Chemosphere 204 (2018) 267–276

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

The persistence of a broad range of antibiotics during calve, pig and broiler manure storage

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HIGHLIGHTS

- A simple and straight forward method is presented to determine the persistence of antibiotics in manure.
- The fate of a broad scope of antibiotics during manure storage of different animals and consistency is reported.
- The between-species and between-animal variation of the persistence of antibiotics in manure is reported.
- Based on the outcomes, we identified which compounds have the highest likelihood to enter the environmental reservoirs.
- This is the most extensive study in this field yielding data that is of critical importance for risk assessment.

ARTICLE INFO

Article history:
Received 19 February 2018
Received in revised form
26 March 2018
Accepted 8 April 2018
Available online 11 April 2018

Handling Editor: Klaus Kümmerer

Keywords:
Manure
Antibiotics
Persistence
Dissipation
Fate
Environment
Reviewer suggestions

ABSTRACT

After administration to livestock, a large fraction of antibiotics are excreted unchanged via excreta and can be transferred to agricultural land. For effective risk assessment a critical factor is to determine which antibiotics can be expected in the different environmental compartments. After excretion, the first relevant compartment is manure storage. In the current study, the fate of a broad scope of antibiotics (n = 46) during manure storage of different livestock animals (calves, pigs, broilers) was investigated. Manure samples were fortified with antibiotics and incubated during 24 days. Analysis was carried out by LC-MS. The dissipation of the antibiotics was modelled based on the recommendations of FOCUS working group. Sulphonamides relatively quickly dissipate in all manure types, with a DT90 of in general between 0.2 and 30 days. Tetracyclines (DT90 up to 422 days), quinolones (DT90 100–5800 days), macrolides (DT90 18–1000 days), lincosamides (DT90 135–1400 days) and pleuromutilins (DT90 of 49–1100 days) are in general much more persistent, but rates depend on the manure type. Specifically lincomycin, pirlimycin, tiamulin and most quinolones are very persistent in manure with more than 10% of the native compound remaining after a year in most manure types. For all compounds tested in the sub-set, except the macrolides, the dissipation was an abiotic process. Based on the persistence and current frequency of use, oxytetracycline, doxycycline, flumequine and tilmicosin can be expected to end up in environmental compartments. Ecotoxicological data should be used to further prioritize these compounds.

1. Introduction

In the European Union antibiotics are widely applied to treat bacterial infections in livestock and in some other regions they are also widely applied as growth promoting agents. The type of antibiotic compounds commonly used depends on the sector and the region. In the Netherlands, the most commonly applied antibiotics in veal calf production are (data of 2015) (Geijlswijk et al., 2016): tetracyclines (mainly oxytetracycline and doxycycline), penicillines (mainly ampicillin), sulphonamides (mainly sulphadiazine) combined with trimethoprim, fluoroquinolones (mainly flumequine) and macrolides (mainly tilmicosin). In pig production these are: tetracyclines (mainly oxytetracycline and doxycycline), macrolides (mainly tylosin) and sulphonamides (mainly

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https://doi.org/10.1016/j.chemosphere.2018.04.042

sulphadiazine and sulphamethoxazole) combined with trimethoprim. In broiler production these are: penicillins (mainly amoxicillin), quinolones (mainly enrofloxacin) and tetracyclines (mainly doxycycline).

After administration, large fractions (generally >50%) are excreted unchanged via urine and faeces (Kim et al., 2011; Chee-Sanford et al., 2009). Antibiotics have previously been detected in faeces from various animals at levels ranging from the low μg/kg range up to the g/kg range (Kim et al., 2011; Berendsen et al., 2015; YongShan et al., 2012; Wang et al., 2017; Van den Meersche et al., 2016). Usually the active compound is excreted unchanged. Only for a small number of the antibiotics it is known that they partly metabolise in the body yielding antimicrobially active and/or inactive metabolites (Boxall et al., 2004). For example, in the liver, enrofloxacin is partly (<25%) metabolized to ciprofloxacin, which is also antimicrobially active. Furthermore, sulphonamides are, for a small fraction, metabolized to N4-acetylsulphonamides which are somewhat less microbially active compared to the parent drugs (Anderson et al., 2012).

Agro-ecosystems are exposed to antibiotics by livestock manure application (Du and Liu, 2012a). Manure can have very different properties, ranging from solid (farm yard manure) to liquid manure (slurries). The most abundantly used type of manure in the European Union is (semi)liquid manure mainly consist of faces and urine from intensive livestock rearing (Weinfurtner, 2010).

In The Netherlands, calve manure is mainly applied onto agricultural land untreated (approximately 75%). For pig, approximately 85% of the manure is applied untreated and for poultry, manure is mainly processed (by composting) before application (only <10% applied to land directly) (Lahr et al., 2017). Effects of manure treatment have only been limitedly studied demonstrating that some antibiotics are removed but others are persistent (Feng et al., 2017). Certainly, agricultural land is exposed to antibiotics, due to the direct application of livestock manure when originating from animals that have been treated with antibiotics. Depending on the physico-chemical properties of the antibiotic and the soil composition, the compounds may remain in the manure fortified soil or are transferred to ground and surface waters by leaching or run off. Furthermore, antibiotics might break down in the soil due to photolysis, hydrolysis or biodegradation. They may also be taken up and accumulate in crops (Pan and Chu, 2017; Berendsen et al., 2013). As a result antibiotics have a relatively high detection frequency in environmental matrices (Carvalho and Santos, 2016; Kümmerer, 2009). Antibiotics and/or their metabolites are known to have adverse effects on agro-ecosystems, soil life, plant growth, water life and non-target organisms (Lindberg et al., 2007; Thiele-Bruhn and Beck, 2005; Rysz and Alvarez, 2004; Ding and He, 2010; Pomati et al., 2004; Keen and Patrick, 2013; Isidori et al., 2005; Christian et al., 2003; Bártíková et al., 2016; Gullberg et al., 2011, 2014; Liu et al., 2011; Bernier and Surette, 2013; Andersson and Hughes, 2014; Du and Liu, 2012b). Also the presence of low concentrations of antibiotic residues in the environment is likely to contribute to the selection of resistant bacteria (Kemper, 2008).

