Science of the Total Environment 543 (2016) 524–535

Contents lists available at ScienceDirect

Science of the Total Environment

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

Fractionation and analysis of veterinary antibiotics and their related degradation products in agricultural soils and drainage waters following swine manure amendment

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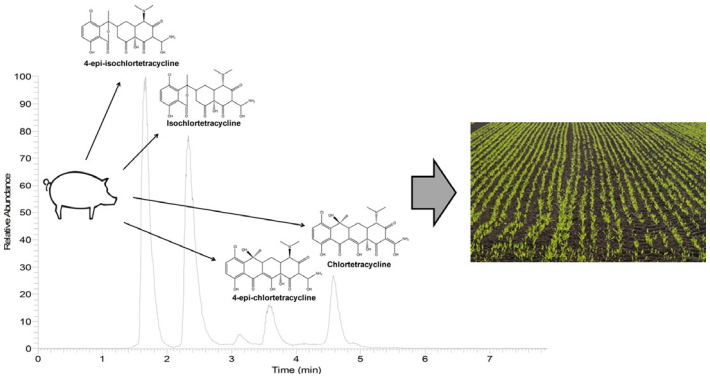
HIGHLIGHTS

- Veterinary antibiotics were analyzed in soils, drainage waters and swine manure.
- Veterinary antibiotics were quantified using high-resolution mass spectrometry.
- Degradation products of tetracyclines were identified and semi-quantified.
- A study of the behavior of these residues in the agricultural area was proposed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 May 2015
Received in revised form 2 November 2015
Accepted 11 November 2015
Available online xxxx

Editor: Kevin V. Thomas

Keywords:
Veterinary antibiotics
Metabolites
Soils
Drainage waters
LC–HRMS

ABSTRACT

The fate of antimicrobial active compound residues in the environment, and especially antibiotics used in swine husbandry are of particular interest for their potential toxicity and contribution to antibiotic resistance. The presence of relatively high concentrations of bioactive compounds has been reported in agricultural areas but few information is available on their degradation products. Veterinary antibiotics reach terrestrial environments through many routes, including application of swine manure to soils. The objectives of this project were first, to develop an analytical method able to quantify and identify veterinary antibiotics and their degradation products in manure, soil and water samples; and second, to study the distribution of these target compounds in soils and drainage waters. A brief evaluation of their potential toxicity in the environment was also made. In order to achieve these objectives, liquid chromatography coupled to high-resolution mass spectrometry was used for its ability to quantify contaminants with sensitivity and selectivity, and its capacity to identify degradation products. Samples of manure, soil and water came from a long-term experimental site where swine manure containing veterinary antibiotics has been applied for many years. In this study, tetracycline antibiotics were found at several hundred μg L^{-1} in the swine manure slurry used for fertilization, several hundred of ng L^{-1} in drainage waters and several ng g^{-1} in soils, while degradation products were sometimes found at concentrations higher than the parent compounds.

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http://dx.doi.org/10.1016/j.scitotenv.2015.11.061
0048-9697/

1. Introduction

As worldwide swine production is constantly increasing, the associated consumption of veterinary antibiotics is also growing. Veterinary antibiotics are widely used to treat diseases and to protect the animals from infections. They can also be used to improve feed efficiency and act as growth promoters. A study estimates that nontherapeutic uses of antibiotics for livestock production alone account for 11,200 t annually in the United States (Mellon et al., 2001). In France, 1349 t were sold in 2007 including 50% for swine breeding (AFSSA-ANMV, 2009). Commercial swine production is the agricultural activity consuming the largest amount of antibiotics compared with other livestock (Sarmah et al., 2006). Several classes of antibiotics exist, among them, tetracyclines (TCs) are the most used, followed by β-lactamines, sulfonamides, lincosamides, diaminopyrimidines and macrolides (Fig. 1) (AFSSA-ANMV, 2009; Sarmah et al., 2006). Due to the large doses used and the fact that the antibiotics are poorly absorbed by swine; the majority of these pharmaceuticals are excreted either unchanged or as metabolites resulting from the biotransformation of the parent compounds. Depending on the substance, the mode of application and the period after administration, it has been shown that excretion rates vary between 40 and 90% for TCs and sulfonamides (Berger et al., 1986; Haller et al., 2002; Halling-Sørensen, 2001). This means that a large quantity of these antibiotic residues is present in swine fecal matter and can thus be released in the environment through the field application of manure. Several studies have revealed the presence of numerous veterinary antibiotic residue classes in different environmental compartments close to agricultural areas and especially in soils, surface waters, ground waters and aquatic sediments (Hirsch et al., 1998; Hu et al., 2010; Jia et al., 2009; Kemper, 2008; Kim et al., 2011; Kümmerer, 2009). The introduction into the ecosystem of substances created to be biologically active raises questions on their potential health or ecotoxicological impact on the environment. Moreover, all these molecules could certainly contribute to bacterial resistance, for example, TCs-resistant genes were found in *Escherichia coli* bacteria isolated from manure of agricultural animals (Chee-Sanford et al., 2001; Sengeløv et al., 2003). Otherwise, their mobility, their sorption and their degradation in the environment is not well known due to their highly variable nature.

However, throughout their transfer from the ingestion by the animal to the environment, the antibiotic molecules could undergo changes. There are an important number of biotransformation by-products (metabolites) excreted by animals (Kuhne et al., 2000; Søeborg et al., 2004). All these metabolites along with their parent molecules will be released in the environment and could subsequently be transformed into primary degradation products. Recent studies have shown that the dissipation in the environment of organic contaminants such as veterinary antibiotics or even pesticides occurred via different physicochemical ways i.e. oxidation, hydrolysis, photo-degradation and reduction reactions (Fenner et al., 2013; Kim et al., 2011). Several factors such as humidity,

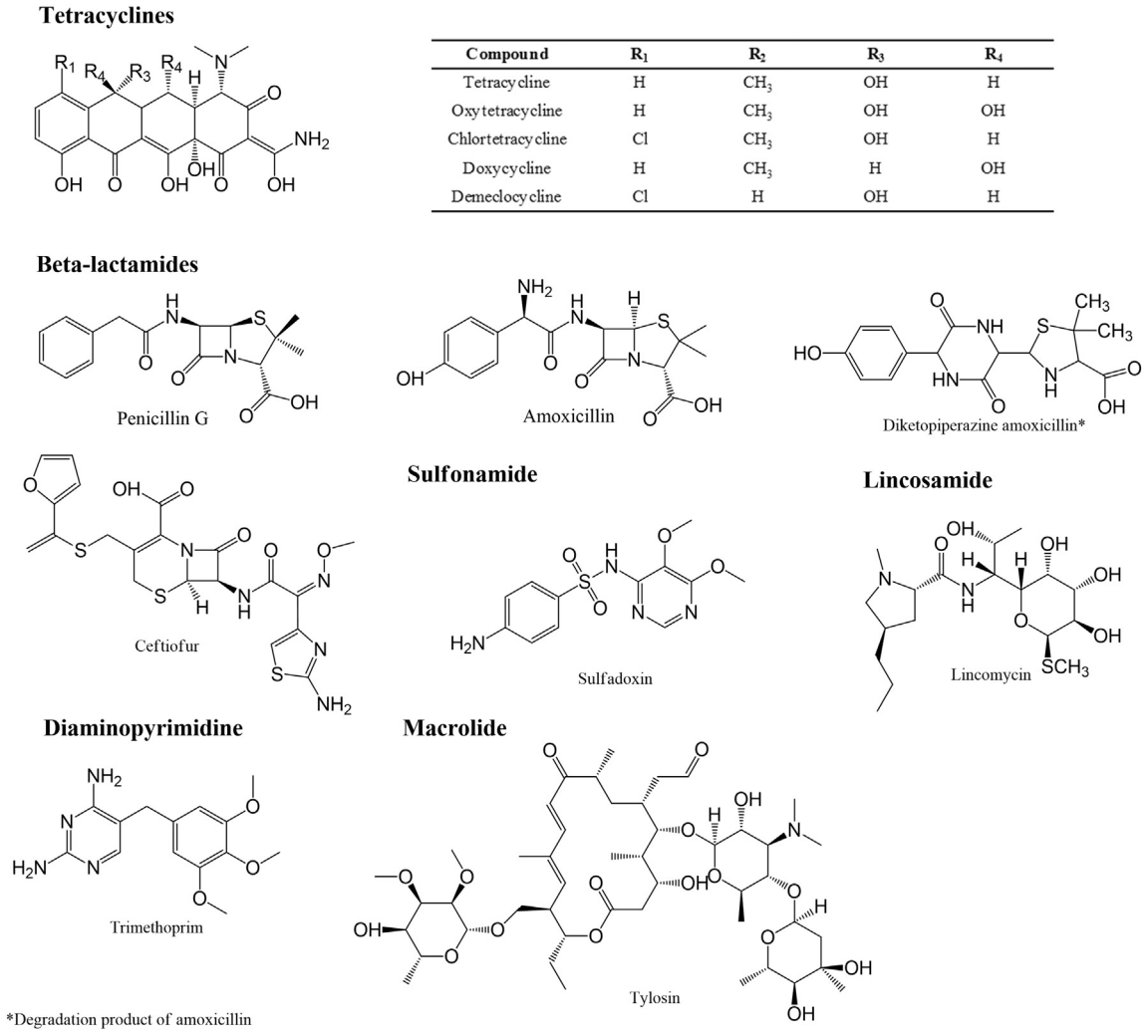

Fig. 1. Chemical structures of target compounds i.e. tetracyclines, β-lactamines, sulfonamides, lincosamides, diaminopyrimidine and macrolides. Tetracyclines and their respective degradation products were all presented in Fig. S1.

