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A strategy to determine the fate of active chemical compounds in soil; applied to antimicrobially active substances

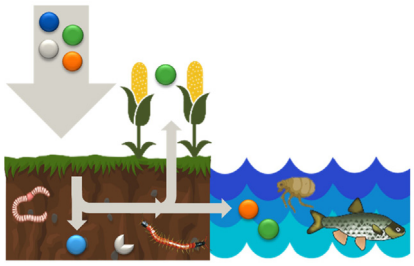
Bjorn J.A. Berendsen*, Gregg Roelofs, Benjamin van Zanten, Wilma D.M. Driessen-van Lankveld, Mariël G. Pikkemaat, Irma E.A. Bongers, Erik de Lange

Wageningen Food Safety Research, Wageningen University & Research, Akkermaalsbos 2, 6708 WB, Wageningen, the Netherland

HIGHLIGHTS

- A tiered fate study strategy for soil including a persistence and mobility test.
- A test to uncover the production of active degradation products is included.
- Application of the fate study strategy to ten antibiotic from 6 different groups.
- New degradation products of tylosin A including kinetics and mobility study.

GRAPHICAL ABSTRACT



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ABSTRACT

Data on the fate of chemical substances in the environment after e.g. manure application is mandatory input for risk assessment in perspective of a more circular biobased economy. Such fate studies include a persistence study to determine a half-life value and a mobility study. It is recognized that not only the native substance should be considered, but that also degradation products should be included that might exert a similar effect as the native substance. We report a tiered fate study strategy that starts with a persistence study. For non-persistent substances a study is performed to determine if degradation products have a similar effect as the native compound. If so, a procedure using high resolution mass spectrometry is suggested to identify the potentially active degradation products. Based on the outcomes, substances are divided into three categories: (I) persistent, (II) degradable to inactive products or (III) degradable to active products. Even though the priority is with category I and III, for all substances and possible degradation products a mobility study is proposed. The fate strategy is successfully applied to ten antimicrobially active substances originating from the tetracyclines, sulfonamides, diaminopyrimidines, fluoroquinolones, macrolides and lincosamides. The fluoroquinolones, tetracyclines and trimethoprim were relatively persistent. The sulfonamides, macrolides and lincomycin (the latter also depending on soil type) degraded relatively quickly. Tylosin A proved to degrade to antimicrobially active degradation products which were tentitatively identified as tylosin C, tylosin A acid, tylosin B acid and tylosin C acid.

1. Introduction

Chemical contaminants end up in agricultural soils after manure

*Corresponding author.
E-mail address: bjorn.berendsen@wur.nl (B.J.A. Berendsen).

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application as a result of disease treatment in animal husbandry (e.g. veterinary drugs), crop production (e.g. biocides) or through irrigation using contaminated surface water (e.g. biotoxins). Manure is known to be one of the major pathways through which antibiotic residues are introduced into the environment (Halling-Sørensen et al., 1998; Chee-Sanford et al., 2009). When further implementing the concepts of a circular biobased economy, other waste streams might also contribute to soil exposure. As the number of chemicals that is (unwillingly) applied to agricultural soil is high, so is the number of organisms that are potentially affected. It is, therefore, impossible to carry out an extensive risk assessment for all chemicals and organisms in all relevant reservoirs. Consequently, it is important to understand the fate of these chemicals in a circular system and use this knowledge to predict the fate of chemicals by translocation modelling. As a result risk assessment studies can be prioritized for the most hazardous chemicals in the reservoirs they occur in most.

The fate of chemical substances in soil depends on their persistence and their mobility. The persistence relates to the kinetic degradation of the native contaminant and the mobility determines the transport of the chemical to other reservoirs. Immobile contaminants are retained in the soil and, if persistent, can accumulate there. Mobile contaminants leach to ground water or run off to surface water (mobile) and are potentially available for plant uptake.

The persistence of a chemical substance depends on the physico-chemical properties of the substance itself and on the soil composition and its biotic and abiotic properties, including the fraction of organic matter, pH and the active microbiome. Several studies investigated the fate of selected antibiotics in manure (Berendsen et al., 2018), or soil after applying manure containing antibiotics (Martínez-Carballo et al., 2007; Brambilla et al., 2007; Burkhardt et al., 2005; Domínguez et al., 2014; Hamscher et al., 2002; Jacobsen et al., 2004; Uslu et al., 2008; Spielmeyer et al., 2017; Stoob et al., 2007; Sukul et al., 2008). Methods applied in this study are partially derived from method 307 of the Organisation for Economic Co-operation and Development (OECD (OCED, 2002)). Many studies focus on the persistence of the native substance. However, it is recognized that degradation products can still exert a negative impact on the environment. Therefore, degradation studies followed by a studies to annotate potential degradation products should be included in strategies to assess the fate of chemicals in the agricultural environment.

The mobility is related to the soil-water partitioning coefficient (K_d), which expresses the potential of a chemical contaminant to be adsorbed by soil. Since adsorption occurs predominantly by partition into the soil organic matter, it is more useful to normalize K_d to the organic carbon content of the soil and express the distribution coefficient as a organic carbon-water partition coefficient (K_{OC}) (Wegst-Uhrich et al., 2014). Additionally, estimation of K_{OC} by K_{OW} (octanol water partition coefficient) leads to significant underestimation of the K_{OC}. Mechanisms other than hydrophobic partitioning, e.g. ionic interactions, complex formation and hydrogen bonding are not accurately accounted for using this approach which results in underestimation (Tolls, 2001). Several methods have been used for estimation of K_{OC}, of which most are derived from OECD method 106 (OCED, 2000) or method 312 (OCED, 2004).

We developed a tiered fate study strategy including the determination of (1) persistence, (2) active degradation products and (3) mobility in soil. The strategy was applied to antimicrobial active compounds that are most frequently detected in animal manure (Berendsen et al., 2015; Van den Meersche et al., 2016; Karaca et al., 2018; Patyra et al., 2020; Zhi et al., 2020). The fate study strategy consists of a simple laboratory scale persistence test, an effect based test (in this case a bacterial growth inhibition test) to study the potential production of active degradation products combined with a high resolution mass spectrometry approach for identification of potential degradation products, and a cost-efficient column format mobility test. According to our knowledge, this is the first publication to combine these tests yielding an efficient tiered fate study strategy, and apply it to ten relevant veterinary antibiotics. This yielded persistence and mobility data for antibiotics of which no data has previously been reported. Additionally, we report the formation of an unknown degradation product of tylosin.

2. Materials & method

2.1. Reference standards and reagents

Oxytetracycline, enrofloxacin, flumequine, lincomycin, tylosin, sulfadoxine, and trimethoprim were purchased at Sigma-Aldrich (St. Louis, MO, USA). Note that the tylosin reference standard consists of a mixture of several forms of which tylosin A is >85%. Doxycycline and sulfadiazine were purchased at Council of Europe (EDQM, Strasbourg, France) and tilmicosin was purchased at Dr. Ehrenstorfer GMBH (Augsburg, Germany).