For effective environmental risk assessment (Kools et al., 2008) a critical factor is to determine which antibiotics can be expected in the different environmental compartments. This depends on their use, their mobility and their persistence in the different compartments. After excretion, the first relevant compartment in intensive livestock rearing is manure storage, which on average lasts a total of 6–9 months (Boxall et al., 2004). Data on the persistence of antibiotics in environmental compartments was reviewed (Kim et al., 2011; Gothwal and Shashidhar, 2015; Schmitt et al., 2017), but data on the fate of antibiotics during manure storage is scarce and limited to a relatively small number of compounds (Chee-Sanford et al., 2009; Boxall et al., 2004; Kolz et al., 2005; Bao et al., 2007; Joy et al., 2014; Kuchta and Cessna, 2008; Loke et al., 2000; Wohde et al., 2016). Data on the dissipation of antibiotics during manure storage is of crucial importance to assess the risk of antibiotics for soil and water life and the risk of leaching to groundwater, and with regard to the selection for and persistence of resistant bacteria in the environment, because the fate during manure storage determines how much of the antibiotics may reach the environment at a later stage. The determination of the fate of veterinary medicinal products in manure is optional in the assessment of the environmental impact of a veterinary medicinal product and is only used to stop the assessment in the first phase if demonstrated that the active substance is mineralised or transformed into products at less than 5% of the total (EMA, 2011).

Because data on the dissipation of antibiotics during manure storage in intensive livestock rearing is limited to a relatively small number of compounds and frequently only available for a single manure type, we studied the persistence of a very broad selection of tetracyclines, sulphonamides, (fluoro)quinolones, macrolides, lincosamides and pleuromutilins (in total 46 active compounds) in different types of manure (originating from different types of species and of different consistency). We applied a straightforward and very simple laboratory approach, mimicking on-farm storage conditions in slurry pits. Penicillins were excluded from this specific study, because instantaneous hydrolysis was observed in manure samples in previous experiments, which is in agreement with published results (Schmitt et al., 2017). Besides the broad scope, the novelty of this work is in the simple approach to determine the dissipation of antibiotics in manure storage, allowing analysis of different types of manure in a cost effective way. The simple approach reported applied does not distinguish between different break down processes in the dissipation process. However, to study the contribution of biodegradation we studied the role of the microbiome on the dissipation process for a selected set of antibiotics. Based on the outcomes, we identified which compounds have the highest likelihood to enter the environmental reservoirs. According to our knowledge, this is the most extensive study in this field yielding data that is of critical importance for risk assessment.

2. Materials and methods

2.1. Reagents and equipment

HPLC grade methanol (MeOH) and acetonitrile (ACN) were obtained from Biosolve (Valkenswaard, The Netherlands). Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 18.2MΩ cm^{-1} (Millipore, Billerica,MA,USA). Formic acid, ammonium formate, citric acid monohydrate, disodium hydrogenphosphate (Na_2HPO_4) dihydrate and disodium ethylenediaminetetraacetate (Na_2EDTA) were obtained from VWR International (Darmstadt, Germany) and lead acetate and trifluoroacetic acid (TFA) from Sigma-Aldrich (St. Louis, MO,USA). The reference standard of the antibiotics and the isotopically labeled internal standards were obtained from various suppliers. The preparation of solutions is presented in supplementary data (S1).

2.2. Persistence experiments

The persistence of the antibiotics was determined over a time period of 24 days and in five different types of manure: three for calve manure (solid, semi-solid and liquid), one for pig manure and one for broiler manure. For calve, three different manure types were selected because in practice we observed large differences in specifically the consistency of calve manure. Consistencies were assessed visually. For calve and pigs, uncontaminated manure was

taken straight from the intestine of a single animal during slaughter. Uncontaminated broiler manure was freshly picked from the floor of broiler houses. Of each type of manure, three different batches (A, B and C, different animals originating from different barns) were studied in duplicate. A schematic overview of the persistence experiments is presented in Fig. 1.

Fresh manure samples were stored a maximum of 30 days at −20 °C before starting the experiment. After homogenizing by stirring with a wooden spatula, for each batch of manure, 14 aliquots (7 in duplicate) of 2 g were transferred into 50 mL PP tubes, resulting in 42 samples per manure type. At the start of the experiment 200 µL of mixed antibiotic solution (resulting in a concentration of 500 µg kg^{-1} of all active substance in manure) was added to 6 aliquots in duplicate. The aliquots were homogenized by stirring. The other 2 remained blank as a control. The tubes were capped but not closed to let air through and placed at room temperature (19–21 °C) in exposure of daylight. In this way manure is stored under conditions that approach storage in a slurry pit in housing systems: the largest part of the manure is anaerobic and the top layer is in contact with air. Because no average temperature for manure storage can be defined due to the large regional, seasonal and system variations (Weinfurtner, 2010), storage at room temperature is considered appropriate. After 10, 17, 20, 22, 23 and 24 days the procedure was repeated and on day 24 the incubation was stopped. This resulted in tubes in which antibiotics were exposed to manure during 24, 14, 7, 4, 2, 1 and 0 days respectively. A maximum incubation time of 24 days was selected because it was expected that the antibiotics would have sufficiently dissipated to allow calculation of the DT50.

After the final additions, internal standards were added and the full aliquots were immediately extracted according to a previously reported procedure (Berendsen et al., 2015). In short, this included an extraction using 4 mL of McIlvaine–EDTA buffer and 1 mL ACN. Next, a clean-up was carried out by adding lead acetate solution