temperature, organic matter content and other soil components strongly affect the degradability of the molecules by initiating these abiotic transformations (Ingerslev et al., 2001; Kim et al., 2011). Various compounds and especially sulfur organic compounds and metal species present in the soil can degrade organic contaminants via direct reduction or electron transfer. Nitroaromatic compounds, a pesticide class, could be reduced through a direct electron transfer in presence of sulfur species after sorption on organic matter (Fenner et al., 2013). In that case, organic matter plays the role of sorbent and initiator of the degradation via reduction or electron transfer (Zeng et al., 2012). Otherwise, sulfonamides could undergo rapid transformation by reacting with metallic species such as Fe(II) generated by microbial reduction of Fe(III) soil minerals (Mohatt et al., 2011). Also, TCs behavior could be affected by the presence of aluminum oxide in the aquatic environment via both adsorption and transformation and promote dehydration of TCs (Chen and Huang, 2010). These examples of abiotic reactions could play a significant role in the fate and transformation of veterinary antibiotics. In addition, microbial populations present in the different compartments of the environment can also contribute, to a lesser degree, to the degradation of veterinary antibiotics (Ingerslev et al., 2001). Antibiotics, along with their associated metabolites and degradation products have different physicochemical properties and therefore, different behavior in the environment. This study focuses on the fractionation and the degradation of the most used TCs antibiotics in swine husbandry and a few other current antibiotic classes i.e. β-lactamines, macrolides, sulfonamides, lincosamides and diaminopyrimidines. Tetracyclines tend to degrade by changing their conformation to form epimers, depending on various environmental conditions such as pH, the presence of chelating ions and light (Halling-Sørensen et al., 2002; Mccormick et al., 1957). These isobaric compounds or epimers were found in large quantities in manure, soil and drainage water samples studied. All these metabolites and primary by-products were grouped under the name of degradation products in this manuscript.

Robust analytical methods are necessary in order to extract and quantitate veterinary antibiotic residues and their degradation products in a variety of biological and environmental matrices. The presence of organic compounds and large amounts of divalent cations in these matrices can significantly decrease the extraction recoveries and the target compounds quantitation by affecting signal intensities (Dams et al., 2003; O'Connor and Aga, 2007). Thus, these interferences could also have significant impact on the selectivity and sensitivity of the method when applied to traces of organic contaminants. To avoid this, an appropriate sample processing is necessary to remove large portions of undesirable compounds while keeping the compounds of interest. The extraction of veterinary antibiotics contained in biological and environmental matrices have been reported earlier and were usually based on sonication or pressurized liquid extraction (PLE) (Blackwell et al., 2004; Jacobsen and Halling-Sørensen, 2006; Yang et al., 2004). The extraction process used here was inspired by a previous study developed at our laboratory to quantitate several TCs in freeze-dried swine manure and was adapted to soils, drainage waters and swine manure (Solliec et al., 2015). The advantage lies in the fact that this method used a sonication extraction with an aqueous McIlvaine buffer wherein veterinary antibiotics are readily soluble. A water solvent extraction will therefore extract less potentially interfering matrix compounds compared to organic solvent such as MeOH (Anderson et al., 2005; Thiele-Bruhn, 2003). This extraction method was used at pH 5 to avoid the degradation of tylosin and TCs (Paesen et al., 1995). The addition of EDTA has been used to prevent the chelation of TCs with divalent cations present in the samples and make them more available (Jezowskabojczuk et al., 1993; O'Connor and Aga, 2007). Drainage water samples were acidified at pH 5 by adding citric acid and ethylenediaminetetraacetic acid (EDTA) before filtration on fiberglass filter. A Strata-X polymeric SPE cartridge was used in previous studies with acceptable recoveries and was selected for its ability to capture large amounts of polar contaminants (Jacobsen et al., 2004; Solliec et al., 2015; Yang et al., 2005).

Veterinary antibiotics are usually analyzed by liquid chromatography (LC) coupled to UV–Vis spectrophotomer, diode array detector (DAD) or triple quadrupole (QqQ) (Blackwell et al., 2004; Kim and Carlson, 2007; Mamani et al., 2009). The UV–Vis detection is still the most common method and achieves relatively low method limit detection (MLD), but has poor selectivity. The QqQ is the preferred instrument for the analysis of complex matrices due to its sensitivity and selectivity owing mainly to the specificity of selected reaction monitoring (SRM). Nevertheless, a recent study has shown that false positives could potentially be generated, especially when using QqQ due to the low resolving power (RP) of the quadrupoles (Kaufmann et al., 2015). The use of high-resolution mass spectrometry (HRMS) could avoid a large portion of false positives due to its high RP measurement. This technology was previously mainly applied in the field of proteomics and metabolomics but there is an increasing interest for environmental studies focusing on small molecules in recent years (Michalski et al., 2011).

The Q-Exactive is a hybrid mass spectrometer which combines the high RP of the Orbitrap with the selectivity of the quadrupole. This combination, coupled to a high-energy collision cell, is a powerful tool for the screening of emerging contaminants and the investigation and identification of degradation products. The use of hybrid quadrupole-Orbitrap operated in a combination of full scan (FS) and fragmentation events named data-dependent acquisition (DDA) has previously been employed for the research of unknowns. Negreira et al. developed a method to investigate and identify unknown transformation products of anticancer drugs with this approach (Negreira et al., 2015). However, degradation products of veterinary antibiotics are "know unknowns" since many have already been referenced (Chen and Huang, 2011; Little et al., 2011). Thus, we used a derived mode of DDA that uses an inclusion list in order to make a targeted identification of degradation products. This approach was used by Wang et al. to obtain high resolution product ion spectra and allowed them to identify and confirm the presence of pesticide residues in fruits and vegetables (Wang et al., 2014). The DDA mode was used in a first approach to screen for veterinary antibiotics and degradation products by scanning a large mass range (m/z 100–1000) using the FS event. In a second event, the quadrupole select target ions from an inclusion list and then carries out fragmentation in the collision cell to generate high-resolution spectra of the product ions. Fragmentation was triggered uniquely if the signal intensity of the target ion compounds included in an inclusion list exceeds the pre-defined threshold. This inclusion list included several exact masses of common veterinary antibiotics and known associated degradation products that are of interest (Table S1). However, we also leave the door open for unknown candidates. When the instrument is not already acquiring a target ion, this mode will be triggered to fragment unexpected ions that exceed the specified threshold but that were not present in the inclusion list. In a second run, target compounds were quantified using the parallel reaction monitoring (PRM). These target compounds were selected based on their ability to trigger fragmentation in the DDA mode when analyzing samples. This mode was already used in previous studies to quantify organic contaminants in complex biological and environmental matrices (Fedorova et al., 2013; Solliec et al., 2015) and it allowed reaction monitoring in a tandem mass spectrometry (MS^2) mode, it improved selectivity and lessened the impact of the matrix on the signal (Berendsen et al., 2015). We used this mode to allow semi-quantification of the degradation products of chlortetracycline (CTC) and demeclocycline (DEC) because absolute quantification would require reference standards that were not available.

This method was adapted to quantify TCs, 7 other veterinary antibiotics from several classes and a few degradation products (Fig. 1 and S1). An evaluation of the analytical methods was done by determining the MLD and quantification (MLQ), linearity, accuracy, precision and matrix effects. Three samples of swine manure, 24 samples of drainage waters and 96 soil samples were investigated in DDA mode and

contaminants were quantified in PRM mode. These samples were provided by the Research and Development Institute for the Agri-Environment (IRDA) and are part of a long term study on the environmental fate of nitrogen following field applications of swine manure.