The internal standards enrofloxacin-d_5, flumequin-$^{13}C_3$ and sulfadoxine-d_3 were purchased at Witega (Berlin, Germany). Doxycycline-d_3, lincomycin-d_3, sulfadiazine-d_4, tylosin-d_3, tilmicosin-d_3 and trimethoprim-d_9 were purchased at Toronto Research Chemicals. and oxytetracycline-13C2215N2 were purchased from RomerLabs Diagnostics (Newark, DE, USA).

Citric acid monohydrate, di-sodium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), acetonitrile (ACN), formic acid (FA), methanol (MeOH), ammonium (25%), were purchased at Witega (Darmstadt, Germany). Lead acetate trihydrate, trifluoric acetic acid (TFA) and ammonium formate were purchased at Sigma-Aldrich (St Louis, MO, USA).

McIlvain-EDTA buffer was prepared by adding 500 mL 0.1M citric acid and 280 mL 0.2M di-sodium hydrogen phosphate to 1 L water into a 2 L volumetric flask. The pH was adjusted to 4.0 using citric acid solution or di-sodium hydrogen phosphate solution and the solution was diluted with water up to the mark.

Stock solutions of reference standards and internal standards were prepared at a concentration at 100 mg L^{-1} for the fluoroquinolones and at 1000 mg L^{-1} for the other compounds. Tetracyclines, sulfonamides and diaminopyrimidines were dissolved in MeOH and (fluoro)quinolones in a solution of 2% 2M ammonium hydroxide in MeOH. The lincosamides and the macrolide tylosin were dissolved in water and the macrolide tilmicosin in ACN. A mixed solution of reference standards and a solution of internal standards was made at a concentration of 10 mg L^{-1} in MeOH for the ten compounds used in the extraction experiments. For the longitudinal experiment, a mixed solution of reference standards was made at a concentration of 20 mg L^{-1} and internal standards at a concentration of 5 mg L^{-1}, both in MeOH.

2.2. Soil samples

To demonstrate the effectiveness of the presented approach, the fate of ten model antibiotics in two different soil types was studied. One soil type was a sandy, the other was a clay soil. These were selected due to being the two major soil types used for agricultural practices. in The Netherlands. The soil type was pH 5.5, with soil organic matter (SOM) of 6.4% and the 16S RNA count was $9.3*10^9$. The clay soil had a pH of 6.8, a SOM of 5.9% and a less abundant microbiome: S9 RNA of $1.8*10^9$.

2.3. Sample preparation and analysis

The soil analysis was carried out using a procedure published before for analysis of manure samples (Berendsen et al., 2018; Jansen et al., 2019). In short, 2 g of soil was extracted with 4 mL of a freshly prepared 0.125% trifluoro acetic acid in acetonitrile and 4 mL of McIlvain-EDTA buffer. In case a larger sample intake was used, extraction volumes were adjusted accordingly. After evaporation of the organic solvent, the extract was cleaned by solid phase extraction (SPE) prior to analysis with liquid chromatography mass spectrometry (LC-MS), either by triple-quadrupole (MS/MS) or by Quadrupole-orbitrap (high resolution MS, hrMS). Water samples (4 mL) were mixed with 1:1 with McIlvain-EDTA buffer and directly subjected to SPE.

Chromatographic separation was done using a Kinetex C18 2.1 × 100 mm 1.7 μm analytical column (Phenomenex), placed in a column oven operating at 40 °C. The mobile phases used were 2 mM ammonium formate and 0.016% FA in water (Solvent A) and 2 mM ammonium formate and 0.016% FA in MeOH (Solvent B). The gradient used at a flow of 0.3 mL min^{-1} was: 0–0.5 min, 1% mobile phase B, 0.5–2.5 min, linear increase to 25% B, 2.5–5.4 linear increase to 70% B, and 5.4–5.5 min linear increase to 100% with a final hold of 1.0 min. Initial conditions are returned within 0.1 min with a final equilibration time of 0.9 min, resulting in a total run of 7.5 min. The injection volume was 5 μL.

LC-MS/MS analysis was carried out using an Acquity UPLC System, coupled to an AB Sciex Q-trap 6500 mass spectrometer. Both liquid chromatography and mass spectrometry settings, including ion transitions, were used as described previously (Berendsen et al., 2018; Jansen et al., 2019). Data processing was done using MultiQuant 3.0.2 software.

For correct quantification of the soil and water fractions analysed, matrix fortified soil and water were used and isotopically labelled internal standards were added to all individual samples before sample preparation. Analytical methods applied were all ISO 17025 accreditated under flexible scope.

For identification of degradation products LC-hrMS was used. The LC system consisted of a Thermo Scientific (San Jose, CA, USA) Ultimate 3000 UHPLC system equipped with the same analytical column as used for LC-MS/MS. The gradient was similar to LC-MS/MS but slower, with a total run time of 15 min and the injection volume was 10 μL. Detection was carried out using a Thermo Scientific Q-Exactive Orbitrap MS operating with a heated electrospray ionisation II source in positive mode. The operating parameters were: electrospray voltage, 3.5 kV; capillary temperature, 255 °C; analyzer temperature, 30 °C; sheath gas, 50 AU; auxiliary gas temperature, 410 °C; heated capillary, 255 °C. All resolutions mentioned are FWHM defined at *m/z* 200. Detection was carried out by full scan analysis (*m/z* 80–1075) at resolution 140,000 alternated with a set of variable data-independent acquisition (vDIA) windows at resolution 35,000 applying an isolation range of subsequently *m/z* 75–205, 195–305, 295–405, 395–505, 495–1205 and a fragmentation NCE of 30 and 80. Data was processed using Xcalibur software (version 4.2.28.14).

2.4. Fate study strategy

An overview of the developed tiered fate study strategy is presented in Fig. 1. The starting point is a laboratory scale degradation study (1). Only if significant degradation of the native compound is observed, a study into the potential formation of active degradation products is carried out (2a). If this test indicates the presence of active degradation products, these are identified (2b). On the basis of these tests, three categories are distinguished: in soil the chemical substance is (I) persistent, (II) degrades to inactive products or (III) degrades to active products. Substances belonging to category I and III are subjected to a mobility study and the final fate is used as input for the translocation model (outside the scope of this paper).

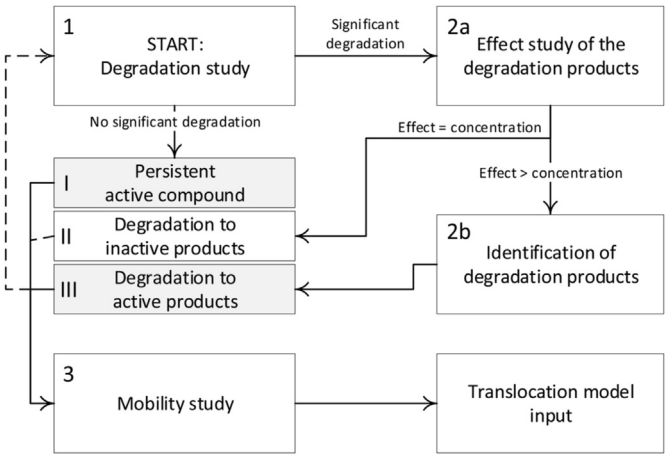

Fig. 1. Overview of the tiered fate study strategy consisting of a degradation study, study for active degradation products and a mobility study.

