), a mobile phase of 70% 10 mM NaH_2PO_4 at pH 3 and 30% acetonitrile at a flow rate of 0.7 ml min^{-1}, and UV detection at 260 nm. For LC-MS/MS analyses, the same column was used with a gradient of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.75 ml min^{-1} (85% A to 30% A in 7 min). Sulfachloropyridazine was detected in positive MRM mode with m/z 285 (Q1) and 156 (Q3). The internal standard, sulfadiazine, was detected as Q1=285 and Q3=156. The declustering potential, focus potential, entry potential and collision potential for the two compounds were 11V, 90 V, -4.5 V, 21 V and 21 V, 120 V, -4 V, 21 V, respectively. The calibration curves in analytical procedures were acquired with SCP obtained from Riedel-de Haën (Taufkirchen, Germany; purity 98.8%). The analytically determined concentrations of SCP in the bacterial inoculum were close to the nominal concentrations (about 80-120%) in the samples with concentrations of 1 mg l^{-1} and higher. At lower concentrations, SCP loss occurred. Analytically determined concentrations were used to derive EC_{50} values.

RESULTS

Influence of nutrient amendment on tolerance development

Background tolerance

The sensitivity of the indigenous microbial community of a given soil to SCP can be termed background tolerance if there was no previous selective pressure of SCP. Figure 2A gives the background community tolerance to SCP of all microbial inocula extracted from soil samples that were not exposed to SCP. Tolerance is shown as the concentration of SCP in the Biolog plate that reduces the plate colouring by 50% (the log EC_{50} based on the averaged colouring of all responding substrates, AUCs of rWCDs). The variation between the background community tolerances of different control soil samples (mean EC_{50}: 14.2 mg l^{-1}, 95% CI 10.6-16.8 mg l^{-1}, coefficient of variation 42%, n=11) was slightly bigger than the within-sample variation of the three (pseudo-) replicates in the Biolog plates, which is indicated by the 95% CI.

From the data in the CLPP experiments, dilutions had been chosen for the PICT experiments that were assumed to lead to a comparable colouring, in order to standardize inoculum densities to the same activity. The actual colouring realized in the PICT experiments is shown in figure 2A as the area under the curve of the total colouring. The total colouring still varied between the samples, but was without a clear effect on the background tolerance. Only the tolerance of the soil extract with the highest activity significantly differed from the grouped background tolerances. The colouring (as AUC of the AWCD) of this sample was more than two standard deviations higher than the average of the rest of the values.

Albeit greatly differing nutrient amendments with pig slurry or alfalfa, the soil nutrient status alone did not show a big influence the tolerance of soil microbial communities to SCP, as shown in Figure 2B. The possibility exists that a decline in sensitivity until an amendment of about 100 kg ha^{-1} occurs, followed by an increase in sensitivity. However, the slightly higher tolerance observed at the highest N application rate is most likely rather linked to its higher inoculum density than to the nutrient amendment, and the correlation with the nitrogen input was not strong enough to yield statistical significance, so that we hesitate from assuming a trend.

Antibiotic tolerance development upon SCP and nutrient amendment

Figure 3A summarizes the influence of pig slurry addition on the development of bacterial community tolerance upon exposure of soil to SCP, again based on the average colouring of the responding substrates. Soil samples to which only water had been added showed similar tolerance for both SCP-amended and control soil samples. Sulfachloropyridazine together with pig slurry increased the bacterial tolerance towards SCP: upon addition of fresh pig slurry at concentrations of 120 and 290 mg N per kg dw^{-1}, the EC_{50} value increased by a factor of 5.8 and 5.1, respectively. The absolute EC_{50} values were 66 (95%CI: 56-78) and 60 (50-71) mg l^{-1} for the treatments with pig slurry and SCP, compared with background tolerances in soil samples only receiving pig slurry of 11 (10-13) and 12 (11-14) mg l^{-1} (see also Table 1).

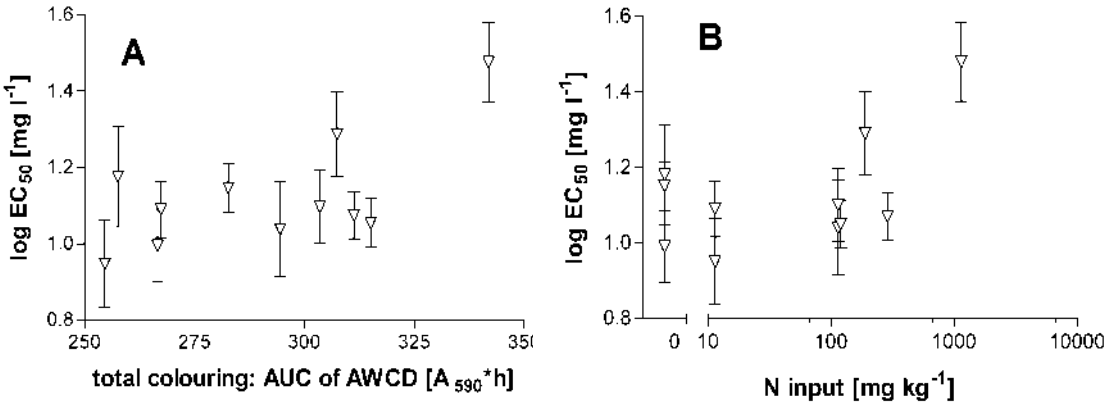

Figure 2. Influence of method parameters on community tolerance determinations. The tolerance of control soil microbial communities to SCP and its dependence on A. inoculum density and B. soil nutrient amendment. Y axis: Community tolerance, shown as log EC50 and its 95% CI, obtained from the averaged area under the curve of the well colour development of responding substrates in Biolog Eco-plates. A. Dependence on inoculum density. X axis: Inoculum density as absolute colouring (area under the curve of the absorbance at 590 nm vs time for the average well colour development). B. Dependence on nutrient input. X axis: Data from A rearranged per kg nitrogen addition per ha.

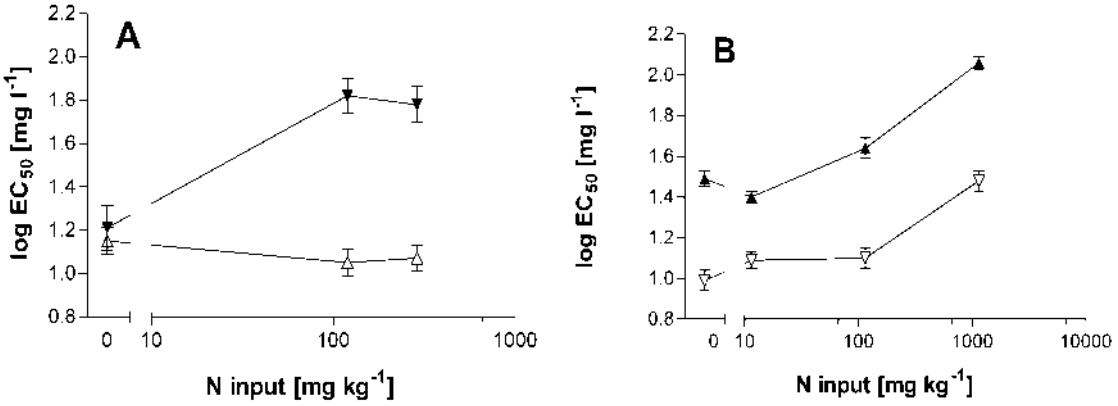

Figure 3. Enhancement of tolerance development of bacterial communities to SCP by soil amendment with different types and amounts of nutrients. Y axis: see Figure 2. A.: Soils amended with fresh manure. Open triangles: soils without SCP addition. Closed symbols: 115 mg SCP / kg dw. B. Soils amended with alfalfa meal. Open symbols: soils without SCP addition. Closed symbols: 100 mg SCP / kg dw.

Amendment with SCP and aged pig slurry sampled from the slurry pit did not result in a clear increase of the tolerance compared to the SCP-free samples. The background EC_{50} values of SCP-free soil samples amended with aged pig slurry were 19 (15-25) mg l^{-1}, and the tolerance in soil samples treated with aged pig slurry and SCP amounted to 17 (13-21) mg l^{-1} .

The amount of alfalfa addition also facilitated the increase in community tolerance upon SCP addition to soil, although slightly less than pig slurry. The highest tolerance was detected at the highest alfalfa treatment (Figure 3B). It has to be noted, however, that both samples with the highest nutrient addition showed a higher inoculum activity relative to the other samples. The inoculum density rather than the nutrient amendment thus probably causes the increased control log EC_{50}.

Duration of soil exposure (PICT selection time)

The influence of time on the community tolerance development in soils was investigated by analysing the development of PICT after 8 and 15 days incubation of soil with SCP and alfalfa. The results did not show a clear trend: for samples with an intermediate nutrient input (0.05%), the increase in tolerance was greatly enhanced at 15 days exposure compared to 8 days exposure, while the opposite was the case for samples with higher nutrient addition (0.5%, Figure 4). The soil samples amended with pig slurry already showed a clear tolerance increase after 8 days' exposure. Some additional visual observations indicated that community changes can occur on time scales even shorter than one week: Fungal hyphae were developing on the soil surface already after two days of soil incubation in samples with high nutrient input. In samples which also received the antibiotic, the inhibition of bacterial growth further promoted fungal growth, as judged by the amount of fungal hyphae on the soil.

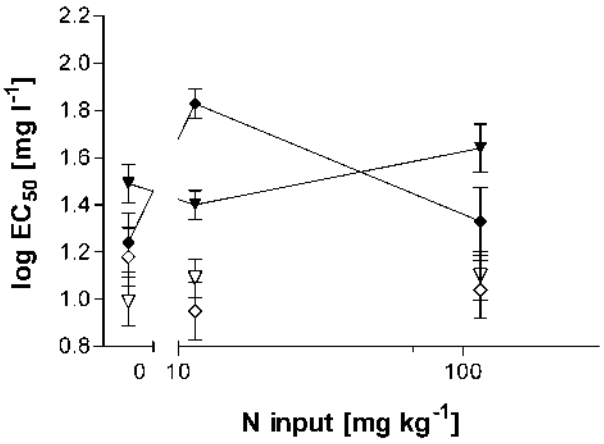

Figure 4. Influence of PICT selection time (duration of soil exposure to SCP) on tolerance increase. Y axis: see Figure 2. X axis: alfalfa input as kg nitrogen addition per ha. Open symbols: control soils without SCP addition. Closed symbols: soil exposure with SCP, triangles: 8 day soil exposure with SCP, diamonds: 15 days soil exposure.

Duration of bacterial metabolic activity measurement (PICT detection time)

The community tolerance of soils was analysed for 6 and 11 days of bacterial activity measurement in Biolog plates. As an example, Figure 5 shows the results for the soil sample

exposed to 100 mg kg^{-1} SCP and 0.05% alfalfa for one week. The PICT effect was more clearly visible at longer plate measurements: The two physiological process sensitivity distributions of the short activity measurement (6 days) overlapped, while the sensitivity distribution of the SCP-treated soil is clearly shifted to the right compared with the non-SCP-exposed soil sample for the longer plate measurement (11 days). The increase in the average EC_{50} (of responding substrates) between the control and SCP treated soil sample was 4 mg l^{-1} (6 day measurement) and 12 mg l^{-1}, respectively (11 day measurement).

CLPP: Confirmation of PICT effects

Multivariate analysis of the physiological fingerprints of soil bacterial extracts showed that both alfalfa amendment and SCP addition changed the bacterial activity profile (Figure 6). In particular, the highest alfalfa amendment led to clear shifts of the profile along the first PCA axis to the right. SCP effects could be seen on the second axis, which separated between control and SCP amended soil samples. When testing the significance of alfalfa and SCP addition with redundancy analysis and Monte-Carlo simulations, alfalfa amendment was highly significant ($p<0.01$), and SCP addition was significant ($p<0.05$).

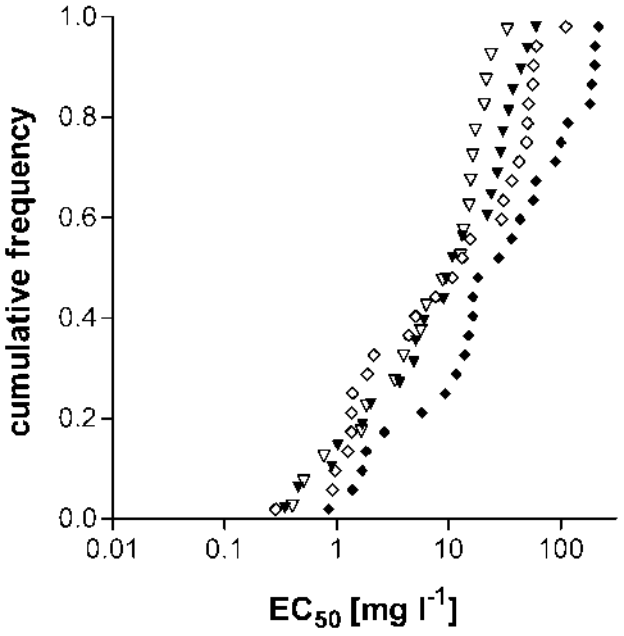

Figure 5. Influence of PICT detection time. Bacterial extracts from soil amended with 0.05% alfalfa with or without SCP measured in Biolog plates for 6 days (open symbols) and 11 days (closed symbols). X axis: EC_{50} values for the single substrates in Biolog plates. Triangles: control soil, diamonds: 100 mg kg^{-1} SCP.

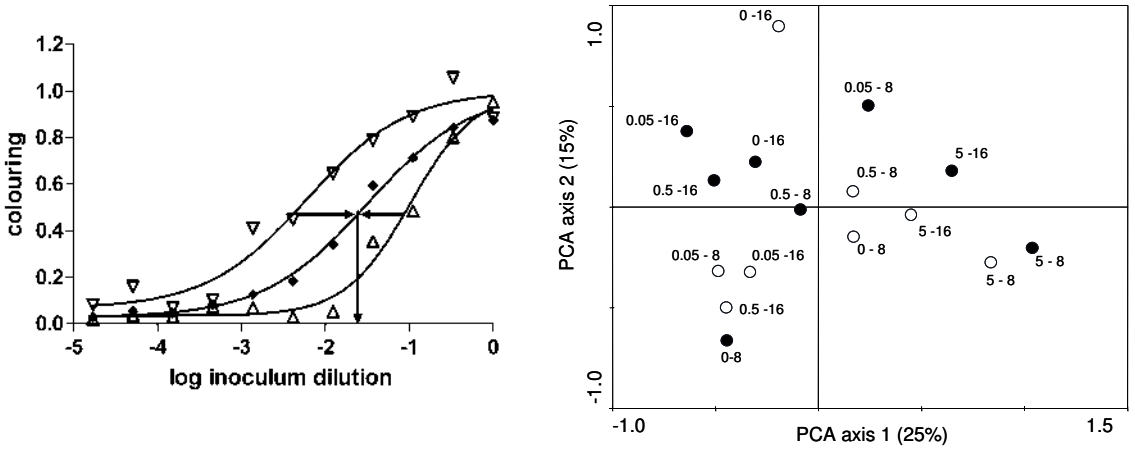

Figure 6. Community-level physiological profiling of soil samples treated with alfalfa and sulfachloropyridazine. Left: calculation of CLPP. Dependence of colouring on the inoculum dilution is shown, with colouring as the area under the curve of the well colour development (AUC of WCD), for the average over all substrates (closed diamonds) and two different substrates (tween 40 and alpha-cyclodextrin, open triangles). The fingerprint input is indicated by the horizontal arrows as the difference between the dilutions that lead to a reduction in colouring by 50% for the substrates (log WCD_{50}) and the average colouring (log $AWCD_{50}$, shown as vertical arrow). Right: Influence of alfalfa and SCP treatment on CLPP. Closed symbols: soil amendment with SCP, open symbols: control soils without SCP. Numbering: first number represents alfalfa input in % soil dry weigth, second number gives the duration of SCP soil exposure (PICT selection time). The first and second axis explain 25 and 15% of all variation, respectively.

DISCUSSION

This study focused on a method optimalisation of the detection of sulfonamide effects by community tolerance testing in laboratory experiments. Adaptations of method parameters such as an increased exposure time had been necessary in previous investigations in order to avoid false-negative results due to the bacteriostatic activity of most antibiotics (Backhaus et al. 1997, Thiele & Beck 2001). We therefore tested the influence of nutrient amendments and measurement time as growth conditions on tolerance testing.

PICT and nutrient addition

The background tolerance of soil samples without antibiotic addition was not dependent on nutrient amendment (Figure 2). The low variance in background tolerance is striking and illustrates the potential of the PICT approach to specifically detect toxicant effects, at least in laboratory settings. These findings are in agreement with Bååth et al. (1998), who also observed that the community tolerance to metals is independent from the soil moisture content. Field investigations based on Biolog plates come to a comparable coefficient of variation between different uncontaminated sites for metals of about 16-55% (van Beelen et al. 2004). The most extreme background tolerance values in periphyton communities ranged between 3 and 6 (summarized in (Blanck et al. 1988), which is also well in agreement with our experiment (4.9). A ratio between the upper and lower 95% confidence interval of zinc

tolerances of periphyton communities of up to 4.3 has been found locally, and Europe-wide, this ratio was up to 8.6 (Blanck et al. 2003), as compared to values of 1.7 in this study.

Sulfonamides are competitive antagonists of p-aminobenzoic acid (pABA), a precursor of folic acid, in protozoa and bacteria. Folic acid is a necessary for the synthesis of nucleic acid (Walsh 2003). The sulfonamide sulfachloropyridazine therefore exerts bacteriostatic effects on cell growth. However, bacterial growth in soils is normally restricted by nutrient limitation, and doubling times are in the range of weeks (Bååth 1998). Nutrient amendments as a trigger for soil bacterial activity peaks (Frostegård et al. 1997) might thus enhance the toxicity of SCP. Sulfachloropyridazine and fresh pig slurry at treatment rates comparable to normal agricultural practice indeed triggered a clear tolerance increase, when compared to soil samples receiving SCP but not pig slurry.

The tolerance increase in the soil samples of about a factor 5 was even stronger than the factor 1.5 seen in a comparable experiment with 115 mg kg^{-1} dw (Schmitt et al. 2004). The maximum community tolerance was 79 mg l^{-1} at a SCP concentration of 1000 mg kg^{-1} dw, which is well in accordance with the maximum EC_{50} observed in this study for pig slurry and alfalfa amendments, 66 and 114 mg l^{-1}. The maximum tolerance gain depends on the variation in SCP sensitivity among all species in the community (Blanck et al. 1988, van Beelen et al. 2001).

Interestingly, the tolerance measured with Biolog plates is in the range of minimum inhibitory concentrations for sulfonamides: the breakpoint for sulfonamide resistance in *Enterococci* is 512 mg L^{-1} (NCCLS 2003), and concentrations of 1000 mg L^{-1} nearly completely inhibit Biolog plate colour development of control soil extracts, but not in SCP-treated soils.

Aged pig slurry also stimulated SCP tolerance, but much less than fresh pig slurry - maybe due to a smaller availability of easily degradable nutrients in the former. Alfalfa was also less effective than fresh pig slurry in increasing SCP tolerance. Alfalfa meal is known to shift the fungal / bacterial ratios in soil towards fungal dominance (Mamilov et al. 2001). A smaller stimulation of bacterial growth might thus be the reason for the smaller sulfonamide-induced tolerance in the bacterial community.

Interestingly, the unfertilised control soils reacted differently to SCP amendment: in the alfalfa experiment, SCP induced a small tolerance increase also without nutrient amendment, but in the soils receiving water in place of pig slurry, tolerance with and without SCP was nearly identical. The soil used for the pig slurry experiment, sampled in March before field fertilization, might have been oligotrophic to an extent that no bacterial growth occurred in the water control and no bacteriostatic SCP effect was detectable.

When extrapolating these findings to the normal exposure route of antibiotics to soil, it is a worrying observation that manure enhances the effects of antibiotics on soil microorganisms. Previous investigations showed that sulfonamide concentrations in soils might not be high enough to affect soil microbial communities (Schmitt et al. 2004), but this might be different for other antibiotics. Further, pig slurry also contains gastrointestinal bacteria of the farm animals, which might form a reservoir of resistance genes (Mathew et al. 1999, Guerra et al. 2003, Vedantam & Hecht 2003, Jackson et al. 2004) possibly transferable to soil bacteria.

Inoculum density

The inoculum density in the microtiter plates might influence the determination of community tolerance, as it has been shown to also affect CLPP profiles (Garland & Mills 1991, Garland 1997, Garland et al. 2001, Preston-Mafham et al. 2002). In our setup, we adjust the bacterial activity by choosing one optimal inoculum density for the PICT step for each bacterial extract, based on the dilution series from the CLPP experiments (figure 6, left part). Generally, this approach was successful, as the background tolerances varied only little with the bacterial activities (Figure 2). However, the tolerance of the control soil with the highest activity, measured as the area under the curve of the colour formation, showed a significantly higher tolerance. This effect might be caused by a too high inoculum density, leading to an increased likelihood that highly tolerant species were present. The inoculum density should therefore be tightly controlled in tolerance testing of communities.

Duration of SCP soil exposure (PICT selection time)

Nutrient addition can lead to rapid changes in microbial respiration and growth (Frostegård et al. 1997, Stenger et al. 2001), possibly enabling the detection of antibiotic effects with nutrient amendment after only a few days. Indeed, in both the pig slurry and alfalfa experiments, effects were already visible after an exposure of 7 or 8 days. However, prolonging the PICT selection time to 15 days did not yield a consistent improvement: The tolerance development of a community exposed to SCP and alfalfa for 15 days was either enhanced or diminished in relation to 8 days exposure, depending on the amount of nutrient amendment.

Tolerance has been shown to decline once the toxicant is removed from the system (Díaz-Raviña & Bååth 2001). With a half-life of 8.5 days as determined in previous experiments, the initial soil concentration is already reduced to approx. 25% after 15 days of soil exposure. The removal might be enhanced under an even higher nutrient input, if bacteria able to degrade SCP are present in the community and experience good growth conditions. Alternative, regrowth of sensitive bacteria might also proceed faster at higher nutrient amendments.

Thus, an optimum PICT selection time for a given antibiotic can be expected, depending on parameters such as abiotic and biotic degradation of the substance as well as nutrient input into the whole system. In our case, 7 days exposure seems to be sufficient to detect sulfonamide effects.

Duration of bacterial metabolic activity measurement (PICT detection time)

The bacteriostatic mode of action of sulfonamides requires testing during cell growth in order to detect the inhibition of synthesis of cell parts – both in the PICT selection and PICT detection step. However, PICT detection based on substrate utilisation in microtiter plates has been questioned due to its relatively long time span (Blanck 2002). Growth and selection processes in the wells (Smalla et al. 1998, Preston-Mafham et al. 2002), might obscure the true community tolerance. If this was true, a longer time of tolerance detection than the 8 days used in previous PICT experiments (Rutgers et al. 1998, Rutgers & Breure 1999) could lead to a reduced apparent community tolerance. This was not the case - A longer PICT detection

time for the substrate utilization detection clearly enhanced the apparent tolerance development. From inspection of the data, it appeared that a longer measurement time better resolves EC50 values of physiological processes with a high tolerance (data not shown).

The mere detection of PICT highlights that differences in the original bacterial community seem to overrule successional adaptations in the multiwell plate. If the net PICT effect resulting from succession in both the control and the adapted community was higher than if measured with a short-time tolerance test, this would enhance the sensitivity of the PICT method. Overall, our data therefore support that antimicrobial tolerance can be measured under circumstances that allow for growth.

CLPP: confirmation of PICT effects

In an analysis of the metabolic community level physiological profile of all alfalfa-treated soils, SCP soils roughly separate from untreated soils along the second axis, and the influence of SCP is a statistically significant parameter. The CLPP data thereby confirm the effects seen with PICT. On the other hand, it can be seen that the nutrient amendment exerts a stronger influence on the physiological profile than the SCP treatment. The background community tolerance in the PICT experiment, in contrast, is not influenced by different nutrient amendments. PICT therefore proves to represent a specific system for the detection of toxicant effects in field samples with greatly varying conditions.

CONCLUSIONS

Based on the results presented in this paper, the following recommendations are made regarding the choice of parameters for testing of antibiotics in PICT experiments with Biolog plates:

1) The antibiotic-induced tolerance development of microbial community microcosms is more pronounced if soils are amended with nutrients.
2) Fresh pig slurry has been shown to facilitate the development of PICT. Slurry also represents the current agricultural practice better than alfalfa, as the normal route of exposure of antibiotics to soil environments is via slurry. As nutrient amendment has been shown to facilitate the occurrence of antibiotic effects, the addition of nutrients, and especially manure, should be considered also when exerting other effect studies with antibiotics.
3) The effects of antibiotics can already be seen after 7 days of soil exposure at 25ºC. Still, a prolonged soil exposure might increase the development of PICT under certain nutrient conditions. Thus, the choice of the PICT selection time is crucial and should be kept constant throughout an experiment, but can be as short as one week.
4) For the detection of antibiotic community tolerance with Biolog plates, extended periods of detection (11 days) proved to be useful.
5) Tolerance investigations with Biolog plates should be carefully adjusted for effects of inoculum density.

ACKNOWLEDGMENTS

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CHAPTER 4

POLLUTION-INDUCED COMMUNITY TOLERANCE OF SOIL MICROBIAL COMMUNITIES CAUSED BY THE ANTIBIOTIC SULFACHLOROPYRIDAZINE

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ABSTRACT

Little is known about the environmental hazards linked to the treatment of farm animals with antibiotics and subsequent spreading of manure, especially regarding soil microbial communities. In this investigation, pollution-induced community tolerance (PICT) of bacteria from soils artificially spiked with the sulfonamide sulfachloropyridazine (SCP) was investigated. Tolerance of the bacterial communities after three weeks' exposure to SCP was determined by analysing the sensitivity of 31 microbial metabolic processes in microtiter plates. Bacterial suspensions extracted from soils containing higher concentrations of SCP showed an increased tolerance of their metabolic activities to this antibiotic. An increase in tolerance by 10% was found at 7.3 mg/kg dw SCP. The PICT effect could be demonstrated by both a shift in the tolerance of the average of all metabolic activities and in a shift of the physiological process sensitivity distributions made up from the single metabolic processes. The PICT effect was accompanied by smaller changes in the community level physiological profile (CLPP). To conclude, PICT has been shown to be a versatile and illustrative method for the detection of the effects of antibacterial agents on soil microorganisms.

INTRODUCTION

The environmental hazards caused by the use of veterinary pharmaceuticals have recently gained more attention (Halling-Sørensen et al. 1998, Kümmerer 2001). Among the various groups of veterinary drugs, the antibiotics deserve special attention. This is due to three characteristics: 1) their mode of action, which can be assumed to affect non-pathogenic bacteria as well; 2) their intensive use, especially in industrial farming; and 3) the potential risks for human health resulting from the increase in resistance genes through veterinary use of related antibiotics (European Agency for the Evaluation of Medicinal Products 1999, Séveno et al. 2002). The environment is mainly exposed to veterinary antibiotics through the application of manure of treated farm animal herds on agricultural soils (Spaepen et al. 1997, Montforts et al. 1999).

Effects of antibiotics on soil microbes have been addressed only in a few studies (Martin & Gottlieb 1952, Patten et al. 1980, Colinas et al. 1994, Pfeiffer et al. 1998, Hansen et al. 2001, Ingerslev & Halling-Sørensen 2001, Halling-Sørensen et al. 2002), with attention to resistance development (Chee-Sandford et al. 2001, Jensen et al. 2001, Aminov et al. 2002). Given the importance of microorganisms for soil processes, the aim of our research was to elucidate whether exposure to antibiotics would affect the structure of the microbial community, which has been proposed to be a sensitive indicator of toxicant effects (Blanck et al. 1988).

Several authors successfully used the pollution-induced community tolerance (PICT) concept for studying effects of chemicals on soil microbial communities (reviewed in (Blanck 2002, Boivin et al. 2002). The PICT concept as introduced into ecotoxicology by Blanck (Blanck et al. 1988) is based on the assumption that long-term exposure of a community will lead to a higher tolerance for this toxicant. Tolerance development can be caused by death of the most sensitive species and replacement by more tolerant ones as well as physiological and genetic adaptations. The shift in sensitivity of the whole community during so-called "PICT

selection" can be analysed with short-term ecotoxicological tests ("PICT detection"). If a community previously exposed to high concentrations in the PICT selection step is subjected to such a short-term test, it is assumed that higher toxicant levels are needed to cause effects than for a community taken from a clean environment. PICT uses as response parameter the tolerance for a toxicant, which is believed to be mainly influenced by the exposure to that toxicant as opposed to other environmental parameters. PICT has therefore been claimed to be highly specific (Rutgers et al. 1998).

For the detection of PICT, several short-term tests can be used, among them the rate of metabolism of simple organic substrates. This method has been highly automated using Biolog® plates, containing 31 different organic substrates, which allows for detection of multiple microbial metabolic activities (Rutgers et al. 1998). Growth on and metabolisation of a substrate can be analysed by measuring the cellular redox state by means of the colouring of the pH-dependent tetrazolium dye contained in the wells.

Community-level physiological profiling or CLPP is another technique for the analysis of bacterial community structure that frequently makes use of microtiter plates with organic substrates (Garland & Mills 1991, Garland 1997, Mills & Garland 2002). The pattern composed of the extent to which a community utilizes the different substrates, the so-called physiological fingerprint, is compared between samples using multivariate techniques. Whereas PICT focuses on toxicant impacts, CLPP is used for the study of a broader range of environmental parameters, but has also been applied to toxicant impacts (Knight et al. 1997, Lehman et al. 1997, Engelen et al. 1998, el Fantroussi et al. 1999, Kelly et al. 1999b, Dahllöf et al. 2001, Ellis et al. 2001, Müller et al. 2001, Barranguet et al. 2003, Garland et al. 2003, Vinther et al. 2003). A combination of both techniques might reveal whether changes in community tolerance are accompanied by changes in the potential physiological capacity, but to date, only few studies have applied both approaches simultaneously (Dahllöf et al. 2001, Müller et al. 2001, Barranguet et al. 2003).

The aim of this study is to investigate the effects of a wide-spectrum sulfonamide antibacterial agent on microbial community composition, We focused on the question whether exposure to an antibiotic would lead to changes in community tolerance and physiological potential. To this end, we analysed the effects of a range of sulfachloropyridazine (SCP, 4-Amino-N-(6-chloro-3-pyridazinyl)benzenesulfonamide, CAS-No. 80-32-0) concentrations on the tolerance and the physiological fingerprint of metabolic activities of an indigenous soil microbial community for this antibiotic.

MATERIALS AND METHODS

The PICT test approach consists of three consecutive steps: soil sampling and exposure of the indigenous bacterial communities to a toxicant gradient in soil microcosms (PICT selection step); extraction of bacterial communities and analysis of the sensitivity of the different communities, using a second toxicant gradient in Biolog plates (PICT detection step); and analysis of all dose-response relationships in order to detect an increased tolerance in the soils with higher exposure. This setup is illustrated in Figure 1. In addition, CLPP analyses were performed using the exact same bacterial extracts.

Soil sampling

A loamy sandy soil was sampled from an experimental agricultural site (soil and site described in detail by Korthals et al. (Korthals et al. 2000)). The site received one portion of NPK fertilizer containing heat-treated cow and chicken manure in the growing season after 20 years of mineral fertilizer treatment only. In the growing season, maize was planted. Samples consisted of 300 soil cores taken from the top 10 cm of the soil with an auger (diameter 2.3 cm, depth 10 cm). All soil cores were combined and stored at field moisture (21% w/w) at 4ºC without further treatment.

Manure was sampled from the manure tank of the Utrecht University pig farm. The 120 sows had no record of sulfonamide treatment during the month preceding. The manure was stored in open glass containers at room temperature for 10 days before usage.

