DISSERTATION

OCCURRENCE, FATE, AND TRANSPORT OF HUMAN AND VETERINARY ANTIBIOTICS IN THE WATERSHED

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SUNG-CHUL KIM ENTITLED OCCURRENCE, FATE, AND TRANSPORT OF HUMAN AND VETERINARY ANTIBIOTICS IN THE WATERSHED BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

OCCURRENCE, FATE, AND TRANSPORT OF HUMAN AND VETERINARY ANTIBIOTICS IN THE WATERSHED

Antibiotics have been used to abate infections for humans, not only to prevent diseases but also to enhance the growing efficiency as growth promoters for animals. However, antibiotics released in the environment can cause adverse effects producing antibiotic resistant bacteria in the ecosystem and potentially human health issues. Consequently, researchers have begun to study antibiotics to evaluate the occurrence, transport, and fate in several environmental compartments.

The first objective of this study was to identify the presence of antibiotics in both aqueous and sediment matrices of the watershed with newly developed solid phase extraction (SPE) methods combined with high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) techniques. The selected watershed, Cache la Poudre River is located in Northern Colorado, and combines effluents from two wastewater treatment plants (WWTP) and runoffs from several animal feeding operations (AFO) making it suitable for studying human and animal antibiotics. The measured concentration levels of antibiotics in water was generally less than 1 μg/L but as much as a 1000 times higher concentration was detected in sediment, indicating the importance of studying this matrix for better environmental risk assessment.

The second objective was to verify transport mechanisms of antibiotics focused on veterinary antibiotics from source to environment. To evaluate rainfall effects on

surface runoff and leaching behavior of veterinary antibiotics, rainfall simulation and column leaching experiments were conducted. Depending on the sorption and persistence of individual antibiotics, different characteristics were observed. In particular, these studies revealed that colloids could act as carriers to move even strongly sorbed antibiotics deeper into the subsurface and possibly cause contamination in groundwater.

The last objective was to compare two animal waste management approaches, stockpiling and composting, for minimizing the release of antibiotics prior to application in the field as fertilizer. Composting was shown to be more effective to degrade antibiotics and can be suggested as an alternative strategy to reduce the antibiotics in the environment.

Although there is no regulation regarding handling or treatment of wastes with residual antibiotics, adverse effects have been identified and there are concerns about possible human health risk. As a result, continued research on antibiotics in the environment should be pursued.

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Chapter 1

1.1 Introduction

Antibiotics and antimicrobials are regarded as emerging contaminants and the increasing concern over the occurrence and fate of these compounds in the environment has been growing. Human used medicines are mainly released into the environment via wastewater treatment plants (WWTP) due to partly removed active compounds in the WWTP process. Other possible entry pathways of human used medicines into the environment are disposal of unused medicine or unintended release from manufacturers. Animal used medicines, veterinary pharmaceuticals, can be discharged into the environment via manure applied in the field as fertilizer and surface runoff or leaching through the subsurface.

One of the major adverse effects of releasing antibiotics or antimicrobials into the environment is the possibility of generating drug resistant bacteria in the ecosystem. Different mechanisms can be involved to produce resistant bacteria in biota. One of the common mechanisms in nature is horizontal gene transfer and this can happen through a variety of mechanisms, such as transformation (taking up DNA from the environment), transduction (virus mediated transfer), and conjugation (bacterium-bacterium contact). Another possibility is the vertical gene transfer by passing along resistance genes to the next generation (Schwartz et al., 2003). Resistant bacteria in the aquatic environment or

solid (soil or sediment) matrix may be transferred to drinking water systems and may increase the risk of human health impacts.

Despite the fact that increased attention has been paid to occurrence, fate and the adverse effects of antibiotics or antimicrobials in the environment, there is still a lack of information available due to the insufficiency of robust and reliable analytical methods for detecting and quantifying residuals of antibiotics or antimicrobials in aqueous or solid matrices. Solid phase extraction (SPE) methods have been widely used to cleanup and to concentrate the residual of antibiotics or antimicrobials in aqueous matrices. For solid matrices in the environment, adequate buffer solution or organic solvent has been used to pre-extract residuals from the solid phase to the liquid phase for further purification of the sample. Conventional detection and separation methods of gas chromatography mass spectrometry (GC/MS) have been replaced with high performance liquid chromatography (HPLC) combined with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) due to the derivatization steps required for GC/MS and the non-volatile or polar characteristic of antibiotics and antimicrobials.

Having developed robust and reliable analytical methods, transport and fate of human or animal derived antibiotics also need to be understood in both aqueous and solid matrices to properly reflect the risks. In particular, veterinary pharmaceuticals have been found in the watershed several miles away from the field. Therefore, verifying the transport pathway or mechanism from the field source to the watershed is necessary.

The purpose of this dissertation is to help understand the effects of human or animal derived antibiotics on the environment and to find possible solutions for minimizing these effects. To achieve this purpose, the first stage is to identify the

occurrence of human and animal used antibiotics or antimicrobials in the watershed including the aqueous and sediment matrices using newly developed analytical methods. The next step is to evaluate the transport and fate of released antibiotics or antimicrobials in the environment with a simulated rainfall event and column leaching experiments. Finally, natural attenuation with abiotic or biotic mechanisms is assessed to reduce high mass loading from source for veterinary antibiotics.

All chapters in this dissertation are presented in a journal format. Some of the chapters have been published already, others are in press, others are in review and still others are still being prepared for submission. The statue of each manuscript is indicated on the first page of each chapter.

Chapter 2 Literature Review

2.1 Background

2.1.1 Concerns and Primary Usage of Antibiotics

Antibiotics were originally designed to inhibit the activity of microorganism, viruses, and eucaryotic cells as therapeutic purpose for humans (Thiele-Bruhn, 2003). For animals, antibiotics are used not only to treat and prevent the illness therapeutically but also to promote growth as a non-therapeutic purpose (Tolls, 2001). However, antibiotics released to the environment are recently regarded as micropollutants and there is an increased concern to introduced antibiotics in environment due to the possibility of production of resistant bacteria in the ecosystem. These resistant bacteria can transfer the resistance genes to other bacteria and eventually render certain antibiotics useless (Ohlsen et al., 2003).

Previous studies have attempted to identify antibiotic resistance genes (ARG) in several environmental compartments (Chee-Sanford et al., 2001) and these researchers have found tetracycline resistance genes in lagoons and groundwater located in two swine production facilities. They concluded in this study that groundwater or soil could be significant reservoirs for distribution of ARG. In addition, ARG has been found in farmland treated with pig manure slurry (Sengelov et al., 2003), wastewater, surface water, and drinking water biofilms (Schwartz et al., 2003), and sewers receiving hospital

effluent (Guardabassi et al., 1998). This results indicate that ARG can be produced anywhere in the environment.

There are two aspects that antibiotics have a high potential to be released into environment and caused the adverse effects. The first factor is enormous usage amount of antibiotics for human and animals. According to a recent report (Mellon et al., 2001), the estimated total annual consumption of antibiotics was 35 million pounds. Among the total usage amount, 3 million pounds that is 9% of total consumption were used for human and 2 million pounds occupied 6% of total consumption was used for animals as therapeutic purpose. However, most of the antibiotics were used in the absence of disease for non-therapeutic purpose mainly as growth promoters for animals and usage amount was estimated to 25 million pounds that is 70% of total consumption.

The second aspect is pharmacokinetic mechanism of antibiotics. Antibiotics are intended to act efficiently at low doses and excreted completely from the body after administration. Consequently, antibiotics can be released up to 50% as a parent compounds or metabolites normally conjugated with glucose or other polar compounds (Ternes, 2001; Thiele-Bruhn, 2003).

As a result of two aspects described above, antibiotics can be introduced in environment and produce ARG into the ecosystem, potentially threatening human health.

2.1.2 Extraction of Antibiotics in Aqueous and Sediment

Antibiotics are mainly comprised of a non-polar backbone with functional groups causing high polarity. Certain antibiotics have more than two acid dissociation constants (pKa) and can be dissociated or protonated depending on pH of media. In addition, physicochemical properties of antibiotics exposed in strong acidic or basic condition can

be altered and limited the proper analysis of antibiotics. Thus, general procedure to analyze the residual of antibiotics in different environmental compartments is difficult to establish and various analytical techniques have been documented in previous studies depending on different target antibiotics and sample matrices.