2.4.1. Degradation study

An aerobic degradation study was carried out during a time frame of over a month. For each time point, 2 g of soil sample was transferred into a polypropelene tube in duplicate. The antibiotics were added as a mixture to all soil aliquots at a concentration of 250 ng g^{-1}. The spiked soil sample aliquots were mixed and the tubes slightly capped, so not to exclude air from the tubes. They were incubated at room temperature under the exclusion of UV light and selected soil sample aliquots (n = 2) were transferred to −80 °C after 0, 1, 2, 4, 7, 11, 16, 22, 29 and 37 days to terminate any potential degradation process. Loss of water during incubation was corrected for by adding water after 2 weeks of incubation on weight basis. After all aliquots were placed in the ultrafreezer, internal standards were added prior to sample extraction, to allow accurate quantification by LC-MS/MS. Additionally a short term experiment was carried out comparing the persistence in sterile and non-sterile soil as was previously described (Berendsen et al., 2018).

The degradation kinetics of the antibiotics was assessed based on the recommendation of the Forum for the Co-ordination of pesticide fate models and their Use (FOCUS) degradation kinetics (kinfit) (FOCUS, 2016). For each of the individual duplicates, the remaining fraction of intact active substance (a.s.) was plotted against the storage time and fitted using single first order (SFO) and three bi-phasic models: first order multi compartment (FOMC), double first order in parallel (DFOP) and hockey stick (HS) (Software: R-studio (Computing, 2017)). When the χ^2-error of the fit to the SFO model was below 5%, this model was used. In case the SFO fit showed a $\chi 2$-error above 5%, the model with the lowest $\chi 2$-error was selected. Next, based on the selected model for each of the antibiotics, the half-life (DT50) of the native compound was calculated.

2.4.2. Activity analysis of degradation products

To determine if produced degradation products show antimicrobial activity an effect assay was applied. The antibiotic of interest was added to soil (n = 2) and was incubated until its degradation was between 70 and 90% (estimated based on the determined DT50). The soil samples were extracted as described and the resulting extract was analysed by LC-MS/MS to determine the exact

concentration of the native compound remaining in the final extract. The extracts were also transferred onto an antimicrobial plate test (Nouws Antibiotic test) to quantify the potency of the degradation products present (Pikkemaat et al., 2008). Briefly, extracts were transferred into wells in a layer of nutrient agar inoculated with a susceptible bacterial strain. The agar plate was incubated overnight at optimal temperature to allow bacterial growth, which turns the agar opaque. If a sample extract contained antimicrobially active substances to which the bacteria are susceptible, the bacteria in the proximity of the well were unable to multiply. A growth inhibition zone became visible, of which the diameter depended on the potency of the antimicrobially active substance present and its concentration.

The concentration of the native substance partial degradation was determined by LC-MS/MS. The inhibition zone corresponding to this concentration was determined using a solvent calibration line on the assay. If the actual inhibition zone of the soil extract exceeded the expected inhibition zone, it was concluded that the native compound (partly) degraded to antimicrobially active degradation products.

2.4.3. Identification of active degradation products

The extracts obtained from the activity analysis experiment was introduced into the LC-hrMS system. The selection of relevant signals and the subsequent tentative identification of the degradation products occurred by several data evaluation procedures. First the data was scanned for previously reported degradation products by producing extracted ion chromatograms based on their exact masses (*m/z* tolerance 5 ppm) and relevant adducts, doubly and triply charged ions thereof. Secondly, if possible, fragment ion flagging (FIF) analysis was carried out, extracting typical structure related product ions of a specific compounds from the vDIA chromatograms. In case a relevant signal was observed, the molecular mass was determined from the full scan chromatogram at the indicated retention time. Finally, the ion chromatograms of the extracts obtained from the spiked soil sample, before and after degradation, were compared visually by inspecting multiple small mass extraction windows, by automated subtraction using Xcalibur software and by statistical analysis using MetAlign software (version 041012, WFSR, Wageningen).

2.4.4. Mobility study

For studying the mobility of a substance, a column leaching test based on OECD method 312 (OECD, 2004). Five grams of fresh soil (corrected for dry weight) was transferred (n = 2) into a polypropylene reservoir (10 mL, 15 mm diameter) that contained a small layer of cotton wool to hold the soil in place. The reservoirs were placed on top of an SPE manifold and the soil was wetted by running 5 mL of tap water through the soil. A 200 μL volume containing 400 ng of a mixture of the antibiotics was transferred onto the soil. After a brief incubation to prevent severe degradation of the antibiotics (unless the mobility of degradation products is to be determined), tap water (10 mL) was run through the soil (during 1 h) and was collected in a centrifuge tube. The content of the reservoir (soil and cotton wool) was transferred to another tube. The soil fraction and the water fraction were analysed by LC-MS/MS individually. Internal standards were added prior to sample extraction, to allow accurate quantification. The K_d was determined by dividing the antibiotic content of the soil fraction by the antibiotic content in the water fraction. K_{OC} was determined by dividing the K_d by the organic content of the soil (Wegst-Uhrich et al., 2014). The mobility study was carried out for all native antibiotics and additionally for the (tentatively) identified degradation products.

3. Results and discussion

The individual experiments were optimised to be applicable in a relatively high throughput set-up. The tiered strategy was then applied to ten antibiotics frequently detected in animal manure. These reflect six different antibiotic groups: tetracyclines: oxytetracycline and doxycycline, sulfonamides: sulfadiazine and sulfadoxine, diaminopyrimidines: trimethoprim, fluoroquinolones: enrofloxacin and flumequine, macrolides: tylosin A and tilmicosin, and lincosamides: lincomycin. The optimisation of the experiments and the results of the individual antibiotics are discussed.

3.1. Degradation study

In the degradation experiment, the antibiotic amended soils were placed at room temperature for a maximum of 37 days. The remaining fraction of the antibiotics during the long term experiment is graphically presented in Fig. 2 for both sand and clay. Note that for enrofloxacin and tilmicosin in clay no data are presented as the analysis yielded unreliable results due to high variability. The calculated DT50 and DT90 values for the degradation of the antibiotics in soil are presented in Table 1. The χ^2-errors of all fits carried out by the kinfit procedure are presented in the supplementary data (S1). The selected model, the model parameters and calculated DT90 values are also presented there. Note that DT50 and DT90 values which exceed the time frame of the study, are considered to be rough estimates.

