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Effects of sulfamethoxazole on soil microbial communities after adding substrate

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ABSTRACT

The effect of the antibiotic sulfamethoxazole (SMX) on soil bacteria was studied using two methods (leucine incorporation and Biolog plates) of estimating pollution-induced community tolerance (PICT). SMX was added to an agricultural soil in a microcosm setup. The addition of different substrates (manure and alfalfa), and a non-amended soil, were also studied over 5 weeks. PICT measurements were validated by comparison with other measurements. Community structure was assessed using phospholipid fatty acid (PLFA) analysis and community-level physiological profiling (CLPP), and bacterial growth was estimated using leucine incorporation. Increased PICT was found at SMX concentrations of 20 and 500 mg SMX kg^{-1} soil in samples containing manure and alfalfa, and at 500 mg SMX kg^{-1} soil in non-amended soil (only concentration tested) using leucine incorporation. No effect was seen at 1 mg SMX kg^{-1} soil. It was not necessary to add any substrate to increase the microbial activity in order to detect the effects of a bacteriostatic toxicant such as SMX when using measures based on bacterial growth. Direct inhibition of bacterial growth 2 days after SMX addition was correlated to PICT. No major changes in PICT due to SMX addition were found when using Biolog plates. However, there was a tendency towards increased PICT at the higher SMX concentrations in the manure-amended soil. Thus, different methods of detecting PICT have different sensitivities in detecting the toxic effects of SMX. The effects of substrate amendment were reflected by changes in the microbial community, estimated using both PLFA and CLPP. SMX was found to have a clear effect at the two highest levels of SMX in the manure- and alfalfa-amended soils, with an increase in fungal and a decrease in bacterial PLFAs. Little difference in the PLFA composition was found in the non-amended soil. CLPP was only affected at the highest SMX concentration. Although different variables showed different sensitivities to the effects of SMX, the results were consistent with an initial decrease in bacterial growth rates of sensitive species, which eventually transformed into more tolerant species, altering the community composition.

1. Introduction

Pharmaceutical antibiotics have been detected in various environmental compartments, among them hospital waste water and sewage water treatment plants (Daughton and Ternes, 1999), and receiving environments such as surface water (Schrap et al., 2003). Also, antibiotics used in veterinary pharmacotherapy have been detected in soils receiving animal waste (Hamscher et al., 2005; Stoob et al., 2006). The effects of this environmental exposure on soil microorganisms have been the subject of several recent investigations (Thiele-Bruhn, 2003). Effects have been identified on microbial biomass (Thiele-Bruhn and Beck, 2005; Zielezny et el., 2006), activity (Vaclavik et al., 2004; Bailey et al., 2003; Thiele-Bruhn, 2005; Rousk et al., 2008) and community structure (Westergaard et al., 2001; Schmitt et al., 2004, 2005, 2006; Kong et al., 2006). The European authorization of pharmaceuticals also requires an evaluation of the environmental 'side effects' of the active ingredients (Koschorreck et al., 2002; Spindler et al., 2007). For new veterinary antibiotics with predicted environmental concentrations above 100 μg kg^{-1}, inhibition of nitrification has to be tested according to the OECD guideline 216 (OECD/OCDE, 2000). However, due to the complexity of environmental bacterial communities, the question arises as to whether nitrification provides a correct approximation of the effects of pollutants on bacterial communities. Furthermore, antibiotics have very specific modes of action compared to more general toxicants such as metals and phenols. Antibiotics result in selective effects, in other words, only part of the microbial community is affected. It is therefore not self-evident that the routine methods used in pesticide ecotoxicological tests are suitable for evaluating the effect of antibiotics

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(Vaclavik et al., 2004). It has indeed been shown that current regulatory methods can lead to false–negative results: while no effects of antibiotics were initially detected, slight adaptations in the test setup revealed an inhibition of soil respiration in a loamy soil (Thiele-Bruhn and Beck, 2005). One aim of this investigation was thus to analyze the effects of antibiotics on the soil bacterial community as a whole, including fungi and bacteria, and to evaluate the suitability of different detection methods to reveal these effects in order to further the risk characterization of antibiotic residues in soil.

Sulfonamides are one of the oldest types of antibiotics, and were in use already in the 1930s. They are still among the most commonly used antibiotics both for humans and animals, with 78 tonnes being used in Europe annually (Thiele-Bruhn, 2003). Sulfonamides are considered to be bacteriostatic, inhibiting the synthesis of folic acid. In the present study we have studied one of many sulfonamides, sulfamethoxazole (SMX). Sulfamethoxazole is an antibiotic that is widely used in both human and veterinary medicine. A number of different sulfonamides have recently been the subject of several soil ecotoxicological studies (Schmitt et al., 2004, 2005; Thiele-Bruhn and Beck, 2005; Thiele-Bruhn, 2005; Hammesfahr et al., 2008; Heuer et al., 2008; Kotzerke et al., 2008).