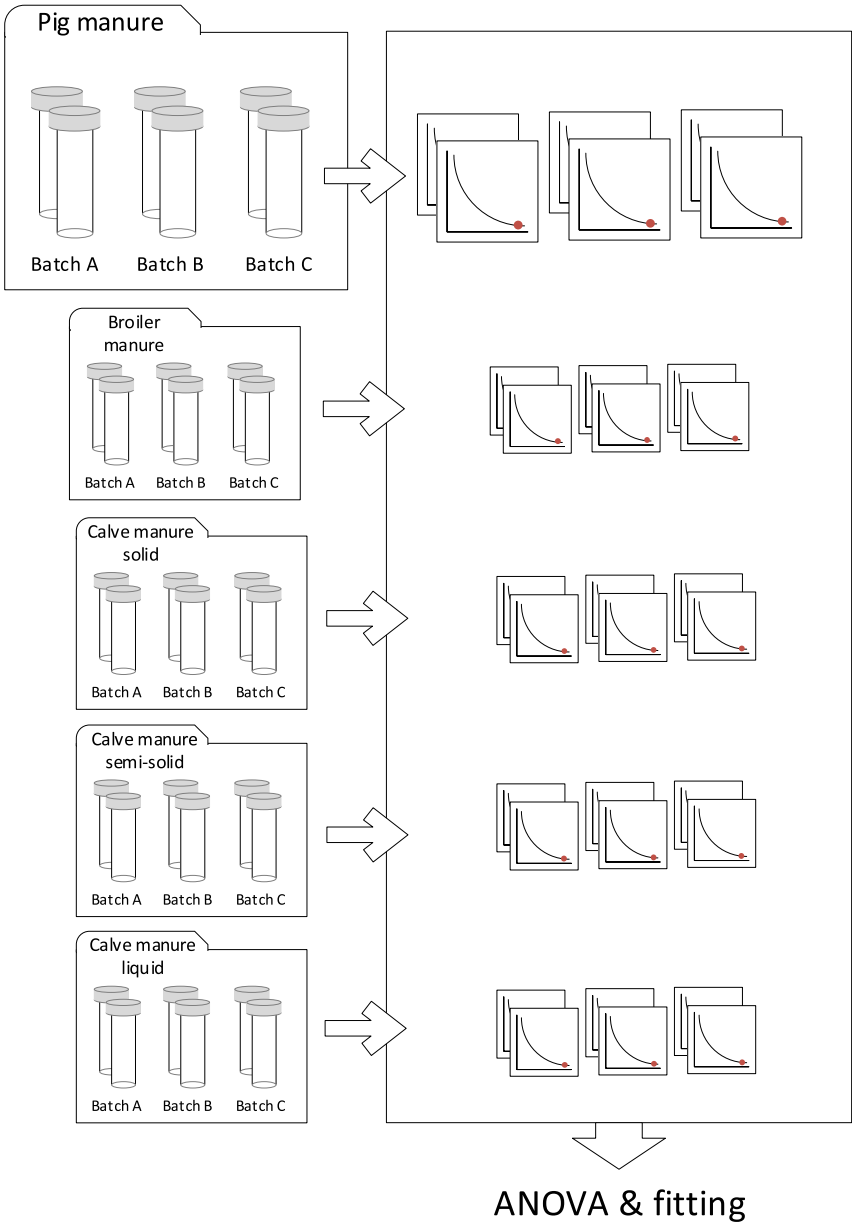

Fig. 1. Schematic overview of the set-up of the persistence study. See the main text for an explanation.

followed by centrifuging. The supernatant was isolated and shaken with EDTA-solution before solid phase extraction clean-up. Detection was carried out by liquid chromatography coupled to tandem mass spectrometry using a validated and ISO 17025 accredited method previously published (Berendsen et al., 2015).

The persistence of the antibiotics was analysed 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 of all batches, 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 χ^2-error above 5%, the model with the lowest χ^2-error was selected. Next, based on the selected model for each of the antibiotics, the half-life (DT50) and time at which 10% of the native compound remained (DT90) were calculated.

Additionally, for each antibiotic, Two-way Analysis of Variance (ANOVA) with replication was carried out on the dataset to determine the influence of the batch and the type of manure on the antibiotic's persistence. To be able to compare the persistence among types and batches using experimental data (independent of any fitting of models as applied in the data evaluation), the remaining fraction of the antibiotics at the end of the experiments, after 24 days, was taken as a representative measure.

2.3. Sterile versus untreated manure

With a selection of the most relevant antibiotics (mostly applied in animal breeding and most frequently detected in manure in routine monitoring programmes) a follow-up experiment in calve manure (semi-liquid) was carried out in order to determine to what extent biological degradation played a role in the breakdown of these compounds. The selected antibiotics were: oxytetracycline, doxycycline, sulphadiazin, sulphadoxin, tylosin, tilmicosin, lincomycin, enrofloxacin and flumequine. A large amount of fresh calve manure was taken from the slatted floor in a barn with untreated animals and was homogenized by ultra-turrax®. The pH of this mixed manure sample was 7.5–8 and the moisture content 85.4%. A part of this batch was sterilized in an autoclave for 1.5 h, of which 15 min at 121 °C. Sterilized manure aliquots were transferred to sterilized tubes in a sterile down flow cabinet. The persistence of the selected antibiotics in both the sterilized and untreated manure was assessed under the exclusion of light by spiking 6 aliquots of 2 g in duplicate of the untreated and sterilized manure samples at 500 μg kg^{-1} and storing them at 15 °C. After 0, 1, 2, 4, 7 and 14 days a duplicate aliquots of untreated and sterilized manure were transferred to −80 °C, at which the antibiotics are assumed to remain stable. The experiment was also carried out in milli-Q water to monitor the stability of the antibiotics in absence of manure matrix. Afterwards, all samples were analysed in a single run. The extraction was carried out using 4 mL McIlvaine-EDTA buffer mixed with 4 mL 0.125% TFA in ACN as the extraction solvent to eliminate binding effects as much as possible. The organic solvent was evaporated at 45 °C before application onto the SPE cartridges for clean-up. To aid statistical analysis, a single first order dissipation model was fitted to the remaining fraction of the active substance in both sterile and untreated manure. Linear regression on the log transformed concentration was carried out according to the least squares approach. The slope of the regression line for the sterilized manure was statistically compared with the slope of the regression line for untreated manure to determine the contribution of biodegradation to the dissipation of the substances.

3. Results

3.1. Persistence of antibiotics in manure

The data about the dissipation of all antibiotics is presented in the supplementary material (S2). Of nine selected frequently applied antibiotics from the different groups, the data is presented in Fig. 2. The DT50 and DT90 values of all antibiotics included are presented in Table 1. The remaining fraction of the active substances after 24 h is presented in Table 2. The χ^2-errors of all fits carried out by the kinfit procedure are presented in the supplementary data (S3). The selected model and the model parameters are also presented there.

3.2. Untreated vs sterile dissipation

For tetracyclines, sulphonamides, lincosamides, pleuromutilins and quinolones no significant differences were observed between the slope of dissipation in sterile and untreated calve manure. Only for tylosin and tilmicosin a significant difference was observed between the dissipation rate in untreated and sterile calve manure (Fig. 3).

Dissipation rates of the antibiotics in untreated calve manure were similar to the dissipation rate determined in the previous experiment even though the manure samples were incubated in exclusion of light (data not shown).

