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Nonextractable residue formation of sulfonamide antimicrobials: New insights from soil incubation experiments

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HIGHLIGHTS

- Formation of nonextractable residues (NERs) of sulfamethazine in soil was studied under different incubation conditions.
- NER formation of sulfamethazine in soils occurs most likely by covalent bonding to reactive quinones.
- Quinone formation by oxidation of organic matter is the rate-limiting factor for NER formation.

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ABSTRACT

Soil incubation experiments using ^{14}C-labelled sulfamethazine were carried out to assess the factors governing its nonextractable residue (NER) formation via nucleophilic addition reactions. Circumstantial evidence on possible mechanisms of NER formation was derived from a selective manipulation of soil samples. The amount of quinones in soil available for nucleophilic addition was a limiting factor as indicated by (i) an (initial) increase of NER formation by adding quinone precursors or enhancing their formation by manganese oxide addition and (ii) a decrease of NER formation by limiting the formation of quinones under anaerobic conditions. A slow NER formation with time under aerobic conditions is likely caused by covalent bonding as well, because no slow NER formation phase was observed under anaerobic conditions.

1. Introduction

Among different classes of antimicrobials, sulfonamides (SAs) are widely used in animal husbandry to treat or prevent bacterial infections (Sarmah et al., 2006). Residues were detected in surface waters (Kolpin et al., 2002), ground waters (Batt et al., 2006; Karthikeyan and Meyer, 2006), surface runoff (Stoob et al., 2007), and soils (Kay et al., 2004). Inevitably, soil is a hot spot for SAs as it receives a large portion of excreted compounds through grazing livestock or the application of manure. Studies on the fate of SAs in soil demonstrated that a large portion forms nonextractable residues (NERs) along with transformation products and a low mineralization (Kreuzig and Höltge, 2005; Schmidt et al., 2008; Förster et al., 2009; Rosendahl et al., 2011). In these studies sequential extraction and fractionation techniques were applied to monitor extractability of ^{14}C-labelled sulfadiazine in long-term soil incubation experiments. Although the extent of NER formation differed among these studies due to different extraction methods used, a common observation was an initially fast formation of NERs up to 50% of the applied amount within a few days. This is indicative of a chemical reaction rather than slow sorption processes, but the complexity of the soil matrix did not allow for making unanimous conclusions on the mechanisms of NER formation in soils.

The most reactive functional group of SAs is their aromatic amino group, for which a direct evidence on covalent bonding was obtained using humic model constituents and humic acids (Bialk et al., 2005, 2007; Bialk and Pedersen, 2008; Gulkowska et al., 2012, 2013). These studies also showed that nucleophilic addition reactions to quinones are predominantly responsible for covalent bond formation rather than radical reactions based on one-electron oxidation of organic matter (OM) or sulfonamides. Most of these reactive quinones are not stable and consequently only a small number of reactive sites is present in humic acids to readily react with SAs (Gulkowska et al., 2013). In contrast, the formation of quinones by oxidants such as MnO_2 or oxidative enzymes greatly enhances covalent bonding. This covalent bonding to humic acids and model quinones in the presence of oxidants was also

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demonstrated for other aromatic amines (Weber et al., 1996; Dec and Bollag, 2000; Thorn and Kennedy, 2002). Our recent studies (Gulkowska et al., 2012, 2013) furthermore indicated that covalent bonds might undergo hydrolysis under harsh extraction conditions, thus covalent bonding does not necessarily mean NER formation.

Oxidants such as oxidase enzymes, Mn oxides and possibly Fe oxides are also abundant in soils. These are likely to play a pivotal role in the NER formation of SAs in aerobic topsoils. Since most of the quinone moieties in SOM are short-lived (Gulkowska et al., 2013), soil oxidants may activate de novo unreactive hydroquinones by oxidizing them to quinones and, thus, determine the extent of SAs binding. Study on other aromatic amines has demonstrated the role of Mn oxides in NER formation (Li et al., 2003), although the reduction of Mn(III)/Mn(IV) was not sufficient to account for all NERs formed.

The objective of our study was to assess the factors governing NER formation of ^{14}C-labelled sulfamethazine in soils. As it is not possible to obtain direct evidence on the nature of the NER fraction of a compound from extraction procedures alone, we link evidence obtained from model systems (Bialk et al., 2005, 2007; Gulkowska et al., 2012, 2013) with a selective manipulation of experimental conditions in soil incubation studies. To this end, we compared an aerobic incubation of untreated soil with an anaerobic incubation or a removal or an addition of manganese oxides and inhibition of oxidative enzymes. The addition reaction itself was targeted by addition of a competing nucleophile and addition of selected model hydroquinones to increase the pool of compounds available to form quinones.