For both fluoroquinolones, the degradation process is best described using simple first order kinetics. For tetracyclines and the sulfonamides the degradation is best described by a double first order in parallel (DFOP) model. These findings comply with the degradation kinetics previously reported in pig and poultry manure (Chen et al., 2019). For the other antibiotics, the best description of degradation kinetics depends on the soil type.

The sulfonamides are very non-persistent in both soils with a DT50 of approximately one day. The tetracyclines are slightly more persistent with a DT50 of around 10 days. The fluoroquinolones demonstrated to be very persistent in both soils with a maximum degradation of 25% after 37 days of incubation. For flumequin a DT50 of over 200 days is estimated in sand. Also, tilmicosin proved to be very persistent in sand with an estimated DT50 of over 100 days. However, if a simple first order kinetic model was used instead of a DFOP model, which also showed a good fit, the DT50 was estimated at 52 days. For trimethoprim, lincomycin and tylosin A, the persistence strongly depends on soil type. Trimethoprim shows a DT50 of 12 days for sand and 75 for clay. In both soils a DT90 of approximately 250 days was estimated. Lincomycin shows fast degradation in sand (DT50 = 1 d) but is more persistent in clay (DT50 = 11 days). Another extreme case is tylosin A showing a DT50 of approximately 3 days in sand and 73 days in clay. In clay, the estimated DT90 for tylosin A is about 250 days. An interesting observation is that the degradation of tylosin A, lincomycin, trimethoprim and tilmicosin in the tested soils, is almost exclusively caused by biotic processes as was demonstrated in an experiment comparing the persistence in sterile and non-sterile soil (data not shown). For tylosin this was reported previously (Sassman et al., 2007). The observation of higher persistance of tylosin A, lincomycin and trimethoprim in clay is therefore most likely contributed to the less pronounced microbiome in clay as is apparent from the 16S RNA determination.

Determined DT50 values in the current study were compared with previously reported values (Supplementary information S2). Interestingly, in the current study, the DT50 of doxycycline was much lower than previously reported (533–578 days) (Walters et al., 2010). As that specific study was carried out in biosolids-

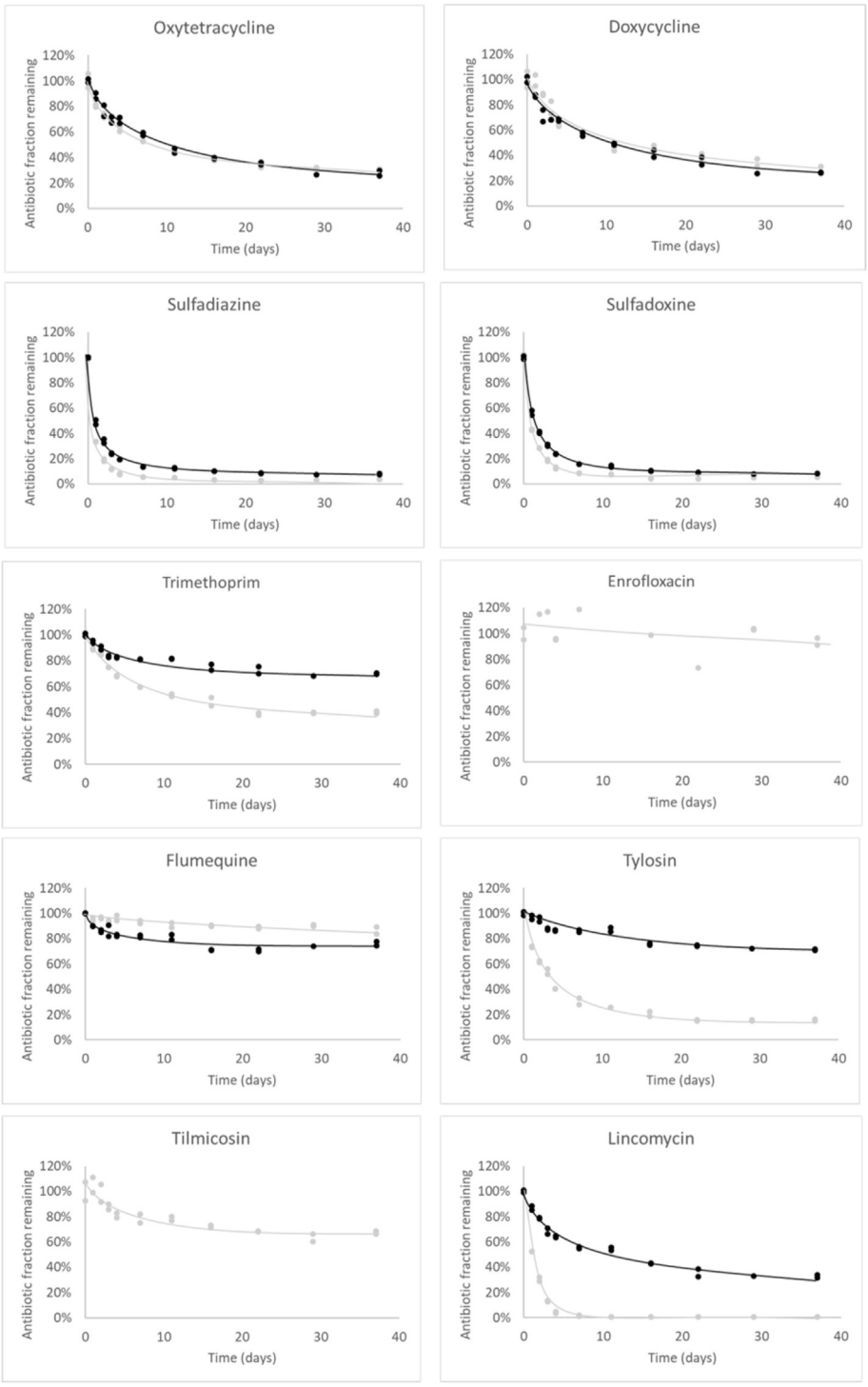

Fig. 2. Degradation of the antibiotics in (grey) sandy soil and (black) clay soil in the long term degradation study. For enrofloxacin and tilmicosin in clay, no data was obtained.

amended soil, the DT50 might be extremely influenced by the different soil composition. It is expected that a high concentration of cations in the soil would influence the adsorption of the tetracyclines and therefore also possibly the degradation rate. Note that it is unexpected that oxytetracycline and doxycycline, which are structurally very similar, show severely different persistencies independent of the soil used (Walters et al., 2010; University of Hertfordshire, 2019; Blackwell et al., 2005; Li et al., 2010; Wang and Yates, 2008; Kay et al., 2004). In that respect the similarity of the DT50 values found in the current study for both tetracyclines is according to expectations. For the sulfonamides, the DT50 determined in the current study is comparable to values reported before (Chen et al., 2019; Hammesfahr et al., 2008; Sittig et al., 2014; Zhang et al., 2017; Förster et al., 2009). The quinolones were reported to be very persistent, with DT50 ranging from 123 to 150 days (University of Hertfordshire, 2019; Thiele-Bruhn, 2003). In the current study, the quinolones are also found to be very persistent. For trimethoprim a wide range of DT50 values is reported in

Table 1
Determined DT50 and log K_{OC} for the antibiotics in sand and clay.