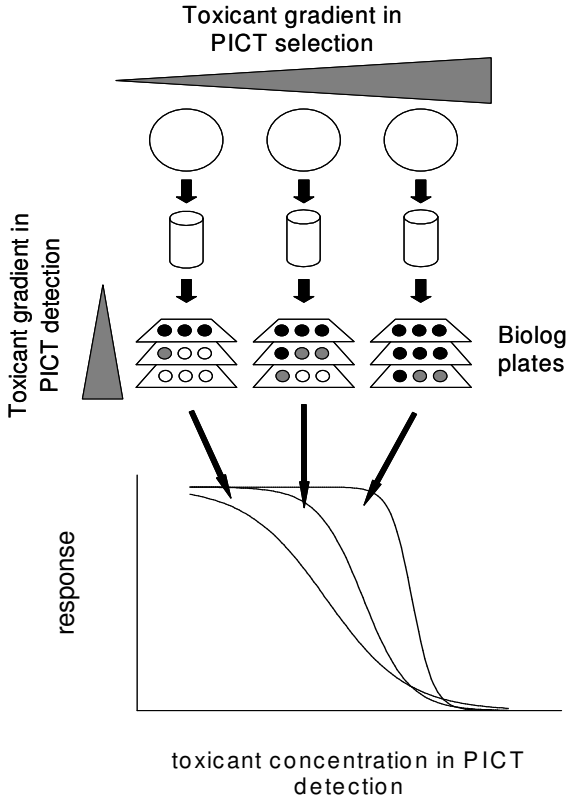

Figure 1. Test scheme for PICT investigations with Biolog plates. Soil samples are exposed to a gradient of a toxicant in the PICT selection step, resulting in shifts in the community composition. Bacterial inocula extracted from each soil are exposed to a second toxicant gradient in Biolog plates in the PICT detection step. PICT assumes that bacterial extracts from the soils exposed to the highest toxicant concentrations show the highest tolerance towards the second toxicant addition (more colouring wells in the Biolog plates). Dose-response analysis is used to show shifts in dose-response curves.

Soil exposure (PICT selection step) and extraction of bacterial inocula

The soil was sieved (8 mm) and subsamples (300 g) were artificially contaminated with SCP (1, 3 and 10 mg/kg soil wet weight as an aqueous solution; 30, 100, 300 and 1000 mg/kg ww as solid compound), and received 5 ml manure each. A control sample received manure only. SCP for soil exposure was provided by Novartis and was more than 98% pure. The samples were then stored at 25ºC in the dark for 20 days in open plastic trays to allow for sufficient oxygen supply. The moisture was kept at 21% (w/w) by regular addition of deionised water. Microbial extracts were prepared from these soils by mixing 20 g soil (wet weight) with 200 mL 100 mM phosphate buffer (pH 7) for one minute in a blender, and centrifuging the soil slurry at 350 g for 10 min. The supernatant was rapidly frozen in liquid nitrogen in 2 mL portions and stored at –20ºC until testing. These frozen extracts were used for both CLPP and PICT tests. Frozen storage of the samples prior to analysis permitted the execution of different types of experiments and repeats on practically the same communities. Previous research in our laboratory demonstrated that there was no significant effect of rapid freezing of samples in liquid nitrogen on the number of colony forming units on agar plates (37), on the CLPP (M. Boivin and M. Wouterse, personal communication), and on the PICT response. Furthermore, any bias introduced by this method is of minor importance, because all the samples received the same treatment (except for the duration of storage of the samples in the freezer), and the results were interpreted only within the framework of this study.

Soil SCP concentrations during SCP exposure were verified with extractions of 1 g of soil in 1 ml of 10 mM Na_2HPO_4 buffer (pH 6) and 1.5 ml acetonitrile. Samples were shaken overnight at room temperature, centrifuged (1188 g, 10 min), and filtered (Millipore GVWP filters 0.22 µm). Concentrations were determined with HPLC (Supelco discovery C18 column (15 cm * 4.6 mm, 5 µm), mobile phase of 70% 10 mM NaH_2PO_4 at pH 3 and 30% acetonitrile, flow rate of 0.7 ml/min, UV detection at 260 nm).

Community level physiological profiling (CLPP)

Prefilled 96-well microtiter plates (ECO microplate™; Biolog, Hayward, CA, USA) which contain triplicates of 31 different organic substrates such as sugars or amino acids, as well as a tetrazolium redox dye and a dried mineral salts medium (Preston-Mafham et al. 2002), were used to determine CLPP. In preliminary investigations of the influence of inoculum density on the colour development in Biolog plates, a series of three fold dilutions of the thawed bacterial extracts were inoculated into Biolog plates, and the absorbance at 590 nm was determined once or twice daily until day 11. A too high inoculum density has to be avoided because it limits the sensitivity towards addition of antibiotics to the plate for PICT tests. A nine-fold dilution resulted in a high average absorption (maximum approx. 1.2), but showed a significant reduction in colouring in the subsequent dilution. This nine-fold dilution was chosen for subsequent CLPP and PICT experiments. For the CLPP experiments, the plates were inoculated with 100 µL per well of 9 fold dilutions of the microbial extracts in 10 mM phosphate buffer (pH 7), containing 9 g/L sodium chloride. For selected soils, duplicate plates were used. The absorbance at 590 nm was determined as above.

CLPP, the fingerprint of the metabolic activities in each soil, was compared between the soil samples using multivariate statistics. To this end, the colour development over time for all 31 substrates per soil sample was characterized by both kinetic and sum-parameters: The lag in colour formation onset, asymptote of colour development and maximum colour formation rate, were obtained by a nonlinear curve-fit of the log-transformed absorption readings with the Gompertz function as described in (Garland et al. 2001), and the area under the curve as sum parameter was calculated from the raw absorption data using the trapezoid method (Guckert et al. 1996). Further, readings at an average absorption (or average well colour development, AWCD) of 0.5 were compiled as described in (Garland & Mills 1991), which included dividing the background-corrected absorption readings closest to an AWCD of 0.5 by the AWCD. The number of substrates utilized (metabolic richness) was calculated as the number of substrates with an average absorption exceeding 1.5 times the absorption of the control well at t=74 hrs.

For PCA analyses, the highest soil treatment was discarded due to its reduced total activity, as it has been shown that Biolog fingerprints are heavily dependent on the total colouring (Preston-Mafham et al. 2002). PCA was performed on unscaled data. Further, redundancy analysis (RDA) was undertaken to analyse the significance of the SCP soil concentration (given as logarithm of the concentration).

PICT detection step

The PICT detection step resembles the CLPP determination, only that additional Biolog plates with SCP amendment were used to determine bacterial tolerance. Bacterial inocula from the range of soils pre-exposed to the SCP gradient were prepared as above, and amended with SCP (Novartis) to yield nominal concentrations of 0, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 mg/L each. Each plate was inoculated with 100 µL per well of bacterial inoculum with a specific SCP concentration. Selected soil samples were analysed in duplicate. The absorbance readings (determined as above) yielded an extensive dataset of approximately 200 000 single absorbance readings.

PICT from single metabolic processes: physiological process sensitivity distributions

The colour development curve of each substrate was summarized into one parameter by calculating the area under the curve (AUC) of the absorbance at 590 nm over time with the trapezoid method. The AUC was chosen as it is able to integrate different changes in the colour formation curves (Guckert et al. 1996), and as it can be calculated without curve-fittings that lead to data loss due to bad fits. Response data consisted of triplicate readings of the AUC per substrate and per antibiotic concentration in the Biolog plates for each soil. A dose-response curve was generated for each soil bacterial extract, yielding effective concentrations of SCP that reduce the AUC in Biolog plates by 50% resp. 10% (EC_{50} and EC_{10}). Curve fits were performed in GraphPad Prism (2002) using a log-logistic fit of the response versus analytically verified SCP concentrations in the Biolog plates (Y=1/(1+10^(log(c)-logEC_{50})*slope)) with c=SCP concentration in Biolog plate).

The slope parameter was replaced by the EC_{10} since the slope can be calculated from the ratio between the EC_{50} and EC_{10} using slope=log(9)/($logEC_{50}$-$logEC_{10}$) as described previously (van Beelen & Fleuren-Kemilä 1999).

Log EC_{50} values with low goodness of fit (r2 values <0.4) and high relative standard deviation of $logEC_{50}$ (>1) were excluded. In order to combine information on the individual metabolic processes into one graph, the EC_{50} values were shown as a physiological profile sensitivity distribution (ppSD): The EC_{50} values were ranked and plotted in a cumulative distribution function using plotting positions of (i-0.5)/n (with i=substrate number, n=total number of substrates), so that the y axis represents the frequency of EC_{50} values which are smaller than a given EC_{50} value on the x axis (Aldenberg & Jaworska 2000).

PICT as seen from the average well colour development curves

The average well colour development (AWCD) was calculated for each measurement time as the average of the colour development of all substrates per plate, and the area under the curve (AUC) of the AWCD over time was determined. Per soil inoculum, non-linear regression was undertaken to analyse the dose-response relationship of the AUC of the AWCD versus the SCP plate concentration as above.

The same analysis was repeated for the responding substrates only, in order to restrict the analysis to the suitable endpoints and to give substrates with different absolute colouring the same weight upon averaging. To this end, the responding substrates were defined as those with a robust log-logistic dose-response curve of their AUC when plotted against plate concentration (r^2>0.4, standard error of $logEC_{50}$ <1, standard error of log EC_{10}<2). For each responding substrate, the AUC in the Biolog plates exposed to SCP was normalised by dividing them by the AUC of this substrate in the control plate without SCP addition. Per plate (thus, per SCP concentration), all AUCs of all responding substrates were averaged to yield average AUCs of rWCDs (responding well colour development). These were fitted against the plate concentrations using a log-logistic dose-response curve as above.

Characteristic effect levels of the modelled curves (EC_{25} and EC_{50}) were compared with soil SCP concentrations, and the dose-effect curve gained modelled with log-logistic regression.

Analysis of SCP concentrations in Biolog plates

The SCP concentrations in the Biolog plates were verified by analysing the SCP-spiked bacterial inocula used for Biolog plate inoculation. Analysis was based on HPLC-UV (conditions as described under soil exposure) or by LC-MS/MS (Supelco discovery C18 column, 15 cm * 4.6 mm, 5 µm, mobile phase A: 0.1% formic acid, mobile phase B: acetonitrile, gradient of 85% A to 30% A in 7 min, flow rate of 0.75 ml/min). The MS/MS method parameters were the following: mobile phase was split before entry into the MS and ionised by electrospray injection with an ionspray voltage of 5200 V. SCP was selected as precursor ion (m/z=285) and detected as product ion (m/z=156) (MRM mode). Sulfadiazine (m/z=251, product ion m/z=156) was used as internal standard. Quadrupole voltage settings were: declustering potential 11 / 21 V, focus potential 90 / 120 V, entry potential –4.5 / -4 V

and collision potential 21 V for SCP and sulfadiazine, respectively. SCP for calibration curves in analytical procedures was obtained from Riedel (purity: 98.8%).

RESULTS AND DISCUSSION

Concentration of SCP in soil and Biolog assay

SCP concentrations in soil as low as 0.3 mg/kg could be quantified. The concentrations of SCP in the spiked soils matched 79-109% of the nominal concentrations at day zero, and SCP disappeared from the soil in an approximate first-order reaction with half lives of around 10 days (25ºC). The final concentrations after 20 days exposure amounted to 13-26% of the initial concentrations (70% for 1000 mg/kg).

Concentrations of SCP in the Biolog plate inoculum were 80-120% of nominal concentrations from concentrations exceeding 1 mg/L. At lower nominal concentrations, deviations were greater and more varied. Actual concentrations were used to derive EC_{50} values.

Community level physiological profiling

The CLPP was based on the control plates only, without antibiotic addition. Principal component analysis of the area under the curve of the colour formation in the differently spiked soils reveals a trend with toxicant concentration (Figure 2): Higher soil SCP concentrations shift the PCA scores of the samples to the lower right. The first two axes explain 29% resp. 24% of the total variation. RDA revealed that the SCP soil concentration significantly influenced the CLPP (logarithm of soil concentration as environmental variable; p=0.044). The pattern is dominated by the soil treated with 300 mg/kg, the highest treatment included in this analysis. This might be explained by the non-linear nature of toxicant effects: while PCA and RDA are suited to visualise linear effects of an environmental variable, toxicant effects are generally non-linear with a sigmoidal shape, and effects of a certain concentration may thus exceed the effects of lower concentrations by far. The remaining variation might be caused by variations in inoculum density or differences in soil conditions between the samples. The usage of the area under the curve has been suggested as sum parameter integrating possible changes in lag time, maximum colour development rate and maximum colouring reached (Guckert et al. 1996, Hackett & Griffith 1997). Still, more specific information on the nature of community changes can possibly be obtained from kinetic parameters (Garland et al. 2001). However, principal component and redundancy analysis of the abovementioned kinetic parameters revealed a much weaker trend of CLPP changes with toxicant concentrations (data not shown), which was also the case for the readings at an AWCD of 0.5. In addition, it was difficult to obtain kinetic parameters of substrates that led to slow colour development, as they did not reach the asymptote of colouring during the experiment. These gave no satisfying curve fits and had to be removed from the analysis or set to extreme values (0 for asymptote, maximum rate of colour development, and reading at A(AWCD)=0.5, and 200h for lag phase), resulting in data loss.

The metabolic diversity was reduced in the highest exposed soil, as no substrate showed colouring at a time where the other soils had reached an AWCD of 0.5 (74 h). In the other

soils, about 15-20 substrates were utilized at this time, with no clear trend with toxicant concentration (data not shown). In contrast to PICT analyses, information on the nature of the single substrates is maintained throughout the CLPP data treatment process. Therefore, community level physiological profiling enables a visualisation of differences in community structure brought about by a toxicant. On the other hand, CLPP has been shown to reflect changes in a range of environmental soil parameters, such as soil moisture or soil type. Community changes can therefore not directly be related to toxicant impacts, and toxicant-induced changes can also be masked by changes in other parameters.

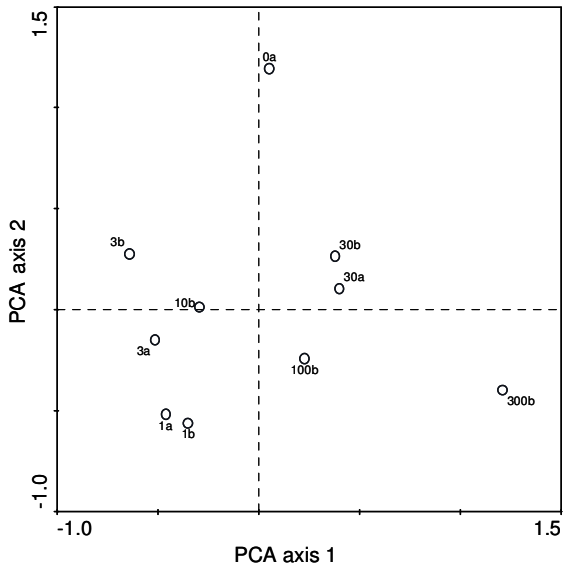

Figure 2. Principal component analysis of metabolic profile of soils with different PICT exposure. a,b: results of duplicate determinations. Numbers give nominal soil concentrations of SCP in mg/kg dw.

PICT comparisons based on physiological process sensitivity distributions

Typically, about 22 out of 31 possible EC_{50} values for the inhibition of single substrate metabolisations could be calculated per soil – the others were not responding in a dose-response manner. The highest soil concentration reduced the activity of the bacterial inoculum in the Biolog plates, so that only 7 EC_{50} values of sufficient quality could be derived. All EC_{50} values of one sample were ranked and plotted in a cumulative distribution function. The resulting physiological process sensitivity distributions were left-skewed, with higher soil concentrations leading to greater steepness (Figure 3). PICT was observed as a shift of the ppSD curves of the soils with higher SCP pre-exposure to higher EC_{50} values. The major community changes can be observed in the sensitive processes: the smallest EC_{50} values of the control soil and the soil with the highest SCP pre-exposure differ by two orders of magnitude. A rationale for this observation would be that these processes with high sensitivity experience the highest selection pressure upon soil SCP exposure– only the sensitive bacteria that are not able to withstand a given soil concentration would need physiological adaptations or would be suppressed by more tolerant species. In summary, process sensitivity

distributions enable a visualisation of the community changes brought about by the exposure to a toxicant.

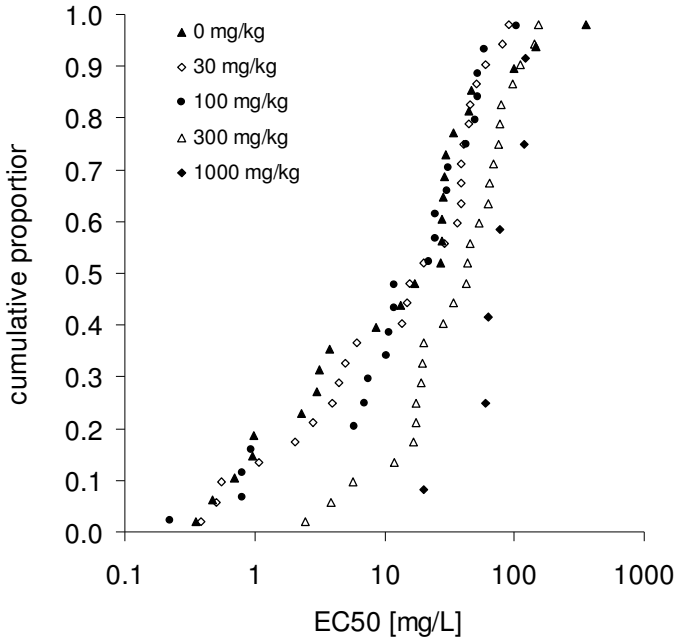

Figure 3. ppSD curves for bacterial communities stemming from increasingly contaminated soil. The x axis gives the EC_{50} values of SCP for one substrate determined in Biolog plates. The y axis gives the proportion of the EC_{50} values stemming from one soil sample that are smaller than the given EC_{50} value

PICT comparisons based on average well colour development curves

Figure 4 shows an example of a dose-response relationship of the influence of SCP on the averaged metabolic activity of soil bacteria in Biolog plates. The EC_{50} for the reduction in metabolic activity of bacterial inocula from control soils was 14.8 mg/L (52 µmol/L, 95% CI: 11.9-18.3 mg/L). The corresponding EC_{10} values were 0.44 mg/L (0.26-0.76 mg/L). A comparison between the dose-response relationships for soils with a different pre-exposure reveals that higher soil pre-exposure shifts the dose-response curves to higher tolerance (Figure 5). The curves show pronounced dissimilarities in the low-dose-range. Again, the selection pressure acting strongly on the more sensitive species might be the reason. As a measure of first effects of SCP, the soil concentration resulting in a 10% increase of the community tolerance (as EC_{50} and EC_{25}) was calculated (Figure 6). 11 mg/kg dw SCP increases the base EC_{50} by 10%. In addition to the EC_{50} values, the EC_{25} are shown (Figure 6), to reflect the more pronounced changes in the more sensitive processes. 7.3 mg/kg dw SCP increases the base EC_{25} to 110%, and the EC_{25} of the control community, being slightly higher than the EC_{25} of the soil samples with low SCP concentrations, would be increased by 10% at a soil concentration of 40 mg/kg.

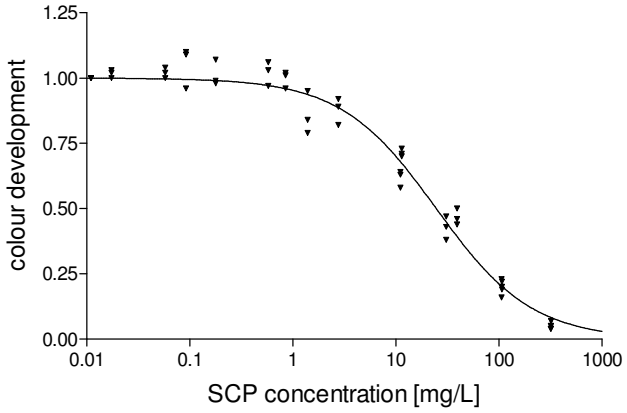

Figure 4. An example of a dose-response curve for the inhibitory action of SCP on the metabolic activity of microbial inocula (isolated from soil pre-exposed to 3 mg/kg SCP). Colour development: averaged area under the curve of the well colour development of responding substrates in Biolog ecoplates. Experimental values and fitted curve given.

When the same analysis was undertaken for the area under the curve of the simple AWCDs (the average of all substrates), the curves showed the same trend, but the effects were slightly less pronounced (data not shown). The latter method is characterized by greater simplicity of calculation, whereas the former focuses on the responding endpoints only. The first method could therefore yield higher sensitivity in cases where a few substrates dominate the total average and where substrates with less total colouring would show more PICT effects. In comparison to the physiological process sensitivity distributions described before, the average well development curves have the advantage that the tolerance of the whole community can be described and quantified in a single value, on the expense of loosing information on changes in the particular substrates.

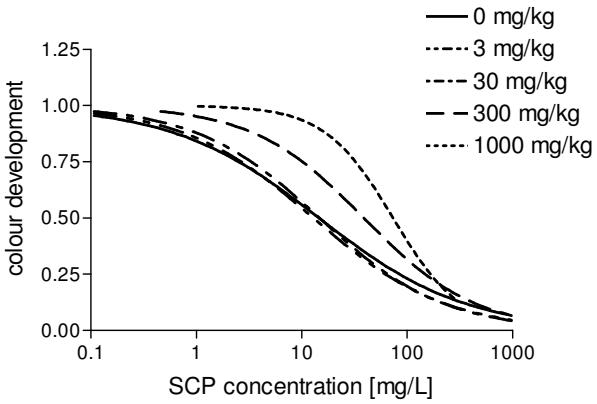

Figure 5. Effect of SCP on the metabolic activity of microbial inocula from soils pre-exposed to different nominal concentrations of SCP, measured as averaged area under the curve of the well colour development of responding substrates of Biolog ecoplates.

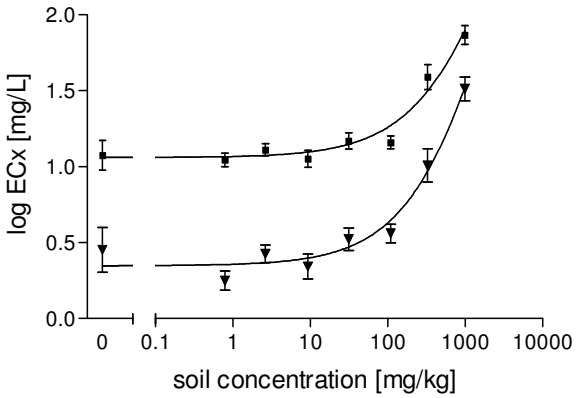

Figure 6. Effect of SCP on the metabolic activity of microbial inocula from soils pre-exposed to different concentrations of SCP. Squares: EC_{50}, triangles: EC_{25}, and standard error of the mean. X axis: measured soil concentration. ECx: Effective concentration of SCP at which 25% resp. 50% reduction in colouring of bacterial inocula in Biolog ecoplates is seen (as the averaged area under the curve of the well color development of the responding substrates).

Effects of sulfonamides on soil bacteria

Our experiments show that antibiotics are capable of inducing community changes in soil microbial communities: an increase in the tolerance of bacterial inocula to sulfachloropyridazine was observed in soils with increasing antibiotic concentrations. This could be seen in a right-shift of the dose-response curves of the AWCD curves (Figure 5) as well as a right-shift in the physiological profile sensitivity distribution curves (Figure 3). In addition, a reduced colour formation rate and thereby reduced metabolic diversity was observed in the soil with the highest SCP exposure.

SCP concentrations also influenced the metabolic fingerprint of the communities, as seen in CLPP (Figure 2). However, changes were only apparent for a sum parameter of colour formation, but not in various kinetic parameters studied, and could therefore not be further characterized. The correlation between the toxicant and the physiological profiling was thus relatively weak. This is in line with investigations that show that CLPP is not the most sensitive method to demonstrate toxicant effects (Kelly et al. 1999a, Dahllöf et al. 2001, Bundy et al. 2002). In other studies, only treatments that resulted in a reduced overall activity (which had been observed only in the highest exposed soil in our experiments as a reduced colour formation rate and an increased lag time) were separated in multivariate analyses of the CLPP pattern (Engelen et al. 1998, Ellis et al. 2001, Vinther et al. 2003). Others found, however, that CLPP results are generally in line with other test results, and are as sensitive as e.g. dehydrogenase essays or phospholipid fatty acid or 16S rDNA analysis (Lehman et al. 1997, Engelen et al. 1998, Kelly et al. 1999b, Müller et al. 2001, Barranguet et al. 2003).

The detection of first PICT effects of SCP at around 7.3 mg/kg dw is in agreement with expected pore water concentrations: K_d values for SCP in the range of 0.9-1.8 L/kg have been reported (Boxall et al. 2002), which would result in a pore water concentration of 3.7-6.8

mg/L for a soil concentration of 7.3 mg/kg dw. These concentrations are in the range of effective concentrations determined for the influence of SCP on bacterial communities from control soils in Biolog plates (see Figure 4).

There are only few publications that address soil and manure concentrations of sulfonamides. Manure and soil concentrations in a German farming area reached a maximum of 2.9 mg/kg in manure and 0.01 mg/kg in soil for sulfadimidine (Höper et al. 2002). A second publication reports maximum manure concentrations as high as 8.7, 2.6 and 12.4 mg/kg ww for sulfamethazine, sulfathiazole, and trimethoprim, respectively (Haller et al. 2002). In order to perform a complete risk characterisation for the environmental effects of sulfonamide antibiotics, we feel that more information is needed regarding environmental-chemical and ecotoxicological properties, though. Especially, the persistence of possible community effects should be determined, ideally under field conditions.

The PICT investigations in our study have shown that first effects of antibiotics on soil microbial communities might occur at concentrations of 7 mg/kg dw. As pointed out by van Beelen (van Beelen & Doelman 1997), a community shift towards greater tolerance coincides with the extinction of sensitive species. This in turn has been shown to have adverse ecological consequences as decreases in for example: competitive abilities, mineralisation rate, biodegradation capacities, cold resistance, and metabolic diversity (van Beelen & Doelman 1997). In our CLPP experiments, which are however based on a cultural method not representative of normal soil conditions, the soil with the highest exposure indeed showed a reduced metabolic diversity.

Further, the PICT method does not differentiate between the disappearance of sensitive species and an increase in physiologically adapted or genetically resistant bacteria. The latter bears the risk that the veterinary use of antibiotics might also add to the building up of a pool of resistance genes in soil.

To summarize, the PICT method has been shown to be a versatile, illustrative and sensitive method for the detection of changes in soil microbial communities brought about by exposure to antibiotics.

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CHAPTER 5

EFFECTS OF VETERINARY ANTIBIOTICS ON SOIL BACTERIA: COMMUNITY STRUCTURE, PHYSIOLOGICAL PROFILE, AND INDUCED TOLERANCE

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ABSTRACT

The veterinary antibiotics oxytetracycline and tylosin are possible soil contaminants, since they are able to reach agricultural soils with manure of treated farm animals. We investigated possible toxic effects of this exposure on the indigenous bacterial community of agricultural soil. Soil microcosms amended with manure as stimulant for microbial growth were spiked with a range of antibiotic concentrations. Changes in community structure were monitored using pollution-induced community tolerance (PICT) and community-level physiological profiles (CLPP) as well as denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal DNA (rDNA). Tylosin effects became apparent by a small tolerance increase and a change in the CLPP pattern. CLPP revealed that some potential physiological abilities disappeared in the soils exposed to high concentrations of tylosin. The influence on the DGGE pattern was marginal for tylosin, possibly because tylosin mainly affected fast-growing heterotrophs. The effects of tylosin were thus only detectable with a combination of techniques addressing different parts of the microbial community. Oxytetracycline changed the overall community tolerance and the DGGE pattern to a great extent, but affected the physiological fingerprint only slightly. The effect pattern of oxytetracycline might be explained by the high soil sorption of oxytetracycline causing a patchy distribution of the substance and a locally varying selection pressure. Oxytetracycline concentrations that can occur in field situations are expected to affect microbial communities.

INTRODUCTION

The role of pharmaceuticals as environmental contaminants has recently gained more attention (American Academy of Microbiology 1999). Among the veterinary drugs, the tetracycline group was ranked highest concerning possible adverse effects on the environment (Boxall et al. 2003). Additional concern has been raised with regard to an increased selection pressure for antibiotic resistance genes, creating gene pools that might ultimately interfere with the treatment of human pathogens (American Academy of Microbiology 1999). The usage volume of veterinary antibiotics, which are one of the major substance classes applied in veterinary medicine, equals roughly half of the amount used for human therapy use in Europe (FEDESA 1999). The application of antibiotics as growth promoters, which is still allowed in several countries, adds to the total quantity used. The main route of entry of veterinary antibiotics into the environment is via the application of manure of treated farm animals. The question arises whether antibiotics exert effects on agricultural soil communities, and ultimately whether some soil functions might be hampered by this exposure.

Previous findings indicate effects of antibiotics on community functioning, e.g. with respiration techniques (Thiele & Beck 2001, Hund-Rinke et al. 2004). Further, tests with single strains (Sengeløv et al. 2003), and incidentally, community characterization methods (Westergaard et al. 2001) have been applied to study effects of antibiotics. Another test system which promises to yield sensitive results regarding possible toxicant effects on communities is the so-called pollution-induced community tolerance or PICT (Blanck et al. 1988). With PICT, it is assumed that toxicant-induced effects such as disappearance and

replacement of sensitive species, genetic or physiological adaptations render the whole community more tolerant (Blanck 2002). The occurrence of an increased community tolerance can thus yield information on whether an exposure affected the community. PICT is thought to be highly specific in the sense that the influence of environmental factors on the community tolerance to one specific toxicant is possibly limited, while other community characteristics can be strongly influenced by abiotic or biotic factors (Boivin et al. 2002). On the other hand, PICT does not give detailed information as to the nature of community changes. In soils, PICT has mainly been studied for metals (Bååth et al. 1998, Rutgers et al. 1998, van Beelen et al. 2001), but also for organic pollutants such as trinitrotoluene (TNT) (Siciliano et al. 2000). For effects on bacterial communities, the sensitivity of the utilization of a range of organic substrates by the bacterial community in so-called Biolog plates (Garland & Mills 1991) has been suggested as a highly automated tool (Rutgers et al. 1998). This approach is limited to the part of the community that is able to grow or respire under the circumstances of the multiwell plates (Preston-Mafham et al. 2002) – however, this is no disadvantage as long as the toxicant tolerance of parts of the community reflects the overall community tolerance or the tolerance of sensitive parts of the community.

The utilization of different substrates in multiwell plates has also extensively been applied for the study of structural changes in bacterial communities. The influence of a toxicant on the pattern of the community-level physiological profile (CLPP) has been among the research questions studied (el Fantroussi et al. 1999, Müller et al. 2001). PICT and CLPP as tests based on the heterotrophic, fast-growing part of the community can be well accomplished by a structural test that is not limited to the cultural part of the community: Denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal DNA (rDNA). DGGE has also been successfully used for studies of toxicant impacts (Ibekwe et al. 2001, Westergaard et al. 2001).

In this investigation, we characterize the effects of two antibacterial compounds, oxytetracycline and tylosin, on the bacterial community of an agricultural soil. In order to study the dose-response relationship for the community effects, soil microcosms were amended with manure and a range of antibiotic concentrations. Pollution-induced community tolerance, PICT, was used as sum parameter, including physiological and genetic adaptations as well as structural changes. The potential physiological diversity was investigated with CLPP, and DGGE was applied to detect changes in the numerically dominant members of the community. Further, the observed effects were related to current agricultural practices.

MATERIAL AND METHODS

Soil sampling

A sandy soil was sampled at two occasions from an agricultural experimental site which has been described previously (Korthals et al. 1996). The site had received organic fertilisers twice (early May 2001 and 2002, NPK fertilizer containing heat-treated cow and chicken manure). About 300 soil cores were taken from the top 10 cm with an auger (2.3 cm diameter), combined and sieved (mesh 8 mm). The soil was stored at 10ºC for a maximum of 4 days prior antibiotic application.

Exposure of soil and extraction of microbial communities

Briefly, subsamples of soil were amended with manure and a range of antibiotic concentrations. Subsequently, they were kept for up to two weeks in order to allow for community adaptation.

The soil samples were also amended with manure as nutrient, as the bacteriostatic effects of antibiotics do not become apparent in oligotrophic environments such as soil (Schmitt et al., in press). Manure was sampled at the farm of the University of Utrecht. For the preparation of a fresh slurry sample, fresh manure and urine were sampled separately from one individual sow, and stored at 4ºC overnight. 15 g of fresh manure was then vortexed with 150 ml urine, resulting in an N and C content of 2.92 and 32 g/kg ww, respectively, and kept frozen before soil manure amendment. Manure amendment was intended to reflect agricultural conditions, so that the N addition equalled 170 kg/ha, the maximum N input in the European Union (European Commission 1991).