In general, solid phase extraction (SPE) technique is the most popular method to extract residual of antibiotics in aqueous matrix. Main mechanism of SPE method is to increase the retention efficiency of antibiotics in SPE cartridge or disk and at the same time, to remove any impurities present in the sample. To obtain optimized condition for SPE method, selection of proper cartridges or disk, pre-conditioning of cartridge prior to load the sample, and choosing appropriate organic solvent to extract residuals in the cartridge need to be considered. Among various SPE cartridges, HLB (Hydrophillic-Lipophillic-Balanced) cartridge has been used commonly to extract various antibiotics in aqueous matrix due to acceptance in wide range of pH and no silanols that can cause irreversible binding with certain compounds in the cartridge (Lindsey et al., 2001; Zhu et al., 2001; Kolpin et al., 2002; Barreiro et al., 2003; Loffler et al., 2003b, a).

Pre-conditioning of cartridge prior to loading the sample can make suitable condition in the cartridge to enhance the recovery efficiency of target antibiotics. Common procedure is to use adequate amount of organic solvents (i.e. methanol or acetonitrile etc.) to remove any organic residuals in cartridge followed by removing any impurities in the cartridge with deionized (DI) water. In certain cases, acidic or basic condition is preferred to retain antibiotics and applied in the cartridge (Lindsey et al., 2001; Kolpin et al., 2002; Miao et al., 2002; Barreiro et al., 2003; McArdell et al., 2003; Wiegel et al., 2004).

To extract retained target antibiotics in the cartridge, various organic solvents are commonly used. Extracted target antibiotics are normally concentrated in adequate temperature to lower the limit of quantification (LOQ) making enable to measure sub-microgram of concentration of antibiotics in environment.

Developing robust and reliable analytical method for solid matrix is more challenging due to complexity of matrix and still lack of information is available. Using traditional extraction method, Soxhlet extraction, has been diminished because this method can produce significant amount of hazardous organic solvent and also require more time and labor (Koester et al., 2003). Instead of this traditional method, demanding of accelerated solvent extraction (ASE) referred to pressurized liquid extraction (PLE), adequate buffer solution extraction, and liquid-liquid extraction (LLE) has been increased. New developed extraction techniques are considered to reduce solvent consumption, sample-handling time, and to increase the extraction efficiency. PLE method uses elevated pressure and temperature at short time under static condition to allow appropriate contact time with extraction solvent and sample for extraction (Ferrer et al., 2002). Application of PLE method to extract environmental solid samples has been documented in previous studies (Ferrer et al., 2002; Golet et al., 2002b; Christian et al., 2003; Jacobsen et al., 2004; Halling-Sorensen et al., 2005).

Buffer solution extraction method is an alternative method to extract target antibiotics in solid matrix. Since very polar and non-polar extractants might cause the incomplete extraction in solid matrix, weakly acidic or basic buffer solution is frequently used (Thiele-Bruhn, 2003). Certain organic solvent can be combined with buffer solution depending on physicochemical properties of target antibiotics. Hamscher et.al.

(Hamscher et al., 2002) used citrate buffer solution (pH 4.7) to extract three tetracyclines from soil and same method was adapted to compare different class of antibiotics in sandy soil (Hamscher et al., 2005). Haller et.al. (Haller et al., 2002) used basic condition (pH 9.0) of buffer solution to extract sulfonamides and trimethoprim in manure. Buffer solution combining with organic solvent was adapted to extract various classes of antibiotics in animal feed (Caballero et al., 2002), manure (Liguoro et al., 2003; Kolz et al., 2005a; Kolz et al., 2005b), and soil (Blackwell et al., 2004b; Aga et al., 2005).

Although LLE method can produce certain amount of organic solvent, this method is still used to extract antibiotics from solid matrix. Schlusener et.al. (Schlusener et al., 2003) used ethyl acetate to extract eight antibiotics from liquid manure and two ionophore antibiotics was extracted from animal feed with methanol (Bertini et al., 2003).

2.1.3 Separation and Detection of Antibiotics

A common technique to separate and detect antibiotics in extracted environmental samples is high performance chromatography (HPLC) combined with mass spectrometry (MS) or tandem mass spectrometry (MS/MS). Before HPLC/MS or HPLC/MS/MS is adapted as routine techniques for antibiotic analysis, gas chromatography mass spectrometry (GC/MS) was used to measure pharmaceuticals in river and wetland (Gross et al., 2004) and also to track the pathway of several pharmaceuticals including antibiotics from sewage treatment plant to watershed (Heberer, 2002). However, GC/MS method requires post-derivatization step that can cause more time, labor and contamination in the sample. In addition, interface of HPLC/MS or HPLC/MS/MS has been developed rapidly making enable to increase sensitivity and efficiency of antibiotic analysis.

Several variables need to be considered to optimize HPLC conditions for better separation of target antibiotics. Mobile phase composition, selection of analytical column, and injection volume are major consideration. Diluted acids or weakly acidic buffers composed with formic, phosphoric, or citric acid in deionized (DI) water is a common practice to avoid possible dissociation of antibiotics and normally combined with organic solvent (i.e. methanol and acetonitrile) (Thiele-Bruhn, 2003). Analytical columns and injection volume can be varied depending on target antibiotics and sample matrix.

Two popular ionization techniques are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Both methods have similar mechanism to produce protonated or deprotonated molecules for mass analysis (Zwiener et al., 2004). ESI technique is wildly used to polar compounds while APCI is more suitable for medium or low polarity compounds (Alda et al., 2003). To resolve complex chromatographic peaks in environmental samples, MS or MS/MS is primarily adapted. Single MS method with selective ion monitoring (SIM) was used in early developing period for environmental samples but has been replaced with more sensitive and selective technique, MS/MS, for complex samples. Disadvantage of applying SIM method is that analysis can be interfered from co-eluting analytes and matrix components fragmented during analysis (Zwiener et al., 2004). Therefore, MS/MS techniques compensate this discrepancy to fragment molecules once more using collision energy to increase the selectivity of target compounds. Recently, more advanced mass analyze technique, time of flight (TOF), is available and adapted in analysis of environmental samples (Ferrer et

al., 2003; Ferrer et al., 2004). This method is superior to identify unknown or metabolites of compounds by avoiding false findings in process.

2.1.4 Pathways and Occurrence of Human and Animal Used Antibiotics

Anticipated exposure pathways of human and animal used antibiotics are different and schematic diagram of different pathways is shown in Figure 2.1. Exposure pathways of human and animal used antibiotics need to be identified because dose of antibiotics and duration of treatment are necessary for environmental loading calculation (Jorgensen et al., 2000).

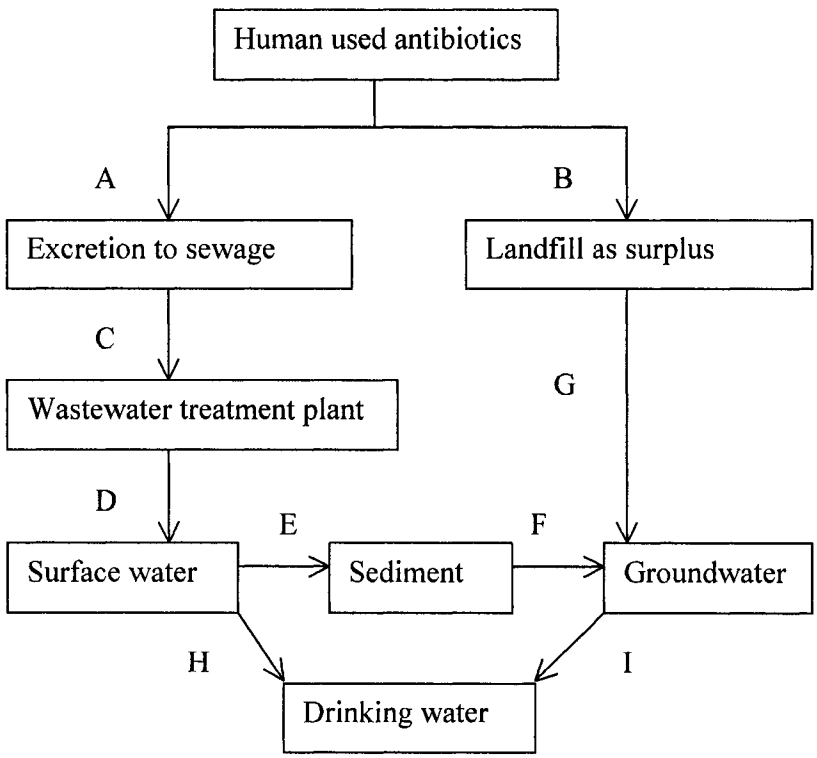

Figure 2.1 Anticipated pathway of human used antibiotics. Pathways are numbered for specific reference in the text.

