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Drying and rewetting events change the response pattern of nitrifiers but not of denitrifiers to the application of manure containing antibiotic in soil

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ABSTRACT

Application of manure for soil fertilization is a common practice in arable and pasture soils. As antibiotics are regularly used in animal husbandry, these compounds often enter the soil via manure application. The effects of antibiotics on microbial communities, however, might depend on soil moisture, as water availability may influence both the fate of the compound and the activity of the microbes. To test this hypothesis, we investigated the effects of the application of manure containing the antibiotic sulfadiazine (SDZ) on the abundance and activity of nitrifies and denitrifies in soil, based on the copy number of marker genes and their related potential activities, as affected by different moisture regimes. We observed significant effects of SDZ on potential denitrification activity, but those were not influenced by the soil moisture regime. Nevertheless, neither SDZ nor changes on moisture significantly affected the abundance of denitrifies. In contrast, both potential nitrification activity and abundance of ammonia oxidizing bacteria were significantly affected by the application of manure containing SDZ and moisture regime. Interestingly, no effects were observed for ammonia oxidizing archaea. Overall, our data show that soil moisture modulates the effects of antibiotics in soil microbial communities, and we recommend to include this parameter in the risk assessment of new chemicals.

1. Introduction

The increasing demand for meat production has caused drastic changes on the animal husbandry. Intensive and large-scale farming industry greatly relies on the use of antibiotics, not only for therapeutic purposes but also as growth promoters (Sarmah et al., 2006). Concerns about the consequences of these practices lead to the prohibition of the use of antibiotics as growth promoters in Sweden, followed by Denmark and lately by all members of the European Union (Pruden et al., 2013). Even though the ban reduced overall antibiotic use (Casewell et al., 2003) this measure was not applied in other countries, e.g., USA, where recent estimates indicate that more than 70% of the administered antibiotics is used for livestock (Pruden et al., 2013).

The use of manure for soil fertilization was shown to be a major route by which antibiotics reach the environment. Many compounds are only poorly assimilated by the animals and are excreted, mostly unaltered, in the feces and urine. If these compounds are not degraded during manure storage, they will enter the soil via manure fertilization (Du and Liu, 2011). This is the case of sulfadiazine (SDZ), a bacteriostatic antibiotic of the sulfonamide class frequently used in pig husbandry (Lamshöft et al., 2010). Once in the soil, antibiotics might alter microbial community structure and function (Ding and He, 2010).

Effects of bacteriostatic agents on microbial communities are mainly dependent on its bioavailability in soils and metabolic activity status of the community, both known to be influenced by environmental parameters. Results obtained by Rosendahl et al. (2011) indicate that the temperature-dependent fate of SDZ in soils

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is modulated by soil moisture. Dissipation rates in bioaccessible and sequestered fractions strongly depend on the temperature and could be predicted by laboratory experiments. Sequestration processes were influenced by soil moisture and could explain differences observed between concentrations measured at field experiments and those predicted based on laboratory data. Thus a prognosis of how antibiotics affect microbial community structure and activities under changing climatic conditions is challenging. Furthermore, beside the bioavailability of the compound, drying–rewetting events also directly influence microbial activity. As shown by (Morillas, 2013), increases on organic matter mineralization are observed in soils that undergo drying–rewetting events in comparison with constantly wet soil. Soils microstructures are often disrupted when rewetted after a drying process (Reatto et al., 2009), exposing physically protected organic matter previously inaccessible to microbes. Additionally, osmolytes (amino acids, polyols, carbohydrates and inorganic solutes) accumulated by the microbes during periods of dryness are rapidly disposed after rewetting.

Therefore, we hypothesized that the short-term effects of the application of manure containing bacteriostatic antibiotics are highly dependent on soil moisture. To test this hypothesis, we performed a laboratory experiment where soils cultivated with *Dactylis glomerata* and treated with manure containing SDZ were submitted to 2 drying–rewetting cycles or kept constantly wet. Effects on the abundance and activity of nitrifiers and denitrifiers were assessed by qPCR of marker genes and potential enzyme activities, respectively. We selected these microbes due to their high relevance to the soil N cycle, and because their different ecophysiology provides ideal test parameters for risk assessment of antibiotics in soils.