4. Discussion

4.1. Evaluation of the test application

The presented procedure to determine the persistence of antibiotics in manure could be a simple and straight-forward alternative for more elaborate procedures (EMA, 2011; Hennecke et al., 2015) when a quick assessment is needed. The dissipation rate of veterinary pharmaceuticals can differ due to differences among types of manure (*e.g.* dry matter content and composition as a result of feeding) (Weinfurtner, 2010). The impact of the different parameters on the dissipation rate is not well known and therefore, it is important to include a large variety of manure samples in a dissipation study (EMA, 2011). Because the method is relatively simple, it allows easy screening of a large number of different active substances and/or manure samples. Furthermore, for compounds that were also studied previously (Kim et al., 2011; Chee-Sanford et al., 2009; Boxall et al., 2004; Gothwal and Shashidhar, 2015; Schmitt et al., 2017; Kolz et al., 2005; Bao et al., 2007), the two approaches yield similar outcomes with respect to the categorization of the dissipation.

4.2. Classification of persistence

Previously, a classification of persistence based on the DT50 was presented (Hollis, 1991). However, because the dissipation of many antibiotics seem to follow a diphasic dissipation process (supplementary data S3), we suggest to use the DT90 as a more representative measure to classify the persistence of antibiotics in manure. Because manure is on average stored for 6–9 months (Boxall et al., 2004), it is realistic to consider this timeframe in the classification of the persistence. Therefore we suggest the following classification: DT90: >1 year, very persistent; half a year to a year, persistent; quarter year to half a year, moderately persistent; a month to a quarter year, slightly persistent; < 1 month, non-persistent.

Note that the maximum dissipation time in our experiment was 24 days. Therefore, DT50 and DT90 values are in some cases

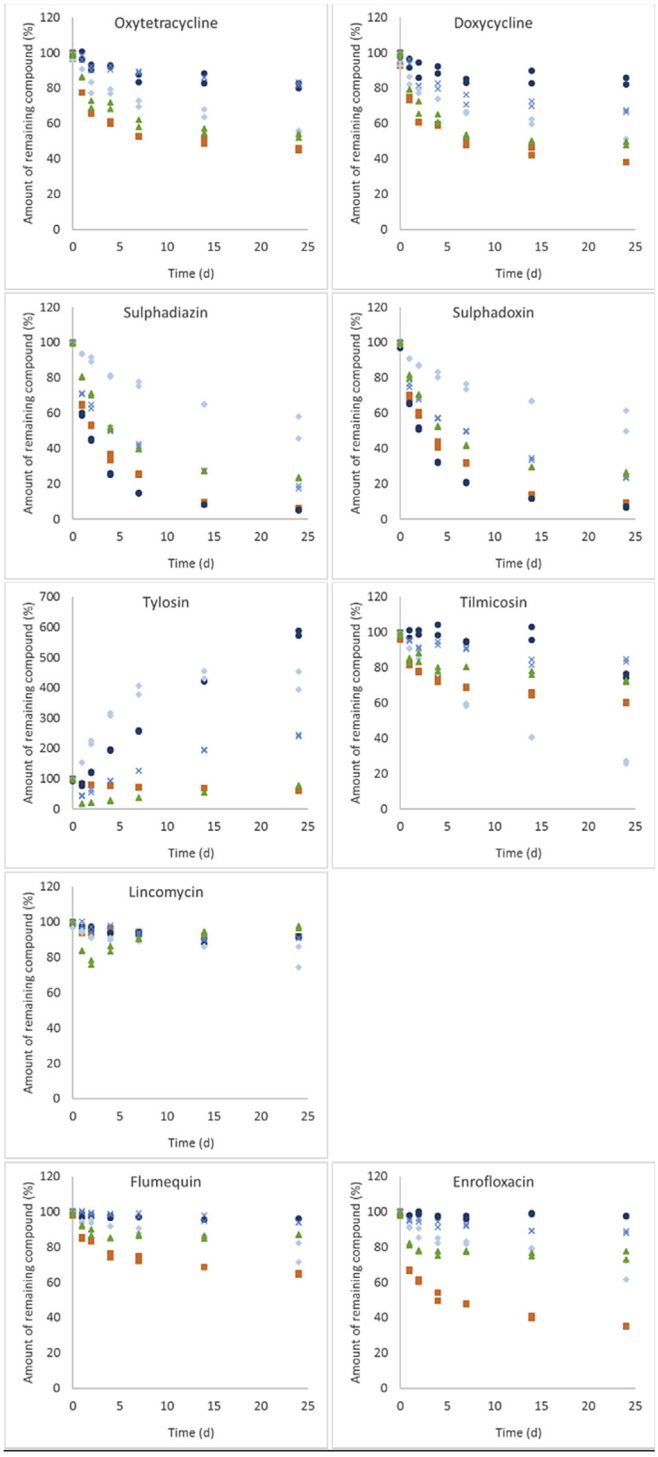

Fig. 2. Dissipation of nine selected antibiotics in time for (dark blue dots) solid calve manure, (middle blue crosses) semi-solid calve manure, (light blue diamonds) liquid calve manure, (orange squares) pig manure and (green triangles) broiler manure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

extremely extrapolated. These values should be considered as an estimation rather than a precise number. It is advised to extend the dissipation time in future studies. However, because of the large variation of calculated DT50 and DT90 values among the different types of manure, estimates of the DT50 and DT90 are considered appropriate for a fit for purpose classification of the persistence of the antibiotics to be used for environmental risk assessment.

4.3. Persistence of the antibiotic classes

The persistence of the individual antibiotics was assessed. In general, within antibiotic classes comparable dissipation rates were observed for the individual compounds (Table 1).

In our experiments, the tetracyclines are moderately persistent to very persistent in most calve and broiler manures and slightly less persistent in pig manure. According to previously reported data, tetracyclines are very persistent, but this was tested in a single type of manure only (type of manure not indicated) (Boxall et al., 2004). At first instance, the tetracyclines dissipate relatively quickly, but the dissipation is relatively slow during the second stage (Fig. 2). A possible explanation could be that a large fraction of the tetracyclines instantaneously binds to solid particles (Boxall et al., 2004) and that, as a consequence, only part is available for the dissipation processes, which has previously been suggested to be an enzymatic process (Bansal, 2012). The strong binding delays the biodegradation (Sukul et al., 2007) and thus tetracyclines can persists in manure for a long time.