Administrated human used antibiotics are excreted to sewage (route A) and mainly end up in wastewater treatment plants (WWTP, route C). Incompletely removed residuals of human derived antibiotics are released directly into surface water (route D). In certain cases, Introduced antibiotics in surface water can be sorbed in sediment (route E) or leached into groundwater depending on physicochemical properties of different antibiotics. Eventually, surface water or groundwater contaminated with residual of antibiotics can be used as drinking water source and threaten human health risk.

From the late 1980s, occurrence of human derived antibiotics has been reported (Richardson et al., 1985) and later more effort has been made to quantify the human derived antibiotics in different environmental compartments. Hirsch et.al. developed analytical method to measure 18 antibiotics in water sample and measured concentration of studied antibiotics at the range of 0.2 – 6.0 μg/L in WWTP effluent, surface water, and groundwater (Hirsch et al., 1998; Hirsch et al., 1999). Lindsey et.al. introduced standard addition method to measure two classes of antibiotics and found concentration range of 0.06 – 1.34 μg/L in surface water and groundwater (Lindsey et al., 2001). Kolpin et.al. surveyed 95 organic waste contaminants including 22 antibiotics derived from WWTP and provided concentration of antibiotics measured in several streams of U.S (Kolpin et al., 2002). Until now, numerous studies have been documented to report occurrence of human used antibiotics in environment and measured concentration was generally less than 1 μg/L with few exception (Farre et al., 2001; Golet et al., 2002a; Heberer, 2002; Miao et al., 2002; Barreiro et al., 2003; McArdell et al., 2003; Stamatelatou et al., 2003; Vanderford et al., 2003; Cahill et al., 2004; Gobel et al., 2004; Gross et al., 2004; Kolpin et al., 2004; Wiegel et al., 2004; Glassmeyer et al., 2005).

Pathways of animal used antibiotics are somewhat unlike with human used antibiotics and anticipated pathway is shown schematically in Figure 2.2.

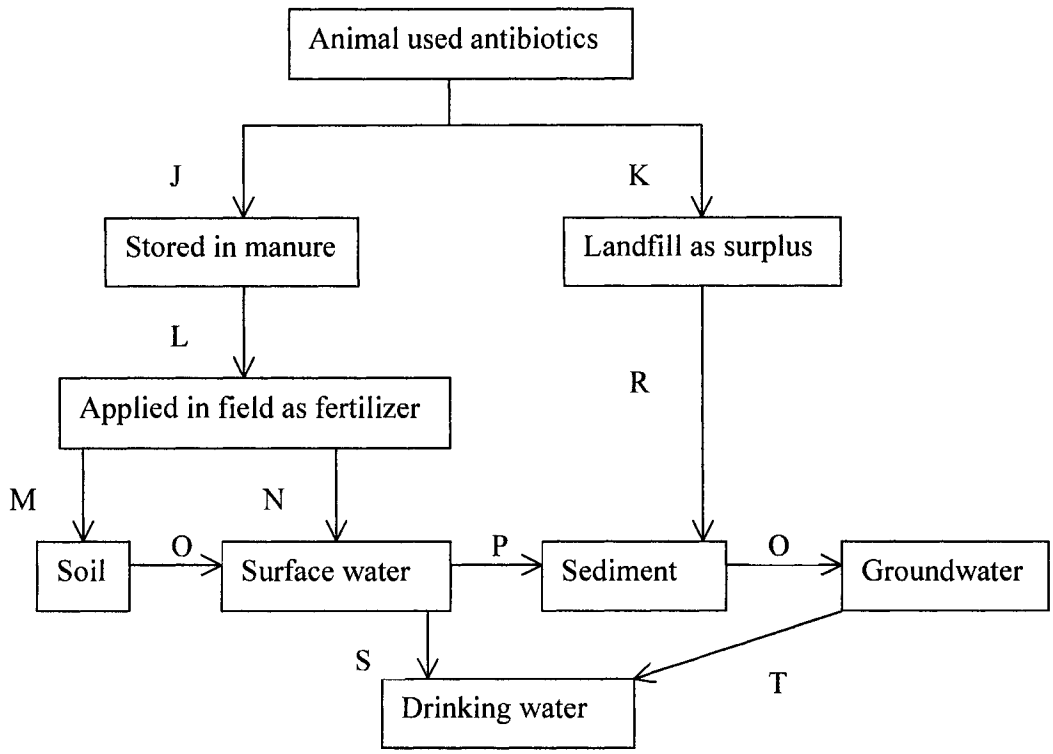

Figure 2.2 Anticipated pathway of animal used antibiotics. Pathways are numbered for specific reference in the text.

Antibiotics are normally administrated to animals with medicated feed or external application and excreted animal used antibiotics are contained in storage as manure (route J). After a certain period, stored manure containing residual of animal used antibiotics can be applied in field as fertilizer. Soil is the main environmental source to be exposed in animal used antibiotics (route M) and introduced contaminants in soil can be transferred to surface water and groundwater via surface runoff and leaching (route O and

Q). For this reason, animal used antibiotics also have a potential to harm the human health after surface water and groundwater are used as source of drinking water.

Presence of animal used antibiotics is mainly reported in soil or manure matrix and much higher concentration compared to reported concentration of human used antibiotics in aqueous matrix was measured for both matrices. Previous studies measuring animal used antibiotics in manure revealed that significantly higher concentration up to 12.4mg/kg is present in manure (Haller et al., 2002; Christian et al., 2003; Liguoro et al., 2003; Schlusener et al., 2003). Hamscher et.al attempted to measure 4 veterinary antibiotics in soil previously fertilized with liquid manure and found 4.6 – 198.7 μg/kg of residuals within 30cm of soil depth (Hamscher et al., 2002). This study also concluded that tetracyclines could build up persistent residuals via repeated fertilization. Another studies also verified that fairly high concentration is present in soil amended with manure (Liguoro et al., 2003; Blackwell et al., 2004b; Jacobsen et al., 2004; Aga et al., 2005; Hamscher et al., 2005).

To demonstrate the occurrence of veterinary antibiotics in surface water or groundwater located in near animal feeding operations (AFOs), several studies reported the measured concentration of animal used antibiotics (Zhu et al., 2001; Blackwell et al., 2004a) and much lower concentration was detected in surface water and groundwater. This result might indicate that a natural attenuation mechanism (biotic and abiotic) may be involved in released antibiotics to environment.

2.1.5 Fate and Transport of Released Antibiotics in Environment

As stated in section 2.1.4, human and animal used antibiotics have been present in different environmental compartments with various concentration ranges. Consequently,

understanding the fate of released antibiotics in environment is also important. Once antibiotics are introduced in environmental compartments, concentration of antibiotics in different matrices depends on biotic or abiotic processes including biodegradability, partitioning to solid matrix, and subsequent movement.

Biodegradation is the primary process to dissipate parent antibiotics released in environment. Microorganisms utilize the antibiotics as substrate and mineralize to decompose the antibiotics. Common experiment method for biodegradation is closed bottle test (CBT) and 18 selected antibiotics were evaluated to assess the biodegradability (Alexy et al., 2004). The result of this study showed that none of the studied antibiotics was readily biodegradable during study period (28 days) and concluded that co-metabolism, microorganisms do not obtain any energy from utilization of antibiotics, might lead to the slow degradation.

For human used antibiotics, fate and removal of released antibiotics was mainly focused on WWTP and drinking water system. In general, human used antibiotics cannot be fully eliminated in WWTP process and removal rate was varied depending on physicochemical properties of antibiotics and different operation process (Ternes et al., 2003; Clara et al., 2004; Kreuzinger et al., 2004a; Kreuzinger et al., 2004b; Castiglioni et al., 2006). Recently, fate and removal rate of introduced antibiotics were evaluated in drinking water treatment system (Ballard et al., 2005; Westerhoff et al., 2005). In Westerhoff et.al. study, adequate amount of 62 different endocrine-dispuptors (ED), pharmaceuticals including antibiotics, and personal care products (PPCP) were manually injected in bench scale drinking water treatment (DWT) followed by general DWT process, coagulation, flocculation, and adding power activated carbon (PAC) to examine

fate of target compounds. This study revealed that removal efficiency was varied polarity of each compounds and more elimination of compounds was observed as amount of PAC was increased.