Subsamples of soil (300g dw at a humidity of 22 and 19.5% for oxytetracycline and tylosin, respectively) were amended with manure and a range of antibiotic concentrations (0, 0, 0.5, 5, 15, 50, 150, 500, 1500 mg/kg). Oxytetracycline (OTC) was applied as dihydrate (Sigma Aldrich, purity > 99%), and the lower concentrations (≤15 mg/kg) were dissolved in 1M HCl before being added to the manure. The lower concentrations of tylosin (tylosin tartate, Sigma Aldrich-purity > 90%, TYL) were dissolved in deionized water before being added to the manure, while the higher concentrations (15 mg/kg and more) were applied as solid compound to the manure. After one minute of mixing the manure and the substances, the soil samples were added and mixed for two minutes. Soil samples were stored in a humid stove at 25ºC. Deionized water was supplied every few days in order to maintain soil humidity.

On day 6 and 14 (oxytetracycline) and 8 and 15 (tylosin), bacterial communities were extracted from the soils. Soil (20 g dw for OTC, 17.5 g dw for TYL) was blended with 200 mL of phosphate buffer (100 mM potassium phosphate at pH=7) for one minute, centrifuged at 350g for 10 minutes. The supernatant was rapidly frozen in portions of 2 mL in liquid nitrogen. These bacterial extracts were stored at –20 ° C, and used for both PICT and CLPP analyses. On the same days, 20g soil samples were taken and stored at –20 ° C until DNA extraction.

CLPP

The community-level physiological profile, CLPP, was determined using 31 different simple organic substrates contained in 96-well microtiter plates, together with a tetrazolium redox dye, a dried mineral salts medium and a phosphate buffer (Biolog ECO plates). In order to compensate for differences in inoculum density, we calculated the contribution of the utilisation of each substrate to the community fingerprint from a dilution series of the original inoculum (van Elsas et al. in press). For the inoculation of the plates, 3-fold serial dilutions of the thawed bacterial extracts were prepared in 100 mM potassium phosphate buffer, pH 7 (oxytetracycline) or 10 mM phosphate buffer with 0.9% NaCl, pH 7 (tylosin). 100 uL of each dilution was pipetted into each of 32 wells of the plates. The colour formation was monitored

over 6 days, with at least two measurements per day. Data analysis was based on the area under the curve (AUC) of the absorbance at 590 nm over 144 hrs. In order to enable curve fitting with colour readings of a maximum of 1, the AUCs were divided by the maximum AUC for each specific well obtained in a range of field experiments in the Netherlands (Wouterse et al. 2002). From nonlinear log-logistic regression with a Hill sigmoid curve of the AUC versus the logarithm of the inoculum dilution, the dilutions were determined that yield half maximum well colour development (WCD_{50}). The same was done for the average of the absorption of all 31 wells of each dilution (the average well colour development, AWCD, yielding an $AWCD_{50}$ value. The contribution of the utilization of each substrate to the total community fingerprint was calculated as the dilution that yields 50% of maximum average colouring divided by the dilution at which colour formation in one respective well is reduced by 50%.

Fingerprint=log ($AWCD_{50}$ / WCD_{50})

Values of >2 or <-2 were set to 2 and –2.

The resulting fingerprints were analysed by principal component analysis (PCA) and redundancy-gradient analysis (RDA) with the statistical package R (R development core team 2003), based on the rda routine. The logarithm of the nominal soil concentrations was used as environmental variable. Further, the diversity and evenness of the CLPP pattern has been calculated in the following way: The contribution of each substrate to the total colouring (or, in other words, the fingerprint input, on normal scale), has been used as "species" counts n_i, neglecting the substrates that needed dilution of less or more than 100 times as much as the average colouring to reduce their colouring by 50%.

$$n_i = \frac{AWCD_{50}}{WCD_{50,i}}, p_i = \frac{n_i}{\sum_i n_i}$$

with i: the number of substrates utilized.

The Shannon-Wiener diversity H' and evenness E were calculated from p_i.

PICT

Prior to the PICT analyses, the activity of the bacterial extracts was determined using the data from the CLPP dilution series. One dilution was chosen for each soil (mostly 3^{-5}) that yielded sufficient colouring (AWCD approximately 1.2 on day 7), and that showed a reduced colouring upon subsequent 3-fold dilution.

PICT analyses were based on the inoculation of the bacterial extracts of one soil into several microtiter plates, each plate amended with a different concentration of antibiotics. For one soil treatment, all plates were inoculated with the same dilution of the bacterial inoculum.

Data analysis proceeded as follows: as an integrated parameter of the colour development curve of each substrate, the AUC of the absorbance at 590 nm over 260 hours (12 days) was determined (H. Schmitt, H. Haapakangas, and P. van Beelen, accepted for publication). Dose-response relationships of the AUC per antibiotic treatment in the plate were created for each soil treatment. From these dose-response relationships, the antibiotic concentrations resulting in 50% reduction of colour formation (EC_{50}) were determined. The dose-effect curves were fitted (van Beelen et al. 1991) using

Y=1/(1+10^((log(c)-logEC_{50})*slope)), with slope=log(9)/(logEC_{50}-logEC_{10}).

Log EC_{50} values with low r^2 values (<0.3) and high relative standard deviation of EC_{50} (>1) were excluded.

The result of this treatment is a row of maximal 31 log EC_{50} values that describe the sensitivity – or tolerance – of one soil sample. These logEC_{50} values have been grouped as a physiological profile sensitivity distribution (ppSD), by plotting the log EC_{50} values in a cumulative distribution function using plotting positions of (i-0.5)/n (with i=substrate number, n= total number of substrates)(Aldenberg & Jaworska 2000).

In order to integrate the data into one "tolerance value" per soil treatment, the log EC_{50} has also been calculated for the average plate colouring. Not all substrates have been taken into account, but only those which respond to the antibiotic amendments to the multiwell plates in a dose-response manner, in order to exclude substrates that are not influenced by antibiotic addition. As described in (Schmitt et al. 2004), the raw AUC values for each dose-response-curve have been normalized to a maximum of 1 by dividing each AUC value by the AUC of the plate without antibiotic addition. The normalized AUC values of each substrate with a logEC_{50} of sufficient quality (as above) were averaged to yield average AUCs of rWCDs (responding substrates). These were fitted to the same logistic function, yielding logEC_{50}ArWCD.

By plotting the logEC_{50}ArWCD against the antibiotic soil concentration, the tolerance induction has been summarized. The resulting relationship has been fitted with a sigmoidal Hill-function in order to be able to calculate the soil concentrations that lead to an increase in community tolerance over the background tolerance (tolerance of the control soil treatments) of 10% and 100%, representing a low effect level and a clear effect.

16S-rDNA DGGE

Soil DNA was extracted using the FastDNA Spin kit for Soil (BIO101, Carlsbad, Calif.) in 0.5 g portions of soil that had been dried overnight at room temperature. The procedure was slightly modified by extending the centrifugation step after bead-beating to 2 minutes, and by eluting the final DNA with 70 uL purified water. DNA was purified using Wizard DNA Clean-Up System (Promega, Madison, Wis.) and diluted in 70 uL purified water. For DGGE analysis, PCR primers F-968 and R-1401, which were described by Nübel et al. (Nübel et al. 1996) were used and amplification was performed in a Hybaid PCR Express thermocycler. PCR mixtures contained 5 µl of PCR buffer 2 (10-fold concentrated; Boehringer), 1.7 mM

$MgCl_2$, 200 µM concentrations of each deoxynucleoside triphosphate, 10 pM primer, and 2.5 U of Expand Long Template enzyme mix (Boehringer), 1 µl of template DNA, and sterile Millipore water up to a total volume of 50 µl. Samples were first denatured at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplification was finished by a final extension step at 72°C for 10 min. DGGE gels were made using the Bio-Rad Gradient Delivery System establishing a gradient from 30% to 60% denaturant. Two 8% (wt/vol) polyacrylamide (ratio of acrylamide to N, N'-methylenebisacrylamide 37,5 : 1) stock solutions were made, one with 30% denaturant containing 0.5× TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8.3]), 12% (vol/vol) formamide, and 2 M urea and another with 60% denaturant containing 24% formamide and 4.2 M urea. Polymerization was achieved by adding 0.29% (vol/vol) ammonium persulfate (10% solution) and 0.1% (vol/vol) N,N,N',N'-tetramethylethylenediamine (TEMED). On top of this gradient gel a 1-cm stacking gel was poured, consisting of 6% polyacrylamide in 0.5× TAE without denaturant.

An approximately 18-µl PCR sample was applied on the gel, and gels were run at 60°C at a constant voltage of 80 V for 16 h in a DCcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, Calif.). Gels were stained in 25 ml of 1:10,000 diluted Sybr Gold (Molecular Probes, Eugene, Oreg.) in 1× TAE for 45 min. Banding patterns were digitalized on a GeneGenius bioimaging system (SYNGENE, Maidenhead, United Kingdom). Banding profiles were analyzed using the Bionumerics program (Applied Maths, Kortrijk, Belgium). Normalized and background-subtracted intensity values of all lanes were used for cluster analysis and statistical analysis. Similarity coefficients calculated according to Pearson were used to construct a complete linkage dendrogram based on the UPGMA algorithm. The cluster consistency of each subcluster was calculated with the cophenetic correlation routine in Bionumerics.

OTC and TYL soil concentrations and plate concentrations

Soil concentrations of oxytetracycline were determined by extracting 800 mg of soil with 4 ml McIlvaine buffer pH 4.1 (60 ml 0.2M citric acid + 40 ml 0.4M NaH_2PO_4), 4 ml 0.1M EDTA and 3.2 ml methanol. The samples were treated in an ultrasonic bath for 10 minutes, and centrifuged for 15 minutes at 2500 rpm. For LC-MS/MS analyses of the supernatant, a Supelco discovery C_{18} column (15 cm * 4.6 mm, 5 µm) was used in a gradient program (90% A to 42% A in 8 min, A: 0.1% formic acid, B: 0.1% formic acid in acetonitrile) at a flow rate of 0.7 ml/min. Oxytetracycline was detected in positive MRM mode with Q1=461 and Q3=426, and its internal standard, tetracycline, as Q1=445 and Q3=410.

Soil concentrations of tylosin were measured by extracting 1000 mg of soil with 4 ml 10mM sodium phosphate buffer (pH5) containing 0.9% NaCl and 4 ml methanol. The samples were treated in a sonic bath for 10 minutes, and centrifuged for 15 minutes at 2500 rpm. For HPLC measurements, a Supelco discovery C18 column (15 cm * 4.6 mm, 5 µm) was used. The mobile phase consisted of 60% 0.18 M $NaClO_4$ in water (pH=2.5 with trifluoroacetic acid) : 40% acetonitrile, at a flow rate of 0.7 ml/min and room temperature. Tylosin was detected with a UV detector at 290 nm. For LC-MS/MS analyses, a Supelco discovery C_{18} column (15 cm * 4.6 mm, 5 µm) was used with a gradient of A and B at a flow rate of 0.7 ml/min (100%

A to 70% A in 3 minutes, followed by 70% A to 5% A in 3 minutes, and 5% A for a further 3 minutes).

Tylosin was detected in positive multiple ion mode with Q1=916, and its internal standard, erythromycine, as Q1=734. The declustering potential, focus potential, entry potential and collision potential for oxytetracycline and tetracycline were 26V, 160 V, -5.5 V, 27 V and 26 V, 160 V, -5.5 V, 23 V, and for tylosin and erythromycine 23 V, 180 V and –5.5 V (no collision potential).

The analytical availability of oxytetracycline in soil decreased from 67% day 1 to 43% on day 14. For tylosin, previous experiments with the same soil had shown that initial concentrations well matched nominal concentrations, and that analytical availability of tylosin was reduced to around 40% in 6 days, according to a first-order reaction with a half-life of around 5 days at 25 °C (data not shown).

Sorption experiment

The sorption coefficients of tylosin and oxytetracycline for the soil used in this study was determined in a procedure which is based on the OECD guideline 121 (OECD/OCDE 2000), with the exception that the soil amount was chosen in a way that the total amount sorbed was in the same order of magnitude as the amount that remained in solution. Further, sodium azide was used instead of calcium chloride in order to prevent complex formation of oxytetracycline with calcium ions. Aliquots of dried and sieved soil (1 mm mesh size) were shaken for two days with sodium azide (10 mM) and spiked with a range of antibiotic concentrations. After two days shaking, the supernatants of the centrifuged samples were analysed by HPLC on a YMC ODS- AQ C_{18} column column (5 cm * 4 mm, 3 µm) with the settings as above. Assuming no substance loss, the sorption coefficients were determined as the ratio of the concentration in soil and water. An average was calculated over a range of antibiotic concentrations.

RESULTS

PICT

The effects of the antibiotics oxytetracycline and tylosin on soil microbial communities were studied first by investigating the increase in community tolerance to these antibiotics. To determine the community tolerance, the influence of antibiotics on colour formation of bacterial soil extracts in Biolog plates was analysed. An example for the stepwise determination of the community tolerance is given in Figure 1. It shows the average well colour development of one soil bacterial extract inoculated in Biolog plates amended with increasing concentrations of oxytetracycline. With higher antibiotic amendments, the substrate utilization proceeded with a longer lag phase and a slower rate, and reached a lower maximum colouring. These changes have been summarized into one parameter by calculating the area under the curve of the colour development curves (or more specifically, the average area under the curve of the responding substrates, average AUC of rWCD, as described in Material and Methods). A typical dose-response relationship obtained from this average

colouring versus the oxytetracycline concentration in the multiwell plate is also shown in Figure 1, together with a sigmoidal curve fit. Substrate utilization was completely inhibited in this soil extract by the addition of 1000 mg/L oxytetracycline to the plates. The concentration at which the colour development reached 50% of the maximum value in the control soils was 0.82 mg/L for oxytetracycline, and 127 mg/L for tylosin.

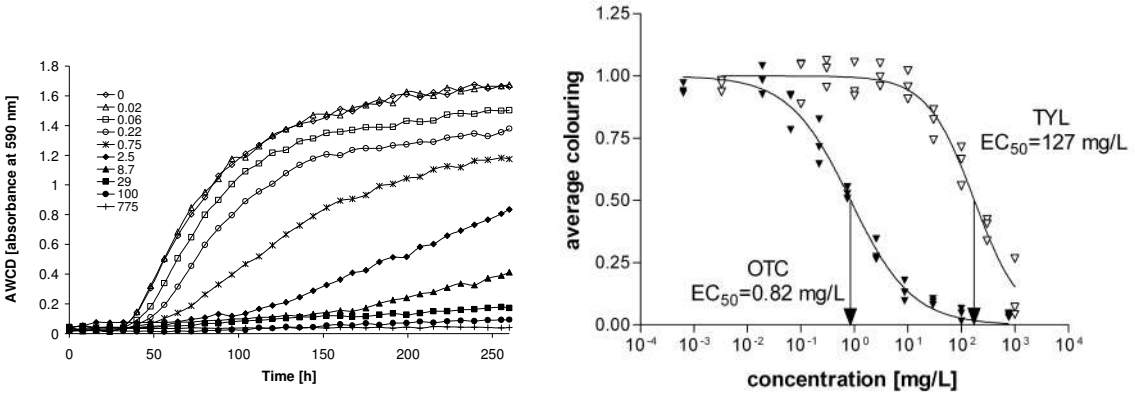

Figure 1. Pollution-induced community tolerance determination: An example of dose-response curves for the treatment of inocula from one soil microbial community in Biolog plates with antibiotics – A: colour development versus time of an inoculum from a control soil for a range of antibiotic plate amendments (0 until 774 mg/L OTC), B: dose-response curves constructed from the area under the curve of the colour development (average of all normalized responding substrates) versus Biolog plate amendment. Solid triangles: oxytetracycline, open triangles: tylosin

While Figure 1 is based on the average over all substrates in the microtiter plate, Figure 2 shows the community tolerance for each single substrate and thus gives up to 31 EC_{50} values for each soil instead of one. The EC_{50}values for the bacterial communities extracted from the different soils are shown as physiological process sensitivity distributions. In this plot, the y axis gives the proportion of all substrates which have a tolerance which is smaller than the respective logEC_{50} value on the x axis. A shift of the sensitivity distributions to the right, thus to higher logEC_{50} values and more tolerance was apparent for soils which had been exposed to higher concentrations of both antibiotics. Still, the two antibiotics show different patterns:

Tylosin leads to a decreased number of substrates with a high sensitivity, and the distributions thus change more in the lower left part of the graph. With oxytetracyline, substrates with high sensitivities can even be found at high soil pre-exposure concentrations. In contrast, the number of highly tolerant substrate utilization processes (in the upper right part) increases much more than with tylosin. The EC_{50} values for a control soil range from 6 to 500 mg/L tylosin, and from 0.06 to 6.8 mg/L for oxytetracycline. Oxytetracycline is therefore more toxic to the bacterial extracts than tylosin.

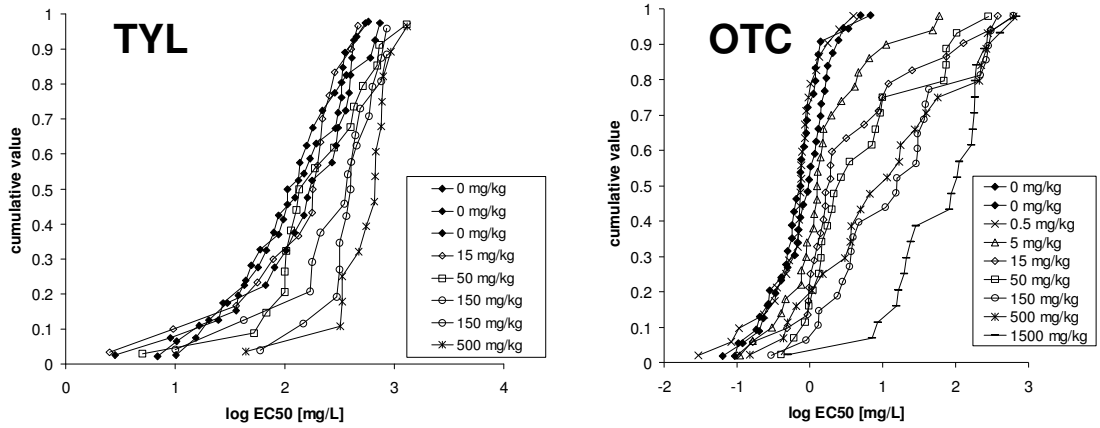

Figure 2. Pollution-induced community tolerance, shown as physiological process sensitivity distributions: sensitivity of colour formation of single substrates in Biolog plates to an amendment with antibiotics. X axis: antibiotic concentration at which colour formation is reduced by 50%, Y axis: cumulative proportion of substrates that are less sensitive than the respective value on the x axis. Left: tylosin-pretreated soils, right: oxytetracycline-pretreated soils

Figure 3 relates the tolerance of each soil to the soil antibiotic concentration. In this figure, the tolerance of each soil community is aggregated into a single logEC_{50} value (the concentration reducing the averaged colouring of the responding substrates to 50%). The maximum tolerance increase caused by tylosin amounts to 0.5 log units or a factor of 3, whereas it is a factor of 100 for OTC. Moreover, the tolerance increase starts at lower concentrations for oxytetracyline in comparison with tylosin. In order to determine and compare the soil concentrations at which a specified increase in tolerance occurs, both relationships have been modelled with a log-logistic function. With this function, the soil concentrations that increase the original tolerance by 10% (as an arbitrary measure for first signs of effects) and 100% (doubling of tolerance as clear effect level) have been calculated, and are shown in Table 1. For oxytetracycline, community tolerance doubled at soil concentrations of 1.3 mg/kg, whereas for tylosin, 58 mg/kg were needed to increase the tolerance by 100%. The effect of soil treatment with oxytetracycline has been analysed after one and two weeks of soil exposure, but the differences between both exposure times are negligible.

CLPP

Community-level physiological fingerprints (CLPP) of soil communities were determined as a measure for structural changes in the heterotrophic, fast-growing part of the soil bacteria.

CLPPs are based on the patterns of utilization of a range of substrates by different bacterial communities and are mostly analysed with multivariate statistical techniques. For both oxytetracycline and tylosin, there is an influence of soil spiking on the substrate utilization fingerprints, as shown in principal component analyses (Figure 4).

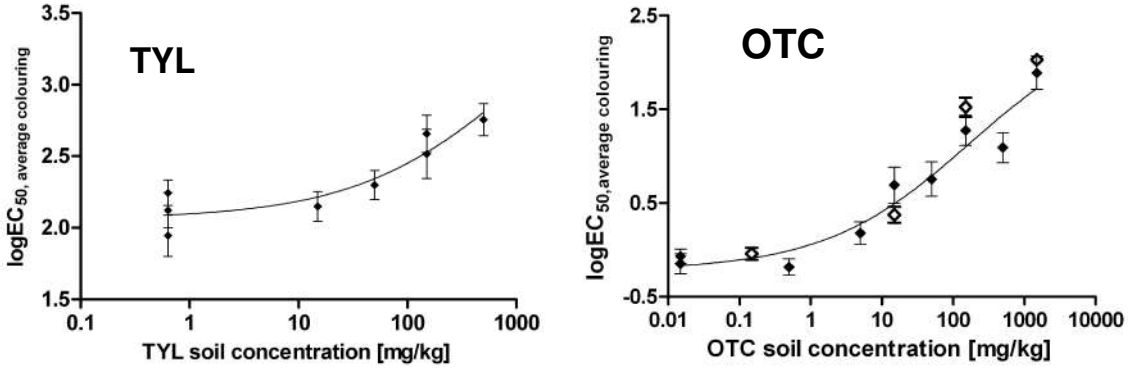

Figure 3. Increase in community tolerance of soil microcosms exposed to tylosin (left) or oxytetracycline (right) for one week. Community tolerance is given as the concentration of antibiotic that reduces colour development of bacterial inocula extracted from the soils in the average responding substrates in Biolog plates to 50% ($logEC_{50}$, average colouring). Soil microcosms have been tested after 1 week exposure (tylosin) and 1 (closed symbols) and 2 weeks (open symbols) for oxytetracycline.

The graphs show that there is a pronounced influence of tylosin on the CLPP pattern, seen as a gradual shift of the fingerprint to the right with the soil concentration. For oxytetracycline, in contrast, first influences of the treatment on the CLPP fingerprint become only apparent at higher soil concentrations, and the lower concentrations form one cluster. The CLPP pattern for oxytetracycline is also dominated by the highest treatment. The variance explained by the first and second axis is also slightly higher for the tylosin treatments in comparison with oxytetracycline. RDA analyses tell that the correlation of the fingerprint with the soil concentration (as logarithm of the nominal concentration) is significant for TYL ($p=0.02$). For OTC, the correlation according to Monte-Carlo simulations is close to be significant ($p=0.07$). The CLPP analyses have also been performed after two weeks of soil exposure (data not shown). After two weeks, the influence of the antibiotic treatment is still apparent for oxytetracycline ($p=0.06$) and tylosin ($p=0.0006$), and the influence of time on the pattern is not significant for both substances.

These observations are backed up in an analysis of the diversity and evenness of the CLPPs for both substances (Figure 5). There is a clear tendency for a decrease with soil tylosin amendment in both the number of substrates utilized, the diversity H' and the evenness E. In contrast, the oxytetracycline treatments only result in a smaller number of substrates utilized at higher soil concentrations, and the diversity and evenness remain stable with only small decreases at the highest soil amendments.

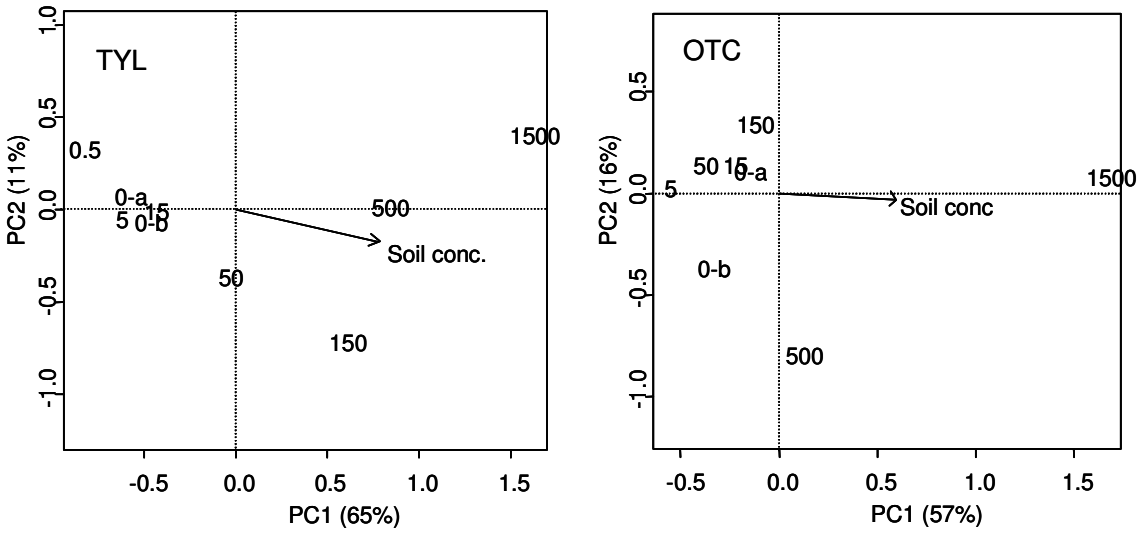

Figure 4. Potential physiological diversity as seen in CLPP experiments: the fingerprints of the utilization of a range of substrates in differently pre-treated soil samples, analysed by principle component analysis. TYL soil samples (left) and OTC (right) are shown, together with the gradient of the soil concentration (on log scale), given as arrow.

DGGE – genetic diversity

In order to investigate the diversity of the most dominant community members, DGGE analyses have been performed, characterising the pattern of the 16S rDNA fragments in each soil treatment. The DGGE patterns for one week of soil exposure for tylosin and oxytetracycline are shown in Figure 6. Despite the high similarity of the 16S rDNA of soils exposed to tylosin (max. 6% dissimilarity between the groups), the influence of tylosin on the DGGE pattern is visible by separate clustering of the low, middle and high treatments.

The cluster analysis of the oxytetracyline-exposed soils also shows that there are gradual changes of the profile with the soil concentration. The highest concentrations (150, 500 and 1500 mg/kg) group together and form a cluster which is separated from the lower concentrations. A second group is formed by the controls together with the two lowest oxytetracycline soil amendments. The overall dissimilarity is much bigger as observed for tylosin.

Sorption coefficients

Table 1 summarizes the sorption coefficients Kd found for the two compounds. Soil sorption is much higher for oxytetracycline than for tylosin.

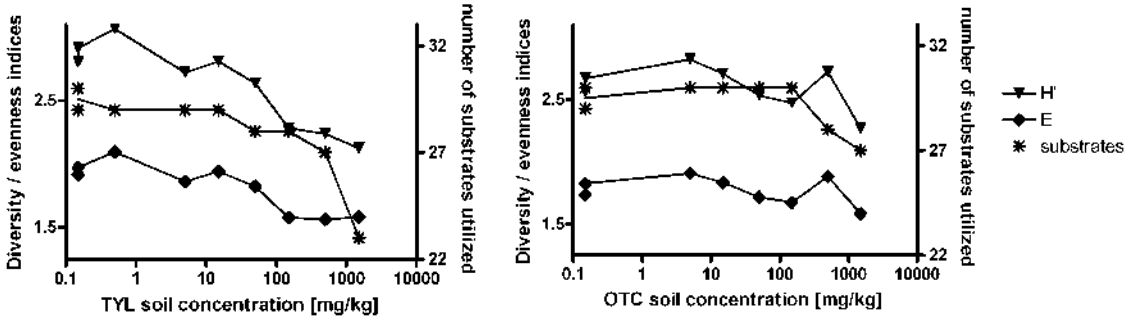

Figure 5. Diversity of potential physiological activity profiles of bacteria in tylosin (left) and oxytetracycline (right)-exposed soils – x axis: TYL/OTC soil concentration. Y axis 1: Diversity as Shannon-Wiener index H' and evenness E, Y axis 2: number of substrates utilised\

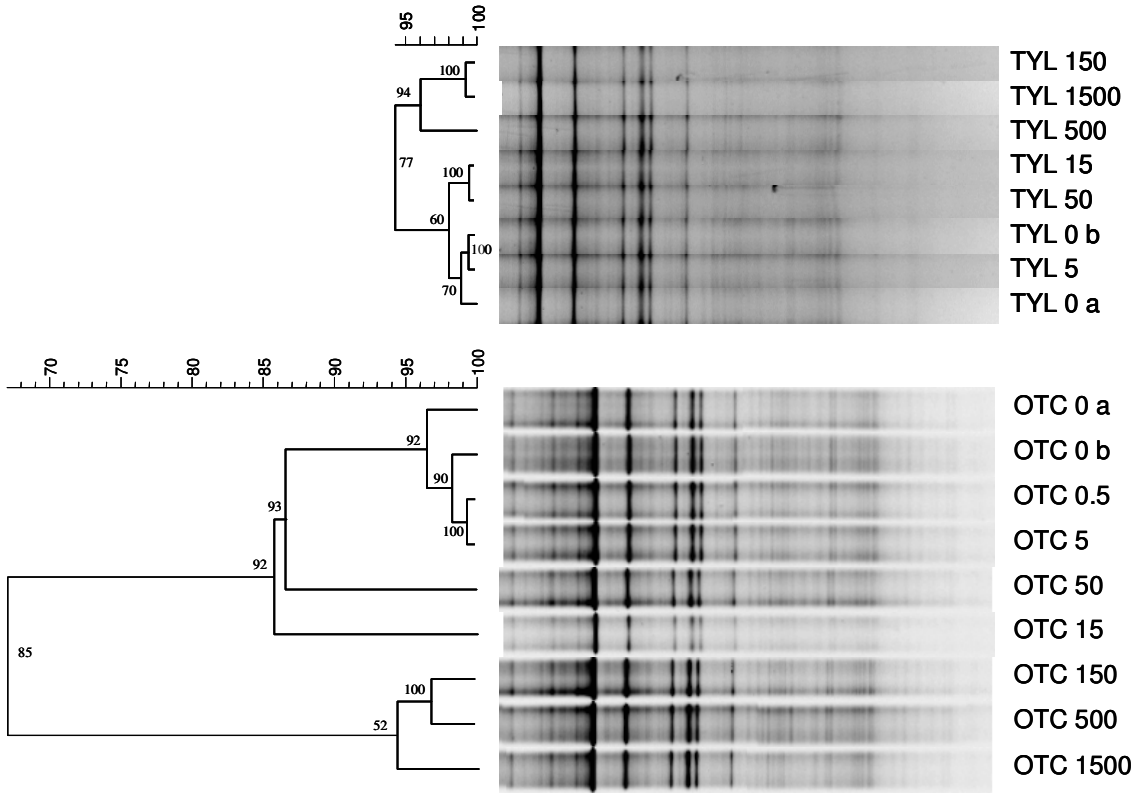

Figure 6. 16S-rDNA DGGE gels of soils treated with a gradient of tylosin and oxytetracycline concentrations. The soil concentrations are shown at the right of the bands [mg/kg], and a cluster analysis of the banding pattern is shown to the left, with the similarity given as the Pearson product-moment correlation coefficient on the scale. The cophenetic correlation is shown for each sub-cluster as a measure for the consistence of the cluster.

Table 1. Pollution-induced community tolerance and soil sorption of two antibiotics

	Soil concentration [mg/kg] [a]		Kd [L/kg] [d]
Increase in community tolerance	10% [b]	100% [c]	
Oxytetracycline	0.009	1.3	2330 ± 604
Tylosin	1.3	58	9.6 ± 1.6

[a] calculated from Figure 3

[b] 10% as sign of first effects

[c] 100% or a doubling of the community tolerance as clear effect measure

[d] Soil sorption coefficient

DISCUSSION

This study investigated the effects of antibiotics on soil microbial community structure. Oxytetracycline and tylosin are able to increase community tolerance in soils, indicating an influence of the antibiotics on the structure or physiological state of the communities. Further, shifts in the community structure were observed when determining community-level physiological profiles and 16S rDNA fingerprints. However, the nature of community changes differs for both compounds.