Sulphonamides show a high dissipation rate in most manure samples. It was previously reported that sulphonamides are non-to moderately persistent in manure (Boxall et al., 2004), which is in agreement with our findings. Photolysis is reported to be a main cause for sulphonamide dissipation (Sukul et al., 2007) and it could also have been a main factor in the current experiment. However, in the second experiment, which was carried out under exclusion of light, also quick dissipation of the sulphonamides was observed, so we conclude that other processes play a major role as well.

Macrolides were found to be moderately persistent to persistent in all types of manure in this experiment, with the exception of natamycin which was non-persistent. According to these data, the macrolides are somewhat more persistent than previously reported (Boxall et al., 2004). For tylosin (focussed on tylosin A), some interesting observations were made. Only for pig manure a slight decrease of tylosin concentration over time was observed (DT90 = 179 d). For the other manure types, surprisingly, a significant increase in the concentration over time was observed (for solid calve manure up to 570%), in most cases after an initial decrease (see Fig. 2). It has previously been reported that tylosin is not persistent (Boxall et al., 2004; Loke et al., 2000; Scott Teeter and Meyerhoff, 2003) which is in contrast to the current observations. It is also reported that tylosin degrades to structurally related products among which tylosin B, tylosin A aldol, tylosin D (Loke et al., 2000; Paesen et al., 1995a; Hu et al., 2009), isotylosin (Paesen et al., 1995b), isotylosin aldol and isotylosin alcohol (Hu et al., 2008). The degradation products that are isomers are not discriminated from tylosin A by mass spectrometric detection. If we assume that these isomers show a higher ionisation efficiency compared to tylosin, formation of such metabolites could explain the increase in the tylosin signal. Tylosin B, C and D are antimicrobially active (Scott Teeter and Meyerhoff, 2003), but whether or not the isomers of tylosin A exert antimicrobial activity is unknown. The results for tylosin dissipation are therefore to be considered with care.

Lincosamides and tiamulin (a pleuromutilin) were very persistent antibiotics in the experiment. The other pleuromutilin included in the study, valnemulin, was found to be moderately persistent in all manure types.

Finally, quinolones, with the exception of ciprofloxacin and norfloxacin, were very persistent in solid and semi-solid calve manure and somewhat less persistent in the other manure types. Ciprofloxacin and norfloxacin were moderately persistent to persistent. Unspecified quinolones were found to be very persistent

Table 1
Calculated DT50 and DT90 for all antibiotics in the different manure types (in days).

Compound		Matrix type				
		Calve solid	Calve semi-solid	Calve liquid	Pig	Broiler
Tetracyclines						
Oxytetracycline	DT50	87	98	31	16	30
	DT90	290	327	103	171	221
Chlortetracycline	DT50	36	35	58	19	18
	DT90	119	118	193	62	61
Doxycyclin	DT50	127	44	26	10	20
	DT90	422	147	117	98	268
Tetracycline	DT50	55	52	12	12	62
	DT90	181	171	86	111	330
Sulphonamides						
Dapsone	DT50	0.9	1.2	1.9	1.2	2.3
	DT90	6	15	20	11	20
Sulphacetamide	DT50	4.4	11	39	1.5	4.9
	DT90	37	36	131	8	100
Sulphachloropyridazin	DT50	1	2.4	12	1.6	2.9
	DT90	8	24	60	15	38
Sulphadiazin	DT50	1.5	4.4	25	2.2	4.4
	DT90	11	33	84	18	83
Sulphadimethoxin	DT50	1.6	4.6	11	3.2	3.4
	DT90	14	35	59	21	37
Sulphadimidin	DT50	1.1	2.5	10	1.8	3.7
	DT90	8	26	58	16	48
Sulphadoxin	DT50	2.1	7	29	3	5
	DT90	16	41	97	25	89
Sulphamerazin	DT50	1.3	3.4	19	1.8	3.7
	DT90	9	29	62	17	45
Sulphamethizole	DT50	1	2.2	13	1	2.4
	DT90	7	21	60	9	23
Sulphamethoxazole	DT50	1.6	3.2	5.7	2.6	2.5
	DT90	12	21	19	22	53
Sulphamethoxypyridazin	DT50	0.9	1.7	9	1.6	2.5
	DT90	7	21	48	14	29
Sulphamonomethoxin	DT50	1.4	3.4	13	2.1	3.3
	DT90	9	28	64	19	44
Sulphamoxole	DT50	0.2	0.4	0.4	0.7	0.4
	DT90	1.3	1.3	1.3	3.5	3
Sulphaphenazole	DT50	0.9	1.8	4.9	1.6	0.7
	DT90	6	19	22	13	3.2
Sulphapyridin	DT50	0.8	1.6	6	1.4	3.2
	DT90	6	20	41	13	41
Sulphaquinoxalin	DT50	0.8	1.6	4.2	3.8	2.2
	DT90	7	24	37	13	30
Sulphathiazole	DT50	0.6	1.1	3.7	1.2	2
	DT90	4	14	24	11	18
Sulphisoxazole	DT50	1.3	2	1.1	1.3	0.7
	DT90	10	10	3.7	10	10
Macrolides						
Tylosin	DT50	–	–	–	42	–
	DT90	–	–	–	179	–
Aivlosin	DT50	5	2.8	3.6	35	0.4
	DT90	57	54	18	159	37
Erythromycin	DT50	24	32	5.8	52	17
	DT90	80	106	19	172	56
Gamithromycin	DT50	37	61	7.3	50	53
	DT90	124	203	24	239	177
Josamycin	DT50	25	27	16	231	43
	DT90	82	89	67	769	141
Natamycin	DT50	2.3	2.6	0.3	5	0.7
	DT90	21	22	1.4	17	18
Spiramycin	DT50	30	31	4.1	20	31
	DT90	100	104	36	113	102
Tildipyrosin	DT50	29	71	1.2	5	16
	DT90	119	236	36	78	106
Tilmicosin	DT50	74	104	11	47	71
	DT90	246	346	38	220	235
Tulathromycin	DT50	32	92	36	6	317
	DT90	142	304	97	89	1053
Lincosamides						
Lincomycin	DT50	214	175	95	269	»
	DT90	711	581	315	892	»
Pirlimycin	DT50	699	142	19	125	443
	DT90	2320	473	135	414	1473
Pleuromutilins						