For animal used antibiotics, sorption properties and mobility of compounds play an important role to decide the fate of released antibiotics and evaluating the fate of veterinary antibiotics in solid matrices is necessary to predict accurate behavior in environment for future environmental risk assessment (ERA). Laboratory scale experiment, batch test and column test, is the common practice to validate the sorption or mobility of antibiotics because parameters are easy to control and experiment can be repeated easily. Although, field experiment requires more attention to control the parameters, it can reflect a more realistic situation than laboratory experiment.

Sorption and mobility characteristic of antibiotics can be varied depending on several variables and soil texture, pH, and organic matter content are the major key parameters (Hari et al., 2005). For instance, tetracyclines possessing acidic characteristic and strongly sorbed in solid matrix tend to decrease the sorption, as pH is increased and more sorptive in clay fraction rather than other texture in soil (Figueroa et al., 2004; Kulshrestha et al., 2004; Jones et al., 2005). In addition, metal complexation can be a part of sorption mechanism for tetracyclines (Gu et al., 2005).

In case of less sorptive antibiotics, sulfonamides, sorption to solid matrices is minimal and shows more mobility (Thiele, 2000; Boxall et al., 2002; Thiele-Bruhn et al., 2004; Gobel et al., 2005). More mobile antibiotics have the possibility to leach into groundwater and might contaminate the drinking water when groundwater is used as drinking water source.

2.2 Hypotheses

1. Human or animal derived antibiotics are present in the studied watershed, Cache la Poudre River (CLP), at varying concentrations depending on the land use of the adjacent landscape and different hydrologic conditions. Both animal and human wastes contribute to the occurrence of antibiotics.

2. The origin of human used antibiotics in the CLP watershed is primarily wastewater treatment plants. The origin of veterinary antibiotics in the CLP watershed is animal feed and grazing operations. The transport mechanism for antibiotics to the river is a combination of overland runoff and sediment-facilitated transport. In addition, leaching to the groundwater followed heavy rainfall can be one of the factors of transporting exposed veterinary antibiotics to environment. Since Northern Colorado does not have a significant network of small streams, irrigation ditches could be a main source of antibiotics to the CLP river.

3. Animal-origin antibiotics mainly come from manure spread in field as a fertilizer. Biodegradation of antibiotics during the composting process can be an practical management method to minimize the release of these compounds to the environment. Also, composting will prevent transport of the veterinary antibiotics during fertilizer application.

2.3 Objectives

2.3.1 Development of Analytical Methods

This stage is mainly focused on developing suitable and reproductive analytical methods to quantify the residual of antibiotics in both aqueous and sediment matrices. An

optimized solid phase extraction (SPE) method was used to cleanup and to concentrate the aqueous sample combined with high performance chromatography tandem mass spectrometry (HPLC/MS/MS) to separate and detect compounds. Adequate buffer solution was used to pre-extract sediment samples into the aqueous phase followed by SPE/HPLC/MS/MS for quantification. Detailed information is presented as a manuscript that was accepted for publication to the Analytical and Bioanalytical Chemistry in Chapter 4.

2.3.2 Monitoring Occurrence of Antibiotics in the Watershed

Long term monitoring of antibiotics in a defined watershed is necessary to characterize the concentration variability due to temperature, runoff, and stream flow. A total of 19 compounds in four different classes of antibiotics were monitored in the Cache la Poudre River watershed with spatial and temporal diversity. Statistical analysis was conducted to examine spatial and temporal concentration variance. Details of this part of the study are presented in two manuscripts. The first manuscript (Chapter 5) was accepted for publication in Water Research. The second manuscript (Chapter 6) has been submitted to Environmental Science and Technology.

2.3.3 Defining Transport Mechanisms and Pathways

Detailed transport mechanisms and pathways for antibiotic release to the environment are still ambiguous. The major transport pathway for human used antibiotics can be assumed to be the effluent of wastewater treatment plants. However, the transport mechanism and pathway of animal used antibiotics from farms to the environment needs to be verified. Rainfall simulation experiments and column leaching tests will be combined to assess the

effect of runoff and leaching on the occurrence of animal used antibiotics in the watershed. The sorption characteristics of 10 compounds and the possibility that mobile compounds reach the groundwater will be assessed in column leaching tests. Two manuscripts will provide a comprehensive understanding. The first manuscript describing transport of veterinary antibiotics via rainfall simulation has been submitted to Chemosphere (Chapter 7) and the second manuscript representing leaching behaviors of various veterinary antibiotics with column study has been submitted to Environmental Science and Technology (Chapter 8)

In addition, quantifying antibiotics in local irrigation ditches interconnected with the watershed and comparing them with concentration in the river is assessed to verify that exposed veterinary antibiotics can be transported through local irrigation ditches. Furthermore, mass loading from irrigation ditches to the river is calculated to estimate the quantity of antibiotics from ditches to river. This result can be used to propose better local irrigation management. Detailed information has been submitted to Environmental Science and Technology (Chapter 9)

2.3.4 Effects of Composting on Veterinary Antibiotics

In order to define better management practices for animal waste handling, the fate of antibiotics in different management techniques need to be compared. Present animal waste management techniques, high and low level manure managements, are adapted to assess the fate of veterinary antibiotics and observed result can be used to guide the better animal waste management in future. The result of this study has been prepared for publication in Journal of Agricultural Food and Chemistry (Chapter 10).

Chapter 3

LC-tandem MS for Quantifying Trace Amounts of Pharmaceutical Compounds in Soil and Sediment Matrices.

Published in the Trends in Analytical Chemistry, 24635-644 (2005).

3.1 Abstract

The occurrence of pharmaceuticals in watersheds has recently received increased attention due to the possibility of adverse effects to humans and animals and potential development of resistance genes in bacteria. Human and veterinary pharmaceuticals can be introduced into the environment through several different pathways depending on usage patterns, hydrology and treatment practices. However, limited information about occurrence and fate of pharmaceuticals in the environment is available and as a result, efficient mitigation strategies have been difficult to define. Therefore, robust and reliable methods need to be developed to measure human-origin or veterinary pharmaceuticals at environmentally relevant concentrations in aqueous and solid matrices. Solid phase extraction (SPE) coupled with high performance liquid chromatography / mass spectrometry (HPLC/MS) or tandem mass spectrometry (MS/MS) has been placed to pre-treat, separate, and to detect the residuals of pharmaceuticals in a wide range of environmental samples. This paper reviews the recent development of analytical methods

for quantifying pharmaceutical residues in a range of solid matrices found in the environment.

Keywords: Pharmaceuticals; Soil; Sediment; HPLC/MS/MS; PEC

3.2 Introduction

Pharmaceuticals including antibiotics, antiphlogistics, lipid regulators, and beta-blockers have received significant attention in the environmental field recently due to the detection of these compounds in a variety of matrices (e.g. surface water, groundwater, soil, and sediment). Pharmaceuticals are originally used to abate bacterial infection for humans and not only to prevent illness but also to promote growth for animals (Christian et al., 2003; Diaz-Cruz et al., 2003). Based on pharmacokinetic data, the excretion rate of pharmaceuticals is sometimes over 50% as the parent compound or metabolites. Metabolites may conjugate with glucose or other polar compounds and be converted to the original parent compound in the environment with microorganism activity (Ternes, 2001). The estimated annual amounts of antimicrobials used in the U.S. for therapeutic purposes in human and animals are 3 million pounds and 2 million pounds, respectively. Meanwhile, the annual consumption in animals for non-therapeutic purposes, mainly as growth promoters, is about 25 million pounds (Mellon et al., 2001). Thus, it is not surprising that pharmaceutical compound residuals can be found in a wide range of environmental matrices.

Another problem with persistent pharmaceuticals in the environment is the possibility of producing resistance genes in bacteria that can render particular antibiotics useless. Resistance mechanisms are often related with transposons or conjugative plasmids as mobile genetic elements and those elements can transfer the resistance genes

from one bacteria to another through horizontal gene transfer (Ohlsen et al., 2003). Among other pharmaceuticals, resistance genes of tetracycline have been reported in lagoon and groundwater underlying two swine production facilities (Chee-Sanford et al., 2001). In addition, the ecotoxicity of doxycycline in aged pig manure was assessed using a multi-species soil system and the tolerance of the soil microbial communities affected by sulfachloropyridazine was investigated (Fernandez et al., 2004; Schmitt et al., 2004).

Despite the fact that numerous pharmaceuticals have been found in environmental matrices, there is still a lack of robust and reliable analytical methods for quantifying the pharmaceuticals in realistic matrices at environmentally relevant concentrations. Consequently, continued development of analytical methods is among the highest priority needs in the field. In particular, development and description of analytical methods in solid matrices such as soil and sediment is needed.

