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Laboratory testing on the removal of the veterinary antibiotic doxycycline during long-term liquid pig manure and digestate storage

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HIGHLIGHTS

- Doxycycline was found in farm fertilizers in concentrations of mg kg^{-1} dry weight.
- Residues were partly removed during long-term storage of manures and digestates.
- Half-lives of 120 d and 91 d emphasized the persistence of doxycycline.

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ABSTRACT

The veterinary antibiotic doxycycline (DOXY) is today frequently applied in conventional pig husbandry for the control of respiratory diseases. After the treatment, pigs excrete major amounts of DOXY as the unchanged active substance. Thus, DOXY residues were found in liquid manures and digestates of biogas plants at concentrations of mg kg^{-1} dry weight. In order to assess the impact of field applications of contaminated manures and digestates on the entry of DOXY residues into arable and grassland soils, thorough information about the removal of DOXY during long-term storage of farm fertilizers is required. Since this aspect has been only less investigated for manures but not for digestates, first long-term storage simulation tests were performed at laboratory scale. Within the 170-d incubation periods under strictly anaerobic conditions, doxycycline was removed in liquid pig manure by 61% and in digestate by 76%. The calculated half-lives of 120 d and 91 d thus emphasized the persistence of doxycycline in both matrices. Due to the substance specific properties of DOXY, this removal was caused neither by mineralization, epimerization nor biotransformation. According to the high affinity of DOXY to manure and digestate solids, however, the formation of non-extractable residues has to be taken into account as the predominant concentration determining process. This was indicated by the sequential extraction procedure applied. Hence, these results confirmed that a full removal capacity for doxycycline cannot be reached through the long-term storage of farm fertilizers.

1. Introduction

Antibiotics are applied in conventional animal husbandry to treat diseases (Venglovsky et al., 2009). Until now, the class of tetracyclines as first-generation antibiotics is of major relevance. From a total of 1238 tonnes delivered in German veterinary medicine in 2014, 342 tonnes of tetracyclines were administered to treat pigs, poultry and cattle (BVL, 2015). In European pig husbandry, tetracyclines were reported to dominate up to 47% of the total frequency of antibiotic applications for treatments of respiratory diseases caused by *Pasteurella multocida* and *Mycoplasma hyopneumoniae* (Bousquet et al., 1998; De Briyne et al., 2014). Besides tetracycline, chlortetracycline and oxytetracycline (Ratsak et al., 2013; Zhou et al., 2013a, b; Spielmeyer et al., 2014), doxycycline (DOXY) recently belongs to the frequently applied antibiotics (Berendsen et al., 2015), particularly to treat fattening and breeding pigs (Callens et al., 2012).

Tetracycline antibiotics inhibit the protein synthesis in the target bacteria by preventing the attachment of aminoacyl-tRNA to the 30S ribosomal acceptor at A-site. Thus, they cause bacteriostatic effects to wide range of Gram-positive and Gram-negative bacteria,

Abbreviations: DOXY, doxycycline; DMC, demeclocycline; MTC, metacycline; epi, epimer; SPE, Solid-phase extraction; LC-MS/MS, liquid chromatography/tandem mass spectrometry; ESI, electrospray ionization.

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as well as typical organisms such as chlamydia, mycoplasmas and rickettsias and protozoan parasites (Chopra and Roberts, 2001). After the administration to pigs, tetracyclines are predominantly excreted as the unchanged parent compounds up to 75% (Halling-Sørensen et al., 2001; Winckler and Grafe, 2001). As a result, tetracycline residues were found in liquid pig manures up to 770 mg kg^{-1} dry weight (DW) (Gans et al., 2010). DOXY residues were detected from 0.01 to 60 mg kg^{-1} DW (Hu et al., 2008; Qiao et al., 2012).

In conventional manure management, liquid pig manures are long-term stored in cellars, silos or lagoons under anaerobic conditions until field application. Alternatively, liquid manures are used as co-substrates in the biogas plants (Saggar et al., 2004). Here, the use of contaminated manures may also cause DOXY residues in digestates as already reported for other tetracyclines (Ratsak et al., 2013; Spielmeyer et al., 2014). Thus, the application of both farm fertilizers can contribute to soil contamination.

These findings raised the question how far DOXY residues can be removed by anaerobic biotransformation during long-term storage of contaminated liquid pig manures and, in particular, of digestates. Here, detailed information is still limited. Fernández et al. (2004) conducted ecotoxicological tests on DOXY using 15-d aged liquid pig manure. Within this short-term aging period, the initial DOXY concentration dropped by 50–60%. However, the concentration determining process was not elucidated. Similar results were reported by Szatmári et al. (2011) and Ho et al. (2013). During composting of pig feces for 112 d and broiler manure for 40 d under laboratory conditions, DOXY was removed up to 70% and 100%, respectively. In these both studies, the removal of DOXY was only characterized through the decreasing concentrations of the parent compound initially applied. Neither the formation of biotransformation products nor of non-extractable residues were taken into account. Furthermore, epimerization was not considered due to the analytical methods applied there.