Table 1 (*continued*)

Compound		Matrix type				
		Calve solid	Calve semi-solid	Calve liquid	Pig	Broiler
Tiamulin	DT50	»	338	43	101	280
	DT90	»	1124	144	335	930
Valnemulin	DT50	17	13	8	42	7
	DT90	57	96	49	179	70
(Fluoro)quinolones						
Enrofloxacin	DT50	1751	162	49	6	103
	DT90	»	540	164	83	343
Ciprofloxacin	DT50	21	61	1.3	6	23
	DT90	102	277	58	85	221
Danofloxacin	DT50	372	106	54	7	58
	DT90	1236	354	180	78	192
Difloxacin	DT50	374	200	68	11	41
	DT90	1243	665	226	99	194
Flumequin	DT50	569	259	75	44	197
	DT90	1890	860	247	146	655
Marbofloxacin	DT50	205	134	77	4.6	90
	DT90	683	447	346	91	300
Nalidixic acid	DT50	1410	614	87	70	388
	DT90	4683	2040	290	295	1290
Norfloxacin	DT50	25	60	1.6	5	18
	DT90	124	254	67	79	179
Oxolinic acid	DT50	348	268	58	36	116
	DT90	1158	889	193	181	387
Sarafloxacin	DT50	»	398	347	562	176
	DT90	»	1322	1152	1867	585

- No data.
» Above 2000.

in previous studies (Boxall et al., 2004) which is in agreement with current findings. Dissipation of the quinolones also seemed to be a diphasic process, similar to the tetracyclines (Fig. 2) and indeed, quinolones are also known to bind to organic particles (Sukul et al., 2007).

Many different parameters and processes influence the dissipation. For instance, one compound may be degraded by biotic processes, whereas others might degrade under the influence of light. Within this study the role of the microbiome and the exposure to light were included. A more detailed study on the dissipation processes would be beneficial to understand the processes completely and predict dissipation rates for 'new' compounds.

4.4. Microbial degradation

Comparison between degradation of selected compounds in untreated and sterile manure demonstrated that dissipation of tetracyclines, sulphonamides, lincosamides, pleuromutilins and quinolones occurred through an abiotic process during the experiment: no significant difference in the slope of regression lines was found. Only for tylosin and tilmicosin a significant difference in the slope of the regression lines was observed (Fig. 3). It was concluded that dissipation (partly) occurs by the microbial communities in the sample. This is in agreement with previously reported findings on the stability processes responsible for tylosin A degradation in agricultural soils (Sassman et al., 2007). Note that fresh manure samples were stored at −20 °C for a maximum of 1 month, which might have affected the microbial community in the samples.

4.5. Manure types and batches

The EMA guidelines (EMA, 2011) considers manure of a single species as comparable for pragmatic reasons. In our experiment three different batches (from different animals originating from different housings) of each manure type (n = 5) were included in the experiment to compare dissipation rates among manure batches of a single species and among different manure types (different species).

For all antibiotics, a clear influence of the type of manure on the dissipation is observed (Table 2). Besides effects of manure type, for almost all compounds an interaction is observed between the batch and the type of manure and especially for the macrolides and pleuromutilins also a significant between-batch variation was apparent. This might be related to the observation that tylosin and tilmicosin (and possibly other macrolides) dissipation involves a biotic process. Variation in the manure microbial community, even within animals of the same livestock species, is likely (e.g. due to different feeding patterns) and might therefore yield variation in different dissipation rates.

Surprisingly, the sulphonamides are more persistent in the liquid calve manure compared to the solid samples, which is in contrast with the observed effects for the other antibiotics. Apparently different processes underlie the dissipation of sulphonamides compared to the other antibiotics, but no specific hypothesis was developed.

Clearly, the persistence of most antibiotics depends on the composition and type of the manure and therefore, a single generally applicable quantitative measure for persistence in 'manure' does not exist. However, based on the large dataset obtained from this study, including different types and batches of manure, the persistence of the individual antibiotics can be categorized.

4.6. Probability of transfer to environmental compartments

To determine the probability of antibiotic residues reaching environmental compartments through manure application, two main parameters are of importance: (1) the frequency of use in livestock rearing and (2) the persistence of the antibiotics in the slurry pit.

Oxytetracycline and doxycycline (both tetracyclines), tylosin and tilmicosin (both macrolides), flumequin and to less extend

Table 2
The remaining fraction of antibiotic after 24 h (relative standard deviation between brackets) in manure including the ANOVA results demonstrating the significance of the influence of the type of manure and batch on the persistence and the interaction between the two factors. Significant parameters are indicated in bold.