The concept of pollution-induced community tolerance (PICT) has recently been used as a sensitive and specific endpoint measure to evaluate the effects of pollutants on different communities in water and terrestrial systems (Blanck, 2002; Boivin et al., 2002). The rationale behind the PICT concept is that organisms exposed to a toxicant only will survive if they tolerate it, while the more sensitive ones will decrease in abundance. This will thus result in a more tolerant community, i.e., an increase in PICT, in the presence of the toxicant. Comparisons between different methods of estimating PICT using different detection methods have, however, been scarce. Detection methods commonly used to investigate PICT in soil systems are bacterial growth measurements, using the thymidine or leucine incorporation techniques (e.g. Díaz-Raviña et al., 1994; Díaz-Raviña and Bååth, 1996a, b; Shi et al., 2002; Almås et al., 2004; Aldén Demoling and Bååth, 2008b, c), and Biolog plates (e.g. Rutgers et al., 1998; Davis et al., 2004; van Beelen et al., 2004; Schmitt et al., 2004). In this study we have compared PICT estimates using both the leucine incorporation technique and Biolog plates to investigate effects of SMX on the soil bacterial community. To validate PICT results they should, however, be compared with other measures (Blanck, 2002). Phospholipid fatty acid (PLFA) analysis and the community-level physiological profile (CLPP) method are commonly used to indicate community structure (Pennanen et al., 1996; Bååth et al., 2005; Schmitt et al., 2005, 2006), where the former method also will detect differential effects on the fungal and bacterial community. Direct measures of bacterial growth have also been used to evaluate changes in PICT and to correlate PICT to direct inhibition using the leucine incorporation technique (Aldén Demoling and Bååth, 2008b, c).

Bacterial growth measurements will be especially useful in the connection with antibiotics that are bacteriostatic, such as sulfonamides. Microbial activity, such as respiration, can still function after adding a bacteriostatic substance. The observed lack of effects of antibiotics under growth-limiting conditions has led to calls for the addition of a substrate to promote bacterial growth in order to be able to detect effects (Thiele-Bruhn and Beck, 2005). The use of leucine incorporation, a direct measure of bacterial growth, and thus equally susceptible to both bacteriostatic and bactericidal substances, would avoid these problems. Thus, we compared the effect of the antibiotic SMX both with and without adding substrate (manure and alfalfa) to the soil in our investigation of community effects of antibiotics.

2. Materials and methods

2.1. Soil

A loamy sand soil (7% clay, 10.4% silt and 78.9% sand) was used for all microcosms. The soil originated from a cattle farm in Wythem, the Netherlands. During the past 10 years, the farm had been under organic production. Some parts of the farm's grassland are used as agricultural fields. The sample location was in one of these fields, where wheat had been grown during the previous 3 years. No additional fertilizer had been used during this time. The soil had a pH-KCl of 4.9, and contained 3.7% organic carbon and 0.1% $CaCO_3$ (both based on dry weight). The soil was sampled by combining about 500 samples taken with a drill (2.3 cm diameter, 10 cm depth) from a 10 × 10 m part of the field. After sampling, the soil was sieved (4 mm) and stored at 10 °C for 17 days.

2.2. Sulfamethoxazole

Sulfamethoxazole (SMX) (CAS 723-46-6) was purchased from Sigma (Steinheim, Germany) (>98%). SMX (MW 253.3 g mol^{-1}) has pK_a values of 1.69 and 5.57 (Lucida et al., 2000), its soil sorption as characterized by its K_d value is 37.6 kg^{-1} (Drillia et al., 2005), and its log K_{ow} is 0.89 (Hansch and Sammes, 1990). At the pH of the test soil (4.9), SMX is thus predominantly present in the soil solution in the uncharged form, enabling efficient uptake by organisms.

2.3. Substrate addition

Substrates were co-applied with the SMX in order to increase the activity of the soil microbiota. Powdered alfalfa (*Medicago sativa*) application resulted in an addition of 0.16 mg total N g^{-1} soil dw and 3.77 mg organic matter g^{-1} soil. To facilitate mixing, a homogenized slurry (alfalfa:water, 1 g:5 ml) was prepared from alfalfa pellets, C/N ratio 13, directly before addition to the soil. Manure was collected from a free-range pig farm in Groenekan, the Netherlands. Antibiotic use on the farm was limited, neither the piglets from whom the manure was sampled nor their mothers had been treated with SMX. Manure was homogenized with water (1.3 g manure:1 ml H_2O). The C/N ratio was 9. Manure addition led to amendment with 38 μg NH_4^+–N g^{-1} soil dw and 4.35 mg organic matter g^{-1} soil. Manure was also taken from piglets that were on continuous SMX therapy (4 days' treatment, 3 days off for 1 month; the last 4 day treatment period was 3 days before manure collection). Both solid manure and slurry from the manure pit were sampled in order to include the SMX excreted with urine. For the final mixture, 228 g solid manure was mixed with 151 g slurry and water. The mixtures of each of the three substrate amendments were stored at 4 °C for 5 days before use.

2.4. Study design

The study was based on three kinds of substrate-amended soils and an unamended control. The first series was amended with manure, the second with alfalfa meal, and the third was amended with manure from SMX-treated piglets. The control soil was incubated without any substrate (only water was added). We aimed for a wide range and high number of SMX concentrations to obtain full dose-response curves. Thus, duplicate samples were prepared for each treatment.

Sub-samples of soil (1500 g dry wt) were amended with the respective substrate and SMX at varying concentrations (see below). Ten microcosms (five duplicates for each treatment) were amended with the manure and SMX at five concentrations: 0, 0.1, 1, 20, and 500 mg kg^{-1} soil dry wt. Alfalfa slurry and the same five