This paper presents a review of analytical methods for quantifying pharmaceuticals in solid matrices found in the environment (e.g. manure, soil, and sediment). In addition, a general review of the occurrence and fate of human and animal pharmaceuticals in the environment is provided.

3.3 Pharmaceutical Usage and Classification

Representative human and animal pharmaceutical compounds used for therapeutic and non-therapeutic purposes are summarized in Table 3.1 including the estimated annual usage amount. According to Mellon *et.al*, (2001), human, agricultural, and companion animal antimicrobial usage now totals over 35 million pounds annually in the U.S. (Mellon et al., 2001). Of the total usage, 14% is used for human and animal therapeutic purposes and 70% of antimicrobials are used in animals for non-therapeutic purpose

mainly as growth promoters. Thus, a major concern is the antimicrobials that are used in animals for non-therapeutic purposes. Human pharmaceuticals found in the aquatic environment, mainly from sewage treatment plants (STPs), were classified in nine categories (analgesics, anti-inflammatory, antibiotics, antiepileptics drugs, beta-blockers, blood lipid regulators, contrast media, cytostatic drugs, oral contraceptives) depending on the usage purpose (Heberer, 2002a). This study also points out that incomplete elimination of active pharmaceuticals in STPs causes most of the contamination in surface water.

Animal pharmaceuticals can be classified as therapeutic, used for treating illness or to prevent illness, or non-therapeutic, used primarily for enhancing the animal's growth increasing the value of the animals with lower cost and in less time (Boxall et al., 2003). Depending on the different groups of animals, the same compound can be used for different purposes. For example, monensin, an ionophore polyether antimicrobial is used in poultry for preventing *coccidiostats* and at the same time used as a growth promoter in beef and dairy cattle (1999).

Table 3.1 Summary of human and animal used pharmaceuticals (1999; Mellon et al., 2001; Heberer, 2002a; Boxall et al., 2003).

Subject	Use	Treatment details	Examples	Estimated annual used amount (thousand pounds)
Human	Therapeutic	Treatment of human diseases	**Analgesics and anti-inflammatory drugs**: Acetaminophen, Acetylsalicylic acid, Diclofenac, Ibuprofen, Aminophenazone, Codeine **Antibiotics**: Clarithromycin, Erythromycin, Roxythromycin, Lincomycin, Sulfamethoxazol, Ciprofloxacin, Norfloxacin, Tetracycline, Oxytetracycline **Beta-blockers**: Metoprolol, Propanolol, Betaxolol, Bisprolol, Nadolol	3,000
	Nontherapeutic	Other human use	Topical creams, soaps, disinfectants	1,500
Animal	Antimicrobials	Treatment and prevention of bacterial diseases	Amoxicillin, Chlortetracycline, Dihydrostreptomycin, Enrofloxacin, Erythromycin, Licomycin, Oxytetracycline, Sulfadiazine, Tetracycline, Tylosin	2,000
	Coccidiostates and antiprotozoals	Prevention of coccidiosis and swine dysentery	Amprolium, Clopidol, Dimetridazole, Lasalocid, Maduramycin, Narasin, Nicarbazin	
	Growth promoters	Increase food digestion	Flavophospolipol, Monensin, Salinomycin	
	Aquaculture treatment	Treatment of sea lice infestations and funrunculosis	Amoxicillin, Azamethiphos, Cypermethrin Emamectin, Florfenicol, Hydrogen Peroxide Oxolinic acid, Oxytetracycline	27,578

3.4 Analytical Methods

3.4.1 Sample Preparation and Extraction

Methods of sample preparation and extraction for pharmaceuticals have been described as early as the late 1980s and have evolved significantly for both aqueous and solid phases since. The traditional sample preparation method, liquid-liquid extraction, has largely been replaced with solid phase extraction (SPE) for the aqueous matrix. The soxhlet extraction method for soil or sediment has been replaced with buffer solution extraction and pressurized liquid extraction (PLE) methods because of the time consuming nature and high usage of hazardous organic solvents. Soil or sediment sample preparation needs to combine additional cleanup or purification steps, mainly SPE after the pre-extraction step in the solvent due to the complexity of environmental samples.

The main mechanism of SPE is to retain the organic compounds onto the cartridge and to extract efficiently using proper solvents. Thus, selecting the most suitable cartridge depending on the polarity of analytes, sample matrix, or solution is important. Reversed phase SPE is normally used with a polar or moderately polar sample matrix and hydrophobic interaction is involved between the carbon-hydrogen bond in analytes and the functional groups on the silica surface of the cartridge. Ion exchange SPE for both cation and anion exchange SPE is used with charged compounds in solution. The main retention mechanism of ion exchange SPE is electrostatic attraction of the charged functional groups of compounds to the functional groups of the charged silica surface in the cartridge.

Several different cartridges such as Lichrolute EN, C_{18}, HLB (Hydrophilic-Liphophilic Balanced), and diol SPE, were used to extract pharmaceutical compounds in STP, surface water, groundwater and to cleanup or purify the pre-extractants in solid matrices (Hirsch et al., 1998; Hirsch et al., 1999; Lindsey et al., 2001; Zhu et al., 2001; Kolpin et al., 2002; Loffler et al., 2003b; McArdell et al., 2003; Schlusener et al., 2003; Wiegel et al., 2004; Yang et al., 2004a; Yang et al., 2004b). Among other cartridges, HLB cartridges are widely used due to the broad range of pH that can be tolerated and no significant interference compared with silica based cartridges.

Furthermore, tandem SPE methods (strong anion exchange SAX + HLB) have been used to remove humic material with SAX and to retain antibacterial agents with the HLB cartridge in surface water and agricultural soil (Kolpin et al., 2002; Jacobsen et al., 2004).

3.4.2 Separation and Detection

Two major techniques to separate and detect the pharmaceuticals in environmental matrix with low concentration are gas chromatography (GC) and liquid chromatography (LC) combined with ultraviolet (UV), mass spectrometry (MS) or even tandem mass spectrometry (MS/MS). GC/MS was used to measure 8 pharmaceuticals and their metabolites in a river and wetland (Gross et al., 2004). Also, Herberer used GC/MS to track the residuals of 6 pharmaceuticals from municipal sewage to drinking water and reviewed the occurrence, fate, and removal of over 80 pharmaceuticals in the 9 different categories in the aquatic environment (Heberer, 2002a, b). Although, GC/MS is still used as a routine analysis tool, there appears to be a migration to LC/MS or LC/MS/MS techniques for pharmaceutical analysis. Most of the pharmaceutical

compounds are not very volatile and some are highly polar containing ionizable functional groups (carboxylic or amino). Therefore, LC/MS or LC/MS/MS is more suitable for separation and detection of pharmaceutical residuals or metabolites in environmental samples (Heinisch et al., 2004). In addition, GC/MS requires additional derivatization steps that may cause more labor, time, and unwanted contamination in the sample.

A detailed and comprehensive explanation of LC/MS analysis is reviewed with a range of emerging contaminants, related pollutants, microorganisms and humic acids (Zwiener et al., 2004a, b). Among varied LC modes (e.g. reversed-phase, ion exchange, size exclusion), reversed-phase mode packed with octadecyl (C_{18}) or octyl (C_8) bonded silica packing, as stationary phase is the most common used techniques for pharmaceutical analysis. Ion exchange and size exclusion method are used to separate ionic compounds and molecules ob the basis of molecular weight respectively. Few studies have been reported for ion exchange and size exclusion method due to high concentration of salt in mobile phase causing pump damage and Ding et al. (Ding et al., 2000) developed ion exchange chromatography using polymeric column and acidic eluent and applied to measure tetracyclines residual in milk and oxytetracycline removal rate in bio-chemical technology WWTP process.

Composition of the mobile phase is an important factor for better separation in LC depending on characteristic of components. An acidic condition with acetonitrile-water and methanol-water mixtures with gradient elution is among the most common to improve the peak shape in chromatography. Non-volatile additives such as oxalic acid should be avoided when electrospray ionization is used and trifluoroacetic (TFA) acid

can suppress the ionization in the electrospray source (Lindsey et al., 2001). Other parameters that are also important include injection volume, column size and packing material.

In terms of the ionization technique, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) producing protonated $[M+H]^+$or deprotonated $[M-H]^-$ molecules are the most widely used methods. Both techniques use atmospheric pressure and temperature to evaporate solvent in a desolvation region and pass the sample (solute) molecules into a mass analyzer without losing sample itself. Only difference in APCI is to use different spraying method and to be needed another ionization region (corona discharge) for enhancing the formation of protonated molecular ions. Those two techniques are often combined with MS or MS/MS to overcome the difficulties of complex sample composition and poorly resolved chromatography (Petrovic et al., 2003).