Antibiotic	Soil type	DT50 (days)	DT90 (days)	Log K_{OC} (L kg^{-1})
Oxytetracycline	Sand	8	110	≥3.0[a]
	Clay	9	85	3.3
Doxycycline	Sand	9	100	3.5
	Clay	11	66	3.0
Sulfadiazine	Sand	0.6	3	0.5
	Clay	1.0	20	0.7
Sulfadoxine	Sand	0.9	7	0.5
	Clay	1.4	23	0.5
Trimethoprim	Sand	12	270	≥4.2[a]
	Clay	75	250	≥4.3[a]
Enrofloxacin	Sand	137	460	≥2.9[a]
	Clay	NR	NR	≥3.3[a]
Flumequine	Sand	226	750	≥4.2[a]
	Clay	97	320	≥4.3[a]
Tylosin	Sand	3	55	≥3.2[a]
	Clay	73	240	2.0
Tilmicosin	Sand	108	620	≥3.2[a]
	Clay	NR	NR	≥3.4[a]
Lincomycin	Sand	1.1	4	2.1
	Clay	11	93	1.6

NR: No Results.
[a] Calculated K_{OC} values are impacted by the limit of detection of the analytical method: actual K_{OC} values are equal or higher than the indicated value.

literature (University of Hertfordshire, 2019; Liu et al., 2009; Lin and Gan, 2011). This complies with the finding that the persistence of trimethoprim depends on the soil composition. The values found in the current study are within the previously reported range. The same was observed for tylosin: reported DT50 values were 2–96 days (de la Torre et al., 2012; University of Hertfordshire, 2019; Blackwell et al., 2005; Thiele-Bruhn, 2003; Liu et al., 2009; Boxall et al., 2004; Dolliver and Gupta, 2008; Topp et al., 2016; Carlson and Mabury, 2006; Hu and Coats, 2007; Schlüsener et al., 2006; Halling-Sørensen et al., 2005). This observation also complies with the finding that tylosin persistence depends on soil composition. For tilmicosin only one paper reported DT50 values (University of Hertfordshire, 2019): DT50 is 64 (University of Hertfordshire, 2019), which is comparable with the estimated DT50 if a simple First Order kinetic model is applied. Also for lincomycin only a single DT50 value was reported previously (Kuchta et al., 2009): this value was severely higher than determined in the current study. Also here, to some extend, soil composition might have caused this difference.

In perspective of the fate analysis strategy, from the persistence study it is concluded that the sulfonamides, lincomycin and tylosin A are very non-persistent with DT50 values of below 5 days in at least one of the soil types. These are prioritized to be subjected to tier 2, a study for potential antimicrobially active degradation products. Tier 2 is also relevant for the tetracyclines and trimethoprim. The other antibiotics are more persistent and additional effect studies have lower priority: in this study these are considered as category I compounds, (relatively) persistent, and are directly subjected to tier 3, the mobility study.

The applied method is a simplified version of method 307 of the OECD (OECD, 2002). The advantage of the current set-up is that the degradation of large numbers of substances in different soil types can be determined in a cost effective manner. Soil aliquots are small to allow experiments if only little soil is available and is facilitates extraction of the complete aliquot after incubation using only limited solvent volumes (green chemistry). Furthermore, a critical homogenisation step of a relatively large batch of soil after incubation is ommitted. A disadvantage is that a small aliquot is less representative for the whole soil lot. Therefore, in the current setup, homogenisation of the soil before start of the experiment is crucial and by increasing the number of aliquots taken (by incorporating duplicates or increasing the number of time points) the contribution of any inhomogeneity of the soil to the measurement uncertainty of the final result is diminished. As demonstrated by the simularity of the data produced by the method presented here and the persistance data available in literature, the current experiment seems to yield a good indication of the DT50 value and thus the persistence of the tested antibiotics.

3.2. Effect study of the degradation products

Sulfadiazine, lincomycin and tylosin A were subjected to a study to determine if during degradation, products are formed that still have antimicrobial properties. The antibiotics were added to sandy soil and incubated to allow a 70–90% degradation. Based on the persistence data the following exposure times were selected: sulfadiazine 5 days, lincomycin 6 days, and tylosin 7 days. The concentration in the final extracts of the incubated soil samples were determined by LC-MS/MS and were respectively 0.15 μg mL^{-1} for sulfadiazine, 0.26 μg mL^{-1} for lincomycin and 0.50 μg mL^{-1} for tylosin A (respectively, 8, 13 and 25% of the added native substance at t = 0).

These extracts were applied on their respective microbiological plate assay and the observed inhibition zones were compared with the inhibition zones of soil extracts spiked around the concentrations determined by LC-MS/MS. In case of sulfadiazine and lincomycin, the inhibition zone of the incubated soil sample was in agreement with the expected inhibition zone at the concentration of the native antibiotic still present in these extracts. Both compounds were classified as II (Fig. 1): substances that degrade to inactive products. For tylosin the extract of the incubated soil sample showed an inhibition zone equal to a tylosin A concentration of approximately 0.95 μg mL^{-1} whereas the actual concentration of tylosin A was 0.50 μg mL^{-1}. From this it is concluded that the extract exerts approximately 60% more antimicrobial activity than expected if only the native compound would be antimicrobially active. It is concluded that a significant antimicrobial effect is caused by degradation products. This requires that for tylosin tier 2b is carried out aiming for identification of the antimicrobial active degradation products.

The presented approach is easy to apply and proved effective for indicating the presence of antimicrobially active degradation products. However, the effect assay is semi-quantitative and thus, to be able to detect active degradation products, they have to be present at significant concentrations. Therefore, it is of utmost importance to establish the experimental setup in such a way that the native substance is degraded to a large extend and that effects of potential degradation products are the most pronounced. To the contrary, if the soil incubation time is too long this might result in the further degradation (e.g. mineralisation) of the active degradation products.

3.3. Identification of antimicrobially active degradation products

The final extracts of the soil sample spiked with Tylosin A at t = 0 and after 7 days incubation, originating from tier 2a, were analysed by hrMS. As only degradation products have been reported that contained the mycaminose ring (Wegst-Uhrich et al., 2014; Sassman et al., 2007; Hu and Coats, 2007), to demonstrate the effectiveness of the reported approach, the extracts were analysed in positive ionisation mode. It is recommended to analyse extracts in both positive and negative ionisation mode in case no *a priori* knowledge on the degradation products is available. Several strategies were applied to select relevant signals in the full scan spectra. First, previously reported degradation products of tylosin A

were targeted by creating extracted ion chromatograms based on the exact masses (mass error of 5 ppm) of these degradation products. For tylosin A in soil, several degradation products have been reported: tylosin B (desmycosin) (Sassman et al., 2007), tylosin C (macrosin) (Hu and Coats, 2007), tylosin A acid (Sassman et al., 2007) and tylosin B acid (Sassman et al., 2007). Chemical degradation products or degradation products formed by other processes (e.g. metabolism by animals) have been reported and are investigated in the current study as well. These are tylosin D (relomycin), dihydro tylosin B and tylosin A aldol (Wegst-Uhrich et al., 2014). Details of all these compounds are presented in Table 2, including the signals observed in the extract before (t = 0) and after degradation (t = 7).