For oxytetracycline, a significant increase of the community tolerance is visible in the PICT investigations, which is indicative for a selective action of oxytetracycline on the soil microbial community. From dose-effect relationships, it can be calculated that the community tolerance doubles at concentrations as low as 1.3 mg/kg. The DGGE pattern mirrors the gradual changes observed in the PICT investigation. The low treatments (0-5 mg/kg) form a closely related group, and the treatments of 150 mg/kg and higher group in a separate cluster. However, the effects on the pattern of utilization of different substrates (CLPP) were less pronounced and mainly caused by a deviating pattern at the highest soil amendment of 1500 mg/kg.

With tylosin, in contrast, the most striking effects observed are those on the pattern of substrate utilization (CLPP), where a gradual shift of the fingerprints along with the tylosin soil concentration can be seen. The shift in the fingerprint is accompanied by a decrease in the diversity and evenness of the substrates utilized. An increase in community tolerance is apparent as well, but less prominent than for oxytetracycline (the tolerance is doubled at a concentration of around 60 mg/kg). The findings of both cultural tests, CLPP and PICT, are therefore in agreement. In contrast, the DGGE patterns show much less effects than had been the case for oxytetracycline.

Community effects of tylosin: sensitive fast-growing heterotrophs

For tylosin, the divergence of the cultural and molecular analyses suggests that the fraction of bacteria that contributes to the colouring of the Biolog plates reacts more sensitive to the

antibiotic than the major part of the bacterial community seen in 16S rDNA investigations. Biolog plates mainly reflect the activity of fast-growing heterotrophs, and these fast-growing bacteria are likely to be affected most by an amendment with the bacteriostatic compound tylosin (Cocito 1979). So, the different results of the CLPP and DGGE studies for tylosin could reflect different sensitivities of the fast- and slowgrowing populations of the community. It has been observed before that the fast-growing fraction of the community sometimes reacts faster to toxicant gradients (Ellis et al. 2003). A different sensitivity of "culture-based" and molecular approaches to toxicant stress has also been noted in previous investigations with other compounds (Kelly et al. 1999), along with examples for a congruent behaviour (el Fantroussi et al. 1999, Siciliano et al. 2000). It can be concluded that the sensitivity of any test system to detect toxicant effects on soil bacteria depends on the compound in question and its potential effects as well as on the experimental set-up and limitations of each method.

The higher tylosin soil concentrations led to clear effects in the CLPP (Figure 4). This includes the disappearance of some functions in the sense that they are outdiluted from the original community much earlier than in the control community, or in other words, a reduced potential physiological diversity. One hypothesis is that the reduced abundance of the bacteria responsible for the utilization of a specific substrate mainly depends on a direct bacteriostatic effect of the soil treatments. The disappearance would then be directly correlated with the sensitivity of the processes to tylosin. Alternatively, the abundance in exposed soils might be determined by the ability to show physiological adaptations or share resistance mechanisms, which would be independent of the sensitivity in an unexposed situation. To test these hypotheses, the correlation of the abundance in the different soils with the "intrinsic" sensitivity of the utilization of each substrate has been analysed. The intrinsic sensitivity can be retrieved from the PICT experiment, namely as the tolerance of the utilization of each substrate in the control soil. Results from the multivariate analysis of the CLPP fingerprints serve as a measure for the gradual appearance of a substrates' utilization from the community, namely the substrate score of the RDA analysis. A correlation, however weak, was found between the RDA score and the $\log EC_{50}$ of that substrate in the control soil in the Biolog sensitivity assay (slope of a linear regression of the substrates' RDA score on the $\log EC_{50}$ is significantly different from zero, $r^2=0.26$, data not shown). Overall, this suggests that the effect of tylosin is at least partly caused by a decrease in abundance of sensitive fast-growing species from the community.

Our findings are in agreement with Westergaard (Westergaard et al. 2001) who also observed tylosin effects in soils spiked with 2000 mg/kg tylosin. Their indications that gram-negative bacteria became more prevalent in the tylosin-treated soil together with the finding that Biolog plate patterns preferentially reflect gram-negative bacteria (Heuer & Smalla 1997) adds to our observations of clear changes in both CLPP and PICT. Further, they observed that tylosin led to slower growth upon substrate amendment, which also might point to a sensitive response of the fast-growing part of the community (Müller et al. 2002). Interestingly, with a different set-up for both CLPP and DGGE analyses, they found that DGGE changes were even more prominent than changes in substrate utilization. This illustrates that CLPP analyses

based on dilution series might detect community changes with a higher sensitivity than a single inoculum density.

Community effects of oxytetracycline: patchy soil distribution?

According to an ecotoxicological paradigm also known as equilibrium partitioning theory, only aqueous concentrations are bioavailable and thus relevant for toxic effects of substances in soil–pore water systems. For both compounds, soil sorption coefficients K_d were determined, in order to make estimates of the bioavailable fraction. For oxytetracycline, soil sorption is very strong (Table 1). Based on the K_d values determined for our soils, the soil concentrations can be calculated that would lead to a pore water concentration equalling the EC_{50} of both antibiotics as observed in Biolog plates. If the equilibrium partitioning theory were assumed to hold true for tetracyclines and their "unusual" sorption behaviour (complexation with clays, and pH dependent soil sorption (Tolls 2001), effects would be expected to occur only at soil concentrations of 1910 mg/kg for oxytetracycline and 1240 mg/kg for tylosin. It becomes apparent that effects of OTC are observed in the PICT experiment orders of magnitude below this calculated soil concentration (at around 1 mg/kg). For tylosin, the difference is not as large (Table 1). In contrast, the effects seen in the CLPP experiment at the 1500 mg/kg soil amendment with oxytetracycline are more in agreement with the calculated effective soil concentration.

First of all, other differences in physicochemical parameters might play a part to explain this contrast. For example, tetracyclines are known to be taken up as positively charged ions (Yamaguchi et al. 1991), and due to the acidity constant of oxytetracycline (pKa_2=7.33, (Mitscher 1978), the proportion of the positively charged species increases from 0. 68 in the Biolog plates, which are kept at pH 7, to about 1 in the slightly acidic soil system. The higher acidity on particle surfaces further adds to this increase in bioavailability in the soil system as compared to the Biolog plates.

A second explanation for this deviation, which also serves to explain the differences in the effect pattern, is an inhomogeneous distribution of oxytetracycline in the soils on a microscale. Such an inhomogeneous distribution appears likely for compounds with strong sorption, where the pore water can only limitedly equilibrate local differences in the distribution of the compound resulting from incomplete mixing. If local peak concentrations of the antibiotic occur on a microscale, the selection pressure exerted on the bacterial community varies accordingly. Indications for an inhomogeneous distribution of oxytetracycline have also been noted by C. Rodriguez (personal communication), when analyzing its bioavailability in soil samples by means of a whole-cell biosensor carrying an inducible gfp fusion. Further support comes from Férnandez et al (Fernández et al. 2004), who noticed that the application of doxycycline-spiked manure to soil microcosms resulted in the retention of doxycycline in the topsoil, whereas the application of doxycycline as watery solution led to mobility of the substance throughout the soil column of 9 kg. Although the microcosms in that experiment were not homogenized, as was the case in our experiments, it highlights that manure might be a strong sorbent and that retention of the substance in manure constituents might lead to an inhomogeneous distribution in the soil.

Inhomogeneity in concentration, in turn, might lead to the observed divergence between the PICT, CLPP and DGGE results. In the PICT investigations, bacteria that obtained a high oxytetracline tolerance during soil exposure to peak concentrations may survive high Biolog plate amendments. Biolog plates amended with oxytetracycline are thus focussing on the highly tolerant population. On the other hand, if populations of the community remained in unexposed microsites of the soil, these might show a higher physiological activity than the exposed subparts. These unexposed populations might therefore dominate CLPP, which is determined in unamended Biolog plates and thus focuses on the bacteria with the fastest respiration and growth. The CLPP pattern obtained would then be identical to the pattern of unexposed soil. Although PICT might therefore on average zoom in on the most severely affected part of the community, the DGGE results highlight that the changes are also apparent in the numerically dominant part of the community. Changes in community composition have also been observed in phospholipid fatty acid analyses (PLFA) with soils that had been spiked with manure and additional tetracycline for 8 and 16 weeks (Hund-Rinke et al. 2004). Hund-Rinke found a decrease in the number of PLFA bands at a tetracycline amendment of 500 mg/kg, accompanied by a reduction in growth of gram positive bacteria and a smaller bacteria-to-fungi ratio. Further, a reduction of the phosphatase activity at soil concentrations of doxycycline greater than 1 mg/kg has been detected by Fernández et al (Fernández et al. 2004).

The small impact of oxytetracycline on the potential physiological abilities might also be explained by changes in the prevalence of antibiotic resistance genes. While the disappearance of processes in tylosin-exposed soils might be caused by the disappearance of tylosin-sensitive bacteria, bacteria exposed to oxytetracycline might also show genetic adaptations. An increased sharing of resistance genes coding for tetracycline-efflux pumps or ribosomal protection proteins might render the bacteria highly insensitive, while at the same time maintaining their physiological capabilities. Interestingly, the average oxytetracycline tolerance we observed is comparable to background sensitivities in enteric culture collections, and after soil exposure, raise to levels observed with high-level resistant strains (Houndt & Ochman 2000). Notably, manure can also function as an input of resistance genes originating from intestinal bacteria which have become resistant during animal therapy (Chee-Sandford et al. 2001, Aminov et al. 2002). These genes might further be horizontally transferred to soil bacteria (Götz & Smalla 1997).

Relevance of observed effects in field situations

The two antibiotics, oxytetracycline and tylosin, have been shown to be able to affect the microbial community of agricultural soils. However, according to risk assessment paradigms, the actual risk of antibiotic use in veterinary pharmacy results from a comparison of the effect concentrations with concentrations which can be expected in the field.

Usage of tylosin is less than of tetracyclines in many European regions (Thiele-Bruhn 2003), and no tylosin has yet been found in field studies (Höper et al. 2002). Further, degradation studies in soil slurries indicate a high potential for tylosin degradation (Ingerslev & Halling-Sørensen 2001). Overall, the potential of tylosin to enter agricultural soils seems to be low.

More data are available for the tetracycline class of compounds. Hamscher et al. (Hamscher et al. 2002) detected chlortetracycline concentrations in manure of up to 4 mg/kg, and in manured soil of up to 199 µg/kg. In soil column studies manured with slurry of animals treated at subtherapeutical doses, 225 µg/kg have been found shortly after manure application (Aga et al. 2003) Oxytetracycline has in some investigations also been observed to form persistent residues (overview in (Thiele-Bruhn 2003). For oxytetracycline, clear changes in community tolerance have been observed at 1.3 mg/kg (this study). The chlorotetracycline concentrations found in soil are thus only slightly smaller than the oxytetracycline concentrations for which effects have been observed in this study.

In summary, we demonstrated effects of the veterinary antibiotics oxytetracycline and tylosin on soil microbial communites with an array of methods based on both molecular and cultural techniques. It was observed that no one method would have been suited in a standalone fashion for the detection of possible environmental effects of both antibiotics discussed here. In contrast, our investigations suggest that when molecular analyses such as DGGE are accompanied by alternative methods, a more complete picture of possible toxicant effects can be gained. For oxytetracyline, effects on the community tolerance were observed at concentrations which are in the range of concentrations to be expected at hot-spot field situations, highlighting the need for further investigations into this class of compounds.

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CHAPTER 6

ON THE LIMITS OF TOXICANT-INDUCED TOLERANCE: CO-TOLERANCE AND VARIATION FOR THE EXAMPLE OF ANTIBIOTIC EFFECTS

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ABSTRACT

Pollution-induced community tolerance (PICT) as an ecotoxicological test system has been claimed to detect pollutant effects highly specifically and sensitively. However, the specificity might be limited by the occurrence of co-tolerance. Another limitation of the application of any ecotoxicological test system lies in variation of the measured responses. We tested the variation and the occurrence of co-tolerance experimentally; using antibiotics as toxicants, soil microcosms as microbial communities, and tolerance determination in Biolog plates as PICT detection test. Bacteria have been discussed as being prone to multiple tolerances, due to the possible accumulation of multiple resistance genes on mobile genetic elements. However, in our experiments, co-tolerance occurred only between antibiotics of the same group (oxytetracycline and tetracycline), as expected from their identical mode of action. Co-tolerance between oxytetracycline and tylosin in soil microcosms exposed to oxytetracycline was low, as was co-tolerance to oxytetracycline in tylosin-exposed microcosms. We conclude that tolerance development to antibiotics in soils rather reflects the actual selection pressure than a general pattern of multiple resistances. Concerning variation, the PICT effect of tetracycline was well reproducible in two consecutive years. The response variation linked to PICT experiments in controlled microcosms was comparable to that of ecotoxicological test systems of equivalent complexity. In conclusion, our results support an application of the PICT methodology as an effective means to study the soil ecotoxicology of antibiotics.

INTRODUCTION

Environmental quality standards are most often based on single-species tests for the characterization of the effects of pollutants. These tests are mostly used as surrogate for higher levels of biological organisation such as communities or ecosystems, which are more difficult to investigate. Still, some test approaches that are based on higher organisation levels exist and bear significant advantages. One such approach is the so-called pollution-induced community tolerance (PICT), which has been applied in ecotoxicology for over 15 years (Blanck et al. 1988) and has been reviewed recently (Blanck 2002, Boivin et al. 2002). PICT is based on the assumption that communities would react to a toxicant stress by replacement of sensitive species or physiological adaptations that would render the community more tolerant. This community tolerance, in turn, can be tested with common laboratory ecotoxicology tests, and be used to characterize an environment as to whether toxicant stresses are present.

One of the main advantages of PICT is its specificity or causality. PICT might establish a toxicant-effect relationship more specifically than other measures of effect, because the influence of confounding factors is reduced in tolerance testing (Boivin et al. 2002). This idea, however, is strongly dependent on the assumption that the tolerance to one agent would not be coupled to tolerance to another substance, a phenomenon discussed under the name of co-tolerance (Blanck 2002) and observed frequently for metals (Díaz-Raviña et al. 1994, Bååth et al. 1998, Soldo & Behra 2000) . The special attention for co-tolerance dates back to the very early days of PICT, when Blanck et al. (Blanck et al. 1988) pointed out that co-tolerance would undermine the PICT approach if it was a frequent phenomenon. Theoretically, co-

tolerance can be expected for compounds that share a common mode of action, and more importantly, a common tolerance mechanism (Blanck 2002).

The phenomenon of co-tolerance has frequently been described in an area of science not directly linked to ecotoxicology, i.e. multiple antibiotic resistance. This “co-tolerance” of bacterial pathogens to several antibiotic compounds has increasingly been found in hospital environments. Bacteria can “accumulate” different resistance genes, especially as these are often located on mobile genetic elements such as transposons, integrons or plasmids (Tennstedt et al. 2003). In addition, certain resistance mechanisms cause resistance to multiple substances at once, such as ABC transporter complexes (Fernandez-Moreno et al. 1998).

Previous investigations in our lab showed that antibiotics could have toxic effects, when they reach agricultural soils with manure from antibiotic-treated farm animals. We observed an increase in the community tolerance to sulfonamide, tetracycline, and macrolide antibiotics in soil microcosms amended with these antibacterial agents (Schmitt et al. 2004), (Schmitt et al., submitted for publication). If co-tolerance in bacteria were frequent, exposure to one antibiotic as environmental stressor would lead to co-tolerance to other antibacterial substances. Alternatively, if the intrinsic co-tolerance of soil bacteria to different antibacterial compound groups would be limited, exposure to one antibiotic would only lead to a tolerance development to one antibiotic class at a time. In this investigation, we experimentally test this hypothesis by studying co-tolerance in between the tetracycline group and between tetracyclines and macrolides.

The human health risks associated with antibiotic resistant bacteria in the environment are largely unknown (American Academy of Microbiology 2002). Still, if co-tolerance would also occur in soil microbial communities, and if it was linked with genetically encoded resistance, this could add to the concerns about an environmental pool of resistance genes.

Variation in the observed response is an important issue when studying soil ecotoxicology, especially for test systems that might be used in regulatory toxicology. PICT detects the community tolerance with short-tem ecotoxicological tests, which have a potentially low variation due to their high degree of standardisation. One such test is based on the respiration of organic substrates contained in multiwell plates (Biolog plates) (Rutgers et al. 1998, Siciliano et al. 2000, Davis et al. 2004, van Beelen et al. 2004). However, little data have been presented regarding the response variation of PICT studies of microbial communities with Biolog plates.

The aim of this publication is to address two possible drawbacks associated with toxicant-induced tolerance testing. First, we investigated the strength of antibiotic co-tolerance for mechanistically unrelated as well as related compounds as a possible bias in toxicant-induced tolerance. This included investigating the PICT effect of tetracycline. Second, we looked at the variation of PICT testing with Biolog plates.

MATERIAL AND METHODS

Soil exposure

Soil was sampled from a controlled field plot (Schmitt et al. submitted) in 2003 and 2004, and manure was obtained from Utrecht University farms, from individual sows that had no record of antibiotic treatment in the last two months. Solid manure and urine were mixed to obtain pig slurries. Microcosms were set up as described previously (Schmitt et al., submitted for publication). In 2003, 6.6 ml of slurry were added to 300 g soil (dry weight), resulting in an addition of 0.45 g of slurry dry matter per soil kg. In 2004, 5 ml slurry with a dry weight content of 71 g/kg were added to 300 g soil (dry weight), equivalent to 1.1 g slurry dry matter per 1 kg soil.

The nitrogen content of the slurry was 5.96 g/kg in 2004, and the nitrogen input to the soil samples in 2004 equalled 91 mg / kg dw. This is equivalent to 186 kg N/ ha at a mixing depth of 20 cm, and is thus in accordance with normal agricultural practice. Simultaneously with the slurry amendment, soils were spiked with a wide range of tetracycline concentrations: 0, 0.1, 1, 10, 100 and 1000 mg/kg dry weigth. Tetracycline hydrochloride (Fluka, purity >97%) was applied as a solid substance or as watery solution. After 6 days (2003) or 7 days (2004), microbial inocula were extracted from the soil by shaking 20 g of soil for 1 min in a blender with 200 mL 100 mM phosphate buffer at pH 7. The inocula were immediately frozen in 2 mL portions in liquid nitrogen and stored at –80 ˚C until further measurements.

CLPP

Community-level physiological fingerprints were determined with an inoculum-dilution based method (van Elsas & Rutgers 2005). In short, 96-well microtiter plates ECO microplates (Biolog, Hayward, CA, USA) prefilled with 31 organic substrates were used for the determination of the bacterial metabolic activity. Substrate utilization over time are detected by redox changes that lead to colouring of a tetrazolium dye in the wells. The fingerprints were determined as the contribution of each organic substrate to the total colouring. Serially diluted inoculi were used in order to correct for inoculum density differences between the samples.

The plates were inoculated with 100 µL per well of serial 3-fold dilutions of the thawed microbial extracts in 10 mM phosphate buffer (pH 7), containing 9 g/L sodium chloride.

Each serial dilution was tested on 31 substrates and a water control well by inoculating one Biolog plate with three serial dilutions. The absorbance at 590 nm was determined once or twice daily until day 6 (144 h). This procedure yielded one colour development curve for each substrate and for 12 inoculum densities. Per inoculum dilution, the colour development curves for each of the substrates (well colour development, WCD) as well as the averaged absorption over all substrates (average WCD, AWCD) were integrated over time to give the area under the curve (AUC, dimension: $Absorbance_{590\,nm}$*h). The inoculum dilution at which colouring reached 50% of the maximum colouring (log WCD_{50}) was calculated from a curve fit of the AUC against the inoculum dilution with a log-logistic function (GraphPad Prism version 3.00, GraphPad Software, San Diego, California, USA). This was done for both the single

substrates and the average colouring (AWCD). CLPP, the fingerprint of the metabolic activities in each soil, was calculated as the relative contribution of a substance to the average colouring, by dividing the $AWCD_{50}$ by the corresponding value for this substance, the WCD_{50}:

Fingerprint=log ($AWCD_{50}$ / WCD_{50})

If substrates had their WCD_{50} at dilutions more than a factor 100 different from the $AWCD_{50}$, their log WCD_{50} were set to 2 or –2, respectively. The relative contributions of all 31 substrates were then analysed using principal component analysis (without further scaling) and redundancy analysis with Monte Carlo permutations.

PICT

Biolog plates were also used for the determination of the tolerance of the soil sample extracts to tetracycline (Rutgers et al. 1998). The plates were amended with tetracycline (TC) to yield nominal concentrations of 0, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 mg/L. From the CLPP experiments, one inoculum dilution was chosen that lead to sufficient colouring (average absorption on day 7 equalling approximately 1.2 in the control soil). Each plate was inoculated with 100 µl per well of one tetracycline / inoculum combination, leading to triplicate measurements of each combination of soil extract and TC amendment for each of the 31 Biolog substrates. One to three times daily for 11 days (260 h), colouring was determined. Per Biolog plate and substrate, the colour formation was integrated over time, yielding AUCs of well colour development. Response data for each soil sample thus consisted of AUCs for 31 substrates in triplicate, measured at 10 different Biolog plate TC concentrations. In order to integrate the colouring of the 31 substrates into one effect parameter, a normalized average of the colouring over all substrates was calculated. As substrates with different absolute colouring have a different weight upon simple averaging (AWCD), and as some substrates are not influenced by antibiotic addition to Biolog plates, only the responding substrates were used for calculating the average. To this end, the responding substrates were defined as those with a robust dose-effect relationship between tetracycline plate amendment and reduction in colouring. The dose-effect relationship was judged from a log-logistic curve fit of their AUC against the plate concentration using GraphPad Prism.

Y=1/(1+10^((log(c)-logEC_{50})*slope))

slope=log9/(logEC_{50}-logEC_{10}) (van Beelen et al. 1991).

A substrate was deemed responding if its log EC_{50} curve fits had r^2 values >0.4 and its log EC_{50} values had a standard deviation of EC_{50} <1. Each responding substrate was normalized by dividing the AUC in the Biolog plates exposed to tetracycline by the maximum AUC for this substrate (AUC in the control plate without tetracycline addition). Per plate (thus, per tetracycline concentration), all AUCs of all responding substrates were averaged to yield average AUCs of rWCDs (average responding well colour development ArWCD), ranging from 0 to 1. These were fitted against the plate concentrations using a log-logistic dose-response curve as above.

The correlation between tetracycline soil exposure and tolerance increase was tested by curve-fitting to a sigmoidal dose-response curve of the tolerance (as $logEC_{50}$ of the averaged, responding substrates) versus the logarithm of the soil concentration.

Co-tolerance

The co-tolerance of one soil exposure to another antibiotic was determined in a similar way as for the single substrates, only that the antibiotic that co-tolerance was studied for was now added to the Biolog plates in the same concentration range. The chemicals used were oxytetracycline dihydrate (Sigma Aldrich, purity > 99%) and tylosin tartrate (Sigma Aldrich, purity > 90%).

Adaptation of microbial communities to oxytetracycline and tylosin was described elsewhere in detail (Schmitt et al., submitted for publication). Briefly, soil microcosms of the same kind as described above had been spiked with manure (2.9 g dry matter / kg dw soil) and a range of oxytetracycline and tylosin concentrations. Tolerance development to oxytetracycline in oxytetracycline-exposed soils and tylosin in tylosin-exposed soils had been tested as described above. For the present study, the microbial extracts stemming from these previous experiments have been tested for co-tolerance to oxytetracycline, tylosin, and tetracycline.

Selective plating

Fresh extracts of the tetracycline-treated soils were plated in 3-fold serial dilutions on agar plates with 1/10 strength tryptone soya broth (TSB, Oxoid). The plates were also amended with tetracycline in concentrations of 0, 1, 10 and 100 mg/L. Plate counts were evaluated after 8 days in order to include slow-growing cultivable bacteria. Statistical significance was evaluated by comparing the log-transformed colony counts between treatments and controls and correcting for multiple comparisons with the Bonferroni correction. In addition, curve-fitting of the logarithm of the CFU counts versus the soil antibiotic concentration with a sigmoidal dose-response curve was performed according to

$y=B+ (T-B)(1+10^{(logEC_{50} - (logc)*slope)})$

With y : log CFU, B and T : bottom and top of the dose-response curve, c : antibiotic concentration in the soil, slope : Hill-slope of the dose-response curve. It was assumed that the top of the dose-response curve was not yet reached (by artificially setting the top to 8).

Sorption experiment

The sorption coefficients of tetracycline for the soil used in this study was determined in a procedure which is based on the OECD guideline 106 (OECD/OCDE 2000). Diverging from the OECD guideline, the soil amount was chosen in a way that the total amount sorbed was in the same order of magnitude as the amount that remained in solution. Further, sodium azide (10 mM) was used instead of calcium chloride which is known to form complexes with tetracyclines. Aliquots of dried and sieved soil (1 mm mesh size) were shaken for two days with sodium azide and spiked with a range of tetracycline concentrations. After two days shaking at room temperature, the supernatants of the centrifuged samples were analysed by

HPLC on a ODS- AQ C_{18} column (5 cm * 4 mm, 3 µm, YMC). As mobile phase, 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) were used at a flow rate of 0.7 ul/min (A:B 85:15), and tetracycline was detected by UV absorption at 360 nm. Assuming that the mass balance is closed and no substance loss occurs, the sorption coefficients were determined as the ratio of the concentration in soil and water. An average was calculated over a range of antibiotic concentrations.

Analysis

Exemplarily for the three substances investigated, the concentrations of tetracycline in soil, in the PICT tolerance test and in the co-tolerance experiment, were determined by HPLC. For the PICT experiment analysis, the remains of the bacterial inocula amended with the antibiotic after inoculating the Biolog plates were filtered (0.45 um), and subjected to HPLC with the same conditions as above. The concentrations of tetracycline well (81-97%) matched the nominal concentrations at nominal concentrations of 0.3 mg/l and higher. The bacterial extracts of the soils exposed to 1000 mg/kg tetracycline contained about 1 mg/l tetracycline stemming from soil exposure. Due to the good agreement of the nominal and actual concentrations, nominal concentrations were applied when calculating dose-response curves.

Soil concentrations of tetracycline were verified after extraction. As extraction buffer, a freshly-prepared mixture of methanol, 0.1 M EDTA (ethylene diamine tetraacetic acid), and McIlvaine buffer (17.6 mM citric acid and 16.5 mM NaH_2PO_4) (2:1:1) (Blackwell et al. 2004) was used (all chemicals: Merck, p.a. quality). 4 gram of soil were extracted three times with 5 mL of extraction buffer by 5 seconds vortexing, treatment in an ultrasonic bath for 5 min, and centrifugation at 3500 rpm for 10 min. The extracts were used directly for HPLC analyses with conditions as above. The analytical availability of freshly-spiked soils was already low for lower spiking concentrations (32% at 1 mg/kg, 48% at 10 mg/kg and 112% at 100 mg/kg), indicating the tight sorption of tetracycline to soils, and mirroring the low recoveries found with the same method (Blackwell et al. 2004). The analytical availability of the tetracycline in microcosm experiment soils amounted to 20-36% on day 1 and 8-13% on day 8.

RESULTS

Variation in response

The first part of this study addresses the variation of community tolerance testing with Biolog plates. One PICT experiment has been repeated in 2003 and 2004: microcosms of a freshly-sampled agricultural soil were amended with slurry and increasing concentrations of tetracycline. After one week, bacterial extracts of the 6 treatments were inoculated into Biolog Eco-plates that had been amended with additional tetracycline, in order to evaluate the overall community tolerance to tetracycline. In this way, differences in the PICT selection step such as different soil or pig slurry properties were also included.

Figure 1 shows the community tolerance in soil samples exposed to increasing concentrations of tetracycline. The community tolerance is given as the concentration of antibiotic causing the utilization of responding substrates (ArWCD) to decrease by 50% ($logEC_{50}$). The

background tolerance, thus the community sensitivity in the control soils and in the soils exposed to small concentrations of tetracycline, varied from 1 mg/L to 3.4 mg/L in 2003, whereas in 2004, values ranged from 1.2 until 1.7 mg/L.

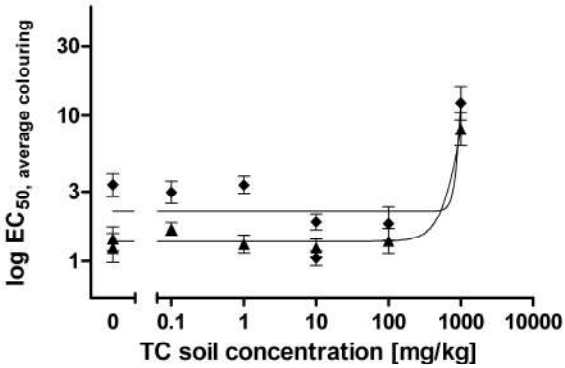

Figure 1. Pollution-induced tolerance to tetracycline in soil bacteria. X axis: tetracycline (TC) concentration in soil, y axis: bacterial tolerance, given as the EC_{50} (the tetracycline concentration needed to decrease the colouring of bacterial extracts in Biolog plates to 50% of the maximum value). Colouring was determined as the averaged area under the curve of the colour development of the responding substrates (ArWCD). Diamonds: experiment performed in 2003, triangles: 2004

The increase in community tolerance beyond the baseline tolerance was nearly identical in both years. It was only the highest concentration of 1000 mg/kg that caused a significant increase in community tolerance of about a factor of 6.

Oxytetracycline tolerance was studied in two different experiments, namely in soils exposed to oxytetracycline (Schmitt et al., submitted) and tylosin (this study). The variance in the oxytetracycline background tolerance of control soils from the two different experiments also yields information on the variation of tolerance determinations in different experiments. The results are shown in Figure 2, which also includes similar data for tylosin. For all measurements, the span between the most extreme values amounted to approximately a factor of 3 (e.g. for tylosin, the range was from 47-147 mg/L).

Additional effect studies: selective plating and physiological fingerprints

Selective plating was used to underpin the findings of the PICT experiment in 2004. The results are shown in Figure 3 for soils exposed to TC for 14 days. Whereas the total counts were nearly identical for all treatments, and also constant over time, a greater proportion of bacteria (as colony-forming units, CFU) able to grow on plates amended with 100 mg/L were visible in soils amended with 100 and 1000 mg/kg (p value for both soil treatments <0.001). An increase in CFU was also observed in plates amended with 10 mg/L, but only for the highest soil treatment. In addition, it is apparent that the control soil that received water instead of pig slurry and tetracycline showed smaller total counts. While the same trends were seen after 7 days of soil exposure, the increase in resistant CFU was less than after 14 days, and data were more variable (data not shown).

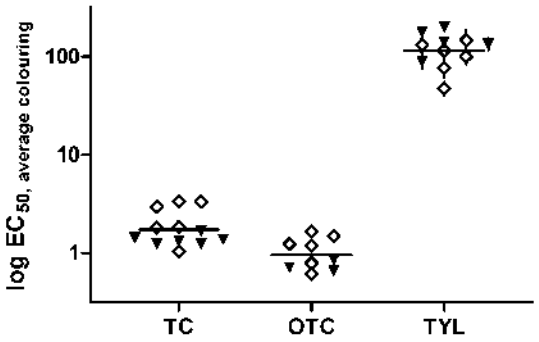

Figure 2. Reproducibility and variance of tolerance testing. The background tolerance of control soils is compared between different experiments. Tetracycline (TC): tolerance experiments performed in 2003 (open symbols) and 2004 (closed symbols), oxytetracycline (OTC): one tolerance experiment with soil exposed to oxytetracycline (closed symbols), and one co-tolerance experiment with soil exposed to tylosin (open symbols), and tylosin (TYL): one tolerance (closed symbols) and one co-tolerance (open symbols) experiment. Y axis: See figure 1 for y axis. The error bars give the standard error of the mean, if extending beyond the symbols. The horizontal lines give the means for each antibiotic.