Mass spectrometry (MS) is originally used to identify and quantify the substance or to confirm its molecular structure. The most widely used LC/MS/MS method adds additional collision energy to fragment protonated or deprotonated ions formed in the several ionization source. This additional fragmentation step may require more analysis time but it enhances the selectivity of the complex-matrix sample by avoiding co-elution of analytes and interferences in samples compared to LC/MS with a single quadrupole. As an example of LC/MS/MS, Figure 3.1 and 3.2 show the chromatogram and tandem mass spectra of three ionophore polyethers (IPs), monensin, salinomycin and narasin. IPs is only used to promote growth for beef and cattle and to treat coccidiostats for poultry and not for human (Schlusener et al., 2003). Thus, existence of IPs residual in

environment can be interpolated as animal-influenced. Each chromatogram and tandem mass spectra was obtained using HPLC/ESI/MS/MS in the positive ion mode.

LC/MS/MS is often applied with triple quadrupole or ion trap mass spectrometry. Triple quadrupole mass spectrometry uses multiple reaction mode (MRM) with fixed m/z values of quadrupole (Q) 1 and 3 while Q2 is used as the collision cell. Ion trap mass spectrometry is the innovative method that has an ability to perform multiple stages of MS/MS to isolate and fragment ions in time and to trap the product ions. While this mass analyzer method has ability to infer the pathways easily for identification of unknowns using MS^n, application to pharmaceuticals in environment has not been found yet. Time of flight mass spectrometry coupled with LC (LC/TOF/MS) is an alternative detection method for pharmaceuticals in the environment. The high-power resolving technique of the TOF/MS method removes the interference signal from the sample making it easier to identify the non-target compounds in a complex environmental sample. This method was reviewed comprehensively and applied with sediment samples for identifying Diphenhydramine (Ferrer et al., 2003; Ferrer et al., 2004).

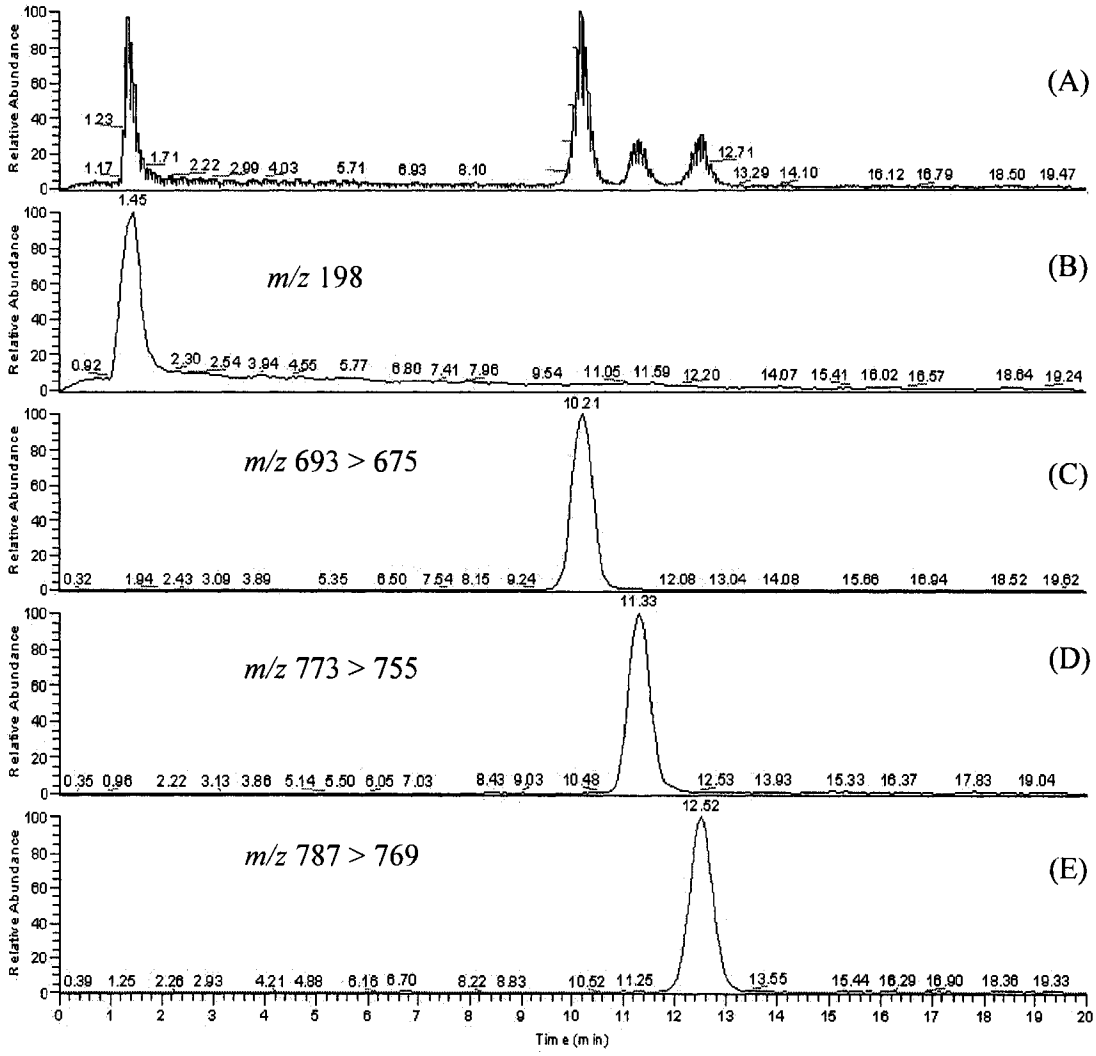

Figure 3.1 Reconstructed chromatogram showing standard solution (2μg/l) of three IPs: (A) total ion chromatogram (TIC), (B) simatone (internal standard), (C) Monensin, (D) Salinomycin, and (E) Narasin.