2. Material and methods

2.1. Experimental design

The experiment was carried out in climate chambers at the Helmholtz Zentrum München (www.helmholtz-muenchen.de/eu) to ensure reproducible climatic conditions during the drying–rewetting cycles. A silt loam soil with no history of manure application, classified as cutanic Luvisol, was taken from an arable field located in Jülich-Merzenhauzen, Germany. The soil had an organic carbon content of 1.2%, a pH ($CaCl_2$) of 6.3, a CEC of 11.4 cmolc kg^{-1} (measured at pH 8.1), 16% clay, 78% silt, 6% sand, and a maximum water holding capacity (WHC_{max}) of 45.8 w/w (Förster et al., 2009). Pots ($9 \times 9 \times 20\ cm^3$) were filled with 1455 g dry mass (dm) of 4 mm-sieved soil to reach a density of 1.2 kg dm^{-3} and sowed with *Dactylis glomerata*. Before manure application, all 140 pots were pre-incubated in greenhouse under 60–70% relative air humidity (RH) and 16 h day light for 11 weeks to achieve dense root masses. Manure was obtained from pigs without history of antibiotic treatment (Agricultural Experimental Station for Livestock Sciences Frankenforst, University of 143 Bonn, Germany). 116 ml of freshly prepared mixtures (1:1 V/V) of dH2O or 100 mg kg^{-1} SDZ (Sigma–Aldrich, Germany) carefully applied at the soil surface, to avoid retaining of SDZ at the surface of the leaves. This corresponded to a manure load of approximately 30 m^3 ha^{-1} and nominal sulfadiazine concentrations of 0 or 4 mg kg^{-1} soil dm. The fertilized pots were then transferred to climate chambers and maintained at a constant temperature of 20 °C, 70% RH and 16 h day light. Directly after manure application, 32 pots of each treatment (labelled as CO-W and S4-W, respectively) were daily watered to maintain the soils at moist conditions throughout the experiment. Therefore, pots were daily weighted and water was added to archive initial weights corresponding to the desired 40% WHC_{max}. Due to increases of plant biomass during the incubation time, keeping variations of water contents at zero was not feasible. However, they never dried out below 27% WHC_{max}. Another 32 pots of each treatment were submitted twice to 7 days of dryness, in which pots were not watered to achieve soil water contents of approximately 10% WHC_{max} (labelled as CO-D, for the treatments with manure without SDZ and submitted to dry–wet regime and S4-D, for the samples where manure was spiked with SDZ and submitted to dry–wet regime). The used minimal moisture level was set up based on pre-experiments, where soils were dried to reach almost air-dry conditions, but avoiding the milting point of the plants. Each dryness interval was followed by 21 days of daily watering, where the system was allowed to recover. The volumetric water content was monitored every hour using an EC-5 sensor (UMS, Germany) in 4 replicates per moisture regime. Daily variations in water content remained under 5%.

Soil samples were taken from the upper 5 cm, where the greatest part of the applied SDZ is expected to be retained, as previously shown by Unold et al. (2010). Sampling was carried out before and immediately after manure application, at the end of each cycle of dryness and 2, 7 and 21 days after rewetting from 4 independent pots per time point and treatment (CO-W, S4-W, CO-D, S4-D). The soil dry mass was determined gravimetrically after every sampling.

2.2. Total dissolved nitrogen (TDN), ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) measurements

Soil samples were extracted with 0.01 M $CaCl_2$ at a soil: liquid ratio of 1:5. ADIMA-TOC 100 (DIMATEC Analysentechnik GmbH, Germany) with TNb module was used for the quantification of the total dissolved nitrogen. Ammonium, nitrate and nitrite were measured by continuous flow analyses with a photometric autoanalyzer (CFA-SAN Plus/Skalar Analytik, Germany) according to Fuß (2009).

2.3. Potential nitrification and denitrification activities

The potential nitrification activity (NEA) was determined using a miniaturized version of ISO 15685 (2004) modified by Hoffmann et al. (2007). Briefly, 2.5 g of soil were incubated with 10 ml reagent solution (1 mol l^{-1} potassium phosphate buffer pH 7.2, 5.63 mol l^{-1} sodium chlorate, and 1.5 mol l^{-1} diammonium sulfate). The suspensions were incubated at 25 °C in an orbital shaker (175 rpm). After 2 h and 6 h, ammonia oxidation was stopped by addition of 1 ml potassium chloride (1 mol l^{-1}). After 20 min residence time, samples were centrifuged at $3000 \times g$ for 2 min. To measure nitrite production, 150 μl of the samples were filled into a microtiter plate in three replicates. Then 90 μl ammonium chloride (0.19 mol l^{-1}) and 60 μl color reagent (0.06 mol l^{-1} sulfanilamide acidified, phosphoric acid and 1.72 mmol l^{-1} *N*-(1-naphthyl)-ethyleneaminedihydrochloride) were added to each well. After 15 min, the absorbance was measured photometrically at a wave length of 520 nm in a microtiter plate reader. The change in concentration over time represents the potential activity.

The potential denitrification activity (DEA) was measured based on the acetylene inhibition method according to Ryden et al. (1979). Three grams of soil were placed into 10-ml gas-tight vials which were sealed with septum caps. DEA was analyzed after adjusting the soil water content to 45% WHC_{max}, adding 40 mg NO_3–(KNO_3) kg^{-1} soil dry mass and exchanging the vial atmosphere to nitrogen. After replacing 10% of the headspace volume with acetylene, the samples were incubated at 20 °C for 24 h. The evolved nitrous oxide was quantified with a gas chromatograph (Shimadzu GC-2014AF gas chromatograph with an AOC-5000 autosampler, Shimadzu, Japan) equipped with an electron capture detector (ECD, 250 °C) and a 1 m × 1/8″ HayeSep Q