We report here the development, the validation and application of HRMS for the quantification of veterinary antibiotics and the identification of degradation products in environmental and biological matrices. This study also emphasizes the behavior and the solid–liquid partitioning of veterinary antibiotics and some of their degradation products in an agricultural field.

2. Materials and methods

2.1. Chemicals and reagents

Amoxicillin (AMX, purity >98.7%), trimethoprim (TMP, purity >99.5%), lincomycin (LCM purity >100%), sulfadoxin, (SFX, purity >99.9%), ceftiofur (CFT, purity >97.7%), tylosin (TYL, purity >84.6%), benzylpennicilin (PEG, purity >99.6%), tetracycline (TC, purity >99.5%), 4-epitetracycline (4-ETC, purity >98.4%), anhydrotetracycline (ATC, purity >99.5%), 4-epianhydrotetracycline (4-EATC, purity >90.5%), 4-epichlortetracycline (4-ECTC, purity >97.0%), doxycycline (DC, purity >98.2%), demeclocycline (DEC, purity >94.5%), oxytetracycline (OTC, purity >97.0%), minocycline (MC, purity >98.7%), chlortetracycline (CTC, purity >93.0%), spiramycin (SPI, purity >94.9%) and simeton (purity >99.0%) were purchased from Sigma-Aldrich (St. Louis, MO). [$^{13}C_3$]-trimethoprim ([$^{13}C_3$]-TMP, purity >99.0%) was purchased from ACP Chemical Inc. (Montreal, QC, Canada). Isochlortetracycline (ICTC, purity >95.0%) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All solvents used were of high-performance liquid chromatography (HPLC) grade from Fisher Scientific (Whitby, ON, Canada) and HPLC grade H_2O was used for dilutions. Individual stock solutions were prepared in methanol (MeOH) at a concentration of 1000 mg L^{-1} and kept at −20 °C for a maximum of 3 months. Individual intermediate solutions were prepared by dilution of the 1000 mg L^{-1} stock solution in H_2O. Given the potential for degradation of the target analytes, working solutions were prepared daily at a concentration of 1 mg L^{-1} by dilution in H_2O from individual intermediate stock solutions. Sodium phosphate dibasic (Na_2HPO_4, purity >99.0%), citric acid (purity >99.5%), formic acid (HCOOH, purity >95.0%) and EDTA disodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, purity >99.0–101%) were purchased from Sigma-Aldrich (St. Louis, MO). Chromatographic mobile phases were prepared daily.

2.2. Water, soil and manure samples

Swine manure, soil and drainage water samples were collected from a long-term experimental field trial conducted at the IRDA research farm located near Quebec City, Canada. Swine manure samples analyzed in this paper came from the indoor pit of a swine facility and were collected during the third field application on September 6th, 2013. Soil samples were taken at 0–10, 10–20 and 20–40 cm depth on November 16th 2011, after swine manure applications at a rate of 54 t ha^{-1} treatments on May 19th. Two years later, soil samples were taken at the same depths on November 20th 2013 after two swine manure applications at rates of 55 t ha^{-1} and 25 t ha^{-1} treatments on June 5th and September 6th 2013 respectively. Soil samples consisted of five subsamples of soil taken at each depth with a 2.5 cm diameter soil core sampler. Water samples were regularly collected after significant drainage occurred following rain events or snowmelt. Water samples analyzed in this paper were collected on September 13th, 2013 and September 18th, 2013, following swine manure application on September 5th and rain events totaling 74 mm between September 5th and September 13th and 28 mm between September 13th and September 18th. Soil samples were collected in plastic flasks and were kept at 4 °C until analysis. Swine manure and drainage water samples were collected in respectively 500 and 100 mL plastic flasks and were kept at 4 °C until analysis. All samples were collected for a previous study dealing with the fertilizer efficiency of swine manure on crops and the partitioning of nitrogen in the environment following manure fertilization. More details and generalities on the field and sample collection are available in supplementary material (Section 1).

2.3. Extraction and sample clean-up

The same extraction was applied to the swine manure and to the soils. Exactly 2 mL of liquid manure were measured and placed into 15 mL conical-bottom centrifuge tubes (Fisher, Rockwood, TN). For soils, exactly 2 g of dried soil were weighted into the tube. We then added 10 mL of McIlvaine extraction buffer at pH 5 containing 0.1 M EDTA to the manure and the dried soil samples. McIlvaine buffer (500 mL) was prepared by mixing 243 mL of 0.1 M citric acid and 257 mL of 0.2 M Na_2HPO_4 (McIlvaine, 1921). The samples were mixed for 1 min on a vortex to suspend the particles and homogenize the solution and they were subsequently placed into an ultrasonic bath for 15 min at 25 °C. They were then centrifuged at approximately 6000 rpm for 15 min. Finally, 9 mL of the supernatants were collected into 15 mL centrifuge tubes. Drainage water samples were filtered on fiberglass membrane filters GF-75 of 0.45 μm porosity (Sterlitech, Kent, WA) and 100 mL aliquots were measured and acidified with citric acid (pH 5) and 0.1 M of EDTA was added before SPE.

The SPE was done using a 12-position manifold manufactured by Phenomenex (Torrance, CA). A polymeric reversed phase Strata-X (Phenomenex, Torrance, CA) cartridge with a total volume of 6 mL with 200 mg bed mass was used to wash the sample extracts. Multiple SPE parameters were optimized: cartridge type, loading step, washing step and sample pH. The SPE cartridges were conditioned with 5 mL of MeOH, 5 mL of H_2O and 5 mL of McIlvaine buffer. Samples were loaded on the cartridge by gravity. The SPE cartridges were washed with 5 mL of H_2O:MeOH (90:10, *v*/*v*). The analytes were eluted with 5 mL of MeOH containing 0.1% HCOOH into conical-bottom centrifuge tubes. The eluates were then evaporated to total dryness under a gentle stream of nitrogen at room temperature with a nine-port Reacti-vap unit from Pierce (Rockford, IL) and then reconstituted into 250 μL with H_2O for LC-HESI-HRMS analysis.

2.4. LC parameters

A Thermo Scientific Dionex Ultimate 3000 Series RS pump coupled with a Thermo Scientific Dionex Ultimate 3000 Series TCC-3000RS column compartments and a Thermo Fisher Scientific Ultimate 3000 Series WPS-3000RS autosampler controlled by Chromeleon 7.2 Software (Thermo Fisher Scientific, Waltham, MA and Dionex Softron GMbH Part of Thermo Fisher Scientific, Germany) were used for analysis. A Hypersil GOLD™ C18 (100 mm × 2.1 mm, 1.9 μm particles) column preceded by a similar guard column (5 mm × 2.1 mm, 3 μm particles) (Thermo Fisher Scientific, Waltham, MA) was used for chromatographic separation of target compounds at 20 °C.

Mobile phase A consisted of H_2O with 0.1% HCOOH. Mobile phase B consisted of MeOH with 0.1% HCOOH. A gradient was used starting from 30% B, and then the MeOH mobile phase was increased to 95% from 0 to 6 min and was held constant for 2 min. Finally, the mobile phase was brought back to initial conditions and maintained for 4 min. A flow rate of 300 μL min^{-1} and an injection volume of 10 μL were employed. The LC gradient is given in supplementary information (Fig. S2).

A heated electrospray ionization source (HESI-II) in positive mode was used for the ionization of the target compounds. The parameters were set as follows: the ionization voltage was optimized at +4300 V; capillary temperature was set at 275 °C; the vaporizer temperature was set to 300 °C; sheath gas and auxiliary gas flow were optimized at 50 and 30 arbitrary units, respectively.