At t = 0, besides tylosin A, also signals corresponding to tylosin D, tylosin B, dihydrotylosin B are present in the sample. These most likely originate from the reference standard used which consists of 87% tylosin A according to the reference certificate. Tylosin A is clearly degraded for approximately 66% as also indicated by the LC-MS/MS experiments. Also the signals that correspond to tylosin D, tylosin B and dihydrotylosin B decrease within 7 days, respectively for about 61, 58 and 91%. Besides degradation of these tentitatively identified compounds (identification confidence 2b according to Schymanski (Schymanski et al., 2014)), some signals corresponding to previously reported degradation product are clearly increasing during the 7 days incubation: in order of decreasing absolute response these are signal belonging to tylosin A acid, tylosin C and tylosin B acid, of which the latter might be a degradation product op tylosin B rather than of tylosin A. Tylosin B itself was not found to be a relevant degradation product in the soil tested, which is in contrast with literature (Sassman et al., 2007). Of course the soil composition might have influence on the degradation processes.

Additionally, the FIF approach was applied to identify signals that might originate from tylosin degradation products. The common fragment *m/z* 174.1125, representing the protonated mycaminose ring, a known product ion for tylosin A, B, C and D was used. Filtering for this specific ion in the vDIA chromatogram of *m/z* 495–1205 could be an effective strategy to select for signals of degradation products that also contain this mycaminose ring. The resulting extracted ion chromatograms is presented in Fig. 3. Unfortunately, the FIF approach does not indicate additional degradation products. As apparently, most relevant degradation products elute around the retention time of tylosin A itself, the spectra at this retention time were investigated in more detail by manual search, subtraction of datasets and pattern recognition. For the soil at t = 0 and t = 7 days, the full scan spectra at the retention time range 10–12 min is presented in Fig. 3. At t = 0 tylosin A (*m/z* 916.5264) is detected and additionally a peak at *m/z* 948.5637 was found, which is deemed to be a methanol adduct of tylosin A. At t = 7, cleary additional signals were observed. These most likely originate from tylosin C (*m/z* 902.5108) and tylosin A acid (*m/z* 932.5213) and an unexpected peak was observed at *m/z* 934.5348, most likely the methanol adducts of tylosin C. A close look (lower panel in Fig. 3) reveals another peaks at *m/z* 918.5086, just next to the $^{12}C_2$ isotope peak of tylosin A. The most likely molecular composition for this compound is $C_{45}H_{75}NO_{18}$. Considering the observed degradation processes occurring for tylosin A, this degradation product could well be tylosin C acid (confidence level 2b (Schymanski et al., 2014)): a degradation product that, to the best of our knowledge, has not been reported previously. After thorough data evaluation, the following degradation products of tylosin have been tentatively identified (confidence level 2b (Schymanski et al., 2014)): tylosin C, tylosin A acid, tylosin B acid and tylosin C acid. It is reported that the antimicrobial activity of tylosin B, C and D is respectively 83, 75 and 35% of the activity of tylosin (Scott Teeter and Meyerhoff, 2003). Sassman et al. (2007) indicated that the aldehyde group in tylosin is important for the antimicrobial properties indicating the acid forms do not exert any antimicrobial effect.

3.4. Mobility study

The mobility of the antibiotics was determined by a column leaching method in which 5 g of antibiotic amended soil was flushed through with 10 mL water. Also sequential extraction procedures were tested to determine the K_d (based on OECD method 106 (OECD, 2000)) but we found that in all cases antibiotics remained in the soil fraction due to the fact that some of the water fraction remains in the soil even after centrifugation. This effect results in an overestimation of the antibiotic concentration in the soil fraction and thus an overestimation of K_d and K_{OC}. Therefore a column set-up was selected.

An important limitation of the mobility study (any set-up in which the distribution of substances over multiple phases is determined) is that it is directly influenced by the analytical performance of the methods used to determine the fraction of the substance in the soil and water phase. Especially if the mobility is very high or very low, the concentration of the substance in either fractions might be below the limit of detection. In the case of a non-detect, the limit of detection should be used as the concentration of the antibiotic to calculate K_d. This affects the lower and upper limits of the K_d value that can result from the chosen approach. For instance, if 500 ng antibiotic is introduced in the test system and the limit of detection of both the soil and the water analysis is 5 ng, the minimum K_d value calculated from the test is 5/495 = 0.01 and the maximum K_d value is 495/5 = 99. As a consequence, given the analytical performance of the currently available methods, very high or very low K_{OC} values can only be determined if antibiotic

Table 2
Tylosin A and (previously reported) degradation products thereof, including molecular formula, exact mass of the protonated ion and retention time and abundance in the soil sample extract at t = 0 and t = 7. Degradation products that were clearly formed in time are presented in bold.

Substance	Molecular formula	Exact mass after protonation (Da)	Retention time (min)	Abundance t = 0	Abundance t = 7	Reference
Tylosin A	$C_{46}H_{77}NO_{17}$	916.5264	11.1	$4.1*10^6$	$1.4*10^6$	
Degradation products reported in soil						
Tylosin B (desmycosin)	$C_{39}H_{65}NO_{14}$	772.4478	10.9	$2.6*10^5$	$1.1*10^5$	Sassman et al., (2007)
Tylosin C (macrosin)	**$C_{45}H_{75}NO_{17}$**	**902.5108**	**11.1**	**$3.8*10^4$**	**$6.0*10^5$**	Hu and Coats, (2007)
Tylosin A acid	**$C_{46}H_{77}NO_{18}$**	**932.5213**	**11.05**	**$6.1*10^4$**	**$8.1*10^5$**	Sassman et al., (2007)
Tylosin B acid	**$C_{39}H_{65}NO_{15}$**	**788.4427**	**10.8**	**-**	**$9.5*10^4$**	Sassman et al., (2007)
Degradation products reported in other matrices						
Tylosin A adol	$C_{46}H_{77}NO_{17}$	916.5264	–	–	–	Wegst-Uhrich et al., (2014)
Dihydrotylosin B	$C_{39}H_{67}NO_{14}$	774.4634	10.5	$3.7*10^5$	$3.5*10^4$	Wegst-Uhrich et al., (2014)
Tylosin D (relomycin)	$C_{46}H_{79}NO_{17}$	918.5421	11.1	$6.4*10^5$	$2.5*10^5$	Wegst-Uhrich et al., (2014)
Tentitatively identified degradation products not previously reported						
Tylosin C acid	**$C_{45}H_{75}NO_{18}$**	**918.5086**	**11.0**	**$3.9*10^3$**	**$5.3*10^5$**	