2. Materials and methods

2.1. Chemicals

Sulfamethazine (^{12}C SMZ) was obtained from Sigma–Aldrich, the stronger nucleophile p-ethoxyaniline (EXA) from Merck. SMZ labeled at the phenyl ring (^{14}C SMZ; 3.7 MBq mL^{-1}, specific activity 2.035 GBq mmol^{-1}, 99% in ethanol) was obtained from American Radiolabeled Chemicals. Stock solutions of SMZ, ^{14}C SMZ, and EXA were prepared in methanol. EXA was diluted in water to the desired concentration and was directly used in incubation experiments. The ^{14}C SMZ solution was diluted with unlabeled SMZ solution in acetate buffer to achieve a starting SMZ concentration of 97.16 μg mL^{-1} and an activity of 33.44 kBq mL^{-1}.

HPLC grade methanol, acetonitrile and water (Acros Organics) were used as solvents for extraction and liquid chromatography eluents. All other chemicals were at least of "pro analysi" grade and obtained from Merck or Sigma–Aldrich.

2.2. Soil sample

An agricultural soil sample classified as sandy loam Cambisol was taken in an intensively farmed area in the northern part of the Canton of Zurich, Switzerland. Details on sampling and soil characteristics are given in Supplementary material.

2.3. Selective manipulation of soil samples

The soil samples were preincubated in the laboratory at room temperature for approximately 30 h to allow a re-growth of the microorganisms after freezing. Two aerobic samples were incubated as control samples and the following soil treatments were carried out to affect NER formation of added SMZ.

(i) *Anaerobic incubation.* The soil was incubated under anaerobic conditions in a glove box under N_2 to prevent a re-formation of Mn oxides from Mn^{2+} and Fe oxides from Fe^{2+} released when these oxidize natural organic matter. Additionally, a re-oxidation of O_2-depending enzymes was inhibited. Incubation bottles filled with soil were left under vacuum for 12 h to exhaust the soil gases prior to pre-incubation for 30 h in the glove box. The SMZ spiking solution and water for water content adjustment were purged with argon before transfer to the glove box.

(ii) *Addition of a hydroquinone mixture*. A mixture of catechol, resorcinol, 3,4-dihydroxybenzoic acid and syringic acid was added to the soil right before SMZ addition, yielding a final concentration of 425 μg g^{-1} of soil.

(iii) *Addition of synthetic birnessite (δ-MnO_2).* Acid birnessite was synthesized according to procedure outlined by McKenzie (1971), for details see Supplementary material. An aliquot of birnessite was added to soil yielding a final concentration of 31 mg g^{-1} and pre-incubated for 3 d at room temperature.

(iv) *Selective removal of Mn oxides* from soil was done according to the method of Li et al. (2003), details are given in the Supplementary material.

(v) *Inhibition of natural peroxidase activity* in soil was done by adding 57 μL of 1.0 M phenylhydrazine per g of soil one day prior to the addition of SMZ (Baldrian, 2006).

(vi) *Addition of EXA one day prior to the addition of SMZ* at a molar ratio of EXA to SMZ of 3:1. The stronger nucleophile EXA competes for sorption and covalent bond formation with SMZ on organic matter. It caused a twofold suppression of SMZ covalent bond formation with humic acid (Gulkowska et al., 2013).

(vii) *Addition of EXA simultaneously with SMZ* at a molar ratio of EXA to SMZ of 3:1.

2.4. Incubation experiment

The batch incubation experiments were carried out in 100 mL glass bottles for 45 d at room temperature and 40% of maximum water holding capacity (MWHC). For each treatment, 60 g of dry weight equivalent soil were used, from which 5 subsamples of 12 g to be sampled after 1, 3, 10, 24, and 45 d of incubation were distributed into 5 different bottles.

Using a 500 μL glass syringe, SMZ standard solution was added dropwise to the soil surface to obtain a final concentration of 3.8 μg g^{-1} soil, corresponding to 1.3 kBq g^{-1} soil. The spiked soil was thoroughly mixed using a stainless steel spatula. Finally, the water content in all sub-samples was adjusted to 40% of MWHC. The incubation bottles were vented every 7 d (except for anaerobic samples) and the water content was readjusted gravimetrically, if necessary.

On each sampling day, the 12 g of each soil sample were mixed thoroughly and divided evenly into two vials. Only one vial was further analyzed, while the other was stored at −20 °C as a back-up sample. To quantify any non-transferred SMZ the incubation bottles were rinsed with methanol and the rinsing solutions were analyzed by liquid scintillation counting (LSC). The analysis showed that in all cases less than 0.1% of the initial SMZ mass added to soil remained in the bottles.

2.5. Sequential extraction and fractionation of soil samples

An overview of the procedure used for the assessment of SMZ extractability and NER formation is presented in Fig. 1. The total extractable fraction of SMZ was determined by pressurized liquid extraction (PLE) based on a method for soil samples (Stoob et al., 2006). The extractable fraction of SMZ was determined by

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