2.5. HRMS parameters

Detection and quantification of target compounds were performed using a Q-Exactive mass spectrometer controlled by the Xcalibur 2.3 software (Thermo Fisher Scientific, Waltham, MA). The molecular formulas of the target compounds were acquired from Chemspider (Royal Society of Chemistry, Raleigh, NC) and exact masses of target compounds were calculated using Qualbrowser in Xcalibur 2.3. Instrument calibration in positive mode was done every 5 days with a direct infusion of a LTQ Velos ESI Positive Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL). The PRM parameters were optimized using individual standards at a concentration of 10 mg L^{-1}. They were infused with a syringe at a flow rate of 10 μL min^{-1} through a T-piece connected to a LC system with a mobile phase flow rate of 300 μL min^{-1}. The product ions were found by increasing the normalized collision energy (NCE) using the Q-Exactive Tune 2.3 software (Thermo Fisher Scientific, Waltham, MA). After choosing the most intense product ions, fragmentation energy scans were carried out to obtain the optimal NCE for complete fragmentation of precursor ions. The exact masses of the product ions were obtained with Mass Frontier software (Highchem, Bratislava, Slovakia). A mass inclusion list for PRM mode was used including the precursor ion masses, their expected retention time (with one minute acquisition time window center on each retention time of target compounds) and their NCE (Table 1). This inclusion list of target compounds referred to the veterinary antibiotics and their associated epimers present in the samples that triggered fragmentation in the DDA mode. All Q-Exactive parameters such as RP, automatic gain control (AGC) and ion time (IT) were chosen relative to the complexity of the matrix study. The different optimization tests were performed with a mix solution containing 250 μg L^{-1} of the 16 target analytes and 100 μg L^{-1} of IS spiked in a blank sample of each matrix submitted to SPE. The Q-Exactive was also operated in DDA mode that included a FS screening and a fragmentation event. The FS event was set at 70,000 FWHM (m/z 200) and m/z 100–1000 scan range wide. The AGC and IT parameters were set respectively at 5×10^5 ions capacity and 100 ms filling maximum time; and 2×10^5 and 55 ms for the fragmentation event of the DDA and PRM mode. A RP of 17,500 FWHM (m/z 200) was used for the measure of product ions. The intensity threshold depends of the filling of the accumulation trap of the Q-Exactive was fixed at 3.6×10^4, as determined as a function of the IT and AGC parameters. An inclusion list was used to force the Q-Exactive to focus on selected common veterinary antibiotics and their known associated degradation products listed in the literature (Table S1). Only 26% of the 39 compounds present in this list triggered the fragmentation and were found present in the samples. The fragmentation was triggered when selected target compound ions present in the list or unexpected ions have intensities above the threshold. The top 5 most intense ions were selected for fragmentation and a single loop count was used for the number of repetitions. The precursor ions were filtered by the quadrupole which operated at an isolation width of 0.4 FWHM (m/z 200). All optimized collision energies, precursor ions and fragment ions are shown in Table 1.

2.6. Degradation products

The degradation of the target antibiotics was monitored in surface waters, soils and HPLC grade water as control solution by spiking a mixture of veterinary antibiotics (500 μg L^{-1}, 500 ng g^{-1} and 500 μg L^{-1} respectively). These samples were exposed to laboratory light at ambient temperature and aged during one month. They were then submitted to sample processing and investigated in DDA mode (Fig. S3). This degradation was studied by comparing the TCs epimerization of spiked surface waters, spiked soils and control spiked HPLC grade water. Only isomeric degradation products of CTC were investigated in this study.

2.7. Method validation

The selected quantification method was validated in three different matrices. The mass tolerance window (MTW) was set to 5 ppm (±2.5 ppm). Target compounds were quantified with PRM mode with the most intense product ion signal and the one in common for each TCs was used for confirmation (m/z 154.0504 − $C_7H_8O_3N$). The instrument response was determined as the ratio of the analytes area to that of the IS. Simeton, SPI and [$^{13}C_3$]-TMP were used as IS and were selected for their capacity to correct the signal variation of their associated target compounds (Table 1). The DDA mode was used for the investigation and identification of degradation products. The fragmentation patterns of unknown chromatographic peaks were compared to those of CTC, 4-ECTC, ICTC, DEC and AMX reference standards (100 μg L^{-1}) for identification. Fragmentation spectra were then interpreted and product ion masses were compared to those predicted by Mass Frontier software that is able to generate fragmentation patterns based on general fragmentation rules. Isochlortetracycline and its associated epimer were semi-quantitative based on the calibration curve of the parent compound. Seven-point calibration curves were obtained by spiking samples between 0 and 500 ng L^{-1} for drainage waters, 0

Table 1
HRMS parameters.

Compounds	Associated IS	Chemical formula	RT (min)	Exact mass $[M+H]^+$ (m/z)	Average experimental mass $[M+H]^+$ (m/z)	ΔM (ppm)[a]	NCE (%)	Quantification ion transition (m/z)	Confirmation ion transition (m/z)
TC	SIM	$C_{22}H_{24}N_2O_8$	1.87	445.1611	445.1600	2.4	30	410.1240	154.0504
4-ETC	SIM	$C_{22}H_{24}N_2O_8$	1.50	445.1611	445.1598	2.9	30	410.1240	154.0504
ATC	SIM	$C_{22}H_{22}N_2O_7$	6.01	427.1505	427.1496	2.2	31	410.1240	154.0504
4-EATC	SIM	$C_{22}H_{22}N_2O_7$	5.82	427.1505	427.1496	2.2	31	410.1240	154.0504
DEC	SIM	$C_{21}H_{21}ClN_2O_8$	2.99	465.1065	465.1052	2.7	34	448.0799	154.0504
OTC	SIM	$C_{22}H_{24}N_2O_9$	2.08	461.1560	461.1550	2.3	30	426.1189	154.0504
MC	SIM	$C_{23}H_{27}N_3O_7$	1.27	458.1927	458.1922	1.1	38	441.1662	154.0504
CTC	SIM	$C_{22}H_{23}ClN_2O_8$	4.45	479.1221	479.1209	2.6	42	444.0850	154.0504
DC	SIM	$C_{22}H_{24}N_2O_8$	5.67	445.1611	445.1601	2.2	30	428.1345	154.0504
TMP	[$^{13}C_3$]-TMP	$C_{14}H_{18}N_4O_3$	1.19	291.1451	291.1451	0.3	50	261.0981	230.1161
SFX	[$^{13}C_3$]-TMP	$C_{12}H_{14}N_4O_4$	2.47	311.0809	311.0808	0.2	35	156.0115	108.0446
LCM	[$^{13}C_3$]-TMP	$C_{18}H_{34}N_2O_6S$	1.23	407.2210	407.2215	1.1	34	126.1277	359.2176
PEG	[$^{13}C_3$]-TMP	$C_{16}H_{18}N_2O_4S$	6.01	335.1060	335.1066	1.7	40	220.0425	217.0640
AMX	[$^{13}C_3$]-TMP	$C_{16}H_{19}N_3O_5S$	1.02	366.1118	366.1124	1.5	35	160.0426	114.0374
CFT	[$^{13}C_3$]-TMP	$C_{19}H_{17}N_5O_7S_3$	5.32	524.0363	524.0367	0.8	20	241.0389	285.0107
TYL	SPI	$C_{46}H_{77}NO_{17}$	6.15	916.5264	916.5274	1.1	30	174.1124	772.4446
SIM (IS)		$C_8H_{15}N_5O$	2.63	198.1355	198.1357	1.2	50	124.0870	None
SPI (IS)		$C_{43}H_{74}N_2O_{14}$	4.96	843.5218	843.5203	1.9	24	174.1120	None
[$^{13}C_3$]-TMP (IS)		$C_{11}^{13}C_3H_{18}N_4O_3$	1.17	294.1558	294.1551	2.2	50	262.1021	None

[a] Mass measurements were performed just after Q-Exactive calibration.

and 500 ng g^{-1} for soils and 0 and 500 μg L^{-1} for swine manure (n = 3). The IS (100 ng L^{-1}, 100 ng g^{-1} and 100 μg L^{-1}) were selected for their capacity to correct the signal variation of target compounds with a RSD ratio < 10% (data not shown). Matrix effects were determined by calculating the ratio of spiked target compounds recorded in sample matrices (soil, water and manure) submitted to SPE relative to a solvent solution (250 μg L^{-1}) and were expressed as a percentage (Table S2). The recovery values of the SPE method were evaluated at two different concentrations by comparing the analyte to IS ratio of the sample spiked before extraction to the analyte to IS ratio of the sample spiked after extraction and was expressed as a percentage (n = 3) (Table S2). Unspiked samples were also extracted to account for their presence in the recovery values. In each set of samples, the IS was added after the extraction and before the reconstitution step. Repeatability (intra-day precision) was evaluated for the three matrices with the analysis of the same spiked sample at two different concentrations on a single workday (n = 5) (Table S3). Reproducibility (inter-day precision) was also calculated for the three matrices by spiking an extracted sample at two different concentrations freshly prepared each day (n = 5) (Table S3). Accuracy values were determined by the relative error (%) and precision values were defined as the relative standard deviation (RSD — %) (Table S3). The MLD and MLQ were determined as 3.3 and 10 times, respectively, the standard deviation of the y intercept divided by the slope of the calibration curve in matrix sample (Table 2). Statistical tests were made using the Statistical Package for Social Science (SPSS 21.0, Chicago, IL) for Windows. ANOVA test and Tukey's post hoc tests were used with statistical significance defined as p-value < 0.05.