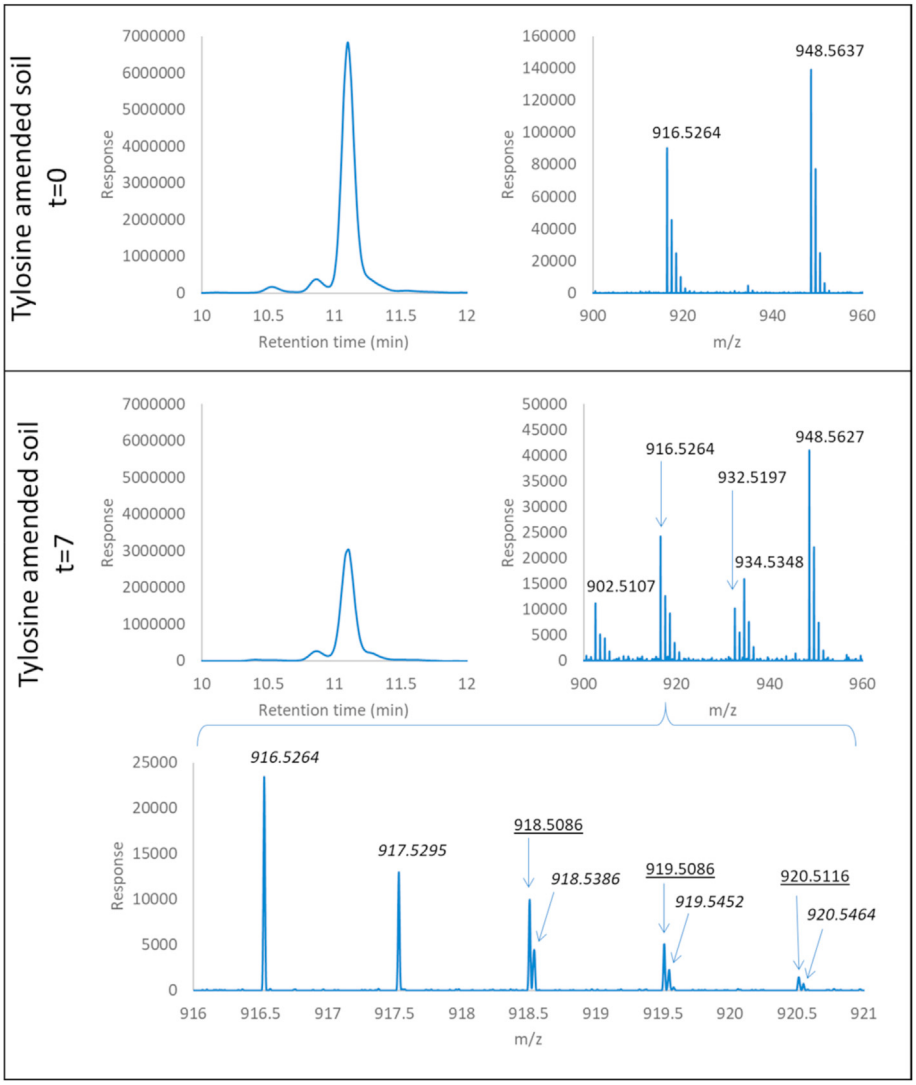

Fig. 3. Tylosin amended soil at t = 0 (upper panel) and t = 7 (lower panel) showing the extracted vDIA chromatogram (*m/z* 495–1205) of *m/z* 174.1125 (5 ppm mass tolerance and the full scan spectrum at mass range *m/z* 900–960 at retention time 10–12 min. A detailed view of the full scan spectrum of the tylosin amended soil at t = 7 days for mass range *m/z* 916–921 is presented in the lower panel; the *m/z* values in italic and the *m/z* values underlined indicate separate clusters of isotopes.

concentrations used in the mobility test are unrealistically high. In the current study, this effect only occurred for the water fraction and thus for the immobile substances. As a matter of fact, with this approach for these compounds an underestimation of the true K_{OC} is obtained.

In the presented set-up, the scale was miniaturized compared to OECD guidelines (OECD, 2004). In the optimisation process, 200 g soil columns were used but we experienced low reproducibility, most likely caused by irregular packing of the columns and difficulties in the homogenisation of the soil after exposure and prior to extraction. The miniaturized set-up proved to be quick and reproducible, allowing the cost-efficient study of many different substances in many different soil samples.

The determined K_{OC} values of the antibiotics is presented in Table 1. The cases in which the analytical limit of detection was the limiting factor in the determination of K_d, this is indicated. Determined K_{OC} values were compared with previously reported values (Supplementary information S2). For most antibiotics, the K_{OC} determined in the current study is in agreement with values previously reported (Sukul et al., 2008; University of Hertfordshire, 2019; Thiele-Bruhn, 2003; Cycoń et al., 2019; Park and Choi, 2008; Boxall et al., 2006; Rabølle and Spliid, 2000). As expected, the tetracyclines show low mobility, which is contributed to the cationic nature of the aminde group. For sulfadiazine, trimethoprim and flumequine clear differences are observed. For sulfadiazine the current study yielded log K_{OC} values of 0.5 for sand and 0.7 for clay, whereas in literature log K_{OC} values ranging from 1.6 to 3.2 are reported (University of Hertfordshire, 2019; Cycoń et al., 2019) (Sukul et al., 2008; Thiele-Bruhn, 2003). Interestingly, for sulfadoxin, which is structurally very similar to sulfadiazine, but slightly more apolar, lower log K_{OC} values are reported: 0.3–1.5 (Cycoń et al., 2019). These are in agreement with the log K_{OC} values for sulfadiazine as determined in the current study. As sulfonamides have pK_a values around pH = 6, their ionisation depends on the soil pH and thus the soil parameters will most likely also influence the sulfonamides' mobility. Trimethoprim showed to be more immobile (log $K_{OC} \geq 4.2$) compared to previously reported results (log K_{OC} = 3.2–3.7) (University of Hertfordshire, 2019; Cycoń et al., 2019). Also, flumequine was found to be more immobile (log $K_{OC} \geq 4.2$) compared to previously reported results (log $K_{OC} = 2.3$) (University of Hertfordshire, 2019). Also here the soil pH could play

a role, but this data is lacking from the previous publication. Note that the K_{OC} reported in literature for flumequine is an estimated value and indicated as 'unverified'.

In the current study, the mobility of the antibiotics tested was similar for the sand and clay soil except for tylosin: in sand log $K_{OC} \geq 3.2$ and in clay it is 2. As for the degradation rate differences between the two soils were observed, it is suggested that degradation and mobility of tylosin are somehow interacting e.g. due to sorption effects.

In perspective of the fate analysis strategy, it is concluded that the sulfonamides are the most mobile antibiotics tested and it is expected that, if they are introduced into the environment, they will leach to groundwater or run off to surface water. The tetracyclines, trimethoprim and flumequin demonstrated to be the most immobile. They will hardly be translocated after introduction into the soil.