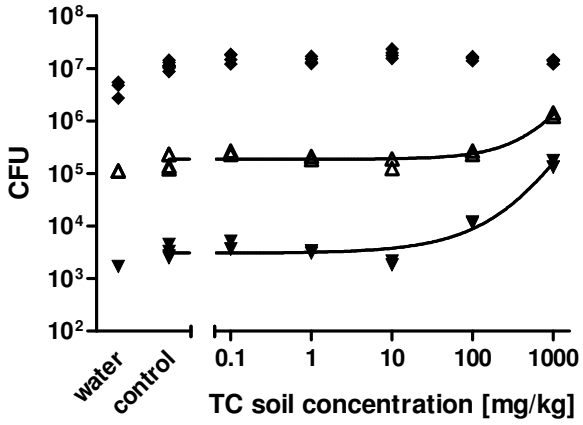

Figure 3. Bacterial counts on selective agar plates of soils pre-exposed to different concentrations of tetracycline after 14 days. Diamonds: total colony forming units (CFU) on TC-unamended plates, open triangles: plates with 10 ug/ml tetracycline, filled triangles: plates amended with 100 ug/ml tetracycline. Water: soil without manure and TC amendment, control: soil with manure but no TC

In order to accomplish the detection of the antibiotic effects with another method addressing the structure of bacterial communities, an analysis of the community-level physiological profile (CLPP) has been performed. Figure 4 gives the changes in the pattern of substrate utilization of soil samples spiked with tetracycline, in two experiments performed in two consecutive years. As judged by Monte-Carlo permutations, the effect of tetracycline is highly significant (p=0.002). Still, the deviation of the soil exposed to the highest TC concentration

(1000 mg/kg) in 2004 dominates the PCA. There was no strong trend in the remaining soil samples, only that the highest treatment in 2003 was also slightly shifted to the right on the first axis. On the second axis, the difference between the soil samples of experiments conducted in two consecutive years becomes apparent, as the treatments of both years are separated.

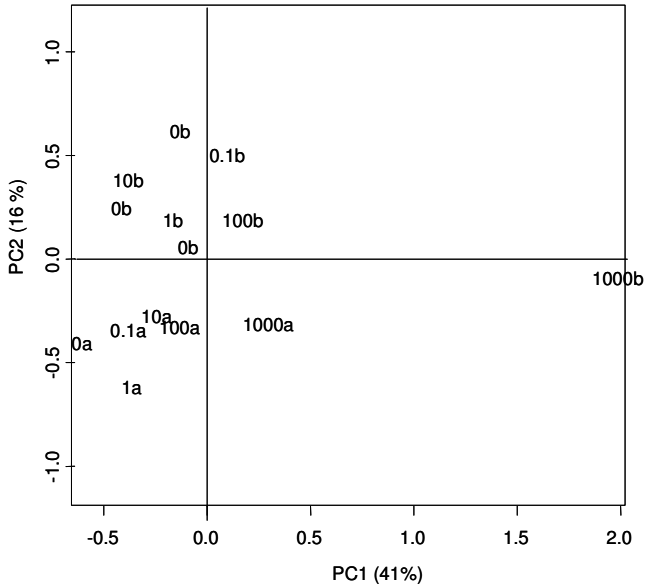

Figure 4. Community-level physiological fingerprint analysis of soils exposed to tetracycline. Principal component analysis of the pattern of utilization of 31 substrates by soil microcosms exposed to different concentrations of tetracyclines (concentrations given in mg/kg). Experiments performed in 2003 are marked with a, and in 2004, with b.

Co-tolerance

The second part of this study addresses co-tolerance of microbial communities that have become adapted to one antimicrobial agent. Figure 5 and Figure 6 show results from co-tolerance experiments with three different combinations of antibiotics. The co-tolerances of oxytetracycline and tylosin, antibiotics with a different mode of molecular action, are shown in Figure 5. For oxytetracycline-exposed soils, co-tolerance to tylosin was limited to a small increase, which is however not significant, as judged from the confidence intervals. In tylosin-exposed soil, the tolerance to oxytetracycline did not change to a great extent. Only in the highest soil treatment, an increase in tolerance by a factor of 4 was found. In order to evaluate a case where co-tolerance can strongly be expected due to common tolerance mechanisms, the co-tolerance of oxytetracycline-exposed soils to tetracycline was evaluated. An increase in the community tolerance to tetracyline started at a soil concentration of 15 mg/kg, as shown in Figure 6. The ultimate community tolerance in a soil exposed to 1500 mg/kg oxytetracycline amounted to 31 mg/L and had therefore increased 10 fold. Figure 6 also includes the results of the tolerance of oxytetracycline-exposed soils to oxytetracycline (Schmitt et al., submitted for publication). It becomes apparent that the tolerance increase was still significantly higher for oxytetracycline than for tetracycline, and that the onset of tolerance increase was at lower

concentrations for oxytetracycline than for tetracycline in the same soils. While there was an increase in oxytetracycline tolerance, but not in tetracycline tolerance in the soil exposed to 15 mg/kg oxytetracycline, a significant increase also in tetracycline tolerance had been found at 150 mg/kg.

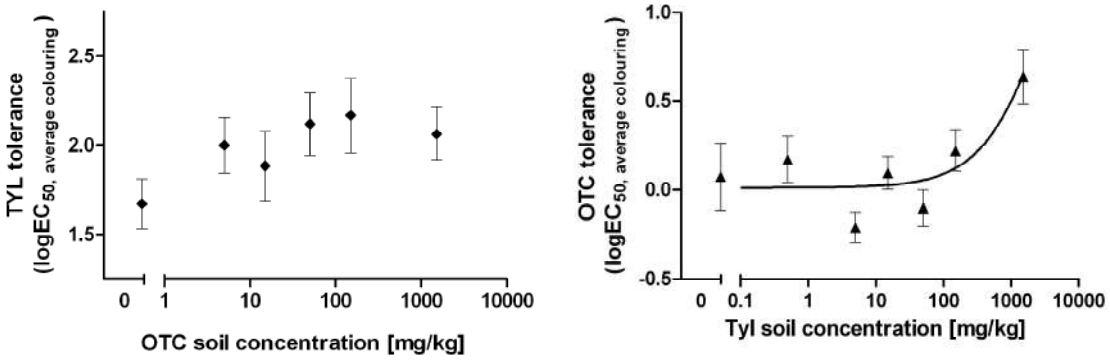

Figure 5. Co-tolerance of soil bacterial communities to a range of antibiotics. Communities stemming from oxytetracycline-exposed soil to tylosin (left) and from tylosin-exposed soil to oxytetracycline (right). Y axis: see Figure 1. The error bars give the 95% confidence interval.

Sorption experiment

In order to be able to compare oxytetracycline and tetracycline with respect to their physicochemical fate in soil and their antimicrobial activity, the sorption coefficient of tetracycline and oxytetracycline has been determined. The sorption coefficient, Kd, of tetracycline determined in this study was 1714 ± 418 L/kg, which is slightly lower than for oxytetracycline (2556 L/kg, Schmitt et al., submitted for publication). The weaker sorption of tetracycline is thus expected to lead to an increased bioavailability of dissolved tetracycline in the soil pore water.

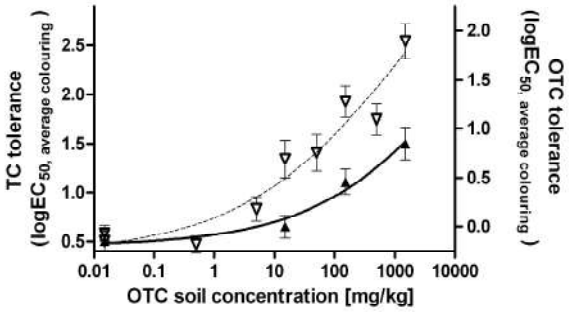

Figure 6. Co-tolerance of soil bacterial communities to a range of antibiotics. Communities stemming from soil exposed to oxytetracycline to tetracycline (left axis, closed symbols) and oxytetracycline (right axis, open symbols).

DISCUSSION

Effects of tetracycline on soil microbial communities

Figure 1 shows that an increase in community tolerance due to soil amendment with tetracycline has been consistently observed in two independent experiments: concentrations of 1000 mg/kg tetracycline led to a rise in community tolerance from about 1.5 mg/L to 10 mg/L. Effects might also occur at lower concentrations, though: after 14 days, an increase in tetracycline-tolerant colony forming units was also seen for the 100 mg/kg soil treatment. The overall community tolerance thus kept increasing after one week of soil exposure with tetracycline and pig slurry.

The effects of tetracycline seen in our study are in accordance with another soil microcosm study (Hund-Rinke et al. 2004), where slight effects of tetracycline in pig slurry-amended soil microcosms at concentrations of about 500 mg/kg were observed. In their investigation, they found a reduction in substrate-induced respiration, and a reduced number of phospholipid fatty acids, after an exposure of 8 and 16 weeks. The community tolerance increase is thus likely to be paired with a reduction in the microbial diversity due to loss of sensitive species or preferential growth of more tolerant bacteria.

Comparison of tetracycline and oxytetracycline effects

Tetracycline and oxytetracycline share the same bacteriostatic mode of action of inhibiting bacterial protein synthesis through interaction with the ribosome (Chopra 2001). Thus, their ecotoxicity could be expected to be comparable, given that their structures and therewith their physicochemical behaviour are also much alike. In contrast, we found that the effects of tetracycline differ greatly from oxytetracycline. In the case of oxytetracycline, we detected a steady tolerance increase, which started in the low mg/kg range (Schmitt et al., submitted for publication), whereas an increase in tolerance occurred at much higher concentrations for tetracycline.

In order to understand this diverging behaviour, we studied the strength of soil sorption, which is an important factor for the bioavailable pore water fraction. Tetracycline (K_d 1714 L/kg) was shown to sorb less strongly than oxytetracycline with a K_d of 2556 L/kg (Figure 6, data taken from Schmitt et al., submitted) and is thus even more available than oxytetracycline. Differences in sorption are thus not the reason for the diverging strength of the microbial effects of both compounds.

The inherent antimicrobial activity of the two substances might also explain the observed differences between oxytetracycline and tetracycline. The EC_{50} values for control, unexposed soil communities determined in Biolog plates amount to about 1 (0.72-1.25) mg/L for oxytetracycline and to about 1.7 (1.34-2.3) mg/L for tetracycline. Thus, oxytetracycline is slightly more effective. Still, the effects of a slightly lower equilibrium sorption coefficient and a slightly lower inherent toxicity of tetracycline as opposed to oxytetracycline should compensate.

Apart from diverging intrinsic properties of the substances, the different results found in both experiments can also be due to experimental parameters like the nutrient input into the system. Indeed, there were differences between both years: In 2003 and 2004, the input into the system was about 0.45 and 1.1 g dry matter / kg dw soil, respectively, and for the oxytetracycline experiment, this was about 2.9 g dry matter / kg dw soil. The reason for this difference was that the pig slurry addition had been adjusted to the nitrogen content of the slurry, according to EU guidelines limiting the nitrogen input to 170 kg N/ha soil. However, the nitrogen content in slurry was variable, resulting in a different carbon input. A more intensive growth of bacteria after the slurry amendment could lead to a stronger induced tolerance, as the bacteriostatic tetracyclines show the greatest effect and therewith the strongest selection pressure during bacterial growth phases (Backhaus et al. 1997, Froehner et al. 2000, Thiele & Beck 2001, Kümmerer et al. 2004, Schmitt et al. in press). The fact that the tetracycline tolerance continued to increase after one week, which was not the case for the oxytetracycline tolerance, adds to the idea that the tetracycline-exposed communities had not yet reached the maximum tolerance gain due to a lower nutrient availability.

Variation in response

Results of two dose-response studies with tetracycline in two consecutive years allowed for an estimation of the intra-laboratory variation. The tolerances for unstressed communities were only slightly higher in 2003 (2.2 mg/L vs 1.3 mg/L). In contrast to the difference of factor 1.7 in the background tolerance, the increase in tolerance detected in both years has been nearly identical (factor 5.5 in 2003 and factor 6.2 in 2004).

The intra-laboratory variance observed between the tolerance and co-tolerance experiments with one substance was in the same order as in the tetracycline experiment (Figure 2). The standard deviations of the pooled EC_{50} values are in the range of 0.15 log units for all experiments (data not shown). The coefficient of variation, a measure used for inter-experiment comparison of variation, amounts to 44.6, 32.9, and 37.5% for tetracycline, tylosin and oxytetracycline, respectively (n=12, 10 and 9). Other investigations based on Biolog plates come to a comparable coefficient of variation for different soil nutrient amendments (42%, in a laboratory experiment with sulfachloropyridazine (Schmitt et al. in press), or 16-55%, at different metal-uncontaminated field sites (van Beelen et al. 2004). Overall, the variation is in the range as observed for intra-laboratory variation in other complex ecotoxicological test systems (Moore et al. 2000, Broos et al. 2005). Although a coefficient of variation of smaller than 30% is generally aimed at, this criterion is frequently exceeded in test systems with difficult environmental media such as soil, with “difficult” polar and degrading test substances, and with non-standardized communities in place of single standardized test species.

Interestingly, the variation seen in the PICT determinations is still smaller than seen in the CLPP experiment. In one experiment, the fingerprints agree with the PICT findings (clear effect of the highest treatment on the metabolic fingerprint), whereas in the other experiment, the effect of the antibiotic treatment was much smaller, probably because other parameters also affected the physiological fingerprints. This is in line with previous experiments, where

differences in the soil nutrient level affected the physiological fingerprint, but not the community tolerance (Schmitt et al. in press). Overall, the results corroborate with the idea that the PICT approach is especially sensitive for the detection of toxicant impacts. In addition, the effects observed in the PICT experiments have been underpinned by the results of selective plating, where again, the PICT experiment showed a more clear effect than the colony counts after 7 days.

In conclusion, although PICT measurements in soil microcosms showed some variation, effects could still well be reproduced in independent experiments. Thus, Biolog plates can be applied for PICT determinations of antibiotics.

Co-tolerance

The second part of this study addresses co-tolerance of microbial communities to different antibiotics. For antibiotics with an identical mode of action and identical resistance mechanisms, co-tolerance was observed as expected: oxytetracycline-exposed soils also showed an increased tolerance to tetracycline. Still, the tetracycline co-tolerance was weaker than the tolerance to oxytetracycline: a doubling in community tolerance occurred at 1.3 mg/kg oxytetracycline, but at 19 mg/kg for tetracycline, respectively (Figure 6). Further, the total tolerance gain was bigger for oxytetracycline (increase in EC50 by a factor of 130) than for tetracycline (factor of 11).

In a PICT investigation with periphyton communities and a range of herbicidal compounds, it appeared that the tolerance gain in diuron-exposed communities varied from a factor 6.5 to 11 for photosystem II inhibitors with a similar mode of action. Co-tolerance was sometimes even higher than the tolerance gain for the original compound, diuron (Blanck & Molander 1991). It has thus been concluded before that co-tolerance seems to be a graded response rather than a presence-absence phenomenon (Blanck 2002). The difference in the level of tolerance to oxytetracycline and co-tolerance to tetracycline observed in our study supports this notion.

Interestingly, the tetracycline tolerance increase in the oxytetracycline-amended soil (Figure 6) was even greater than in the tetracycline-amended soil (Figure 1): tolerance increased by a factor of 10. Further, the soil amended with 150 mg/kg oxytetracycline already showed a clear tolerance increase, whereas no effect had been seen at 100 mg/kg tetracycline amendment. Again, that points to the possibility that the conditions in the oxytetracycline-experiment were in favour of a stronger tolerance development, for example due to a stronger bacterial growth in that system. Still, a stronger effect of an alternative selection pressure has also been observed by Díaz-Raviña et al.(Díaz-Raviña et al. 1994) for nickel tolerance in copper-exposed soils.

Our experiments do not resolve whether the observed co-tolerance was due to an increase in tetracycline-resistant bacteria. Still, the observed co-tolerance between different tetracyclines supports the current policy of avoiding the use of compounds of the same antibiotic class in different application areas, such as growth promotion and human medication.

For antibiotics with a different mode of action, co-tolerance was limited. This finding suggests that the reservoir of soil bacteria with multiple antibiotic resistances is not big

enough to easily render the whole community co-tolerant when exposed to one antibiotic. It has previously been speculated that co-tolerance could be more prominent among bacteria than among higher species, due to the ease of transfer of genetic elements and to the co-location of resistance genes on mobile genetic elements such as plasmids (Blanck et al. 1988). Unfortunately, there is little data on multiple resistances of soil microorganisms to both tetracyclines and macrolides to compare our results with. In isolates of farm animals, multiple resistances to tetracyclines, streptomycin and sulfonamides are most frequently reported (Guerra et al. 2003). The level of resistance to macrolides in strains isolated from farm animals or slurry samples is sometimes in the same range as tetracycline resistance (Cotta et al. 2003), and sometimes lower (Hayes et al. 2003). Further, macrolide and tetracycline resistance genes sometimes co-reside on conjugative transposons found in *Bacteroides* species (Courvalin & Carlier 1986, Caillaud et al. 1987). Compared with bacterial isolates from farm animals, the presence of resistant bacterial strains in environmental compartments not impacted by antibiotics is controversial: whereas some investigators report a lower frequency of resistance in remote areas (Esiobu et al. 2002), some studies find highly resistant bacteria also in situations without antibiotic pressure (Stanton & Humphrey 2003). Thus, it is difficult to say whether the limited co-tolerance observed in our study is due to a limited occurrence of easily transferable genetic elements with multiple antibiotic resistances or due to other reasons, such as a low transfer frequency or a predominance of other tolerance mechanisms.

In our study, co-tolerance between different compound classes has been observed in one soil microcosm: the highest concentration of tylosin caused an increase in the oxytetracycline tolerance as well. As this effect only occurs with the highest concentration of tylosin, this co-tolerance might be caused by selective depletion of the community at that concentration and the intrinsic tolerance of the remaining soil bacteria. Tylosin is indeed known to have selective activity: it belongs to the narrow-spectrum macrolides that affect mostly Gram-positive cocci and bacilli as well as Gram-negative cocci (Nakajima 1999). Oxytetracycline, in contrast, is a broad-spectrum agent (Chopra 2001).

Our results confirm other studies that showed a limited co-tolerance for structurally unrelated compounds, for example for different uncouplers and photosynthetic inhibitors (Blanck & Molander 1991, Blanck & Wängberg 1991). On the other hand, co-tolerance has been frequently observed for metals, for example between copper, cadmium and zinc, as observed in field and microcosm experiments (Díaz-Raviña et al. 1994, Gustavson & Wångberg 1995, Bååth et al. 1998, Soldo & Behra 2000). Although metal co-tolerance has often been described, the reasons for this phenomenon are not generally agreed upon. The same holds true for the co-tolerance observed between two structurally unrelated compounds, diuron and TBT, in periphyton communities (Molander et al. 1992).

Overall, the small co-tolerance between different antibiotic agents was small. However, this does not relieve the notion that co-tolerance might be a limiting factor for the application of PICT to investigate the relevance of a certain toxicant in a given system. Still, co-tolerance should not be assumed as a general rule, but rather be evaluated for toxicants suspected to act as selecting stressors on a case-by-case base.

In summary, we observed that the variation linked to the results of PICT determinations in controlled microcosms is comparable to normal variation in ecotoxicological test systems of equivalent complexity, which points to the suitability of the use of Biolog plates for PICT determinations in soil microcosms. Further, in our experiments, PICT was specific for chemical substances with comparable tolerance mechanisms, but little co-tolerance was observed between different groups of antibiotic compounds.

ACKNOWLEDGMENTS

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CHAPTER 7

TETRACYCLINES AND TETRACYCLINE RESISTANCE IN AGRICULTURAL SOILS – MICROCOSM AND FIELD STUDIES

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ABSTRACT

The influence of the use of antibiotics on the prevalence of resistance genes in the environment is still poorly understood. We studied the diversity of tetracycline and sulfonamide resistance genes as influenced by fertilization with pig manure in soil microcosms and at two field locations. Manure contained a high diversity of resistance genes, regardless from whether it stemmed from a farm operation with low or regular use of antibiotics. In the microcosm soils, the influence of fertilization with manure was clearly visible by an increase in the number of resistance genes in the soil after manuring. Spiking of the tetracycline compounds to the microcosms had only little additional impact on the diversity of resistance genes. Overall, the tetracycline resistance genes *tet*(T), *tet*(W) and *tet*(Z) were ubiquitous in soil and pig slurries, while *tet*(Y), *tet*(S), *tet*(C), *tet*(Q) and *tet*(H) were introduced to the microcosm soil by manuring. The diversity of tetracycline- and sulfonamide resistance genes on a Swiss pasture was very high even before slurry amendment, although manure from intensive farming had not been applied in the previous years. The additional effect of manuring was small, with the tetracycline- and sulfonamide resistance diversity staying at high levels for the complete growth season. At an agricultural field site in Germany, the diversity of tetracycline and sulfonamide resistance genes was considerably lower, indicating regional differences in gene diversity. This study shows that there is a considerable pool of resistance genes in soils. Although it is not possible to conclude whether this diversity is caused by the global spread of resistance genes after 50 years of tetracycline use, or due to the natural background in soil resistance genes, it highlights that environmental reservoirs might play a role in resistance gene capture.

INTRODUCTION

Antibiotic resistance of pathogenic bacteria has been recognized as a global public health concern (WHO 2001). Recently, the non-human application of antibiotics has also been discussed in connection with pathogen resistance (American Academy of Microbiology 2002, FAO/OIE/WHO 2003). The application of antibiotics as veterinary growth promoters has already faced restrictions in the recent years, due to concerns that it might promote transmission of antibiotic resistant microbes between animal and human populations (Witte 1998). Still, the total veterinary use of antibiotics is comparable to human use, according to the latest available usage figures from Europe (3465 tonnes veterinary therapeutic use vs 5460 tonnes human use in 1997 (FEDESA 1999, Ungemach 2000)).

It is widely acknowledged that major data gaps complicate an analysis of the problem of antibiotic resistance. One of them is the role of environmental bacteria as resistance reservoir, linked to the role of antibiotics in the environment as selective pressure for the development of resistance (American Academy of Microbiology 1999, 2002).

A major route of the dissemination of antibiotics to the environment is the application of slurry of treated farm animals on agricultural soils (Halling-Sørensen et al. 1998, Rooklidge 2004). Antibiotics entering the soils as contaminants in the manure (Hamscher et al. 2002, De Liguoro et al. 2003) might induce selection for resistant soil bacteria. Sub-inhibitory

concentrations of antibiotics are generally thought to select for resistant populations (Brady et al. 1993), and the relatively small concentrations of antibiotics detected in soils (Hamscher et al. 2002, De Liguoro et al. 2003, Sczesny et al. 2003, Kay et al. 2004, Hamscher et al. in press) might therefore contribute to an increase in resistance. Still, one has to bear in mind that the bioavailability of many antibiotics in soil may be greatly reduced due to strong soil sorption (Tolls 2001). The manure might also contain faecal bacteria carrying resistance genes, possibly on mobile genetic elements. These might subsequently be horizontally transferred to soil bacteria (Sengeløv et al. 2003, Hund-Rinke et al. 2004), a process which is facilitated by high nutrient availability such as provided by manure (Götz & Smalla 1997). Resistance genes might even persist in soils without an additional selective pressure. There are indications that the costs of resistance are often overcome by compensatory mutations that relieve the fitness decrease of the bacteria carrying the resistance genes (Jones et al. 2000, Andersson 2003).

The importance of animal manures for the occurrence of resistance genes in the environment has been shown for soils or ground waters below a pig farm (Chee-Sanford et al. 2001, Aminov et al. 2002, Hund-Rinke et al. 2004). However, little is known regarding the role of antibiotics as an additional selective pressure for the maintenance of antibiotic resistance in the soil environment due to veterinary antibiotic use (Esiobu et al. 2002, Heuer et al. 2002, van Overbeek et al. 2002). For example, previous investigations of aminoglycoside antibiotics were not completely conclusive as to the role of selection pressures in the environment. A high diversity of gentamycin resistance genes in pig manure has been noted (Heuer et al. 2002), and streptomycin-modifying genes were abundant in many habitats with and without selective pressure (van Overbeek et al. 2002). Thus, more information is needed from habitats with a clearly characterized selective pressure.

Microcosms enable studying the effects of selective pressures under controlled circumstances. In previous studies, it has been shown that the exposure of soil microcosms to oxytetracycline leads to adaptations of the bacterial community, as judged from an increase in the community tolerance and from 16S rDNA denaturing gradient gel electrophoresis (Schmitt et al., submitted for publication). Here, we build upon these experiments by studying the role of resistance gene diversity in these community changes. Tetraycline and sulfonamide resistance genes have been chosen, because these represent the most important veterinary compound classes per volume.

We investigated the effects of both manure and tetracycline amendment on genetically-encoded tetracycline resistance in soil, by performing microcosm studies with manure and a wide range of antibiotic concentrations. Further, tetracycline and sulfonamide resistance was studied in field situations with agricultural soils undergoing fertilization with manure. The goal of the field studies was to evaluate the input of resistance genes with manure and to study the persistence of these genes in the soil.

MATERIAL AND METHODS

Setup of soil microcosms

Microcosms were set up by amending agricultural soil samples with pig manure and a range of tetracycline or oxytetracycline concentrations. Soils were sampled from an experimental agricultural site located in Bennekom, the Netherlands (Garbeva et al. 2003). The soil is a loamy sand with a pH of 5.5 – 6.5. In previous investigations, the soil texture was determined as 3% clay, 10% silt and 87% sand, and the cation exchange capacity was 5.6 $cmol_c\ kg^{-1}$ (Korthals et al. 1996). Pig manure was sampled from the University stables of the Utrecht University, by collecting manure and urine from a healthy sow that underwent no antibiotic treatment during the last 1.5 months, and preparing a slurry by mixing manure and urine in a mixer. Soil microcosms consisted of 300 g dry weight subsamples, and were amended with pig manure. The slurry quantities approximated the maximum nitrogen soil input for nitrate vulnerable zones in the European Union of 170 kg N ha^{-1} (nitrogen input: 101 and 91 mg N kg^{-1} dry weight, equivalent to 157 and 186 kg N ha^{-1} at a mixing depth of 20 cm, and slurry dry matter input: 2.9 and 1 g kg^{-1} dry weight, for the first and second microcosm series, respectively). In the first microcosm experiment, soils were spiked with oxytetracycline (oxytetracycline dihydrate, Sigma Aldrich, purity > 99%), resulting in soil concentrations of 0, 0, 0.5, 5, 15, 50, 150, 500, 1500 mg kg^{-1} dry weight. In the second microcosm experiment, soil samples were amended with oxytetracycline (0.1, 1, 10 and 100 mg kg^{-1} dry weight) or tetracycline (tetracycline hydrochloride, Fluka, purity >97%, at tetracycline concentrations of 0.1, 1, 10, 100 and 1000 mg kg^{-1} dry weight). Spiking was performed by mixing the abovementioned manure with the solid compound (or aqueous solutions for the lower amendments) in a kitchen mixer, and then adding the soil subsample to the manure / substance mixture, again mixing with a kitchen mixer for two minutes. Two soils amended only with manure and one soil amended with water in place of manure served as controls for the second microcosm series. The microcosms were kept in climate chambers at 25 ˚C, and deionised water was added every few days in order to maintain the same soil humidity (119 and 150 g kg dw^{-1}). Soil samples were taken at day zero directly after set-up of the microcosms, on day 6 and on day 14 in the first experiment, and on day 8 in the second experiment.

Field experiments

One field experiment was set up in Switzerland. Liquid manure was sampled from a farm where pigs are routinely treated with chlortetracycline, sulfamethazine and tylosin. The manure was applied on two adjacent parcels of grassland where no manure from intensive pig farming was applied in the last decade to the best of our knowledge. The soil was classified as loamy Eutric Cambisol (FAO) with a pH_{CaCl2} of 5.5 to 6 and a total organic carbon content of 3.6% by weight (analysed in 105°C dried samples with a CN-Analyser from Thermoquest).

The manure application took place in the end of March and beginning of May 2003 using a band spreader. The application rate was 30 m^3 per hectare (equivalent to 93kg N/ha) following Swiss agricultural practice. A few hours before application, the manure was spiked

with additional sulfonamide compounds in order to distinguish between the two parcels and the two applications, respectively. Samples taken from the manure spreader were homogenized with a kitchen blender and stored in the dark at –20˚C until DNA extraction.

Soil samples (top 5 cm) were taken before and after manure applications using a conventional 5 cm diameter split tube core sampler. 25 cores were taken according to the band spreading pattern from the manure application on each field and mixed to one composite sample in order to have a sample representing the average concentration of the parcel. Samples taken two weeks and 6 months before the first manuring event served as controls. Samples taken after 1, 4, 11, 24, 45, 46, 49, 56, 73 and 86 days were used to investigate the pattern of the resistance genes in the time course and the concentration of sulfonamide antibiotics, respectively.

Soil samples were stored in the dark at -20°C, milled (under frozen conditions, with dry ice), homogenized and divided into aliquots with a sample splitter (Rentsch, GmbH & Co, Haan, Germany) before analysis. The determination of the sulfonamide concentration of the soil samples was done by pressurized liquid extraction (ASE 200, Dionex, Sunnyvale, CA, USA) followed by liquid chromatography tandem mass spectrometry (TSQ Discovery, Thermo Finnigan, San Jose, CA, USA).

Field soils were also obtained from a German experiment, in which the leaching behaviour of veterinary compounds after the application of spiked pig manure was tested on two different soils. One soil was a sand (83.5% sand, 4.9% clay, 1.0% organic carbon, pH_{CaCl2} 5.7), which had regularly been fertilized with cattle manure in the last two years. This soil did not contain any tetracyclines above the detection limit of 2 µg kg^{-1} (Hamscher et al. 2002) before the experiment. The other soil was a silty loam (4.3% sand, 41.3% clay, 2.6% organic carbon, pH_{CaCl2} 7.4) which had been regularly fertilized with pig manure in the last two years and contained 41 and 27 µg tetracycline kg^{-1} dry soil in the layers 0-10 and 10-20 cm, respectively. Mixed soil samples were taken in spring (in April), shortly before manure had been applied, from the layer 0-10 and 10-20 cm. An area of 9 m² was supplied with 1 L m^{-2} of pig manure (10 m^3 ha^{-1}, 2% dry matter content) that had been spiked with tetracycline and chlortetracycline. Resistance genes were determined in a sample of manure before spiking. The concentrations of tetracycline and chlortetracycline in manure were 9.5 and 6.4 mg kg^{-1} pig manure for the sandy soil, and 10.0 and 8.9 mg kg^{-1} for the silty clay, respectively. Both soils were sampled within 2 hours after slurry application. Soils were stored at –30ºC until DNA extraction.

DNA extraction and polymerase chain reaction

DNA was extracted from 0.5 g of soil by using a commercial kit (FastSpin kit for soil, Qbiogene, Irvine, CA) according to the specifications of the manufacturer with slight modifications (centrifugation for 2 min after bead-beating, and elution in 70 µL water). Genomic DNA from manure samples was extracted using a commercial kit (QIamp® DNA Stool Mini Kit, Qiagen, Valencia, CA), following the instructions of the manufacturer. Raw DNA was purified by use of a resin column (Wizard DNA clean-up system, Promega, Leiden, the Netherlands) following the protocol of the manufacturer, apart from that 70 µL water were used for DNA elution.

The concentration of DNA in the extracts was determined for selected samples by comparing the fluorescence of PicoGreen® amended DNA with the fluorescence of a standard curve generated from phage λ DNA (PicoGreen® dsDNA kit, Molecular Probes, Breda, the Netherlands).

Amplification of tetracycline resistance determinants by polymerase chain reaction (PCR) was based on a primer set described previously by Aminov et al. (Aminov et al. 2001, Aminov et al. 2002). The determinants tested in this study included *tet*(A), *tet*(B), *tet*(C), *tet*(E), *tet*(H), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), *tet*(W), *tet*(Y), and *tet*(Z). Sulfonamide primers were taken from (Grape et al. 2003) (*sul*(I)), (Chu et al. 2001) (*sul*(II)), and (Perreten & Boerlin 2003) (*sul*(III)). PCR was typically performed in a volume of 25 µL, with 10-40 µM of each primer, 200 µM for each deoxynucleoside triphosphate, 1.25 U of Expand Long Template enzyme mix containing Taq and Tgo DNA polymerase (Roche Applied Sciences, Almere, the Netherlands) and its accompanying reaction buffer 2. Genomic DNA (1 µL, undiluted) served as template. As positive control, DNA extracts of control strains harbouring tetracycline resistance genes described in (Aminov et al. 2001, Aminov et al. 2002) were used. Deionised water served as negative control. Reactions were performed in a Hybaid PCR Express or Hybaid Px2 Thermal Cycler, with PCR amplification (25 cycles) consisting of initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 20 s, 30 s of annealing at the annealing temperatures shown in Table 1, 50 s of extension at 72°C, and a final extension step at 72°C for 7 min. Aliquots (5 µL) were analysed by gel electrophoresis on agarose gels (2.5%, Cambrex, Verviers, Belgium) after staining with SybrGold (Molecular Probes, Breda, the Netherlands. Further, in order to prevent false-negative results due to PCR-inhibiting substances such as humic acids, a second DNA extraction was performed for the Swiss field-soil samples that were negative in the first PCR round, and PCR reactions were additionally undertaken for the new soil DNA extracts and for a 10 fold dilution of the original extracts.