3. Results and discussion

3.1. Validation of the analytical method

The analytical method with optimized parameters was applied to the three matrices. The calibration curves in the different matrices showed good linearity, with coefficients of determination (R^2) ranging from 0.967 to 0.999. The calculated MLD from the calibration curves ranged from 2.0 to 27 ng L^{-1} for drainage waters, from 1.0 to 7.4 ng g^{-1} for soils and from 3.6 to 12 μg L^{-1} for swine manure (Table 2). The MLD results in swine manure were comparable with those reported previously (Jacobsen et al., 2004). Matrix effect is an important parameter to control considering the diversity and the complexity of the analyzed matrices. Drainage waters had the lowest impact on target compounds intensity with matrix effect ranging from 75 to 105%. Soils and swine manure had similar matrix effects with a range of 65 to 110% for soils and 62 to 112% for swine manure (Table S2). These matrices contained an important quantity of organic compounds which can lead to an underestimation or overestimation of the measured concentration. Otherwise, isobaric interferences or false positives could occur due to the presence of organic compounds from the matrices with closely related exact masses (Berendsen et al., 2015; Kaufmann et al., 2015). Furthermore, the Q-Exactive was used in PRM mode, a mimicked SRM mode, to quantitate target compounds. This mode enjoys the benefits of the quadrupole and the collision cell and allows tandem mass spectrometry with high RP measurement on the product ion. A recent study stated that selectivity with HRMS is mostly dependent of the selective mass defect of certain compounds containing halogens or that are highly unsaturated (Berendsen et al., 2015). This type of compounds such as veterinary antibiotics has molecular structures that lower the chances of interfering signals with matrix compounds and thus false positives. The selectivity of the method also depends of several factors such as matrix type, selected transitions, sample clean-up procedure, mass defect and RP according with Berendsen.

The analytes were extracted using SPE and gave extraction recoveries ranging from 40 to 111% in all matrices for all target compounds (Table S2). Recovery is an important parameter but could be highly variable when looking at multi-residues in a single method. Considering the differences of physicochemical properties of analytes, acceptable recoveries cannot be reached for all target compounds. Degradation products of tetracyclines, ATC and 4-EATC, have low recoveries in soils (40 and 41%) and swine manure (64 and 65%) but acceptable recovery in drainage waters (103 and 102%). Tetracyclines are known to complex with divalent ions such as Ca^{2+} and Mg^{2+} as well as with substances of low and high molecular weight (Gu et al., 2007; Wessels et al., 1998). By dehydration, TC becomes ATC and this change in conformation tend to influence its capacity to complex with divalent ions and thus its sorption capacity with humic substances, minerals and clays present in both swine manure and soil matrices. Tetracycline and ATC differ by their chelation sites that seems to influence their complexation capacity and results in low recovery for ATC and its associated epimers (Wessels et al., 1998). Moreover, TCs are well known to be difficult to remove once adsorbed onto organic matter according with their high K_d (Tolls, 2001). In the context of environmental analysis, according to US Environmental Protection Agency and European Commission, recoveries for ATC and 4-EATC were not acceptable for an accredited method. Acceptable recoveries should be in the range 70–130%. However, US EPA has also determined similarly low recoveries for ATC and 4-EATC in soils (European, 2002; Englert, 2007).

Several filter materials were tested for drainage waters filtration: fiberglass, polycarbonate, cellulose, nitrosamine, nylon and mixed

Table 2
Method validation parameters of target compounds in swine manure, soil and drainage water samples including coefficient correlation (R^2), sensitivity, the MLD and the MLQ and recoveries.

Compounds	R^2			MDL			MQL		
	Manure	Soil	Water	Manure (μg L^{-1})	Soil (ng g^{-1})	Water (ng L^{-1})	Manure (μg L^{-1})	Soil (ng g^{-1})	Water (ng L^{-1})
TC	0.989	0.997	0.998	4.1	2.3	3.0	14	7.4	10
4-ETC	0.983	0.993	0.999	8.0	1.0	3.5	27	3.3	12
ATC	0.974	0.986	0.993	6.1	2.0	11	20	6.8	37
4-EATC	0.982	0.963	0.994	7.2	2.7	13	24	9.1	42
DEC	0.977	0.999	0.999	7.1	7.4	2.1	24	25	7.0
OTC	0.987	0.998	0.999	9.4	3.6	2.3	31	19	7.7
MC	0.967	0.977	0.986	8.2	6.6	5.6	27	22	18
CTC	0.998	0.998	0.999	5.7	5.6	2.0	19	19	6.5
DC	0.986	0.988	0.997	5.9	5.3	9.0	20	18	30
TMP	0.999	0.996	0.999	4.6	1.1	2.8	15	3.6	9.4
SFX	0.997	0.996	0.998	6.3	1.8	4.4	21	6.1	15
TYL	0.972	0.961	0.983	16	3.1	27	53	10	91
LCM	0.999	0.999	0.999	8.6	1.2	6.1	29	4.2	20
PEG	0.993	0.991	0.994	12	1.3	10	41	4.4	34
AMX	0.988	0.991	0.998	3.6	1.1	7.6	12	3.6	25
CFT	0.996	0.999	0.999	12	2.2	2.9	42	7.2	9.8

cellulose esters. Fiberglass filters had the best recoveries for all target compounds (Fig. S4).

Accuracy was determined at two concentration levels as percent bias (%) between the concentration added and the one found in the samples (n = 3). It was acceptable with bias under 15% for each concentration levels (50, 250 ng L^{-1}–ng g^{-1}–μg L^{-1}) for all compounds (Table S3). Precision for intraday and interday was expressed using relative standard deviations (RSD − %). Intraday precision values calculated for two concentration levels (n = 5) were under 7% (Table S3). Interday precision calculated for two concentrations levels (n = 5) was under 14% (Table S3).

3.2. Degradation of TCs and formation of degradation products

The metabolism of veterinary antibiotics can be divided into several stages. Primary metabolites result from the biotransformation of the parent compounds by the animal, whereas secondary metabolites result from the biotransformation by the bacteria present in the ecosystem. All these compounds are then subjected to abiotic physicochemical degradation when released into the environment which further complicates the environmental fate of these substances. This mixture of metabolites, degradation products and parent compounds has been measured in the different samples, thanks to LC separation. Several peaks eluted before the TCs standard compounds with greater intensity especially for TC, CTC and DEC chromatographic separation (Fig. S5). Those peaks are constitution isomers of the parent compounds called epimers and could be formed in mildly acidic conditions (Kennedy et al., 1998). Chlortetracycline could form several different degradation epimers: 4-ECTC, ICTC, 4-epiisochlortetracycline (4-EICTC), ketochlotetracycline (KCTC) and 4-epiketochloretracycline (4-EKCTC) (Kennedy et al., 1998; Søeborg et al., 2004). Also, TC and DEC could produce 4-ETC and 4-epidemeclocycline (4-EDEC) (Halling-Sørensen et al., 2002). This epimerization of TCs is a steric rearrangement in the configuration of dimethylamine group and oxidation of the phenolic group present on the TC nucleus (Liang et al., 1998). Several of these degradation products have a concentration level above their parent compounds in the samples suggesting the increased environmental stability of the latter. The identification of some degradation compounds may be difficult due to the unavailability of standards. It was thus based on the interpretation and comparison of retention times and fragmentation patterns of these constitution isomers with the available reference standards.

Three major chromatographic peaks (FS mode *m/z* 479.1209) eluted before CTC (4.45 min) at 1.65, 2.32 and 3.62 min respectively and reached relatively high intensity in the swine manure sample (Fig. 2). Two of these chromatographic peak retention times and fragmentation patterns matched with those of ICTC (2.31 min) and 4-ECTC (3.50 min) reference standards and were also easily identifiable. Chlortetracycline and 4-ECTC reference standards produced three major fragment ions at HCD 30 (%): *m/z* 444.0850, 462.0939 and 154.0504 corresponding to $[M + H\text{-}NH_3\text{-}H_2O]^+$, $[M + H\text{-}NH_3]^+$ and $C_7H_8NO_3^+$ respectively (Fig. 2) (Vartanian et al., 1998). However, the ICTC reference standard that eluted at 2.31 min produced the same fragments with a greater intensity for *m/z* 462.0939. The same fragmentation pattern has been noticed for the chromatographic peak that eluted before ICTC at 1.65 min (Fig. 2). Related epimers have a similar fragmentation pattern due to their close structure; fragment ions of the unidentified epimer peak showed a close relation with ICTC, and allowed us to propose that this peak is 4-EICTC.