Within the present study, therefore, fate and behavior of DOXY was monitored in laboratory simulation tests on liquid pig manure and digestate storage up to 170 d in order to meet the long-term storage conditions practiced at farm scale. Due to the unavailability of DOXY as a ^{14}C-labeled radiotracer, these biotransformation tests under strictly anaerobic conditions were accompanied by residue analysis applying liquid chromatography/tandem-mass spectrometry (LC-MS/MS). Hence, it was to be additionally investigated how far losses during sample preparation and matrix effects during electrospray ionization could be compensated by surrogate standard technique and single-point standard addition, respectively. It was finally studied how far the removal of DOXY was allocated to the formation of epimers and its corresponding metabolite metacycline (MTC) or to the formation of non-extractable residues.

2. Materials and methods

2.1. Sampling and matrix characterization

Liquid pig manure and digestate samples were taken from 3 pig husbandry farms with farm-own biogas plants in Lower Saxony, Germany, in September 2013. The involved farmers cooperated on the condition that farm specific data, including the locations of the farms, were only used in anonymized form. The liquid pig manures were sampled from cellars of animal houses using a probe sampler (2 m length, 53 mm ID), while digestate samples were taken from silos via outlet valves. From each 8-L sample taken, 300-mL aliquots were transferred into polyethylene bottles which were then transported in cooling boxes to the laboratory.

There, pH values and redox potentials (E_h) of all samples were measured using a microprocessor pH meter equipped with pH-glass electrode SenTix61 (pH 535 MultiCal; Weilheim, Germany) and redox electrode (Inolab Redox Single-rod Measuring Cell; Mettler Toledo, Giessen, Germany), respectively. The dry weights (DW) of the samples were determined using a moisture analyzer (DLB-A, Kern and Sohn GmbH, Balingen, Germany).

2.2. Storage simulation tests using liquid pig manure and digestate samples

The long-term storage tests under anaerobic conditions were performed in accordance with the reference-manure concept for testing of veterinary medicines and biocides (Kreuzig, 2010; Kreuzig et al., 2003, 2010a). Thus, laboratory batch systems, traced back to the OECD guideline 304A (OECD, 1981), were used which were equipped with internal traps with inlet and outlet valves allowing for a discontinuous gas exchange. Even though DOXY was tested unlabeled, the internal traps were filled with KOH (8 mL 0.1 M) which was exchanged every week to meet the standard conditions for testing the biotransformation of ^{14}C-labeled test substances.

In order to simulate the manure and digestate storage, 75-g samples were filled into the 300-mL Erlenmeyer flasks of the laboratory batch systems for each incubation interval of 0, 7, 35, 84, 120 and 170 d. Batch tests were conducted in duplicate. According to the findings of tetracycline antibiotics in real manure and digestate samples, manure samples were spiked with DOXY at 51.3 mg kg^{-1} DW, while the digestate samples were spiked at 20.0 mg kg^{-1} DW. For matrix characterization, additional series of samples spiked at the same concentration levels were prepared as well. Additionally, samples only spiked with methanol were used for recovery tests at the termination of each incubation interval. Afterwards, the batches were incubated at 20 ± 0.1 °C in the dark.

To describe the degradation kinetics of DOXY during the storage simulation tests, the first-order kinetic model was applied and the half-lives of DOXY were calculated in both matrices (Ho et al., 2013).

2.3. Analytical procedure

2.3.1. Reference chemicals and standard solutions

The reference chemicals, namely the test substance DOXY-hyclate ($C_{22}H_{24}N_2O_8 \cdot HCl \cdot 0.5\ H_2O \cdot 0.5\ C_2H_6O$) and the surrogate standard demeclocycline-HCl (DMC; $C_{21}H_{21}ClN_2O_8 \cdot HCl$) were purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany, with purities >97.5%. Their corresponding epimers 6-epi-DOXY-HCl and 4-epi-DMC-HCl as well as the metabolite MTC-HCl ($C_{22}H_{22}N_2O_8 \cdot HCl$) were obtained from the European Directorate for the Quality of Medicines and Healthcare, Strasbourg, France.

All reference chemicals were individually dissolved in methanol at 1 μg μL^{-1}. Before use, all glassware was rinsed using 3–5 mL saturated ethylenediamine tetraacetic acid disodium salt dehydrate solution (EDTA; Carl Roth GmbH, Karlsruhe, Germany) in methanol and finally air-dried. The stock standard solutions, directly used for spiking of batch tests or corresponding fortification tests, were diluted for preparing the working standard mixture of DOXY, DMC and MTC as well as the standard mixture of the epimers at 50 ng μL^{-1}. Those standard mixtures were individually prepared in methanol every two months. The daily standard mixtures for single-point calibration were prepared from a consecutive dilution of the 50-ng μL^{-1} standards to 50 pg μL^{-1} using a mixture of water/acetonitrile (1/1, v/v) with 0.1% formic acid (HCOOH). The standard mixture of 1.0 ng μL^{-1} in acetonitrile was prepared for single-point standard addition. All organic solvents and water were of HPLC grade (VWR Chemicals Prolabo, Fontenay-sous-Bois, France) while