For the microcosm samples, a first round of PCR reactions was performed for selected samples (one control and three samples with high concentrations of antibiotic spiked to the soil), and only the tetracycline resistance determinants that gave positive results in at least one sample in the first round were analysed in all microcosm samples.

RESULTS

First microcosm experiment – effect of spiking with oxytetracycline over time

The first microcosm experiment revealed that the amount of detected genes was nearly constant over the whole oxytetracycline dose range. The gene diversity was thus not strongly influenced by an additional spiking of the slurry-amended soils with oxytetracycline (Table 1 and Figure 1). Analysis of the resistance pattern over time showed that some genes, that gave strong signals after 6 days exposure to manure and oxytetracycline, were less clearly detectable directly after soil spiking and in the control soil without oxytetracycline amendment at day 14. Resistance determinant *tet*(C) was not consistently found on both days, *tet*(T) was not detected at day 0, and *tet*(H) and *tet*(Q) were not found at day 14. Further,

tet(Y) seemed to disappear after 14 days of soil exposure in all oxytetracycline treatments (although it gave a weak signal in the control soil). At day zero, directly after application of oxytetracycline and manure, the resistance gene abundance was slightly lower than at day 6.

Second microcosm experiment – effect of manure versus spiking with antibiotics

When testing soil and manure separately, relatively few genes were found in soil samples before tetracycline and manure treatment, but the pig manure contained all 12 tetracycline resistance genes that it was tested for (Table 2 and Figure 2). This difference persisted after setting up the microcosms. Three tetracycline resistance genes were detected both in the water-treated control soil after 7 days and in the soil before treatment. In the water-treated soil, though, there was a weak signal for *tet*(Y), but *tet*(Z) could not be detected. In the manure-treated soils, about 6-8 *tet* resistance genes were found, all of which had also been detected in the manure. Among the genes that failed detection, *tet*(S) was the most frequent. There was no apparent relationship between the tetracycline or oxytetracycline spiking concentration and the resistance pattern.

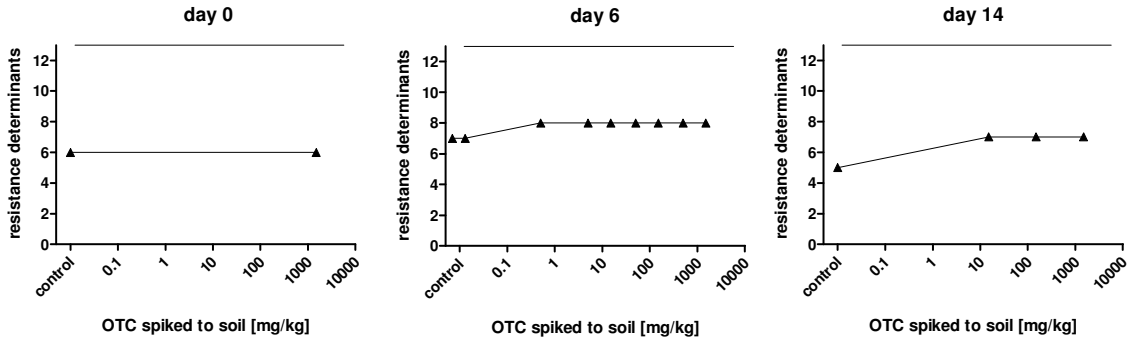

Figure 1: Number of positive findings among 13 tetracycline resistance genes (see Table 1) in the first microcosm study with slurry-amended soils, additionally spiked with oxytetracycline. The microcosms have been analysed three times, directly after setup of the microcosm (day 0), after one week, and after two weeks.

Table 1. Tetracycline resistance in soils microcosms spiked with pig manure and oxytetracycline. First microcosm experiment: influence of soil exposure duration. Results of positive findings of tetracycline resistance determinants per soil sample and oxytetracycline concentration, after 0, 6 and 14 days of soil exposure. *Tet*(B), *tet*(E), *tet*(M), and *tet*(O) had not been found in selected samples with high spiking concentrations.

soil exposure [days]	0		6									14			
OTC concentration [mg kg^{-1}]	0	1500	0	0	0.5	5	15	50	150	500	1500	0	15	150	1500
tet(C)	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+
tet(H)	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
tet(Q)	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
tet(S)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(T)	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
tet(W)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(Y)	-	+	+	-	+	+	+	+	+	+	+	+	-	-	-
tet(Z)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

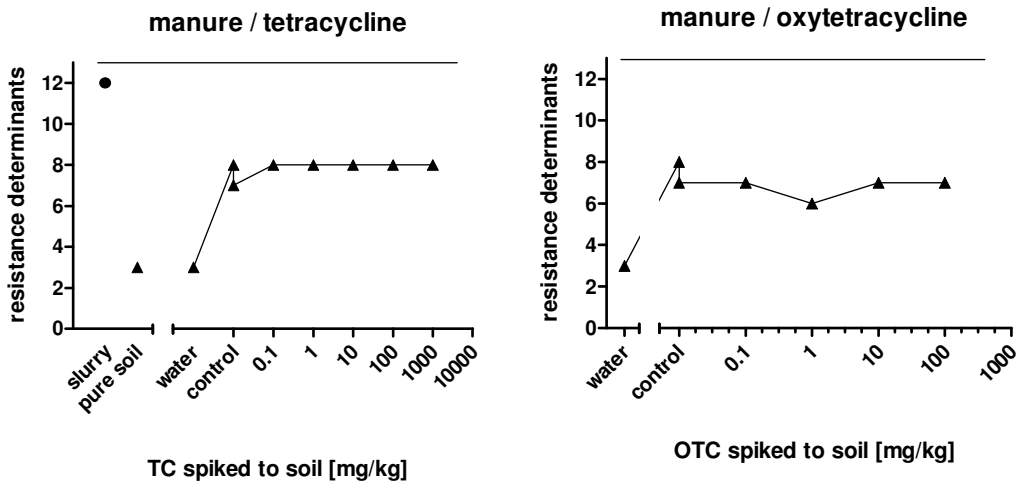

Figure 2: positive findings among 13 tetracycline resistance genes. Second microcosm study: influence of manure, tested with oxytetracycline and tetracycline amendment. Slurry and pure soil: material used to set up the microcosms. Water: soils amended with water in place of pig manure, control: soils amended with pig manure only, all other treatments: soils amended with pig manure and additional antibiotics

Table 2. Tetracycline resistance in soils microcosms spiked with pig manure and oxytetracycline or tetracycline. Second microcosm study: influence of manure versus antibiotic amendment. Detailed results of positive findings of tetracycline- resistance determinants per soil sample. Pure soil: soil before microcosm setup, slurry: slurry before microcosm set up, water control: control without slurry amendment after 8 days, slurry control: soil with slurry amendment after 8 days, oxytetracycline and tetracycline treatments: after 8 days exposure with slurry amendment. In pig manure, *tet*(B), *tet*(E), *tet*(M), and *tet*(O) were also found. These four were also tested in selected soil samples (highest or second highest OTC and TC treatments), but were not found in any of these.

	control							Oxytetracycline [mg kg^{-1}]				Tetracycline [mg kg^{-1}]				
	Pure soil		slurry		water	slurry		0.1	1	10	100	0.1	1	10	100	1000
tet(C)	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
tet(H)	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
tet(Q)	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+
tet(S)	-	-	+	+	-	+	-	+	-	-	-	+	+	+	+	+
tet(T)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(W)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(Y)	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
tet(Z)	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+

Field experiments – time course of resistance genes after manuring of a Swiss soil

The manure used in the Swiss field experiment stemmed from a farm where chlortetracycline, sulfamethazine and tylosin are used on a regular basis. A great variety of resistance determinants were found (Table 3 and Figure 3). All three sulfonamide and eleven tetracycline resistance genes were detected. However, *sul*(III) and *tet*(Z) were only detected in one of the manure samples.

The soil samples taken before manure application showed a varying diversity of resistance genes: The samples taken in autumn of the previous year gave only weak signals for many resistance genes, and *tet*(O), *tet*(M), *tet*(B) and *tet*(S) and *sul*(III) could not be detected in these soils (Figure 3, Table 3). In contrast, the spring soils two weeks before manure application gave signals for all resistance determinants except *sul(*III) and *tet*(O). There was little difference between the soil samples taken before and after both manuring events, in spring and summer.

Most resistance genes were consistently detected over the whole sampling period. Only *tet*(M) gave no signal in autumn, one day before the second manuring (day 45), and on day 56 and 84. Further, *tet*(B) and *tet*(S) once gave a negative signal after manuring. When calculating the total number of genes detected, there is thus little variation between all soil samples, with the exception of the autumn samples (day –199 in Figure 3).

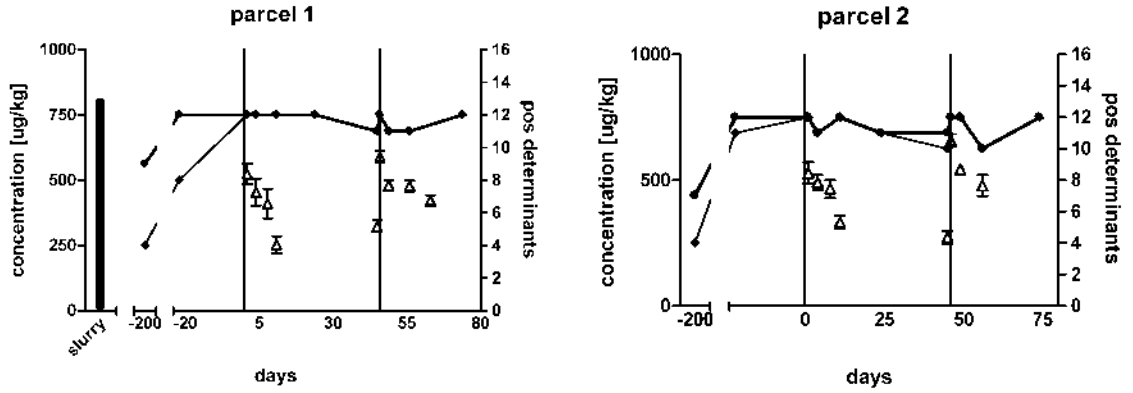

Figure 3: Sulfonamide and tetracycline resistance on two parcels of a Swiss manured field soil. Total counts of positive (circles) and weak-positive (diamonds) findings among 14 tetracycline- and 3 sulfonamide resistance genes in two adjacent meadows of the Swiss field study. Pig manure has been applied twice, on day 0 and day 46, indicated by a solid line. The resistance genes determined in the pig manure are given as solid bars. The results of the analytical determination of sulfamethazin in soil, which was used in the treatment of the pig herd, are also given (open triangles).

Table 3. Sulfonamide and tetracycline resistance in two manured parcels of a Swiss field soil. Detailed results of positive and weak-positive findings (strong PCR bands: +, weak PCR bands: (+)) of tetracycline- and sulfonamide resistance determinants per soil sample and time. The determinants are ordered according to the frequency of positive findings. Slurry gives the determinants in the slurry of the first application (day 0) and second application (day 46).

	Slurry		Parcel 2												Parcel 1											
days	0	46	-199	-13	1	4	11	24	45	46	49	56	73	86	-199	-13	1	4	11	24	45	46	49	56	73	86
sul(I)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(II)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
sul(III)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tet(B)	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
tet(C)	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(H)	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+
tet(M)	+	+	-	(+)	+	+	+	+	-	+	-	-	+	-	-	+	+	+	+	+	-	+	+	-	+	-
tet(O)	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tet(Q)	+	+	(+)	+	+	+	+	+	+	+	+	+	+	(+)	(+)	+	+	+	+	+	(+)	+	+	-	+	+
tet(W)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(S)	+	+	-	(+)	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+
tet(T)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tet(Y)	+	+	(+)	(+)	+	+	+	+	+	+	+	+	+	-	-	(+)	+	+	+	+	+	+	+	+	+	+
tet(Z)	-	+	(+)	(+)	+	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+

Field experiments – resistance in agricultural soils in Germany

Ten tetracycline and sulfonamide resistance determinants were detected in a soil that had received pig manure in the last two years, among them *sul*(I), *sul*(II), *tet*(Y), *tet*(S), *tet*(C), *tet*(Q), *tet*(T), *tet*(H), *tet*(Z) and *tet*(W) (Table 4). In pig manure used for manuring this soil, all of these genes were found, plus *tet*(M) and *tet*(B). These genes were however not found in the soil two hours after manuring. The soil under cow manure amendment was sampled directly before and 212 days after manuring in order to get an impression of the background of resistance present in this soil. In this soil, *tet*(S) and *tet*(C) were not detected.

DISCUSSION

First microcosm experiment – effects of manure versus spiking with antibiotics

In previous studies using the exact same soil samples as in this study, the oxytetracycline-spiked soil samples showed profound community changes (Schmitt et al., submitted for publication).

Table 4. Sulfonamide and tetracycline resistance in manured field soils in Germany. Detailed results of positive findings of tetracycline- and sulfonamide resistance determinants per soil sample and time. The determinants are ordered according to the frequency of positive findings.

	Pig manure history			cow manure history			
				1 hour before manuring		212 days after manuring	
	1 hour before manuring	Pig manure	2 hours after manuring	0-10 cm	10-20 cm	0-10 cm	10-20 cm
sul(I)	+	+	-	+	-	-	+
sul(II)	-	+	+	+	+	+	+
sul(III)	-	-	-	-	-	-	-
tet(A)	-	-	-	-	-	-	-
tet(B)	-	+	-	-	-	-	-
tet(C)	+	+	+	-	-	-	-
tet(E)	-	-	-	-	-	-	-
tet(H)	+	+	+	+	+	+	+
tet(M)	-	+	-	-	-	-	-
tet(O)	-	-	-	-	-	-	-
tet(Q)	+	+	+	+	+	+	+
tet(S)	+	+	+	-	-	-	-
tet(T)	+	+	+	+	-	+	+
tet(W)	+	+	+	+	-	-	+
tet(Y)	+	+	+	+	+	+	+
tet(Z)	+	+	+	+	+	-	+

There, an increase in the community tolerance to oxytetracycline was observed at a concentration of 1.3 mg kg^{-1}, and changes in the molecular diversity were also apparent, as seen in a 16S rDNA denaturing gradient gel electrophoresis analysis (Schmitt et al., submitted for publication). Thus, there is a surprising divergence between the community changes observed previously and the lack of an oxytetracycline effect on the diversity of tetracycline resistance genes in this investigation (Figure 1).

One reason could be that the resistance genes introduced with manure outnumbered the resistance genes present in the soil, possibly masking an increase in the soil-borne genes upon spiking with oxytetracycline. PCR as a qualitative technique will overlook quantitative increases in gene prevalence upon soil antibiotic exposure. Manure had been applied to stimulate bacterial growth and therewith to provoke the bacteriostatic effects of oxytetracycline. As the manure was sampled from a farm with a low-use profile of antibiotics, a high input of resistance genes with the manure was initially not expected. Second, it is possible that other tolerance mechanisms are more important for the tolerance increase observed. There are over 30 tetracycline resistance genes known today (Chopra 2001), and other more general defense mechanisms exist, such as multidrug efflux pumps (Magnet et al. 2001). Still, it is interesting that clear community tolerance changes seem to occur without changes in the diversity of a wide range of tetracycline resistance genes.

Second microcosm experiment – effects of manure versus spiking with antibiotics

In order to test whether manure served as a source of resistance genes, slurry and soil used to set up the microcosms were tested apart in a follow-up experiment. There, manure amendment indeed turned out to be the most dominating factor for the soil content of resistance genes (Figure 2). Several tetracycline genes (*tet*(S), *tet*(C), *tet*(Q) and *tet*(H)) were introduced into the soil via manure. Again, in spite of community changes observed with the PICT methodology and in selective plating experiments (Figure 2, Schmitt et al., submitted for publication), no tetracycline-related changes in resistance gene diversity were observed.

In the manure, all 12 tetracycline resistance genes that were tested for were positive. The manure had been sampled from a pig that had not received antibiotic treatment during at least the previous 1.5 months, and originated from a herd where tetracyclines are only used therapeutically in an incidental manner and in a strictly controlled fashion. Resistance in healthy swine and low-use or no use circumstances has also been documented occasionally (Lee et al. 1993, Mathew et al. 1999, Heuer et al. 2002, Stanton & Humphrey 2003, Stanton et al. 2004), maybe due to the persistence of resistance genes after ending their use (Smith 1975). Even swine feed components might provide a source for resistance genes (Aminov et al. 2001). However, resistance in bacteria from farm animals has more frequently been linked to the usage patterns of antibiotics (Huysman et al. 1993, Mathew et al. 1999, Lanz et al. 2003, Agersø et al. 2004, Bryan et al. 2004, Jackson et al. 2004, Rajic et al. 2004).

Still, there were indications that the antibiotics contributed to the persistence of the genes in the soil microcosms. Between 6 and 14 days of soil exposure, the diversity of resistance genes declined most in the manured soil without additional oxytetracycline spiked. The genes not detected on day 14 (*tet*(C), *tet*(Q), *tet*(H) and *tet*(Y)) were identical with the ones that were

absent or only limitedly found in pure soils and in control soils without pig manure amendment. Possibly, spiking with 15 mg kg^{-1} oxytetracycline maintains the selection pressure necessary for survival of their hosts in soil, or for horizontal transfer. The genes *tet*(C), *tet*(Q) and *tet*(H) have been frequently detected in isolates associated with the gastrointestinal tract of pigs or other farm animals, such as *Bacteroides* and *Prevotella*, gram-negative enterobacteria, and *Pasteurella* (Lee et al. 1993, Hansen et al. 1996, Leng et al. 1997).

In addition, there was a slight increase in the resistance gene prevalence between day 0 (directly after manure and oxytetracycline application) and day 6. This might suggest preferential growth of the bacteria carrying these resistance genes in the soil samples, which is enhanced in the samples spiked with oxytetracycline.

Not all resistance determinants detected in the manure were found in the soils. The prevalence of *tet*(B), *tet*(M), *tet*(E) and *tet*(O) in the pig manure could have been so low that they fell below the detection limit when diluted into the soil, or the bacteria carrying these genes might have disappeared upon the change to an aerobic environment. *Tet*(E) mostly resides on nonmobile plasmids, making its transfer to soil bacteria unlikely (DePaola & Roberts 1995, Chopra 2001). For *tet*(M) and *tet*(B), the detection in pig manure but not in soil is more surprising, given that they have the broadest host range known among the tetracycline efflux genes and can be carried on mobile plasmids (Chopra 2001). It remains thus difficult to conclude from the occurrence of a certain gene in cultured and identified species to the likelihood of occurrence or persistence in other environmental media.

A range of genes seems to be ubiquitous both in the soil and pig manure samples tested: *tet*(T), *tet*(W) and *tet*(Z). One of those genes, *tet*(W), has been detected in bacteria of the gastro-intestinal tract (such as the obligate anaerobes *Butyrivibrio* (Roberts 2003) and *Megasphaera* (Stanton et al. 2004)) but also in bacteria typically connected with the soil habitat (such as *Actinomyces*, *Streptomyces*, and *Bacillus*) ((Chopra 2001) and updates of information therein at http://faculty.washington.edu/marilynr/). *Tet*(T) has only been found in the pathogen *Streptococcus pyogenes* up to now (Clermont et al. 1997), but for *tet*(Z), the occurrence in the soil bacterium *Corynebacterium* has been proven (Tauch et al. 2000).

Field experiments

The effect of manure fertilization was also investigated in two field studies. The first study was located in Switzerland, on grassland that had received pig manure from non-intensive farming during the last years. Antibiotics were not detected in the soils previous to the experiment. For the field study, manure from a farm operation with routine antibiotic application was used. Again, pig manure proved to be a reservoir of resistance genes: all tested genes gave positive signals. Both tetracycline and sulfonamide resistance genes were tested, as sulfamethazine had been used in addition to chlorotetracycline during swine rearing. However, this time, an influence of manuring on the soil diversity was not apparent. The main reason was the high prevalence of resistance genes in the soils already before pig manure application. Only a few genes that were detectable in pig manure did not reach concentrations high enough to be detected in soil: *tet*(O) and *sul*(III).

However, an indication for an impact of manuring on the resistance gene quantity might be inferred from the intensity of the PCR bands. Some genes found both in pig manure and soils were detected with only a weak signal in the spring soil sample of one plot before pig manure spreading, but gave strong signals after manuring (*tet*(M), *tet*(S), *tet*(Y) and *tet*(Z)) (Figure 4). Further, *tet*(M) and *tet*(S) had not been detected six months before pig manure spreading, and at some later sampling events. These genes might reside in soil at quantities close to the detection limit, and their prevalence might increase after fertilisation. Interestingly, these results mirror the findings from the microcosm experiment, where *tet*(M) was only detected in pig manure, and *tet*(S) and *tet*(Y) only in pig manure and manured soil samples. In order to confirm quantitative change upon manuring in resistance determinants, real-time quantitative PCR analysis is underway.

The only clear changes in gene prevalence were apparent between the sample taken half a year before pig manure spreading in September and all other samples, taken between March and June in the following year. The smaller prevalence of tetracycline resistance genes might be related to a different soil nutrient status and a different standing community of microorganisms. Profound changes in the bacterial community in one soil over the different seasons have been found earlier (Andersen et al. 2001, Smit et al. 2001, Griffiths et al. 2003).

We can only speculate about the reasons for the high prevalence of resistance genes in the Swiss soils. The fields had been fertilised with manure from extensive pig farming in the previous years, and it was only in the year of study that pig manure from a high-usage farm was used. For habitats other than soil, it has been found that a higher prevalence of resistance genes was due to a faecal contamination (Andersen & Sandaa 1994, Jensen et al. 2001, Jensen et al. 2002, Onan & LaPara 2003, Agersø et al. 2004). However, it has also been suggested that antibiotic resistance genes would be dispersed globally (Witte 1998). Further, soil might represent a natural reservoir of resistance genes (Riesenfeld et al. 2004), as it is generally thought that antibiotic resistance genes originate from soil-borne antibiotic producers.

In the second field study, located in an area with intensive farming in Germany, the number of positively detected resistance genes was smaller than in Switzerland. Analytical studies in the same region had shown that little tetracycline residues were found in cows' manure, whereas pigs' manure regularly contained concentrations of tetracyclines in the range of mg kg^{-1} (Höper et al. 2002). A higher antibiotic treatment frequency in pig farms would lead to a higher selection pressure and possibly resistance gene prevalence in pig manure. Indeed, the number of tetracycline resistance genes detected was smaller in soils under cow slurry than under pig slurry fertilisation in our study. There was some overlap between the genes found frequently in the German field study and in the microcosm experiments: *tet*(W), *tet*(T) and *tet*(Z) were found in un-manured soils in the microcosm experiment, and also in field soils before the first fertilization of the year.

Again, an impact of manuring on the diversity of resistance genes could not be detected. Still, the difference between gene prevalence in the soil and in the manure was small, as only *tet*(M) and *tet*(B) were additionally detected in the manure, but not in the soil. These were two genes that also failed detection in the microcosm soil despite occurrence in the manure.

Overall, differences between tetracycline resistance gene occurrence in environments and regions have been shown. This can also be seen from the comparison with other investigations. In non-manured soils, Hund-Rinke et al. (Hund-Rinke et al. 2004) reported only incidental findings of *tet* resistance genes, which is in contrast to our studies. Furthermore, in their study, *tet*(M) and *tet*(O) were detected in manured microcosms, while we only detected *tet*(O) in manure. Our limited detection of *tet*(M) in the microcosm and one field experiment is also in contrast to a study on resistance genes in bacteria of the Bacillus group in a manured farm soil in Denmark, where *tet*(M) was most prevalent (Agersø et al. 2002). There is one caveat, though, as detection limits have not been determined in most of these studies, and differences might thus also be caused by diverging sensitivities of the PCR reactions. Most notably, there are also many congruencies, especially with an investigation on groundwater samples impacted by a pig farm (Chee-Sanford et al. 2001, Aminov et al. 2002): *tet*(W) and *tet*(Q) are elements detected in all studies when tested, and *tet*(H), *tet*(T), *tet*(Z) and *tet*(M) were regularly observed in at least 4 out of the 7 locations (Chee-Sanford et al. 2001, Agersø et al. 2002, Aminov et al. 2002, Hund-Rinke et al. 2004).

In conclusion, manure proved to be the determining factor for the diversity of resistance genes in microcosm studies. The contribution of even high concentrations of antibiotics to the resistance gene diversity was comparatively minor, although the antibiotics caused other community adaptations. This suggests that other tolerance mechanisms dominate in the microcosms, or that the community tolerance is induced by quantitative changes in the resistance gene prevalence. In contrast, we failed to detect a clear influence of manuring on the diversity of tetracycline resistance genes in field soils. The main reason was the similarity of the resistance gene patterns between manure and soils in both regions, such that an additional impact of the manure was difficult to observe. The diversity of tetracycline resistance in some soils was high. This could be caused by a global spread of resistance genes, or it could highlight the role of soils as a natural reservoir of resistance genes.

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CHAPTER 8
GENERAL DISCUSSION

Once the cow is out of the barn, closing the door will not get it back

Lipsitch et al. 2002

OUTLINE

In this thesis, the effects of veterinary antibiotics on soil microbial communities were investigated. In the general discussion, three main issues will be addressed. First, the test methodology applied in this study will be evaluated. Advantages and drawbacks of these toxicity tests in environmental toxicology will be highlighted, resulting in comments on their adequacy for the effect assessment of antibiotics. Second, a synopsis of the effects observed and the compounds investigated will be presented. Third, the effects will be evaluated with regard to their relevance from a risk assessment point of view.

EVALUATION OF METHODOLOGY APPLIED

Environmental microbiology and its limits

The soil habitat is marked by an extremely high diversity- each gram of soil might contain more than 10 000 species of bacteria (Torsvik et al. 1990, Torsvik et al. 1998). Any attempt to approximate the impact of toxicants on this vast community by studying the effects on a single selected species is thus in vain. Microbiological tools aimed at studying the whole community exist, but are subject to a methodological debate. The "great plate count anomaly" (Staley & Konopka 1985) is the reason for this debate. Only a small proportion of environmental bacteria can be cultured under laboratory circumstances and therewith be studied in depth (Amann et al. 1995). In soil, this proportion ranges from less than 1 to approximately 10% (Balestra & Misaghi 1997, Dykhuizen 1998, Janssen et al. 2002). Molecular techniques have been claimed to be able to overcome the bias towards the so-called culturable bacteria, by extracting DNA from the whole community, and analysing inter alia the 16S rDNA or rRNA sequences (Woese & Fox 1977).

This thesis combined the two approaches. We studied antibiotic effects in assays limited to the culturable part of the community, and applied molecular techniques to investigate the general genetic diversity and the diversity of selectable genetic traits. In order to help an evaluation of the results, limitations and advantages of each method will be discussed in the following paragraphs.

Bacterial community testing based on substrate utilisation in multiwell plates (Biolog)

Biolog plates provide a highly automated method for studying the respiration pattern of bacteria. They consist of multiwell plates filled with a range of carbon sources, and were originally designed for the characterisation of single bacterial strains (Bochner & Savageau 1977), but are now frequently used for community analyses. This application is not without controversy. It was observed that the number of strains in single wells after 48 hour incubation is smaller than the number of strains detected in the inoculum, and CFU analyses show population growth during incubation (Smalla et al. 1998). It was concluded that selective growth takes place during incubation, a concern also discussed by Preston-Mafham (2002). In fact, it would be surprising if selective growth would not occur, given that growth is also a normal response of indigenous soil microbial communities to triggers such as fertilisation (Frostegard et al. 1997). But surely, care must be taken that bacterial growth does

not mask possible toxicant effects, if adaptive processes take place during the assay (van Beelen et al. 1991).

To summarise the debate, any method based on Biolog plates reveals information on 'the diversity of organisms capable of rapidly growing on substrates under culture conditions' (Smalla et al. 1998), with a bias towards Gram negative species (Heuer & Smalla 1997). Still, 'the results obtained will ultimately reflect the response of a portion of the original inoculum' (Guckert et al. 1996, Ellis et al. 2001).

It has also been argued that changes in the fast-growing part of soil microbial communities can be detected more sensitively than changes in the numerically dominant community members (Dunfield & Germida 2003). Due to their higher activity, the culturable bacteria might sometimes react more sensitive to stressors (Ellis et al. 2003). The active part of the bacteria might thus be worth testing in its own right (Girvan et al. 2003), with the loss of diversity of functional groups as an undesirable effect (Lawrence et al. 2004).

Community-level physiological fingerprint (CLPP)

The main application of Biolog plates in bacterial community studies is to generate profiles of substrate utilisation or so-called community level physiological fingerprints (CLPP). In this thesis, the effects of sulfachloropyridazine, oxytetracycline, tylosin, and tetracycline have been studied with CLPP (Chapter 2, 3, 4, 5 and 6). However, a few additional remarks can be made about the applicability of CLPP for the detection of toxicant effects.

CLPP, such as any other detection method for rather general parameters of communities, bears the disadvantage that any parameter varying between locations or treatments would influence the CLPP pattern, such as soil humidity or nutrient status (the latter shown in Chapter 3). On the other hand, the variance explained by the different environmental parameters can also serve to scale the toxicant impact with respect to other soil properties.

There are also a few technical concerns with CLPP analyses. As outlined in a recent review (Preston-Mafham et al. 2002), standardisation of the inoculum density and data treatment are most urgent subjects for debate. Still, these can be overcome with the methodology set up at the Netherlands' National Institute for Public Health and the Environment (RIVM), described in Chapter 2 and applied in Chapter 3, 5 and 6.

Further, CLPP analyses have been observed to be less sensitive to toxicant-induced changes, when compared to other techniques (Kelly et al. 1999, Bundy et al. 2002, Brandt et al. 2004, Tandy et al. 2005). However, this could also be related to different ways of data handling (Chapter 2). Still, in our own investigations, CLPP was also less sensitive for the detection of antibiotic effects than PICT for a range of compounds - despite the use of the sensitive inoculum-dilution approach (oxytetracycline (Chapter 5) and tetracycline (Chapter 6)).

There has been a considerable debate as to whether the CLPP method yields information on community function. (Garland & Mills 1991, Ellis et al. 1995). It is now mainly agreed on that the functional potential displayed in the CLPP assays does not reflect the actual environmental functions of a community, but that CLPP information is still of value for comparing the similarity of communities (Mills & Garland 2002). This is due to the selective

conditions in the plate, as discussed above. However, the CLPP patterns of sludge-impacted soils were partially correlated with patterns of in situ respiration of a range of substrates, the latter representing a true functional assay (Campbell et al. 2003). If this was regularly the case, CLPP might have a potential to be used as a surrogate for a functional test even if not being a functional assay in itself.

With respect to the investigations of this thesis, we conclude that CLPP analyses can be used to show structural differences between the microbial communities of environmental samples. Still, the ecological meaning and relevance of these differences is not completely understood. CLPP might not be the most sensitive method for the detection of toxicant effects, but it is a relatively quick and easy technique to perform. CLPP has shown to be of value for the detection of toxicant effects in the active part of the community (see Chapter 1 for references). As CLPP is influenced by many parameters, we advise that proper control sites be used, when effects of a toxicant are investigated in field tests. In this respect, the application of CLPP in controlled environments such as microcosms (Chapter 2, Chapter 4, Chapter 5) is less controversial –to study, for example, the influence of a toxicant as sole variable.

Pollution-induced community tolerance (PICT)

It has been shown in Chapter 4, 5 and 6 that pollution-induced community tolerance (PICT) can be used as a methodology to detect the effects of antibiotics on soil microbial communities. However, some technical drawbacks have not been mentioned explicitly there, which should be taken into account when planning a test strategy for soil ecotoxicology.

First of all, in depth PICT testing applied in the studies of this thesis is neither quick nor cheap. As with any investigation based on the PICT methodology, a complete dose-response curve has to be tested for each environmental sample in place of one single test. For a microcosm dose-range study, about 100 plates have been used in the set-up described here, and the price for a single Biolog ECO-plate is approximately 14 Euro in 2005, not included labour. Cost calculations will therefore frequently forbid testing of more replicates than the three pseudo-replicates convened for in the Biolog ECO-plate.