The low intensity degradation products were not formally identified because of the unavailability of the standards. Otherwise, a study reported the presence of KCTC and its conjugate epimer 4-EKCTC in swine tissues that produced a major fragment at *m/z* 462.0939 and a minor fragment at *m/z* 444.0850 (Cherlet et al., 2006). These results were compared with the DDA spectra of the spiked surface water solutions and the control HPLC grade solution waters incubated at ambient temperature and laboratory light. After one month, a major part of CTC was transformed into its constitutive isomers. The spiked surface water solutions contained only two isomers of CTC at 1.67 and 2.30 min and they corresponded respectively to 4-EICTC and ICTC according to their retention times and their fragmentation patterns. However, the control solution in HPLC grade water showed a slower degradation of CTC (Fig. S3). In this solution, the five constitutive epimers of the CTC were present. The epimers at 2.54 and 3.06 min are related due to their close fragmentation pattern and are certainly related to 4-EKCTC and KCTC, keto formed of both enol 4-ECTC and CTC.

Only one chromatographic peak eluted before DEC (3.01 min) and belongs to 4-EDEC according to its fragmentation pattern. The fragmentation of DEC and its related epimer both showed peaks at *m/z* 448.0799 and 430.1 related to a loss of H_2O followed by a loss of NH_3. A common TCs product peak was also present at 154.0504 ($C_7H_8NO_3^+$) thus confirming the presence of 4-EDEC in the sample (Fig. 3). The 4-ETC (1.50 min) and TC (1.87 min) reference standards showed the same product ions at *m/z* 410.1240 and 154.0504 and similar retention times were also observed between samples and standards.

The β-lactamines AMX, PEG and CFT were not found in the samples despite being widely used in agriculture. The absence of this antibiotic family could be explained by the poor stability of the β-lactam ring. Indeed, this ring can be opened by β-lactamase, an enzyme family linked to bacterial resistance, or by a chemical hydrolysis (Kemper, 2008). However, three chromatographic isomeric peaks (1.51 min, 2.08 min and 2.87 min) appeared in the extracted ion chromatogram of swine manure sample for protonated AMX at *m/z* 366.1124 (Fig. 4). While reference standard of AMX showed a main peak intensity at 1.02 min and a lower ne at 2.09 min in standard solution (Fig. 4). The fragmentation of AMX reference standard resulted in the formation of two major product ions at *m/z* 160.0426 and 114.03740. Those fragments assigned to molecular structures with Mass Frontier software and correspond to $C_6H_{10}NO_2S^+$ and $C_5H_8NS^+$ respectively (Fig. 4). The same fragmentation pattern was observed for the chromatographic isomeric peak and could be assigned to diketopiperazine amoxicillin which is known to be a degradation product of AMX (Nagele and Moritz, 2005). It was shown that this compound considerably affects microbial activity and its presence confirmed that AMX was actually used during swine husbandry (Kemper, 2008).

One important issue in quantifying TCs degradation products is their lack of stability and the relative ease with which they form epimers. Pure ICTC reference standard is unstable and readily transforms to its conjugate epimer to form a stable mixture of ICTC and 4-EICTC which further complicates the quantification (Kennedy et al., 1998). In addition, 4-EICTC standard is not available. This unavailability coupled with the instability of TCs epimers lead us to consider the use of a semi-quantification approach. Thus, 4-EICTC and ICTC were semi-quantified based on the calibration curve of CTC. This approach by using an analogous compound to propose estimation has already been used in several studies that study metabolites or degradation products (Du et al., 2012). The accuracy of these data relies on the assumption that the response factor for degradation products was comparable with the response factor for the parent compound. Because of different factors such as matrix effect causing ionization suppression, detector dynamic range or difference in the structure of epimer, semi-quantification could be viewed as a general indication of relative abundance.

3.3. Occurrence, mobility and efficiency of veterinary compounds

The use of large quantities of veterinary antibiotics in swine husbandry has led to the occurrence of some TCs and other often encountered pharmaceuticals in swine manure, soils, drainage waters as well as surface waters and ground waters. The quantity of swine manure spread on the field in 2011 was 54 t ha^{-1} and soil samples were collected 6 months after. However, swine manure samples from 2011's spreading were unavailable; therefore, no data on this manure was

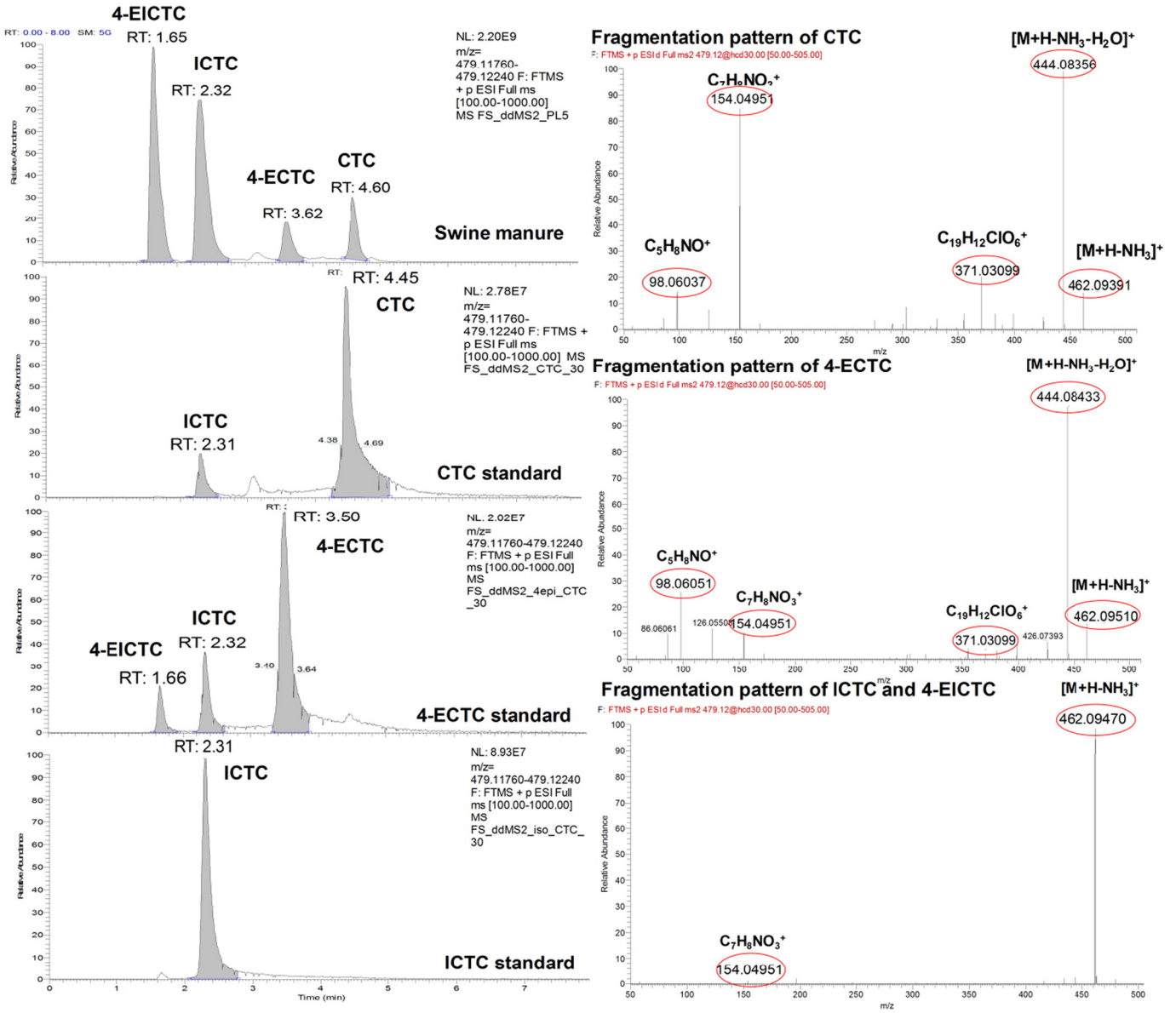

Fig. 2. Chromatographic separation of CTC and its degradation products with their fragmentation patterns.

recorded. A summary of concentrations found in the different samples was presented in Table S4. Traces of TCs, TMP, LCM, SFX and TYL close to the MLD were found in the 2011 soil samples with higher concentrations for DC ranging between 16 and 46 ng g^{-1}. Otherwise, degradation products of TCs formed by a loss of water were present in large amount with concentrations comprised between 17 and 1020 ng g^{-1}. The degradation products of CTC i.e. ICTC and 4-EICTC were semi-quantitative and ranged between 16 and 145 ng g^{-1}.