	Remaining after 24 days (% of active substance)								
	Calve solid	Calve semi-solid	Calve liquid	Pig	Broiler	RSD analysis	P(interact)	P(batch)	P(type)
Tetracyclines									
Oxytetracycline	82 (6)	82 (15)	53 (20)	47 (11)	54 (18)	6%	**0.000**	**0.001**	**0.000**
Chlortetracycline	74 (26)	69 (16)	75 (18)	43 (13)	45 (14)	15%	**0.011**	0.483	**0.000**
Doxycycline	86 (9)	74 (21)	51 (11)	40 (6)	49 (6)	8%	**0.001**	0.073	**0.000**
Tetracycline	73 (6)	73 (14)	39 (13)	42 (7)	62 (30)	7%	**0.000**	**0.000**	**0.000**
Sulphonamides									
Dapsone	2 (12)	6 (61)	9 (46)	3 (8)	9 (20)	20%	**0.000**	**0.023**	**0.000**
Sulphacetamide	13 (18)	18 (59)	65 (20)	2 (13)	23 (16)	25%	**0.026**	0.729	**0.000**
Sulphachloropyridazine	3 (14)	12 (57)	33 (32)	5 (6)	16 (13)	30%	**0.011**	0.494	**0.000**
Sulphadiazine	5 (17)	18 (65)	53 (30)	6 (7)	24 (14)	27%	**0.002**	0.601	**0.000**
Sulphadimethoxine	5 (42)	20 (39)	33 (34)	11 (6)	17 (26)	24%	**0.003**	0.216	**0.000**
Sulphadimidine	4 (12)	13 (63)	32 (38)	5 (11)	19 (16)	24%	**0.000**	0.121	**0.000**
Sulphadoxin	7 (18)	24 (54)	56 (26)	9 (7)	26 (18)	22%	**0.001**	0.662	**0.000**
Sulphamerazine	4 (13)	15 (61)	43 (30)	6 (9)	20 (17)	28%	**0.004**	0.362	**0.000**
Sulphamethizole	3 (18)	9 (81)	35 (33)	3 (13)	10 (11)	30%	**0.001**	0.319	**0.000**
Sulphamethoxazole	5 (16)	9 (53)	11 (81)	8 (4)	17 (29)	11%	**0.000**	**0.000**	**0.000**
Sulphamethoxypyridazine	3 (14)	10 (61)	27 (39)	5 (6)	14 (21)	25%	**0.000**	0.055	**0.000**
Sulphamonomethoxine	4 (14)	14 (57)	36 (33)	6 (12)	19 (13)	30%	**0.009**	0.370	**0.000**
Sulphamoxole	1 (62)	2 (43)	0	1 (7)	1 (63)	18%	**0.000**	**0.000**	**0.000**
Sulphaphenazole	2 (38)	8 (50)	9 (56)	3 (9)	0	35%	**0.000**	0.063	**0.000**
Sulphapyridine	3 (13)	10 (59)	22 (39)	5 (9)	17 (19)	22%	**0.000**	**0.037**	**0.000**
Sulphaquinoxaline	3 (22)	12 (40)	18 (40)	9 (7)	15 (12)	20%	**0.001**	0.157	**0.000**
Sulphathiazole	2 (19)	6 (54)	10 (46)	4 (8)	9 (32)	27%	**0.002**	**0.024**	**0.000**
Sulphisoxazole	5 (11)	3 (74)	0	3 (18)	5 (92)	44%	**0.001**	0.336	**0.000**
Macrolides									
Tylosin	615 (28)	430 (62)	437 (25)	62 (10)	80 (18)	20%	**0.000**	**0.001**	**0.000**
Aivlosin	22 (91)	31 (84)	6 (32)	58 (8)	12 (45)	7%	**0.000**	**0.000**	**0.000**
Erythromycin	47 (77)	63 (52)	5 (70)	70 (8)	33 (54)	10%	**0.000**	**0.000**	**0.000**
Gamithromycin	62 (63)	80 (40)	7 (85)	62 (6)	59 (44)	4%	**0.000**	**0.000**	**0.000**
Josamycin	46 (57)	66 (64)	32 (61)	101 (28)	58 (33)	25%	**0.001**	**0.002**	**0.000**
Natamycin	7 (44)	15 (95)	1 (59)	5 (59)	6 (75)	29%	**0.000**	**0.000**	**0.000**
Spiramycin	54 (46)	60 (48)	20 (52)	47 (8)	49 (41)	4%	**0.000**	**0.000**	**0.000**
Tildipyrosin	55 (49)	77 (9)	19 (20)	33 (11)	44 (18)	11%	**0.000**	**0.000**	**0.000**
Tilmicosin	73 (51)	82 (24)	26 (13)	62 (6)	73 (13)	5%	**0.000**	**0.000**	**0.000**
Tulathromycin	57 (34)	81 (15)	62 (48)	37 (9)	85 (29)	17%	**0.000**	**0.065**	**0.000**
Lincosamides									
Lincomycin	93 (4)	92 (7)	82 (21)	92 (2)	97 (3)	7%	**0.052**	0.216	**0.015**
Pirlimycin	95 (3)	90 (7)	47 (15)	83 (6)	86 (3)	5%	**0.009**	0.952	**0.000**
Pleuromutilins									
Tiamulin	106 (30)	100 (16)	61 (41)	87 (28)	90 (17)	21%	**0.229**	**0.012**	**0.008**
Valnemulin	31 (42)	39 (62)	24 (42)	45 (36)	34 (26)	28%	**0.005**	**0.010**	**0.018**
(Fluoro)quinolones									
Enrofloxacin	98 (7)	89 (10)	67 (14)	36 (7)	76 (12)	9%	0.398	**0.021**	**0.000**
Ciprofloxacin	49 (41)	68 (8)	26 (22)	35 (4)	50 (9)	6%	**0.000**	**0.000**	**0.000**
Danofloxacin	95 (9)	85 (14)	70 (12)	35 (10)	68 (5)	9%	0.052	0.340	**0.000**
Difloxacin	94 (5)	93 (12)	75 (14)	40 (6)	60 (28)	10%	**0.018**	0.087	**0.000**
Flumequine	96 (3)	94 (6)	77 (14)	66 (5)	87 (3)	8%	0.861	0.343	**0.000**
Marbofloxacin	94 (13)	88 (12)	67 (13)	36 (10)	74 (14)	11%	**0.042**	0.453	**0.000**
Nalidixic acid	99 (4)	98 (3)	81 (16)	69 (5)	93 (1)	8%	0.941	0.753	**0.000**
Norfloxacin	52 (36)	67 (11)	29 (21)	34 (6)	48 (5)	6%	**0.000**	**0.000**	**0.000**
Oxolinic acid	94 (2)	92 (10)	73 (15)	56 (3)	82 (3)	8%	0.373	0.261	**0.000**
Sarafloxacin	103 (11)	95 (9)	90 (14)	95 (4)	91 (4)	3%	**0.000**	**0.001**	**0.000**

enrofloxacin (both fluoroquinolones) are among the most frequently applied antibiotics in calve and pig production (Geijlswijk et al., 2016). For all of these, a significant fraction persists in manure for over 4 months in most manure types we have tested. Therefore it is likely that these antibiotic residues can reach environmental compartments. Terrestrial ecotoxic effects for enrofloxacin (Scott Teeter and Meyerhoff, 2003) and aquatic toxic effects for flumequin (Paesen et al., 1995a) have been reported at concentrations in the low μg kg^{-1} range. For the other most frequently applied compounds, the ecotoxicological predicted no effect concentrations (PNEC) have not been reported or are somewhat higher. Furthermore these most frequently applied antibiotics in calve and pig production all have a PNEC for selection of resistant bacteria in the low ppb (μg L^{-1}) range down to the sub ppb range (Bengtsson-Palme and Larsson, 2016). This demonstrates that all of the most frequently applied compounds in calve and pig production are compounds of concern.