Second, tolerance testing in Biolog plates requires detailed and careful data evaluation. In Chapter 4, 5 and 6, the correct inoculum dilution for the PICT detection step was identified in preliminary serial dilution experiments. Still, in each experiment, there was at least one dilution for which the divergence between the estimated and real colouring intensity in the PICT detection step was so big that erroneous EC_{50} values were determined (as discussed in Chapter 3). Rules for the exclusion of inaccurate measurements would profit from a more general investigation of the dependence of tolerance determination on the inoculum density, beyond work described in Chapter 3.

As described above, the determination of substrate utilisation in Biolog plates is accompanied by bacterial growth. This, in turn, might be a methodological drawback linked to the theory of PICT. The long incubation time could facilitate community changes in the PICT detection step - through enrichment of bacteria tolerating both the substrate and the toxicant (Blanck 2002). We argued in Chapter 3 that such a second succession could also increase the

sensitivity of the detection method, if it proceeds faster in the toxicant-adapted communities. However, we ultimately agree that the original tolerance distribution might be skewed by growth in Biolog plates. Still, in the special case of bacteriostatic antibiotics, the short-term PICT detection step might even require bacterial growth in order to observe a meaningful dose-response relationship at all, as discussed in Chapter 3 and a range of other publications (Backhaus et al. 1997, Froehner et al. 2000, Thiele & Beck 2001, Kümmerer et al. 2004). Indeed, in our experiments, an increased incubation time was found to increase the sensitivity of the PICT approach (Chapter 3).

The most interesting feature of PICT remains its causality: theoretically, PICT is exquisitely suited for the identification of toxicant-induced effects, since few parameters are expected to be able to influence community tolerance as strongly as the toxicant itself. Indeed, since the beginning of its application, PICT has been claimed to be a highly specific method for the detection of toxicant effects in the field (Blanck et al. 1988, Díaz-Raviña & Bååth 1996, Bååth et al. 1998a, Rutgers et al. 1998a, Rutgers et al. 1998b, Blanck 2002). However, it has also been recognised early that the occurrence of co-tolerance could limit the causality of PICT. A way to systematically deal with co-tolerance is to describe it as a graded phenomenon, dependent on the similarity of mechanisms of action or tolerance mechanisms (Blanck & Molander 1991). Unfortunately, such theoretical reasoning is yet hampered by a lack of information on common tolerance mechanisms. This is illustrated by the fact that "unexpected" co-tolerances have been documented, such as co-tolerance of algal communities adapted to diuron to TBT and vice versa. TBT and diuron are two compounds with different mechanisms of action, and no common tolerance mechanism is known (Molander et al. 1992). Chapter 6 thus concludes that co-tolerance still has to be evaluated on a case-by-case basis, when co-tolerances are expected. Still, the limited co-tolerance between antibiotics with different modes of action found in Chapter 6 contributes to diminishing the concern that co-tolerance would be frequent with bacteria. This concern was expressed with respect to the ease of exchange of genetic material and the possible co-location of genes coding for different resistance mechanisms on mobile genetic elements (Blanck et al. 1988).

Linked to the specificity of PICT is its potential to be a sensitive endpoint for the detection of toxicant effects. A congruence between PICT and other methods has been found for the sensitivity of single species (van Beelen et al. 2001), for substrate respiration (Witter et al. 2000), and for metal effects on PLFA patterns (Bååth et al. 1998a, Bååth et al. 1998b). These studies indicate that PICT is certainly not less sensitive than these community endpoints. In fact, PICT has proven to be the clearest and most constant effect indicator for all our compounds tested in our studies (Chapter 4, 5 and 6). Most interestingly, PICT has been observed for oxytetracycline in spite of missing effects on resistance gene diversity. Tylosin led to PICT effects, but changes in the molecular diversity (16S ribosomal DNA denaturing gradient gel electrophoresis, 16S rDNA DGGE) were only minor. In a field study with metals, PICT has also been shown to be more sensitive than a wide range of other tests (van Beelen et al. 2001).

To summarise, PICT analyses have turned out to be a sensitive and laborious tool for the detection of antibiotic effects. It is concluded that the tests are not hampered by selective

growth of the communities in the plates, as antibiotics require testing under growth circumstances. Further, co-tolerance proves not to restrain the detection of effects, at least in laboratory settings. Still, the ultimate advantage of PICT testing, namely its specificity in field situations, has not been made use of in thesis, as effects expected at field concentrations were thought to be small.

Molecular methods - general

The advent of molecular techniques has opened up new opportunities to study soil microbial communities (Muyzer 1999, Kent & Triplett 2002). However, it is recognised that molecular methods targeting the whole microbial community are bound to their own biases and limitations, as summarised below (v. Wintzingerode et al. 1997, O'Donnell & Görres 1999, Kanagawa 2003). Still, efforts are being made to overcome the biases linked to PCR-based techniques, and new applications linking structure and function of microbial communities are emerging (Wellington et al. 2003, Handelsman 2004).

Microbial diversity detected from 16S rDNA

The application of molecular detection methods has without doubt revolutionised the analysis of the diversity of the microbial world. An illustrative example was the observation that a then unknown and uncultured marine bacterium, SAR11, whose 16S rDNA was first detected in the Sargasso Sea (Giovannoni et al. 1990), proved to be one of the most abundant micro-organisms on Earth (Morris et al. 2002b). The picture of soil bacterial diversity has been changed to a comparable extent (Kent & Triplett 2002, Oren 2004, Schloss & Handelsman 2004). Still, recent work also suggests that biases in widely used procedures might lead to an overestimation of bacterial biodiversity by a factor of 2.4 (Acinas et al. 2004), highlighting the need to shortly reflect on methodological shortcomings.

First of all, one pitfall common to each polymerase chain reaction (PCR)-based technique is an incomplete or biased extraction of DNA from soil samples, depending on bacterial size or cell wall structure. Still, some extraction protocols have found extraction efficiencies in the order of 90%, and little bias towards gram negative bacteria (van Elsas et al. 1997).

Second, possible mismatches between the primers used to target 16S rDNA and the template DNA would lead to a decrease in PCR efficiency (Brunk et al. 1996, Baker et al. 2003). “Universal” primers are thus a prerequisite for a proportionate detection of all templates. Further, for a small template diversity, a wrong representation of rare and numerous species might occur (Suzuki & Giovannoni 1996). Other artefacts consist of the so-called chimeras (an extension of two highly homologous DNA sequences) and heteroduplexes composed of two heterogeneous genes (Speksnijder et al. 2001). Misincorporations can occur in the extension step, and the total error rate for misincorporations, heteroduplexes and chimeras can be as high as 20% (Qiu et al. 2001). As an example for possibly biased results, Leser et al. (2002) found that one out of two replicate clone libraries of one environmental sample failed to detect the whole *Bacteroides* group, which constituted 22% of the other clone library, although the coverage of both libraries was greater than 64%. Last, the number of rDNA copy

numbers varies between 1 and about 12, such that one species can be represented by multiple bands in one 16S rDNA DGGE gel (Fogel et al. 1999).

Most important, though, is the fact that DGGE gels have a limited resolution of about 100 bands, due to both the sensitivity of staining, the resolution power of the gradient and the detection limit for rare species in the community. Thus, the DGGE gels only detect the most numerous members of the community, which are present in proportions greater than about 0.5-1.5% (Gelsomino et al. 1999). Another caveat of diversity analyses for the identification of toxicant effects is the need of a proper control, as DGGE differences potentially reflect many soil parameters aside to the presence of a toxicant.

To conclude, DGGE of 16S rDNA is judged to be a valuable tool to study the diversity of the numerically dominant members of the bacterial community, bearing in mind that it is not without biases. It proved to be useful in this thesis for distinguishing between effects of a toxicant on the total community and on active parts of the community.

PCR of specific genes

The detection of genes specific for a certain function can be used as a measure of toxicity, since levels of bacteria carrying these genes could increase as a result of the selection pressure, and since expression of the genes could be enhanced. Examples of genes (and their products) generally responsive to stressors are the universal stress protein (Kvint et al. 2003) and cold shock and heat shock proteins (Ramos et al. 2001). Antibiotic resistance genes might be another prime example of "functional" genes selectively responding to a single stressor (antibiotics).

One drawback of resistance testing is the limited coverage of the total spectrum of resistance by the resistance genes known from human and veterinary pathogens. New resistance genes are continuously being found, and even for sulfonamides which have been used for many decades, one third of a population of resistant pathogens happened to carry a novel resistance gene when investigated thoroughly (Perreten & Boerlin 2003). Especially soil might harbour a great variety of yet unknown resistance genes (Riesenfeld et al. 2004).

The extent of resistance in soils not impacted by antibiotics through human activities is largely unknown. To our knowledge, there is no detailed investigation on the "background" resistance in various soils. Any study on resistance caused by veterinary antibiotics is therefore limited to detecting the relative increase in resistance gene diversity. This is highlighted in Chapter 7, where the diversity of resistance genes in one agricultural soil before manure application is already so high that an impact of fertilisation events could not be detected.

PCR tests are not suited to detect quantitative changes in gene prevalence. PCR amplification cycles end with a plateau phase, resulting in a nearly identical amount of DNA product in samples differing in their initial template concentration (Kainz 2000). Thus, PCR can distinguish the gene content of different samples only in a certain window of template concentrations. Real-time quantitative PCR would be a method of choice to overcome this

problem and has been shown to be suitable for the analysis of resistance in the environment (Smith et al. 2004).

To conclude, the analysis of the diversity of resistance genes with PCR is a potentially sensitive method and was indeed able to detect the impact of manure amendments in microcosms. However, in the field studies described in this thesis, the discriminatory power of the qualitative PCR was not high enough to resolve differences between samples before and after fertilisation.

SYNOPSIS OF EFFECTS OBSERVED IN THIS THESIS

Summary of results

The summary of results described in Chapters 4-6 is given in Table 1.

Table 1. Summary of results from Chapter 4, 5 6 and 7.

	CLPP effect concentration [mg/kg], (p value) [6]	PICT tolerance increase [mg/kg]		16S rDNA DGGE	Co-tolerance	Resistance gene diversity
		10%	100%			
Sulfachloro-pyridazine	300 (0.062)[1]	**7.3- 11**[2]	**110-183**	n.d.[3]	n.d.	n.d.
Tylosin	**50 (0.02)**[4]	**1.3**	**58**	no	to OTC at 1500 mg/kg	n.d.
Oxytetra-cycline	1000 (0.07)	**0.009**	**1.28**	**yes**	not to TYL **to TC**	little influence
Tetracycline	1000 (0.002)	279-670 [5]	617–861[5]	n.d.	n.d.	little influence

1 CLPP for sulfachloropyridazine (SCP) was based on different data than for tylosine (TYL), oxytetracycline (OTC) and tetracycline (TC). Further, the p value reported in Chapter 4 is calculated with a different program than in 0 and 6, therefore the p value is recalculated for comparability with these chapters.

2 For SCP, the 10% and 100% increase in tolerance was determined from the single EC_{25} values in order to capture the pronounced effects on sensitive processes. The tolerance increases based on the EC_{50} values are given as the second number.

3 not determined

4 Bold type stands for a strong positive reaction of that test to the antibiotic.

5 For TC, PICT has been determined twice, and both values are given.

6 The deviating concentration is based on visual observations, while the p value gives the significance of the overall toxicant impact

As discussed in the previous paragraphs, the tests applied mostly reflect structural changes in the bacterial community. For all compounds tested, a change in community structure has ultimately been detected at high concentrations.

The fast-growing bacteria were the most clearly affected part of the community, as shown by the PICT experiments. In addition, oxytetracycline led to changes in the structure of the numerically dominant community members, as apparent from 16S rDNA DGGE (Chapter 5).

While oxytetracycline led to an increase in community tolerance at soil concentrations in the low mg/kg range, the other antibiotic classes as well as tetracycline affected the communities to a lesser extent. The community changes due to oxytetracycline exposure were also apparent in the increase in co-tolerance to tetracycline. In contrast, the co-tolerance between different classes of antibiotics was limited. CLPP sensitively detected tylosin effects, and changes in the fingerprint were so strong that a shift in the culturable bacteria up to a possible loss of species can be expected (Chapter 5). For the other substances, though, CLPP only reacted at the highest soil antibiotic concentrations of 300-1500 mg/kg (Chapter 4, 5 and 6). Changes in resistance gene diversity were not apparent for oxytetracycline and tetracycline, but this might rather be caused by an over-proportional influence of the manure amendment, which is superimposed on any antibiotic-related genetic change in the soil community (Chapter 7).

Conclusions – community methods in soil microbial ecotoxicology

Regarding the performance of the test systems applied, both 16S rDNA DGGE and CLPP compare favourably with respect to the ease and speed of effect detection. However, if applied solely, both CLPP and 16S rDNA DGGE would have failed to detect the effects of one or several of the antibiotics that were tested. These tests, addressing different parts of the microbial community, such as the numerically dominant community members (DGGE) or the culturable bacteria (CLPP), should thus be applied concomitantly. PICT, in contrast, responded to amendments with all antibiotics, and was at least as sensitive as the other test systems. The potential of this method to detect toxicant effects specifically and sensitively has therefore been confirmed by our studies. On the other hand, the complexity of PICT investigations exceeds that of the other systems, as the characteristics of each sample have to be determined from a whole dose-response range instead of a single determination. The detection of resistance genes with PCR did not prove to be a sensitive indicator of the antibiotic effects, but it highlighted the importance of the manure characteristics.

What is the relevance of the observed effects?

Current risk assessment paradigms are based on the possible effects of a toxicant (detected in the so-called effects assessment step) and the concentration of the toxicant that can be expected in the environment (exposure assessment). These two are combined in order to yield a risk characterisation (European Commission 2003a). This section gives an overview on the presence of veterinary antibiotics in soils as well as an evaluation of the relevance of the observed effects, both for soil functioning and human health.

Concentrations of antibiotics in soil and their toxicity

Little data are available on the occurrence of veterinary antibiotics in soil. However, some recent studies address the compounds studied in this thesis. In an agricultural soil located in an area with intensive pig farming, maximum concentrations of tetracycline of 0.3 mg/kg were found (Hamscher et al. 2002). In a subsequent investigation in the same area, the mean concentrations at different soil depths and sample times were in the range of 35-295 µg/kg (Hamscher et al. in press). Soil concentrations directly beneath dried liquid manure aggregates reached 1.4 mg/kg chlortetracycline. In contrast, chlortetracycline concentrations did not exceed 10 µg/kg. Further, the tetracyclines were persistent in soil, leading to accumulation over one year (Hamscher et al. 2002). Another field study with oxytetracycline-spiked manure confirmed its slow degradation in a period of 280 days (Kay et al. 2004). In contrast, a Danish field study with soils receiving slurry of tylosin- and chlortetracycline treated pigs showed maximum chlorotetracycline concentrations of 15 µg/kg. The concentrations declined to about 0.5 µg/kg during 5 months (Jacobsen et al. 2004). From these studies, it can be concluded that soil concentrations of tetracyclines might reach several hundred µg/kg in high-usage situations.

In contrast, tylosin degradation in manure seems to be so rapid that tylosin is less likely to be transferred to soils. In one field study with slurry of tylosine-treated pigs, tylosin could not be detected (Kay et al. 2004), which is consistent with a degradation half life of tylosine in slurry in the range of 3-8 days (Loke et al. 2000, Ingerslev & Halling-Sørensen 2001, Teeter & Meyerhoff 2003). Another study resulted in soil concentrations below 57 µg/kg (Jacobsen et al. 2004), declining over 150 days to less than 15 µg/kg.

For sulfonamides, concentrations in manure have been found in a comparable range as for tetracyclines: Swiss pig farms applying sulfonamide-enriched medicinal feed yielded manure concentrations of sulfathiazole of up to 12.4 mg/kg, and sulfamethazine of up to 8.7 mg/kg (Haller et al. 2002). For tetracyclines, a maximum of 45 mg/kg has been detected in cow slurry (Höper et al. 2002) and of up to 24 mg/kg in pig slurry (Winckler & Grafe 2000). Soil concentrations resulting from sulfonamide residues in manure reach a maximum of 0.5 mg/kg for sulfamethazine (Chapter 7). Sulfonamides degrade more rapidly than tetracyclines: they were found to dissipate over a few months in a field study (Kay et al. 2004).

In conclusion, maximum concentrations to be expected in field soils for tetracyclines and sulfonamides are in the range of several hundred µg/kg. Tylosin concentrations might be lower, but the findings are not totally conclusive for this substance. A comparison of the effect concentrations causing 10% increase in community tolerance and the concentrations observed in the field reveals that for both sulfonamides and tylosin, it is rather unlikely that environmental concentrations would affect agricultural microbial communities. The ratios of the observed soil concentration by the effect concentration are 0.04 (0.057 mg/kg / (1.3 mg/kg)) for tylosin and 0.07 (0.5 mg/kg / (7.3 mg/kg)) for sulfachloropyridazine, respectively. In this case, it is assumed that sulfachloropyridazine soil concentrations would equal sulfamethazine concentrations. In contrast, tetracyclines might reach concentration levels that cause an increase in community tolerance to oxytetracycline of 49% in our experiments, if

calculations are based on soil tetracycline concentration of 300 µg/kg and the dose-response relationship described in Chapter 5, figure 3.

Ecological relevance of bacterial biodiversity in soil

When discussing the effects tetracylines might have on bacterial community structure in the field, the "so what" question may be asked. The effects determined show a shift in community structure, which is likely to be linked with a decline in bacterial diversity in the sense that sensitive bacteria become less abundant or disappear from the community. Is the maintenance of structural diversity in microorganisms of agricultural soils a protection goal, and can an effect on community structure be called adverse? From a regulatory point of view, the effect of toxicants on the function of the soil micro-organisms is sometimes believed to be more important than the effect on the diversity (European Commission 2000, 2002). This is most likely linked to the assumption that there is a considerable amount of functional redundancy in the soil microflora, at least for general functions (Degens 1998, Griffith et al. 2000).

Still, the importance of biodiversity lies in the potential linkage of diversity with ecosystem stability and functioning. Due to this reason, diversity has become a protection goal on its own, as initiated with the Rio Convention on Biological Diversity in 1992. With regard to soil in particular, this is mirrored in the following statement from the Technical Guidance Document (European Commission 2003b): "The protection of the soil community requires protection of all organisms playing a leading role in establishing and maintaining the structure and the functioning of the ecosystem". The importance of the bacterial community structure per se has also been raised in scientific publications on soil ecotoxicology testing (Johnsen et al. 2001).

In the following, we highlight findings on the relationship between community diversity (structure) and functioning that would further support the relevance of maintaining soil diversity. Publications addressing this debate are numerous (Morris et al. 2002a). Still, the debate might be resumed by the striking comment of Thomas P Curtis: "Perhaps, biodiversity is not all Mumbo-Jumbo" (Curtis & Sloan 2004).

One of the most attractive hypotheses for the relation between diversity and function from a precautionary viewpoint is the insurance hypothesis, stating that a high diversity can act as insurance for the maintenance of community functioning under fluctuating conditions (Yachi & Loreau 1999, Norberg et al. 2001)[7]. The importance of diversity within functional groups, consisting of different species exerting the same function, has especially been highlighted (Tilman et al. 1997). Experimental evidence is not without controversy (Loreau et al. 2001), but for certain environments, literature overviews conclude species diversity has indeed beneficial effects on ecosystem properties like resilience or productivity (Schläpfer & Schmid 1999).

[7] Key parameters for ecosystem stability are resistance and resilience (in the sense of the ability of a system to withstand the influence of a perturbation and to recover and return to its original state after a perturbation)

Still, in microbiology, the link between diversity and function has only recently started to be investigated, especially on a molecular level (Wellington et al. 2003, Handelsman 2004). This results in only incidental proof of the relevance of biodiversity, such as in an investigation of the revegetation pattern and plant succession taken as functional parameter along a soil reclamation gradient (Yin et al. 2000). More mature sites showed a greater diversity of bacteria within functional groups, such that diversity within functional groups was suggested as a possible indicator of soil quality (Yin et al. 2000). Soil disease suppressiveness has also been discussed to depend on microbial diversity (Garbeva et al. 2004). Regarding toxicants and soil functions, it has been found that toxicants might decrease the degradation of rare substrates (Reber 1992, Burkhardt et al. 1993, Doelman et al. 1994, van Beelen et al. 1994) and even commonly utilised substrates (Nordgren et al. 1986). These findings highlight that rather specialised soil functions might be affected by toxicants such as heavy metals, possibly caused by a loss in diversity. Still, more research in that area is surely needed to more firmly establish the link between microbial diversity and soil functioning (Johnsen et al. 2001).

The envisaged level of environmental protection should be oriented towards the nature of the area in question. For agricultural soils, a range of functions should be preserved, of which a number depend on the soil microflora (adapted after (Breure 2004)):

- nutrient cycling (including nitrogen and carbon mineralisation and nitrogen fixation)
- water regulation
- supply of clean groundwater
- soil disease suppressiveness

These functions should show both resistance and resilience to the impact of a stressor, the latter signifying the ability to recover from the perturbation.

Most toxicants that diminish soil microbial diversity are not likely to affect general soil functions like carbon respiration, for which the functional redundancy is very high, at lower concentrations. Still, functions exerted by fewer bacterial species might be affected when diversity is decreased within this functional group. These are for example some processes in the nitrogen cyclus, the capacity to degrade xenobiotic substances, or soil disease suppressiveness. Therefore, it is suggested here that the maintenance of the soil microbial diversity be seen as a relevant protection goal for agricultural soils.

Human health relevance of antibiotic resistance genes in soil

The occurrence of antibiotic resistant bacteria or resistance genes touches upon an area that goes beyond the original, ecotoxicological focus of this research. There is a well-documented human health problem connected with the occurrence of multiple resistant pathogens (WHO 2001a). The question thus occurs whether antibiotic usage in farm animals (FAO/OIE/WHO 2003) and the occurrence of resistance in other environmental media might contribute to the problem of resistant pathogens. As there are many processes possibly leading to an increase in the resistance prevalence in the human population, this problem is of a very complex nature. Figure 1 illustrates the influence of three contributing factors, namely the compounds

themselves, the resistant bacteria proliferating under selection pressure in animals and the environment, and the genes that carry the resistances. In the following, information gained in this thesis and elsewhere is used to evaluate some of the steps shown in Figure 1 (the number of the process in Figure 1 is shown in bold in the following text).

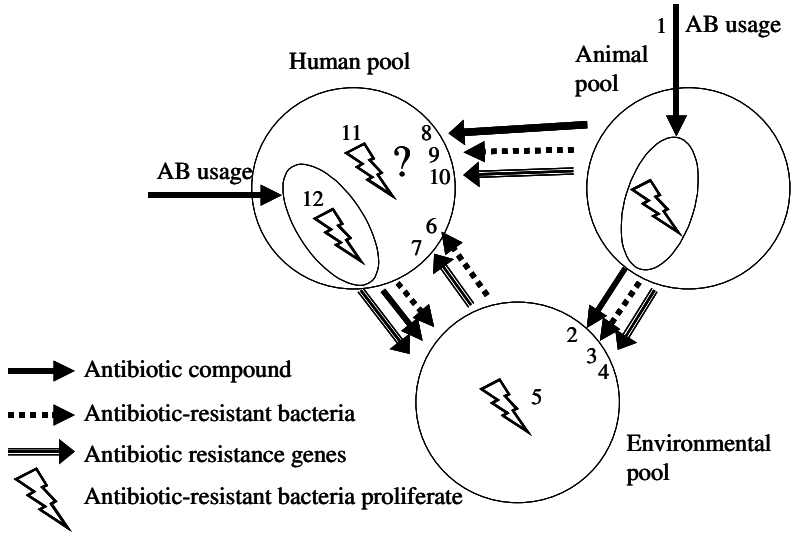

Figure 1. Potential routes leading to an increased prevalence of antibiotic resistant human pathogens. The ultimate "protection goal" is the prevention of an increase in the occurrence of resistant pathogens, denoted by the question-marked flash in the human bacterial pool (**11**). Relevant contributions might consist of **1, 2, 5, 8, 12**) the impact of the antibiotics themselves (during animal treatment, and potentially also during exposure of environmental compartments to antibiotic residues), **3, 6, 9**) the impact of resistant bacteria stemming from animal pools and persisting in the environment and the food chain, and **4, 7, 10**) the impact of resistance genes that developed in animals or the environment and are again transferred via the food chain. All these contributions have to be weighed against the occurrence of resistant bacteria through treatment of humans themselves. AB: antibiotic, ABR; antibiotic resistant / resistance

A range of studies addresses the transfer of bacteria between the different compartments (numbers **3, 6, 9** in **Figure 1**). In general, the survival of enteric bacteria from animal operations in both manure and fertilised soils (**3**) has been well documented (Cools et al. 2001, Unc & Goss 2004). Antibiotic-resistant bacteria specifically have been detected in fertilised soils (Sengeløv et al. 2003). In experimental set-ups, the transfer of pathogens introduced into soil via manure to agricultural commodities such as lettuce has also been shown, enabling entry into the food-chain (**6**, (Islam et al. 2004).

With respect to the flow of bacteria from animal husbandry to humans (**9**), infectious diseases transmitted by meat-borne zoonotic pathogens such as *Salmonella* and *Campylobacter* are well-known examples (Engberg et al. 2001), for which also risk assessment procedures exist (Parsons et al. 2005). An example for antibiotic resistant pathogens is connected with avoparcin, a glycopeptide antibiotic closely related to the human drug vancomycin. The use of avoparcin as growth promotor in Europe enhanced the frequency of vancomycin-resistant enterococci isolated from animals (**1**) and meat (**9**) (Bager et al. 1997). The frequency of

resistance in isolates of healthy humans rose concomitantly, such that an overlap between the human and animal compartment has been suggested (**9**, **10**, **11**) (Bonten et al. 2001). Interestingly, after ending the use of avoparcin and after an initial sharp decrease in the level of resistance, resistance did not drop to zero, but remained at a certain level (Wegener 2003). In the United States, in contrast, vancomycin was extensively applied in intensive care units, but not in animal farming. There, vancomycin resistance was frequently and only found in hospitalised patients.

Less is known regarding the flow of antibiotic resistance genes (**4, 10**) between the compartments. In this thesis, a high prevalence of resistance genes in animal manures has been observed, which can further enter soils and persist for at least two weeks (Chapter 7). Further, an influence of the veterinary use of antibiotics on resistance gene prevalence through manure spreading has been suggested for soil (Agersø et al. 2004) and ground water (Chee-Sanford et al. 2001, Aminov et al. 2002) (**4**). Human antibiotic use might be linked to an increase in resistance genes in wastewater treatment plants (Schwartz et al. 2003, Volkmann et al. 2004). Gene transfer to environmental bacteria seems likely to occur, e.g. in the typically waterborne *Aeromonas* (Goni-Urriza et al. 2000).

In our studies, information was not conclusive as to whether the treatment of animal herds with antibiotics leads to an increase of the diversity of resistance in animal manures (**1**). In fact, it was observed that manure from a farm with low antibiotic use still had high tetracycline resistance gene diversity. This finding has occasionally been observed before (see Chapter 7). Still, other investigators suggest a link between antibiotic use and resistance development in farm animals (Wegener et al. 1999, Aarestrup et al. 2000, Lanz et al. 2003, Rajic et al. 2004). Compared to the input of resistance genes with manure, the selective effect (**5**) of residual antibiotic compounds (**2**) in soils can be regarded as small (Chapter 7).

Thus, there is at least incidental data for many of the processes in Figure 1. Still, major questions remain controversial and make the contribution of the agricultural antibiotic use to the overall occurrence of resistant pathogens unclear, as recognised in a number of discussion papers stimulated by international agencies (European Agency for the Evaluation of Medicinal Products 1999, WHO 2001b, American Academy of Microbiology 2002). These main questions are:

1) What is the evolutionary origin of resistance genes, and what is the background level of resistance in environments without antibiotic selective pressure?
2) Under which circumstances is the selective pressure of antibacterial agents in the different pools, especially in the environment, strong enough to be causative for resistance development?
3) What is the importance of horizontal gene transfer, and do commensal and environmental bacteria play a special role in this process?
4) Is the contribution of the agricultural use of antibiotics significant compared to resistance development upon human medication?

Regarding the fourth point, the influence of agricultural use might be limited once resistance has reached equilibrium in the human population. However, the same modelling approach suggests that the use of identical compound classes in animal and human pharmacotherapy decreases the time before resistance becomes manifest in human pathogens. An earlier onset of resistance might shorten the human use by several years (Smith et al. 2002).

Expert meetings conclude that the occurrence of resistance requires regulatory approval of antibiotics (WHO 2001b). Ideally, a risk assessment to be performed as part of the approval should encompass the risks of both, the compound themselves, the resistant bacteria proliferating under selection pressure in animals and the environment, and the genes that carry the resistances (Salisbury et al. 2002). Still, such risk assessments are still in their infancy (Bailar III & Travers 2002, Claycamp & Hooberman 2004).

The demand for (precautionary) action such as prudent use has in turn often been expressed (Gorbach 2001, FAAIR Scientific Advisory Panel 2002), and is even institutionalised through non-governmental organisations, such as the Alliance for the Prudent Use of Antibiotics. Still, there is a lively and rather political debate as to whether and how the use of antibiotics in agriculture should be reduced (Barber et al. 2002, Sundberg 2002).

Risk management options, if intended, are manifold. They range from changes in the authorisation procedure (see below), a ban on the use of antibiotics as growth promoters, as executed in the European Union (Scientific Steering Committee 2001), the application of antibiotics as prescription-only-medicines when the clinical need has been established (Scientific Committee on veterinary measures relating to public health 2003), the surveillance of both antibiotic use and resistance among veterinary pathogens (WHO 2001b), and finally, scientific research to shed light on open scientific questions (American Academy of Microbiology 2002). Another option is to reduce not only the use of antibiotics as growth promoters, but also the therapeutical use of antibiotics, by adopting good agricultural practice policies.

To conclude, data from this thesis have contributed to the ongoing debate by showing that: a) there might be significant background diversity of resistance genes in soil, and b) there is still a possible role of manure in the spread of resistance genes to the environment. A cautious use of antibiotics in intensive farming is thus recommended, such as limiting the use of growth promoting agents, and decreasing the usage of the same compound groups in animal farming and human health.

Conclusions regarding the risk assessment of veterinary antibiotics

In Europe, the marketing of antibiotics requires an authorisation. The current authorisation of veterinary pharmaceuticals in the EU also contains an environmental risk assessment (EMEA 1997). Possible risks to environmental microorganisms are covered by nitrification studies according to an OECD guideline (OECD/OCDE 2000). Nitrification can be a sensitive ecotoxicological endpoint, but is sometimes not responsive to antibiotics (Landi et al. 1993, Gomez et al. 1996, Campos et al. 2001). Possible toxic effects on soil microbial communities might thus be overlooked. A polyphasic approach including the assessment of community diversity and function has already been suggested in the context of risk assessments of

genetically modified bacteria in the environment (van Elsas et al. 1998). In addition, in this thesis, PICT was presented as a sensitive methodology to detect effects of veterinary antibiotics.

Microbial effect studies are only required if the predicted environmental concentration exceeds 0.1 mg/kg soil (VICH 2000, Montforts & de Knecht 2002). In this thesis, effects at concentrations close to this exposure trigger were documented, highlighting that this approach might not be protective in all cases.

Antibiotic resistance is not covered as an endpoint in environmental risk assessment, in constrast to statements of the European Commission (Meijer 2001). However, another VICH guideline (VICH 2004b) requires the characterisation of the potential to select for antimicrobial-resistant bacteria of human health concern. These include food-borne pathogens and commensal organisms (VICH 2004b).

In light of the results presented in this thesis, it is suggested

- to evaluate the sensitivity of OECD guidelines for the detection of toxic effects on soil microorganisms, and to compare their performance with alternative approaches, including PICT
- to investigate whether a) the soil environment as a reservoir of resistance genes, and b) manuring practices, contribute to the problem of resistant human pathogens, and
- to evaluate whether the assessment of the potential to induce genetically-encoded resistance presented in (VICH 2004b) should be amended with an assessment of the environmental occurrence of resistance.

As a general caveat beyond the focus of the work presented here, I fully adhere to recommendations to reduce overuse and misuse of antimicrobials in food animals (WHO 2001a), and to give emphasis to the prevention of infectious diseases in animals as a way to decrease the quantities of antimicrobial agents used (European Commission 2001).