The same experiment was repeated in 2013, with 2 manure applications of 55 t ha^{-1} and 25 t ha^{-1}, 3 months apart. In this experiment, the drainage waters were collected one week after manure spreading. The swine manure spread on the field was analyzed and large amounts of TCs were found at concentrations ranging from 53 to 137 μg L^{-1}, also, LCM was also found up to 28 μg L^{-1}. While, the degradation products of CTC and DEC were present at concentrations between 118 and 663 μg L^{-1}. In soil samples collected in 2013, several veterinary antibiotics were present at trace levels. Also, TCs and their epimers were found between 3.4 and 333 ng g^{-1}. A rainfall event following manure spreading was recorded by a weather station near the field one day before the sampling of drainage waters. The drainage waters generated by the rainfall carried away part of the antibiotics present in the soils surface. The rainfall waters passed through soils and could have brought the antibiotics deeper in the soils. The concentrations of the different families of antibiotics found in the different soil layers (2011 and 2013) seems to stay in the surface layer (0–10 cm), while a portion of antibiotics infiltrates into deeper layers (10–40 cm) (Fig. 5). Moreover, the concentration of the parent compounds was low compared to the degradation products of TC, CTC and DEC which ranged from 17 to 3290 ng L^{-1}. Tetracyclines could readily degrade in aqueous environments under acidic or alkaline conditions (Halling-Sørensen et al., 2002). The pH of the drainage waters was measured at 7.1 and this is compatible with the formation of iso-TCs degradation products (Kennedy et al., 1998). The same phenomenon was observed in the aging surface waters spiked with target compounds; TCs were rapidly degraded while degradation was slower in the control HPLC grade water solution. Otherwise, CTC was still present in the aging soil samples test and seems to persist longer in this matrix (Fig. S3).There are many ways for such antibiotic residues to be released into the environment. The most obvious way is through the land application of swine manure as fertilizers on agricultural fields. Once the application is

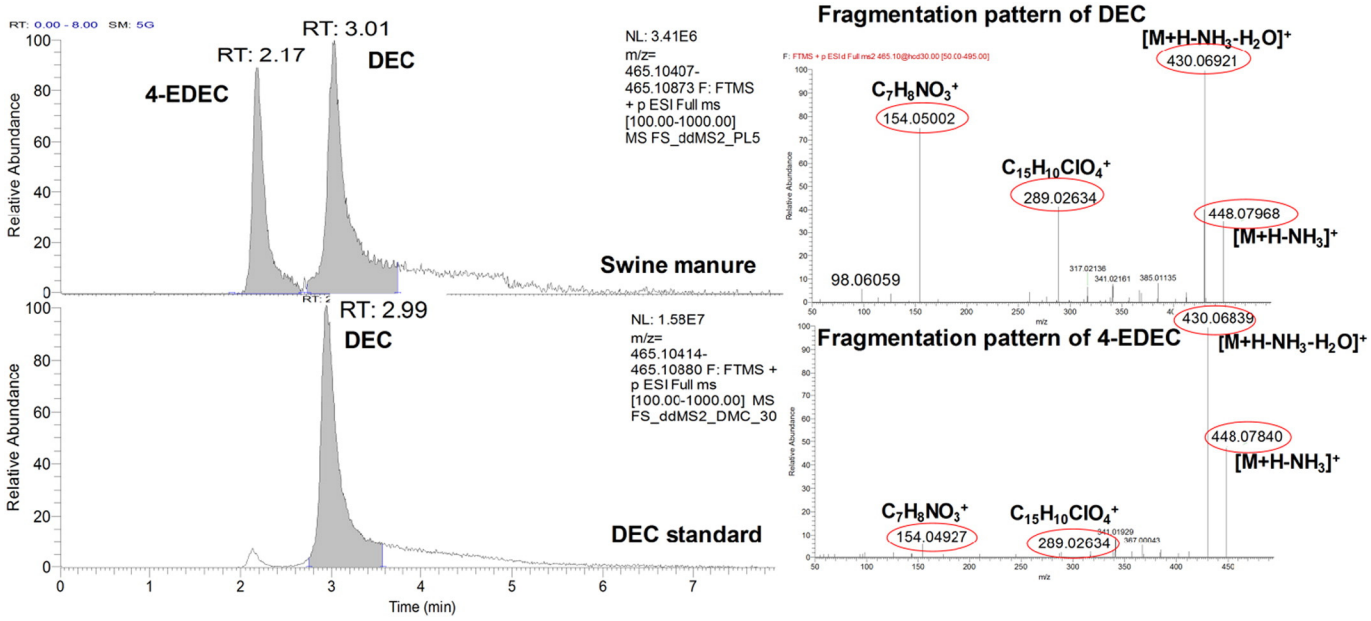

Fig. 3. Chromatographic separation of DEC and its degradation products with their fragmentation patterns.

made, the antibiotics contained in the manure could migrate to the soils and transit via drainage systems. Veterinary antibiotics are mainly hydrophilic with a log *P* below 1, thus allowing them to travel relatively easily through soils (Thiele-Bruhn, 2003). These antibiotics could infiltrate through soils and could eventually reach surface waters or seep into ground waters. Moreover, depending of the type of soil, TCs have a certain affinity with the solid phase (especially with the organic matter and mineral particles) which could modify their potential mobility (Thiele-Bruhn, 2003). This wide range of sorption coefficients reflects the variability in the binding of TCs to soil particles. Otherwise, this binding is based on the ability of the TCs to form complexes with double-charged cations (Mg^{2+}, Fe^{2+}, Zn^{2+} and Ca^{2+}) which are abundant in soils (O'Connor and Aga, 2007). The sorption is less important in the case of the metal–chelate complexes due to a charge competition, thus, making complexed TCs less susceptible to be bound to soil surfaces and therefore increase their mobility (Tolls, 2001). The epimerization of TCs is inhibited by the presence of these cations which explain the persistence of TCs in soils compared to drainage waters. Moreover, the chelation reduces the bioavailability and may therefore reduce the antibacterial effect (Halling-Sørensen et al., 2002). Complexation is an important parameter for assessing their environmental fate and effects, especially when considering that divalent metals are often present at high concentrations in the studied matrices. The temperature and the humidity level of the medium, as well as the pH could greatly influence the mobility of the TCs in soils. It has been demonstrated that under more acidic condition, the adsorption of TCs decreases (Sassman and Lee, 2005). The analyzed soil samples have a pH between 5 and 6 which could indicate a moderate mobility of TCs.

The effects of TCs on pathogenic bacteria have been reported in many studies but the toxicity of their associated degradation products has not been clearly evaluated. Generally, metabolites are less potent than the parent compounds, as illustrated in the case of doramectin (Pfizer, 1996). But in some cases, metabolites may still have significant activity (Halling-Sørensen et al., 2002). The major forms of degradation products of TCs in the matrices were 4-ETC, ATC, 4-EATC, 4-ECTC, ICTC, 4-EICTC and 4-EDEC (Jia et al., 2009; Kennedy et al., 1998; Søeborg et al., 2004). Several of these degradation products were found to be more toxic and potent than their expected parent compounds (Halling-Sørensen et al., 2002). For example, ATC that has a hydroxyl group missing relative to its parent molecule, had an EC_{50} value for sludge bacteria approximately 3 times lower than the EC_{50} value for the parent

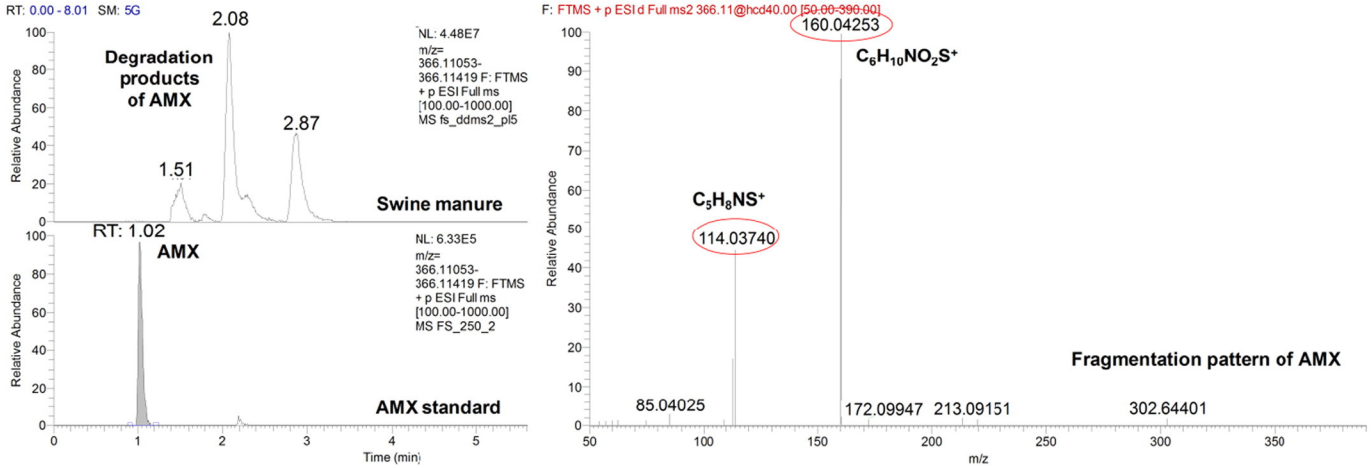

Fig. 4. Chromatographic separation of AMX and its degradation products with their fragmentation patterns.