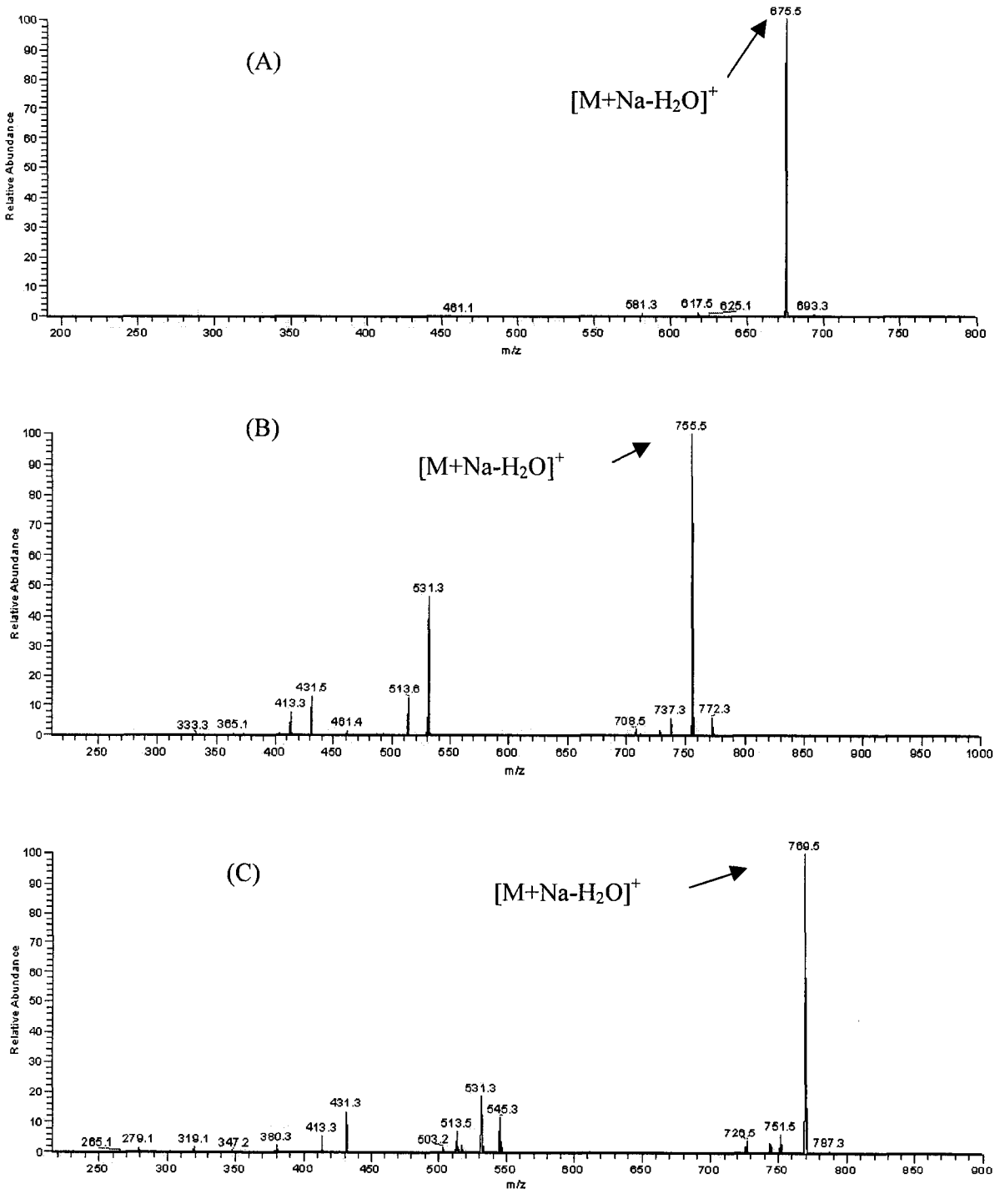

Figure 3.2 Full scan tandem mass spectra of: (A) Monensin, (B) Salinomycin, and (C) Narasin.

Application of LC combined with several ionization sources and mass analyzers described above have been reported to measure pharmaceutical residuals in environment. Table 3.2 summarized recently published literature review for pharmaceuticals in solid matrix including extraction process and detection method with LC. The most primary combination of LC/UV was applied to manure and soil to evaluate the transferring of oxytetracycline and tylosin from farm to soil (Liguoro et al., 2003) and also used to pig slurry and soil for quantifying 3 veterinary antibiotics using ultrasonication as pre-ectraction (Blackwell et al., 2004b). Haller et al. (Haller et al., 2002) used LC/ESI/MS with selective ion monitoring (SIM) mode to measure 7 veterinary antibiotics in manure and reported 100μg/kg as limit of quantification (LOQ). Tandem mass spectrometry (MS/MS) combined with ESI or APCI is the most common combination for pharmaceutical analysis in environment related solid sample. ESI/MS/MS method was used to characterize the persistence of tetracyclines in soil fertilized with liquid manure and ESI/MS^3 was examined in this study for further confirmation of product ions produced from ESI/MS/MS (Hamscher et al., 2002). In addition, Jacobsen et al. applied the two different ESI/MS/MS method to quantify the 8 antibiotics from three different classes in soil and reported LOQ is ranged from 1.1 to 12.8μg/kg for examined compounds in two different soils (Jacobsen et al., 2004). Loffler et al. used two different methods, APCI/MS/MS for 10 acidic pharmaceuticals in negative mode and ESI/MS/MS for 7 antibiotics in positive mode, to determine residuals in river sediment (Loffler et al., 2003b). This study illustrates that different ionization method can be adapted depending on characteristic of examined compounds. Another study to use APCI/MS/MS is to

Table 3.2 Literature review of extraction process and detection method for pharmaceuticals in LC.

Class and Compounds	Sample Matrix	Pre-extration Cleanup	Detection	Recovery (%)	LOQ[a] (μg/kg)	Detected Level (μg/kg)	Reference
2 Fluoroquinolone	Sewage sludge Soil	ASE[b] SPE	FLD[c]	82 – 94 75 - 92	450 180	1400 – 2420 270 – 400	(Golet et al., 2002b)
5 Tetracyclines	Animal Feeds	ACN[d]/Water (pH 3.0)	Diode array	52 - 96	100 – 400 (μg/L)	8000 - 57000	(Caballero et al., 2002)
4 Antibiotics	Soil	Citric buffer (pH 4.7)/EtOAc[e]	ESI/MS/MS	33 - 86	5	4 - 199	(Hamscher et al., 2002)
7 Antibiotics	Manure	LLE[f] (EtOAc)	ESI/MS	47 – 89	100	100 - 12400	(Haller et al., 2002)
8 Antibiotics	Manure	LLE (EtOAc), SPE	APCI/MS/MS	75 – 123	1 - 93	11 - 43	(Schlusener et al., 2003)
18 Pharmaceuticals	Sediment	Ultrasonication	APCI/MS/MS ESI/MS/MS	56- 151	0.4 - 20		(Loffler et al., 2003b)
2 Antibiotics	Manure Soil	McIlvaine buffer/Methanol Phosphate buffer/Methanol	UV (355nm, 282nm)	74 - 80 81 - 82	100 5 - 10	2110 – 19000 6 - 7	(Liguoro et al., 2003)

Table 3.2 (Continued)

Class and Compounds	Sample Matrix	Pre-extration	Detection	Recovery (%)	LOQ[a] (µg/kg)	Detected Level (µg/kg)	Reference
29 Antibiotics	Manure Soil	Ultrasonication ASE	ELISA MS/MS			1000 – 1100 15	(Christian et al., 2003)
8 Antibiotics	Soil	PLE[h] SPE (SAX+HLB)	ESI/MS/MS	31 – 143	1 -11	1 - 57	(Jacobsen et al., 2004)
3 Antibiotics	Soil	Ultrasonication SPE (SAX+HLB)	UV/FLD	27 - 105	18 - 40		(Blackwell et al., 2004b)
2 Antibiotics	Sediment	McIlvaine-EDTA buffer SPE	MS/MS	88 - 93	0.012 – 0.061	0 - 579	(Lalumera et al., 2004)
Erythromycin	Soil	LLE	ED[i]				(Kim et al., 2004)
3 Antibiotics	Soil	Buffer solution/methanol SPE (SAX+HLB)	UV (285nm, 355nm)	35 - 65	10		(Kay et al., 2004)
7 Pharmaceuticals	Sludge	Ultrasonication SPE (MCX)	APCI/MS/MS				(Ternes et al., 2004)

[a] Limit of Quantification, [b] Accelerated Solvent Extraction, [c] Fluorescence Detection, [d] Acetonitrile, [e] Ethyl Acetate, [f] Liquid-liquid Extraction, [g] Radioimmunoassay, [h] Pressurized Liquid Extraction, [i] Electrochemical Detection,

measure manure (Schlusener et al., 2003) and sewage sludge for determination of solid-water distribution coefficient (Ternes et al., 2004).

Alternative method for detecting the pharmaceuticals is fluorescence detection (FLD) for fluoroquinolone determination (Golet et al., 2002b), diode array spectrometry for tetracyclines in animal feeds (Caballero et al., 2002), and electrochemical detection for erythromycin A degradation (Kim et al., 2004).

3.5 Occurrence and Fate of Pharmaceuticals in the Aquatic Environment

As shown in Figure 3.3, the effluent from sewage treatment plants (STPs) is one of the main sources of human-origin pharmaceuticals in the environment. Unchanged parent compounds or slightly modified forms of human-origin pharmaceuticals are collected in STPs and only partially removed with the treatment processes (Heberer, 2002b; Miao et al., 2002; Barreiro et al., 2003; Loffler et al., 2003b, a; McArdell et al., 2003; Stamatelatou et al., 2003; Vanderford et al., 2003; Andreozzi et al., 2004; Blackwell et al., 2004a; Gobel et al., 2004; Gross et al., 2004).

During the last decade, numerous studies have been published discussing the occurrence and fate of pharmaceuticals and endocrine disrupting compounds in the environment (Ternes, 2001; Heberer, 2002a; Kummerer, 2003; Petrovic et al., 2003; Rooklidge, 2004). The measured level of pharmaceuticals in the environment has varied depending on such variables as proximity to wastewater treatment plants, the density and proximity of agricultural feed operations and hydrology. X-ray contrast media such as iopamidol, iopromide, diatrizoate, and iomeprol has shown some of the highest concentrations with ranges from 3.8 to 15 μg/L followed by the antiepileptic carbamazepine (6.3 μg/L) and the erythromycin metabolite (erythromycin-H_2O), an antibiotic (6.0 μg/L) (Ternes, 2001).

In 2002, the U.S. Geological Survey (USGS) reported the first nationwide reconnaissance of the occurrence of pharmaceuticals, hormones, and other wastewater contaminants (OWCs) in 139 streams during 1999 and 2000 using newly developed methods (Kolpin et al., 2002). In terms of pharmaceuticals, most of the compounds were detected with concentrations at less than one microgram per liter. Trimethoprim showed the highest detection frequency (27.4%) followed by a metabolite of erythromycin (erythromycin-H_2O, 21.5%).