3.5. Persistence and mobility of tylosin degradation products

To finalize the complete fate strategy as depicted in Fig. 1, additional studies were done for the tylosin related substances (including degradation products): tylosin B, C and D and tylosin A, B and C acid. The degradation study was repeated in the sandy soil to determine the persistence of these substances. To allow adequate separation of the signals of the structurally related compounds, the following MS transitions were monitored: Tylosin B, 788.5 > 71; Tylosin C, 902.5 > 83; Tylosin D, 918.5 > 83; Tylosin A acid, 932.5 > 174; Tylosin B acid, 788.4 > 59 and tylosin C acid 918.5 > 74. The results of the degradation study are presented in Fig. 4.

In this study, the response for tylosin B and D decreased over time as expected. In the contrary to the observations in the hrMS experiment (Table 2), also the signal of tylosin C decreased over time. The degradation kinetics were modelled (data presented in supplementary data S1) and just like tylosin A in sandy soil, also tylosin B, C and D show FOMC degradation kinetics. The determined DT50 values are tylosin B, 6 days; tylosin C, 90 days and tylosin D, 4 days. Tylosin C has severely higher DT50 because its concentration seems to plateau after an approximate 40% degradation.

The mobility study was repeated in sandy soil. Tylosin was applied to soil columns in duplicate and incubated for 7 days. After that, water was flushed through the soil column to determine the log K_{OC} of the degradation products. The fraction of the degradation products in the aqueous phase was compared with the fraction that remained in the soil phase, assuming similar response for both matrices. From this study it was found that the mobility of tylosin C and D is very similar to tylosin A with log K_{OC} values of 3.5 and 3.2 respectively. The acid forms are slightly more mobile: Log K_{OC} of Tylosin A acid is 2.5, tylosin B acid 2.1 and tylosin C acid 1.8. Only Tylosin B was mainly detected in the aqueous phase assuming it is far more mobile compared to the other forms, showing a log K_{oc} of 0.6. No explanation was found for this observation.

3.6. Input translocation study

By applying the fate strategy reported, data is obtained to be used as input for translocation models. Based on these model it can be determined to what extend agricultural soils, ground water or

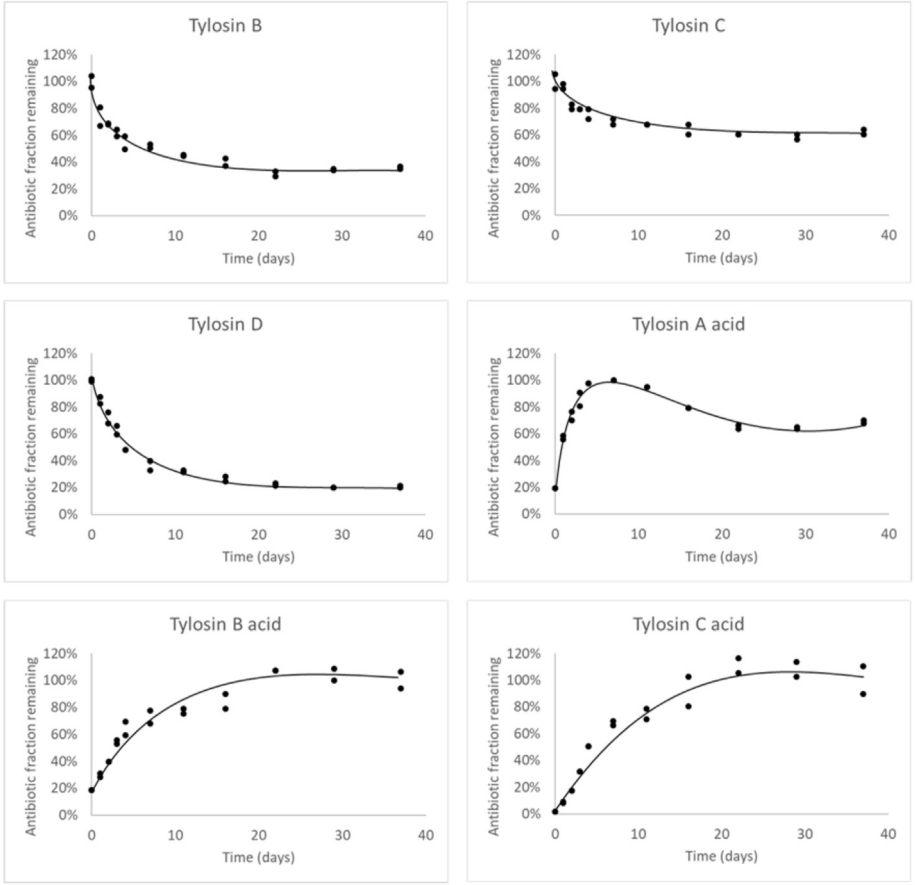

Fig. 4. Kinetics study of the tylosin degradation products in sandy soil.

surrounding surface waters are exposed to antibiotics. Based on the outcomes of the fate study the sulfonamides degrade quickly in soil. However they are quickly translocated to other reservoirs (e.g. surface water). Trimethoprim, enrofloxacin, flumequin, tilmicosin and, in clay also tylosin, are very persistent: for the quinolones and tilmicosin, the estimated DT90 is over one year in sand. Especially trimethoprim, the quinolones and tilmicosin have a relatively low mobility; these will remain in agricultural soils for a long time after introduction and have a high risk of contaminating agricultural soils. Tylosin in clay is slightly more mobile and is therefore expected to be partly translocated to the aqueous environment. Based on these findings specific substances (and their degradation products) can be prioritized for risk assessment in specified reservoirs.

4. Conclusions

The reported fate study strategy consists of several tests in a tiered approach and includes a degradation and a mobility study. The risk of the formation of active degradation products during soil incubation should not be overlooked as was demonstrated by the results for tylosin. Therefore, an assay to determine if produced degradation products show biological activity is mandatory. The presented approach was successfully applied to ten model antibiotics of which, for some, no fate data has been reported previously. The data can be used as input for translocation models and together with input on the antibiotic usage data, excretion models, persistence data in manure (Berendsen et al., 2018) and manure distribution data, the spatial distribution of antibiotics over agricultural soils can be predicted. Additionally, the applied strategy can easily be applied to other substances. In that case an appropriate effect assay has to be applied in tier 2a.

CRediT author statement

Bjorn J.A. Berendsen – Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Supervision, Project administration, Funding acquisition. Gregg Roelofs – Methodology, Investigation, Writing - Review & Editing. Benjamin van Zanten – Methodology, Investigation, Writing - Review & Editing. Wilma D.M. Driessen-van Lankveld – Investigation, Writing - Review & Editing. Mariël G. Pikkemaat - Data Curation, Writing - Review & Editing. Irma E.A. Bongers – Investigation, Writing - Original Draft , Writing - Review & Editing, Supervision. Erik de Lange – Investigation, Writing - Original Draft , Writing - Review & Editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.130495.

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