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SYNOPSIS

Chapter 1

1. With respect to their environmental relevance among the veterinary pharmaceuticals, antibiotics stand out due to their usage volume and their inherent activity against bacteria.
2. As the bacterial diversity is too high to be covered by one experimental test system, an analysis of the environmental effects of the use of veterinary antibiotics should combine complementary approaches from bacterial ecotoxicology.

Chapter 2

3. The determination of community-level physiological profiles (CLPP) of bacterial communities with Biolog multiwell plates based on an inoculum dilution approach was independent from the choice of the inoculum density. This method reflected an environmental impact more sensitively than conventional CLPP methods.
4. According to simulation studies, the number of bacterial strains captured in a CLPP assay is similar to the number of visible bands apparent in a whole-community genetic diversity profile (16S rDNA DGGE). Still, both numbers are extremely low compared with the true diversity of the bacterial community.

Chapter 3

5. The background tolerance in soil samples with greatly differing nutrient status was very similar, reassuring that the concept of pollution-induced community tolerane (PICT) focuses on a toxicant impact rather than other soil parameters.
6. The detection of antibiotic effects with the PICT approach required amending of the soil samples with nutrients and increasing the PICT detection time. Antibiotics mainly interfere with the bacterial growth and effects thus need time and nutrients to manifest.

Chapter 4

7. Sulfachloropyridazine, a sulfonamide compound, led to a 10% increase of the tolerance of the bacterial community (PICT) at concentrations of 7.3 mg per kg soil dry weight.
8. This increase was apparent both from the single physiological processes and from the average of all physiological processes.
9. Effects were most pronounced for the most sensitive physiological processes.
10. The sulfonamide-related changes also became apparent in changes in the CLPP pattern, which were however less pronounced.

Chapter 5

11. Oxytetracycline had pronounced effects on the tolerance of the bacterial communities. A doubling of the oxytetracycline tolerance occurred at 1.3 mg/kg (and a 10% increase in tolerance was calculated to occur at less than 0.1 mg/kg).
12. Tylosin also caused PICT effects, however, at higher concentrations (doubling of the tolerance at 58 mg/kg dw).

13. While tylosin led to clear changes in the CLPP and to a decrease in the physiological diversity of extracts of bacteria from soil, these effects were much less pronounced for oxytetracycline.
14. The molecular diversity, in contrast, was much clearlier affected by oxytetracycline than by tylosine, although there was a dose-related shift in the 16S rDNA DGGE for both compounds.
15. Tylosine thus mostly affects the part of the bacterial community which can be assessed with Biolog plates, which is thought to consist of (Gram-positive) fast-growing heterotrophs.
16. The effect pattern of oxytetracycline might be caused by a patchy distribution of this highly sorptive compound in the soil, leading to a co-existence of highly tolerant and unaffected bacterial communities.

Chapter 6

17. The response variation in the background antibiotic tolerance of bacterial communities stemming from different soils was small, reinforcing the applicability of the PICT approach for the specific detection of antibiotic effects.
18. The reproduceability of a PICT study with tetracycline in two consecutive years was good.
19. Although bacteria might be prone to an accumulation of resistance to different antibiotic compounds, the co-tolerance to antibiotics from different substance classes was small.
20. In line with the identical mechanism of action and similar tolerance mechanisms, a clear co-tolerance between two compounds from the tetracycline class has been observed.

Chapter 7

21. Veterinary antibiotics might pose a human health risk, if their use led to an increase in the occurrence of antibiotic-resistant bacteria in the environment and if the frequency of resistance among human pathogens increased likewise.
22. In microcosm studies, manure amendment led to an increase of the diversity of the detectable tetracycline resistance genes in soil.
23. Spiking of the microcosms with additional tetracyclines did not increase the diversity of resistance genes further, highlighting that the presence of antibiotic residues in soil was less important than the spreading of resistant bacteria with manure.
24. In field situations, the diversity of resistance genes can be high without a clear human impact, such that an additional impact of manuring on the diversity of resistance genes was difficult to be detected.
25. The occurrence of resistance genes in soil might be related to the origin of resistance from soil microorganisms or to the global spread of resistance genes due to the widespread human and veterinary use of antibiotics. Therefore, the above findings are difficult to interpret from a risk assessment viewpoint.

Chapter 8

26. All techniques applied in the course of this thesis are limited by focusing on a certain part of the bacterial community.
27. In contrast to PICT, CLPP proved not to be specific for the detection of toxicant impacts, but also reflected other changes in soil status.
28. In this study, PICT was the most sensitive technique in the sense that all investigated substances ultimately led to a tolerance increase, but not necessarily to detectable effects on the physiological or genetic diversity.
29. With respect to the ease and speed of the analysis, CLPP and 16S rDNA DGGE compare more favourably than PICT.
30. From the compounds investigated, sulfachloropyridazine and tylosine are not likely to reach soil concentrations that could affect bacterial communities.
31. Tetracyclines, in contrast, have been detected in soils at maximum concentrations of several hundred ug/kg, which is about a factor of 10 smaller than the oxytetracycline concentration leading to a doubling in community tolerance in microcosms. This compound class might thus cause effects in the environment, as there might be large variations from soil to soil regarding the bioavailability of the tetracyclines, and the sensitivity of the soil microbial community.
32. The relevance of bacterial biodiversity for soil health and soil functioning is not yet fully agreed upon. Still, the possibility exists that adverse effects of antibiotics on the soil bacterial diversity might be linked to a decline in soil functioning.
33. The environment, and soil in particular, might form a reservoir of antibiotic resistance genes. Due to the complexity of the processes involved, the relevance of this reservoir for the occurrence of resistant human pathogens is still largely unknown.
34. Further research might shed light on the correlation of antibiotic resistance in soil with resistance in humans.
35. If the environment proves to be an important factor for the occurrence of resistance in human pathogens, the risk assessment procedures for veterinary and human pharmaceuticals have to be adapted accordingly.
36. In the meantime, antibiotic use in livestock should be limited to the minimum possible.

NEDERLANDSE SAMENVATTING

Geneesmiddelen kunnen neveneffecten voor het milieu kunnen hebben. Farmaceutische stoffen worden grootschalig ingezet, en ze zijn ontworpen om in levende organismen een werking uit te oefenen. Dus zullen deze (bij-)werkingen ook in het milieu op kunnen treden en er tot nadelige effecten kunnen leiden. Er zijn al voorbeelden van milieueffecten bekend: het gebruik van de pijnstiller diclofenac in runderen in Pakistan leidde ertoe dat gieren door niervergiftiging stierven.

De weliswaar meest gebruikte groep van de diergeneesmiddelen zijn antibiotica, dat zijn middelen die tegen bacterie-infecties ingezet worden. Micro-organismen in het milieu kunnen door antibiotica in principe net zo geremd worden als ziekteverwekkende kiemen in de mens. Anders dan pathogene bacteriën hebben milieubacteriën echter belangrijke functies. Zo bevorderen zij de afbraak van dood materiaal in de grond en sluiten daarmee de natuurlijke kringloop van nutriënten.

Farmaceutische stoffen worden in het lichaam niet totaal afgebroken, maar kunnen vaak zonder verandering weer uitgescheiden worden. Het gebruik van antibiotica leidt dus tot hun aanwezigheid in de mest van behandelde dieren. Deze mest wordt vervolgens op landbouwgronden uitgereden en antibiotica bereiken zo de bodem.

In dit proefschrift wordt onderzocht of de contaminatie van mest met antibiotica schadelijke gevolgen voor bodembacteriën met zich mee brengt.

De bodembacteriën worden gekenmerkt door een bijna onvoorstelbare veelsoortigheid Het leek dus niet zinvol het effect van antibiotica vast te stellen door hun effect op enkele specifieke soorten bacteriën te testen. Daarom is getracht het effect op de levensgemeenschap van bacteriën in haar geheel te onderzoeken. Hoewel er een aantal toetsen bestaat dat ingezet wordt om bacteriëngemeenschappen te onderzoeken, is toch geen enkele methode in staat om de gemeenschappen in hun hele complexiteit weer te geven.

Een techniek die in vele hoofdstukken van dit proefschrift gebruikt werd, staat bekend onder de naam pollution-induced community tolerance (PICT). Dat betekent: door een giftige stof veroorzaakte tolerantie-ontwikkeling in de gemeenschap. Een toxische stof, bijvoorbeeld een antibioticum, kan gevoelige bacteriën aantasten of doden. Daardoor ontstaat ruimte voor het groeien van minder gevoelige, oftewel meer tolerante soorten bacteriën. Daardoor leidt de aanwezigheid van antibiotica ertoe dat de bacteriëngemeenschap als geheel meer tolerant wordt. PICT berust nu op de gedachte dat deze tolerantie-toename gebruikt kan worden om te testen bij welke bodem concentraties een stof een schadelijke werking heeft. Als na blootstelling aan een antibioticum de tolerantie van een gemeenschap toeneemt, kan daardoor de toxische werking op gevoelige bacteriën worden aangetoond.

Een andere techniek die in dit proefschrift gebruikt wordt, onderzoekt het “voedingspatroon” van de bacteriën (Community level physiological profiling, CLPP). Daarbij wordt gekeken welke organische substraten de bacteriëngemeenschap goed kan verteren. Een verandering in het patroon van de afgebroken voedings-substraten kan verorzaakt worden door een toxisch effect van een antibioticum.

De omstandigheden, die de bodembacteriën in deze testen ervaren, zijn heel anders dan het leven in de gewone grond. De grote meerderheid van de bodembacterien reageert daarom niet op deze testen. Over de effecten van antibiotica op deze bacteriën kan om deze reden dus ook geen uitspraak worden gedaan. Er wordt daarom ook gebruik gemaakt van een andere techniek die zich richt op de bacteriën die in de grootste aantallen voorkomen. Deze methode berust op een analyse van de diversiteit in de samenstelling van het DNA van de gemeenschap en heet 16S rDNA DGGE.

Dit proefschrift begint met een evaluatie van de geschiktheid van de boven genoemde toetsen voor het onderzoek naar de effecten van antibiotica. In het tweede hoofdstuk wordt aangegeven hoe CLPP analyses door veranderingen in praktische details en in de dataverwerking gevoeliger en eenvoudiger uitgevoerd kunnen worden. Het derde hoofdstuk laat zien dat PICT toetsen ook geschikt zijn om de effecten van antibiotica in de bodem te onderzoeken. De bodemmonsters werden met extra voeding zoals mest voorzien en de testduur zo ingesteld dat effecten op de groei van de bacteriën zichtbaar werden.

In hoofdstuk vier en vijf worden deze methoden gebruikt om het effect van drie verschillende antibiotica op bodembacteriën te schetsen, namelijk van sulfachloropyridazine, tylosin, en oxytetracycline. Deze stoffen behoren tot de respectievelijke klassen sulfonamiden, macroliden en tetracyclinen. Alle onderzochte antibiotica leiden uiteindelijk tot een toename van de tolerantie van de gemeenschap. Met de PICT-methodologie werden dus effecten van deze drie groepen van stoffen aangetoond. Veranderingen in het voedingspatroon (CLPP) waren bijzonder duidelijk voor tylosin, maar niet voor oxytetracycline. Andersom was de genetische diversiteit door oxytetracycline het meest aangetast en had tylosin maar een klein effect op 16S rDNA DGGE patroon. Daarom luidt een conclusie dat geen van deze laatste twee methodes geïsoleerd ingezet zou moeten worden om effecten van stoffen te onderzoeken.

Van oxytetracycline was maar een kleine hoeveelheid nodig om de tolerantie te verhogen. Bij 1.3 mg oxytetracycline per kg grond was de tolerantie al verdubbeld. Sulfachloropyridazine en tylosin waren minder toxisch – er waren dus grotere bodemconcentraties nodig, om effecten te kunnen meten. Verder werden door andere onderzoekers tetracycline-antibiotica in akkergronden aangetoond in concentraties tot meer dan 200 µg per kg grond. Een tweede conclusie is dus, dat tetracyclines eerder milieuproblemen zouden kunnen veroorzaken dan de andere twee klassen (sulfonamiden en macroliden).

Er waren duidelijke verschillen tussen de werking van oxytetracycline en tylosin te zien. Aangenomen wordt dat tylosin de snel-groeiende, Gram-positieve bacteriën sterk beïnvloedt, terwijl het minder toxisch is voor andere groepen. Oxytetracycline daarentegen bleek toxisch voor de meeste bacteriën. Maar omdat oxytetracycline heel sterk aan bodemdeeltjes hecht en daardoor waarschijnlijk niet gelijkmatig over de bodem verdeeld wordt, zouden de effecten van oxytetracycline op kleine schaal verschillend uit kunnen pakken en aangetaste en niet-aangetaste bacteriën naast elkaar kunnen voorkomen.

In het zesde hoofdstuk wordt een ander aspect van de PICT methode onderzocht. PICT staat namelijk bekend als een toets die toxische effecten meer specifiek aan kan tonen dan andere

toetsen. Maar deze specificiteit wordt beperkt door het optreden van co-tolerantie. Als bijvoorbeeld een tetracycline-tolerantie niet alleen door blootstelling aan tetracycline, maar ook door blootstelling aan koper veroorzaakt kan worden, is een verhoogde tetracycline tolerantie geen bewijs meer voor een aanwezigheid van tetracycline in een milieumonster. In tegenstelling tot enkele andere onderzoeken werd aangetoond dat co-tolerantie bij de onderzochte antibiotica beperkt is tot stoffen uit dezelfde klasse, die ook hetzelfde werkingsmechanisme en dezelfde bacteriële afweermechanismen hebben. Verder was de reproduceerbaarheid van de PICT proeven goed, wat bleek uit een herhaling van een compleet experiment en uit een vergelijking van de achtergrond-toleranties in verschillende experimenten.

Het zevende hoofdstuk opent een ander onderzoeksterrein: de menselijke gezondheid. De genetisch vastgelegde afweermechanismen van bacteriën tegen antibiotica werden onderzocht. Deze staan onder de naam antibiotica-resistentiegenen bekend. Antibiotica resistente ziekteverwekkers kunnen met name in ziekenhuizen grote problemen veroorzaken, omdat zij slecht behandeld kunnen worden. Er is de laatste tijd een discussie gaande of het gebruik van antibiotica ook in het milieu tot een toename van resistente bacteriën zou kunnen leiden. Daardoor zou het ontstaan van resistente humaanpathogenen mogelijk versterkt kunnen worden.

In het zevende hoofdstuk wordt beschreven dat tetracycline- en sulfonamide resistentiegenen in DNA uit varkensmest aangetoond werden. In het laboratorium bleek dat deze genen na bemesting in de grond komen en daar een toename van de diversiteit van resistentiegenen kunnen veroorzaken. Dat terwijl de mest afkomstig was van een boerderij met een gering gebruik van tetracycline. De mest bleek een belangrijkere bron van resistentie te zijn en derhalve meer impact op resistenties in de bodem te hebben dan de antibiotica. In tegenstelling met deze waarneming kon in veldgronden geen invloed van de bemesting op de diversiteit van resistentiegenen aangetoond worden, omdat er al een relatief hoge achtergrond van resistentie aanwezig was voordat de bemesting plaatsvond. Enerzijds zou de grond een natuurlijk reservoir voor resistentie genen kunnen zijn. Anderzijds zal de mest de hoeveelheid van de verschillende genen kunnen beïnvloeden. Dit verschil was met de gebruikte methode (polymerase chain reaction, PCR) niet aan te tonen.

De discussie in hoofdstuk acht begint met een beoordeling van de gebruikte technieken voor een risicoevaluatie van antibiotica. PICT bleek een gevoelige, maar arbeidsintensieve techniek te zijn, die effecten van alle onderzochte stoffen kon aantonen. CLPP en 16S rDNA DGGE daarentegen kunnen waardevol zijn voor het snel karakteriseren van effecten, maar zijn minder geschikt om met zekerheid te bepalen of een stof zonder schadelijke invloed op de bacteriëngemeenschap is. Deze laatste twee technieken zijn evenwel niet specifiek voor effecten van toxische stoffen, maar kunnen ook door andere bodem-eigenschappen worden beïnvloed.

In het vervolg wordt de relevantie van de aangetoonde effecten besproken. Omdat de veelsoortigheid van de bacteriën in de bodem zo groot is, zal een verandering in de samenstelling van de gemeenschap niet meteen tot een ineenstorten van algemene bodemfuncties zoals het afbreken van planten-materiaal leiden. Er zijn echter ook belangrijke

andere bodemfuncties, die door minder bacteriënsoorten worden uitgevoerd en daarom gevoeliger op een verandering in de bacteriële biodiversiteit kunnen reageren. Het verband tussen blootstelling aan antibiotica en een minder goed functioneren van de bodem kon nog niet direct aangetoond worden. Toch wordt aanbevolen antibiotica in de veeteelt zo terughoudend mogelijk in te zetten, zodat de antibiotica concentraties in de bodem niet tot effecten op de bacteriele gemeenschappen kunnen leiden.

Wat het risico van antibiotica-resistenties in het milieu betreft, is het duidelijk dat een heel complex netwerk van processen tot een toename van resistente ziekteverwekkers bijdraagt. Het optreden van resistentie in de bodem is maar een van deze mogelijke bijdragen. Het is onbekend of het risico van resistentie in het milieu even groot is als het risico van het humane gebruik van antibiotica. Het verdient aanbeveling om het opkomen van resistenties in het milieu grondiger te onderzoeken en hiervoor de risicoevaluatie van antibiotica uit te breiden. Ondertussen wordt een restrictief antibioticabeleid aanbevolen.

DEUTSCHE ZUSAMMENFASSUNG

Die Umweltrisiken von Chemikalien wie z.B. Pestiziden sind gut bekannt und oft untersucht worden. Weniger bekannt ist, dass auch Arzneimittel "Nebenwirkungen" für die Umwelt haben können. Pharmazeutika werden jedoch in relativ grossen Mengen eingesetzt, und sie dienen dazu, in lebenden Organismen Wirkungen zu entfalten. Daher liegen Effekte auf Lebewesen in der Umwelt nahe. Beispiele für solche schädlichen Effekte liegen vor: so führte der Gebrauch des Schmerzmittels Diclofenac bei Rindern in Pakistan dazu, dass Geier von den Rückständen in Kadavern tödliche Nierenvergiftungen erlitten.

Die am meisten eingesetzten Tierarzneimittel sind Antibiotika (Mittel gegen Bakterien). Antibiotika können die Krankheitserreger, gegen die sie eingesetzt werden, im Prinzip genauso hemmen wie Bakterien in der Umwelt. Bakterien erfüllen in der Umwelt jedoch wichtige Rollen, zum Beispiel im Nährstoffkreislauf.

Wie kommen die Arzneimittel in die Umwelt? Pharmazeutische Stoffe werden im Körper oft nicht abgebaut. Sie werden im Gegenteil zu grossen Teilen unverändert ausgeschieden. Der wichtigste Eintragspfad für Tierarzneimittel ist das Ausbringen von Gülle von behandelten Tieren auf landwirtschaftlichen Nutzflächen.

Diese Doktorarbeit untersuchte, ob die mit der Gülle ausgeschiedenen Antibiotika Bodenbakterien beeinträchtigen können.

Bodenbakterien existieren in einer beinahe unvorstellbar grossen Vielfalt. Es schien daher nicht sinnvoll, die Effekte von Antibiotika anhand von einigen wenigen Bakterienarten zu untersuchen. Diese Arbeit hatte deshalb zum Ziel, die Bakteriengemeinschaft als Ganzes zu erfassen. Es besteht jedoch keine Methode, mit der Wirkungen auf die gesamte Bakteriengemeinschaft in ihrer Vielfalt festgestellt werden können. Daher wurden in den hier beschriebenen Experimenten mehrere verschiedene Techniken eingesetzt.

Eine besonders wichtige Technik für diese Doktorarbeit ist unter dem Namen pollution-induced community tolerance (PICT) bekannt. Das bedeutet ungefähr: durch einen Giftstoff verursachte Toleranz der Gemeinschaft. Antibiotika können besonders empfindliche Bakterien abtöten und damit ein verstärktes Wachstum weniger empfindlicher Bakterien ermöglichen. Das Absterben der empfindlichen Bakterien bewirkt, dass die Gemeinschaft als Ganzes toleranter wird. Die Idee hinter dem PICT-Konzept ist, dass diese Toleranzentwicklung umgekehrt genutzt wird, um zu testen, ob ein Stoff die Gemeinschaft schädigt oder nicht. Wenn eine Chemikalie auf empfindliche Bakterien toxisch wirkt, sollte das durch einen Anstieg der Toleranz der gesamten Bakteriengemeinschaft kenntlich werden.

Eine andere Technik, die im Laufe der Untersuchungen eingesetzt wurde, untersucht den "Nahrungsmittelkorb" der Bakterien (CLPP, community-level physiological profiling). CLPP untersucht, welche Nahrungs-Substrate die Bakteriengemeinschaft gut umsetzen kann. Eine Veränderung im Muster der Substrate, die die Bakterien abbauen können, kann den Einfluss eines toxischen Stoffes anzeigen.

Die Verhältnisse, unter denen die Bakterien in diesen beiden Testen leben, sind sehr verschieden von den normalen Bodenverhältnissen. Daher kann die grosse Mehrheit der

Bakterien mit diesen Testen nicht “gesehen” werden. Eine andere Technik wird oft eingesetzt, um zumindest am häufigsten vorkommenden Bakterien einer Probe zu erfassen. Diese Technik basiert auf Unterschieden in der Zusammensetzung der DNA der Bakteriengemeinschaft (also dem Erbgut aller Bakterien) und heisst 16S rDNA DGGE.

Diese Arbeit beginnt mit einer Bewertung der oben genannten Teste für die Untersuchung der Effekte von Antibiotika. Im zweiten Kapitel wird gezeigt, wie die CLPP-Methode durch neue Datenverarbeitungsmethoden und praktische Details verbessert werden kann. Das dritte Kapitel veranschaulicht, dass PICT-Untersuchungen auch für Antibiotika genutzt werden können. Es wird vorgeschlagen, die getesteten Böden mit zusätzlichen Nährstoffen zu versehen, wie zum Beispiel mit Gülle. Die Dauer der Testung sollte lang genug sein, um die wachstumshemmenden Effekte der Antibiotika anzeigen zu können.

In den beiden folgenden Kapiteln werden diese Methoden eingesetzt, um den Effekt von drei verschiedenen Antibiotika auf Bodenbakterien zu untersuchen: Sulfachloropyridazin, Oxytetrazyklin und Tylosin. Diese gehören zu den drei Stoffklassen der Sulfonamide, Tetrazykline und Makrolide. Eine Zunahme der Toleranz wurde sowohl für Sulfachloropyridazin als auch für Tylosin und Oxytetracyclin festgestellt. Die PICT-Methode wies also für alle untersuchten Stoffe Veränderungen in der Bakteriengemeinschaft nach. Die anderen Teste, CLPP und DGGE, zeigten jedoch jeweils für einen Stoff keinen Effekt. Eine erste Schlussfolgerung war daher, dass Umwelteffekte von chemischen Stoffen auf Bakterien mit mehreren Testen gleichzeitig untersucht werden sollten.

Nur eine kleine Menge des Antibiotikums Oxytetrazyklin war nötig, um die Bakteriengemeinschaft zu verändern und toleranter werden zu lassen. Bei 1.3 mg Oxytetrazyklin per kg Boden trat eine Verdoppelung der Toleranz auf. In Ackerböden wurden Konzentrationen von Tetrazyklin-Antibiotika gemessen, die nur geringfügig kleiner sind (mehrere hundert µg pro kg Boden). Die Effekte der anderen beiden Antibiotika-Klassen waren erst bei höheren Konzentrationen messbar. Eine zweite Schlussfolgerung ist also, dass Tetrazyklin-Antibiotika wahrscheinlicher Umweltprobleme verursachen können als die anderen zwei Klassen.

Drittens wurde gezeigt, dass Antibiotika die Bakteriengemeinschaft auf verschiedene Weise beeinträchtigen. Während Tylosin die schnell wachsenden Bakterien besonders stark hemmt, wirkt Oxytetrazyklin auf die gesamte Bakteriengemeinschaft. Oxytetrazyklin ist wahrscheinlich im Boden nicht gleichmässig verteilt, weil es sehr stark an Bodenpartikel bindet. Es kann daher für lokal unterschiedliche Effekte sorgen.

Im sechsten Kapitel wird ein neuer Aspekt der PICT Methode untersucht. PICT gilt nämlich als besonders geeignet, um Effekte eines Stoffes spezifisch nachzuweisen. PICT ist aber nur dann spezifisch, wenn keine Kotoleranzen auftreten. Wenn Bakterien zum Beispiel durch Antibiotika nicht nur Antibiotika -tolerant, sondern auch Kupfer-tolerant werden würden, könnte man die Kupfertoleranz nicht als spezifisches Anzeichen für eine Kupferbelastung werten. So eine Ko-toleranz wurde für andere Stoffe schon nachgewiesen. Die untersuchten Antibiotika zeigten jedoch keine “unerwartete” Kotoleranz: die Kotoleranz war beschränkt auf zwei Antibiotika aus derselben Gruppe mit gleichem Wirkmechanismus, deren Ähn-

lichkeit schon aus ihrem Namen zu lesen ist (Oxytetrazyklin und Tetrazyklin). Weiter wurde im sechsten Kapitel gezeigt, dass die PICT-Untersuchungen gut zu reproduzieren waren.

Das siebte Kapitel öffnet ein anderes Untersuchungsgebiet und ist eher auf die menschliche Gesundheit gerichtet als auf die Umwelt. Dort werden die genetisch festgelegten Abwehrmechanismen der Bakterien untersucht. Die Gene, die für diese Mechanismen sorgen, heissen Resistenzgene. Das Auftreten von Resistenzen ist aus Krankenhäusern bekannt. Dort können Krankheitserreger, die mit keinem Antibiotikum mehr zu behandeln sind, für grosse Probleme sorgen. Neuerdings wird diskutiert, ob die Anwendung von Antibiotika auch zu einem Auftreten von Resistenzen in der Umwelt führen kann. Das könnte die Resistenzbildung in menschlichen Krankheitserregern noch verstärken.

Resistenzgene wurden in der Tat in Schweinegülle nachgewiesen. Diese Gene konnten in Mikrokosmos-Experimenten auch auf den Boden übertragen werden und dort eine Zunahme von Resistenzen bewirken – obwohl die verwendete Gülle aus einer Schweinezucht stammte, wo wenig Antibiotika eingesetzt wurden. Im Gegensatz dazu war in Feld-Studien kein Effekt der Begüllung auf die Resistenzgen-Vielfalt bemerkbar. Dies lag an der grossen Vielfalt der Resistenzgene im Boden schon vor der Begüllung. Der Boden könnte ein natürliches Reservoir für Resistenzgene sein. Allerdings konnte die eingesetzte Technik nur Veränderungen in der Art, aber nicht in der Menge der Resistenzgene nachweisen. Mögliche quantitative Veränderungen in der Resistenz konnten also nicht erfasst werden.

Die Diskussion beginnt mit einer Bewertung der eingesetzten Techniken für die Risikobewertung von Antibiotika. PICT erwies sich als eine empfindliche, aber arbeitsintensive Technik, die für alle untersuchten Stoffe Effekte anzeigte. CLPP und 16S rDNA DGGE hingegen waren geeignet für eine schnelle Charakterisierung der Effekte, zeigten aber nicht für alle Stoffe Effekte an.

Die Relevanz der gefundenen Effekte wird im zweiten Teil der Diskussion betrachtet. Weil die Bakterienvielfalt im Boden so gross ist, führen Verschiebungen in der Zusammenstellung der Bakterien wahrscheinlich nicht zu Beeinträchtigungen in allgemeinen Bodenprozessen wie z.B. dem Abbau von Pflanzenmaterial. Es gibt aber auch Bodenfunktionen, die von wenigen Bakterienarten abhängen und daher empfindlicher auf Änderungen in der bakteriellen Biodiversität reagieren könnten. Ein direkter Zusammenhang zwischen Antibiotikabelastung und wichtigen Bodenfunktionen konnte noch nicht nachgewiesen werden. Trotzdem wird angeraten, die Bakterienvielfalt als Schutzziel anzusehen und Konzentrationen von Antibiotika zu vermeiden, die zu Veränderungen in der Bakterienvielfalt führen könnten.

Was das Risiko von Resistenzen in der Umwelt betrifft, wird diskutiert, dass viele verschiedene Pfade zu einem gehäuften Auftreten von Resistenzen in Krankheitserregern führen können. Das Auftreten von Resistenzgenen in landwirtschaftlichen Böden ist nur einer der möglichen Pfade. Die Relevanz des Bodens als Resistenz-Reservoir kann im Moment noch nicht abschliessend bewertet werden. Daher wird nahegelegt, Resistenzbildung in der Umwelt weiter zu untersuchen und in Übereinstimmung mit dem Vorsichtsprinzip den Einsatz von Antibiotika in der Viehzucht in der Zwischenzeit auf ein Minimum zu beschränken.

LIST OF ABBREVIATIONS

16S / 23S	small / large subunit
AUC	area under the curve
AWCD	average well colour development
$AWCD_{50}$	concentration of an agent leading to a decrease in the AWCD by 50%
CAS	chemical abstracts services
CFU	colony-forming units
CLPP	Community-level physiological profile
DGGE	Denaturing gradient gel electrophoresis
DNA	Desoxyribonucleic acid
dw	dry weight
EC_{50} / EC_{10}	Concentration of an agent causing a 50% / 10% effect in a test parameter
HPLC	High performance liquid chromatography
K_{OW}	Octanol-water partitioning coefficient
LC_{50}	Median lethal concentration
LC-MS/MS	Liquid chromatography / mass spectrometry
m/z	Mass/charge ratio
MW	Molecular weight
OECD	Organisation for Economic Co-operation and Development
OTC	Oxytetracycline
PCA	Principle component analysis
PCR	Polymerase chain reaction
PICT	Pollution-induced community tolerance
pK_a	Acidity constant
ppSD	Physiological process sensitivity distribution
RDA	Redundancy analysis
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCP	Sulfachloropyridazine
TC	Tetracycline
TYL	Tylosin
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products
WCD	Well colour development
WCD_{50}	concentration of an agent leading to a decrease in the WCD by 50%
WHO	World Health Organization

DANKWOORD

Jedem Anfang wohnt ein Zauber inne, or, poorly translated, in all beginnings dwells a magic force (H. Hesse) – this is something which I truly experienced during my PhD project. I often changed places, have got to know new supervisors and many laboratories. Thus, I would like to thank the people first who have accommodated me in their groups. It is very distinctive for the Netherlands, as I think, that I always felt welcome.

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But there is a connection between beginnings and partings – and I am less acquainted with the latter, as I realise when looking back.

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Although I became too busy with my PhD to really take part in the PAN activities in the last years: I enjoyed working with you self-willed people, Ute, Stephanie and David, Hans and Catherine, Carina and the rest. The best of luck for the next years!

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CURRICULUM VITAE

Heike Schmitt was born in Hamburg in 1972. After finishing high school at the Gymnasium Vosshagen, she started to study chemistry. She obtained her Vordiplom at the University of Hamburg and continued her studies in Bremen with a focus on marine chemistry. Her diploma thesis (Diplomarbeit) was performed at the Umweltforschungszentrum Leipzig-Halle, under supervision of Rolf Altenburger, Gerrit Schüürmann and Bernd Jastorff. She graduated in 1998 from the University of Bremen. During her studies in Bremen, she obtained a grant by the "Studienstiftung des Deutschen Volkes".

After having worked for PAN Germany and PAN Europe for two years, she continued her scientific activities with her PhD thesis at the Universiteit Utrecht, Institute for Risk Assessment Sciences IRAS (2001-2005). During her PhD studies, she also completed her postgradual studies in toxicology at the University of Leipzig to become a "Fachchemikerin für Toxikologie". The Dutch equivalent, the postgraduate opleiding in toxicology POT, is still underway. She also served on the board of PAN Europe and the section of environmental toxicology of the Nederlandse Vereniging voor Toxicology.

Her PhD thesis was performed in close co-operation with the National Institute for Public Health and the Environment RIVM, namely with the laboratories for ecological risk assessment (LER) and microbial health prevention (MGB). The project was further associated with the EU project ENVIRPHARM. Parts of the research were undertaken at the laboratory of Jim Tiedje at Michigan State University, East Lansing, USA, during a guest visit.

In 2005, she will continue as a junior UD at the Utrecht University and the RIVM. Focus of her work will be antibiotic resistance and a EU project dealing with the environmental risks of pharmaceuticals (ERAPharm), with special attention to antibiotics.

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