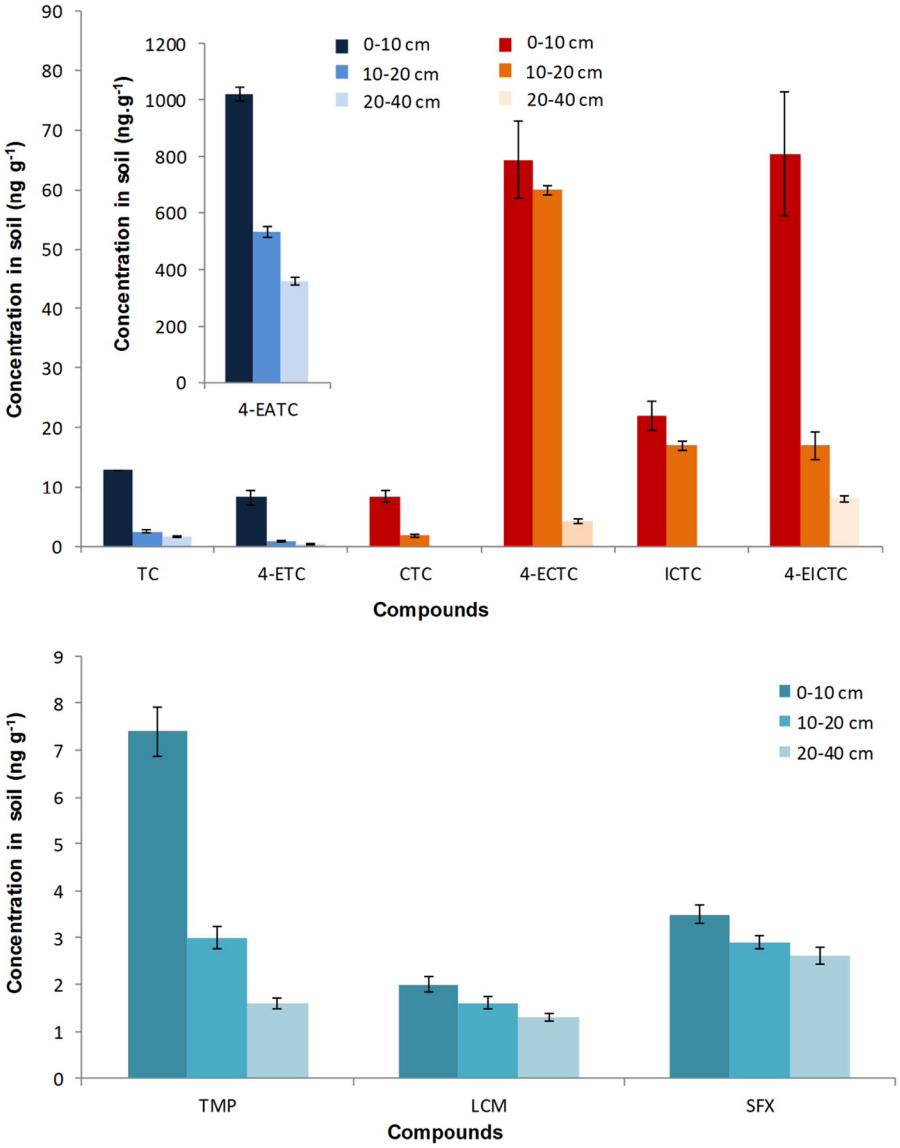

Fig. 5. Distribution of veterinary antibiotic concentrations (ng g^{-1}) from different families as a function of depth sampling in 2011 and 2013 (n = 3).

compound. Relative to CTC, ICTC lost more than 99% of the potency of its parent (EC_{50} = 36 mg L^{-1}) (Halling-Sørensen et al., 2002). All other degradation products had EC_{50} values less or around 1 mg L^{-1}, in the same range as the parent compounds. The concentrations of these degradation products found in soil and drainage water samples were substantially below EC_{50} values. However, the concentrations of TCs degradation products in swine manure samples were above their EC_{50} values (Fig. 6). This means that these molecules were still active on tetracycline-resistant bacterial strains. Moreover, the degradation products behavior could differ from the parent compounds. ATC has a lower sorption coefficient relative to TC and is therefore likely to be transported more readily with drainage waters (Pils and Laird, 2007; Sassman and Lee, 2005). The constant land application of manure containing TCs residues can exert selective pressure on the soil microorganisms and could probably promote the selection of resistant microbes.

4. Conclusion

LC–HRMS is an interesting tool to study the occurrence and the fate of organic contaminants in complex environmental and biological samples. This hybrid mass spectrometer allowed a selective and sensible quantification of veterinary antibiotics while providing the investigation and identification of several degradation products by using the DDA mode. This study revealed the presence of veterinary antibiotics and several of their related degradation products in agricultural soils, drainage waters and swine manure. Even if the concentrations of most of these compounds have been found at trace levels, several degradation products occurred at relatively high concentrations, reaching 1000 ng L^{-1} for drainage waters, 100 ng g^{-1} for soils and 100 μg L^{-1} for swine manure. Other antibiotics such as TMP, SFX, LCM and TYL were present at trace levels in the samples and the occurrence of AMX was confirmed by the presence of one of its degradation product. Following the spreading swine manure and the rain event, veterinary antibiotic residues were distributed in the various soil depth layers of the field but seem to stay mainly at the surface. However, these organic residues can travel in the nearby environment via drainage waters. These constitutive isomers, found in the samples, only represent a small portion of the total potential degradation products. The degradation pathway of these molecules is complex and could result in bioactive compounds, stable, mobile in the environment with potentially higher toxicity than their parent. All these compounds could have a significant impact on both living organisms and microbial communities even at relatively low levels of exposure. Thus, it should be important to consider

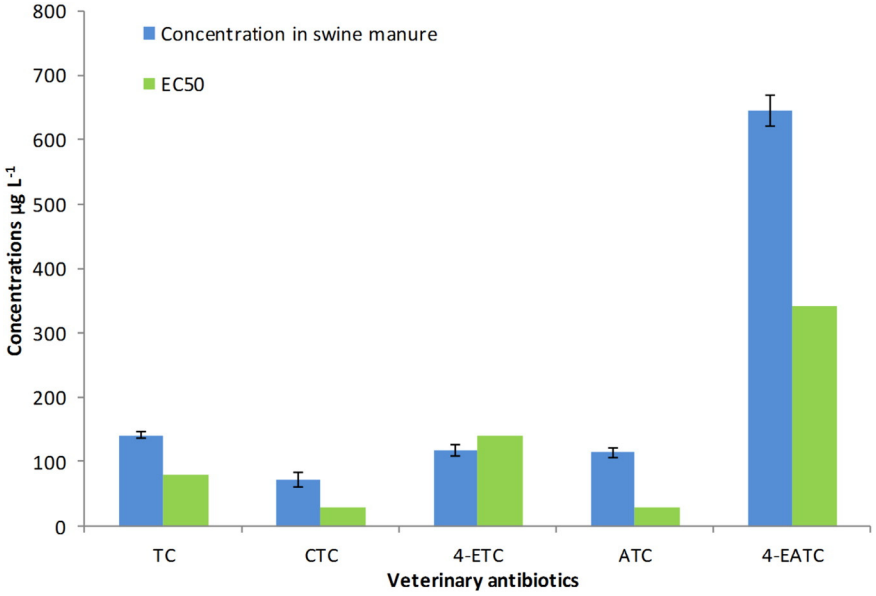

Fig. 6. Concentrations of some TCs in swine manure (μg L^{-1}) compared to their relative EC_{50} on aerobic sludge bacteria (n = 3) (Halling-Sørensen et al., 2002).

the degradation products of veterinary antibiotics and not focus solely on the fate of the parent molecules.

Acknowledgments

The Natural Sciences and Engineering Research Council of Canada (NSERC) (n°249543-2010) is acknowledged for financial support. We thank Thermo Fisher Scientific for their technical support. We also thank Sung Vo Duy for his technical help and scientific support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.11.061.

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