The probability of environmental exposure to sulphonamides through manure application may be low compared to tetracyclines, but cannot be excluded because these compounds are frequently applied in all livestock species and treatments can also occur just before manure is removed from the slurry pit and applied to agricultural land. Because of their high solubility these compounds may also leach to ground- and surface water. A recent paper indicated that, in soil, sulphonamides are degraded to (among others) oxidated transformation products that are more toxic than the parent compound (Zhang et al., 2017). It is unknown if this also occurs in manure itself and therefore if this aspect requires additional

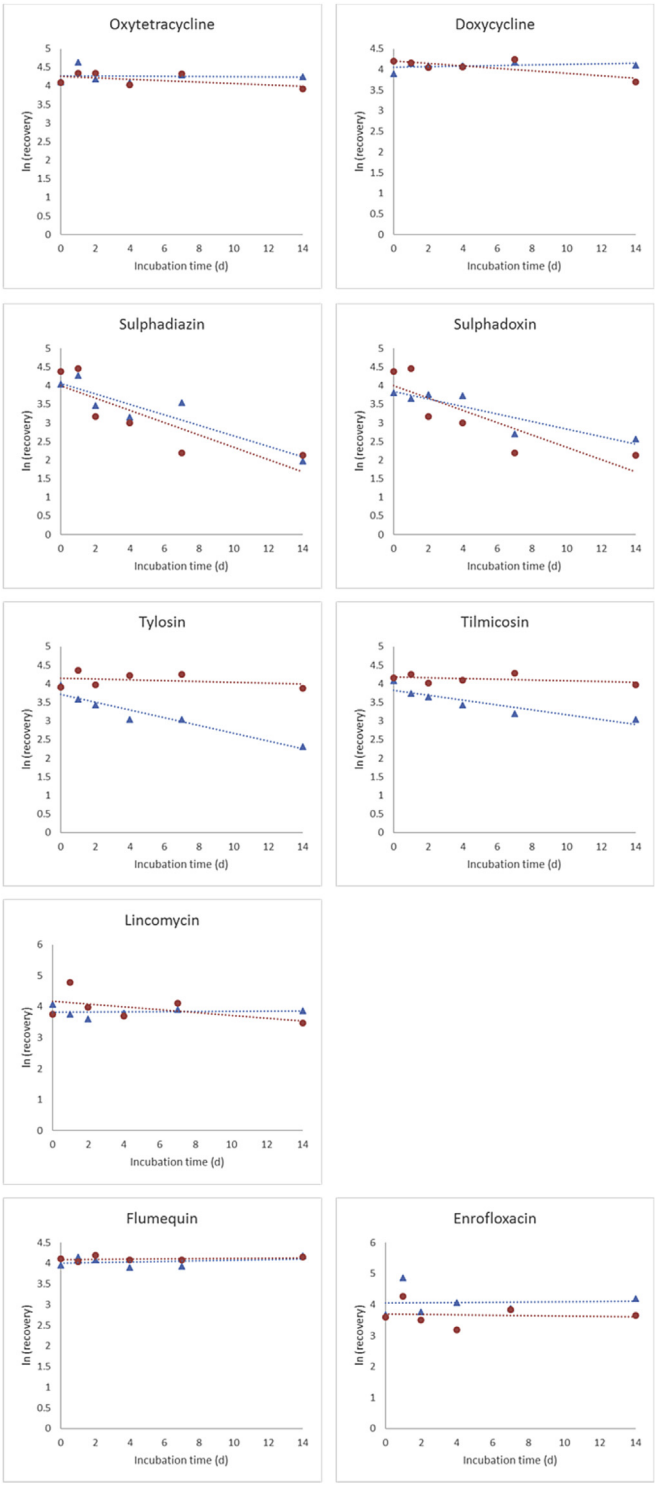

Fig. 3. Linear regression lines of the ln transformed recoveries of nine selected antibiotics during 14 day incubation. The untreated manure is indicated in blue, the sterilized manure in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

research.

Lincomycin is the most frequently used lincosamide in the Netherlands, and is mainly applied in poultry production (in combination with spectinomycin, which was not tested in this study). Because of its high persistence lincomycin is expected to remain present in the manure during manure storage. This is in agreement with previous studies on lincomycin (Kuchta and Cessna, 2008). Because the largest fraction of poultry manure is treated before application by composting and because the volume of poultry manure is only a fraction of the total manure volume, environmental presence of lincomycin as a consequence of its use in poultry rearing is expected to be limited in the Netherlands.

In summary, based on the persistence only, the probability of reaching the environment through manure applications is highest for enrofloxacin, flumquine, lincomycin, pirlimycin and tiamulin residues. However, if the frequency of use in animal production is also taken into account, environmental presence is mainly expected for flumequine, oxytetracycline, doxycycline, tilmicosin and tylosin. These findings comply with the high frequency of detecting these compounds in recent monitoring of environmental samples (data not published). The particular environmental compartments in which these compounds are expected depends again on other factors, of which their adsorption capacity and solubility in water (in other words: their mobility) are of key importance. With additional information of these properties the exact fate of antibiotics in the environment can be further determined.

5. Conclusions

We present a simple experiment to determine the dissipation rate of antibiotic residues in manure, which allows easy screening of a large number of different active substances or manure types. After antibiotics are excreted in manure by livestock, the first compartment is manure storage. If antibiotics dissipate during the manure storage period, contamination of the environment will be limited when manure is spread on land. From the current study we conclude that antibiotic dissipation varies considerably depending mainly on the manure type and to a lesser extent on the manure batch (same manure type, but from a different animal). The sulphonamides dissipate quickly in all manure types, with a half-life of in general between 0.2 and 30 days. In general the tetracyclines, quinolones, macrolides, lincosamides and pleuromutilins are much more persistent in manure, especially lincomycin, pirlimycin, tiamulin, enrofloxacin, difloxacin, flumequine and sarafloxacin with more than 10% of the native compound estimated to remain after a year in most manure types. Comparison between degradation of untreated and sterile manure demonstrated that, with the exception of tylosin, dissipation occurred mostly through abiotic processes. Based on the persistence and global frequency of use, currently, environmental exposure to oxytetracycline, doxycycline, flumequine and tilmicosin seems most likely. Ecotoxicological studies can be used to further prioritize these compounds.

Acknowledgement

This work was funded by the Dutch Ministry of Agriculture, Nature and Food Quality under the Global One Health theme (KB-31-001-005 and 018)

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.04.042.

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