Kolpin *et. al.* surveyed the concentration level of pharmaceuticals and other OWCs in streams with different flow conditions and concluded that the concentration of pharmaceuticals in urban areas can be affected by different flow conditions, especially during low flow conditions because of the dilution factor (Kolpin et al., 2004).

Pharmaceuticals can also be found in groundwater depending on the mobility of different compounds. Laboratory experiments have been conducted to examine the potential mobility of pharmaceuticals from STPs to groundwater (Cordy et al., 2004; Kreuzinger et al., 2004b). This study revealed that 4 compounds (sulfamethazine, sulfamethoxazol, carbamazapine, and cotinine) have the potential to reach groundwater and that the two sulfonamides were detected in the field with a range of 0.16 – 0.47 μg/L (Hirsch et al., 1999; Lindsey et al., 2001). Other pharmaceuticals including analgestic drugs, antiepileptic drugs, and their metabolites were also found in groundwater of bank filtration sites with concentrations of sub-microgram per liter (Heberer et al., 2004; Kreuzinger et al., 2004b).

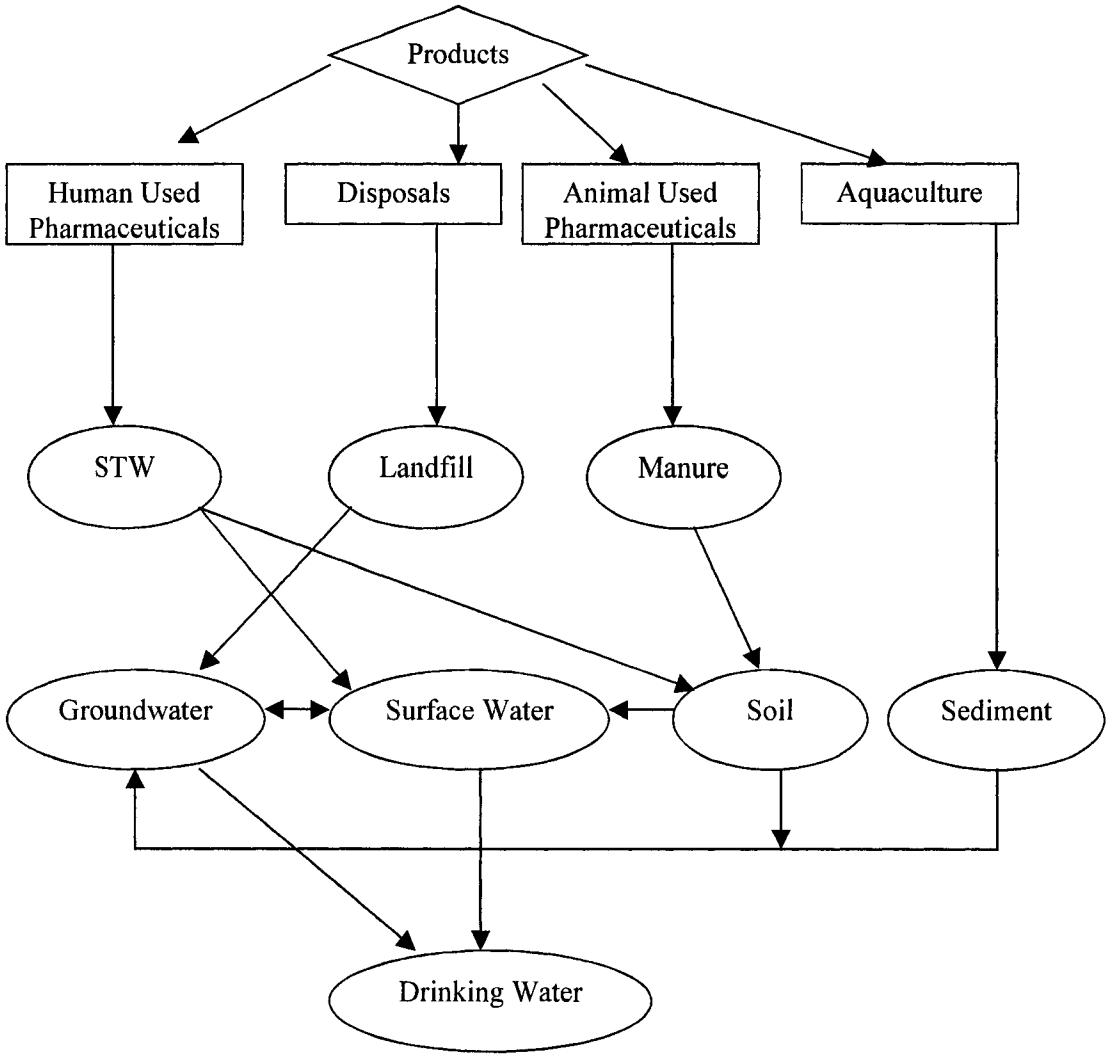

Figure 3.3 Transport pathways of pharmaceuticals in environment

In contrast to the aquatic environment, the occurrence and fate of pharmaceuticals in solid matrices such as soil and sediment has not been well studied. Animal-origin pharmaceuticals including aquaculture-derived compounds contribute significantly to the occurrence of pharmaceuticals in solid matrices due to the application patterns. Studies have been conducted to review the behavior of pharmaceuticals in solid matrices including analytical methods, physicochemical properties, and concentration of human and animal-origin pharmaceuticals in the environment (Petovic et al., 2001; Tolls, 2001;

Diaz-Cruz et al., 2003; Thiele-Bruhn, 2003; Beausse, 2004). After animal-origin pharmaceuticals are excreted and stored in storage or manure, the residual can enter into the environment through leakage from storage facilities, runoff from agricultural fields, or even directly through grazing livestock. However, the transport mechanisms of animal-origin pharmaceuticals into the environment still needs more study. For example, the usage of manure or sewage sludge as fertilizer could be an important source of pharmaceuticals introduced into environment.

Measured concentrations of animal-origin pharmaceuticals in manure have been reported in several studies. Among grab samples of 6 manure sites, only 2 sulfonamides (sufamethazine and sulfathiazole) were found with range of 0.10 – 12.4 μg/kg (Haller et al., 2002). Another study showed the concentration of 2 antibiotics among 8 measured compounds with different groups (macrolides, ionophore polyethers) at 11 and 43 μg/kg of tiamulin and salinomycin respectively (Schlusener et al., 2003). Another study found only sulfadimidine at levels of 1 mg/kg among 29 examined antibiotics in manure (Christian et al., 2003).

As a result of applying manure in agriculture fields, tetracycline and chlortetracycline were measured at soil depths of 0 – 30 cm with a concentration range of 86.2 – 171.7 μg/kg and 4.6 – 7.3 μg/kg respectively (Hamscher et al., 2002). This study also measured the concentrations of oxytetracycline and tylosin in soil but neither of these compounds was found possibly indicating faster degradation or strong sorption to soil or manure particles. (Liguoro et al., 2003). Furthermore, three representative groups of antibiotics (tetracyclines, sulfonamides, and macrolides) were quantified in soil using

tandem SPE (SAX+HLB) followed by HPLC/MS/MS and with a concentration range of 0.6 – 57.4 μg/kg (Jacobsen et al., 2004).

Direct deposition of excreted pharmaceuticals can also occur in aquaculture. The concentration of commonly used pharmaceuticals in aquaculture shown in Table 1 was reported to be as high as 246.3 and 578.8 μg/kg d.w. for oxytetracycline and flumequine, respectively (Lalumera et al., 2004). Pharmaceuticals used in fish farms be can be transported into surface water causing adverse effects in the ecological biota.

The fate and degradation pathway of pharmaceuticals released in the environment will vary depending on the physicochemical properties of the compounds. Mobility of compounds is highly dependant on water solubility, octanol/water partitioning coefficient and organic carbon contents of the sorbent (Diaz-Cruz et al., 2003). For example, tetracyclines show the highest sorption coefficient compared to other major antibiotics and sulfonamides are usually relatively mobile (Tolls, 2001). These trends help predict where compounds may be found in the environment. For example, tetracyclines have been found in high concentrations in the soil or sediment matrix indicating not only strong sorption characteristics but also the tendency to accumulate and persist in solid matrices. Of the major antibiotic classes, sulfonamides are most commonly detected in groundwaters due to their high mobility.

Several complex processes can be involved in the sorption mechanism of pharmaceuticals in solid matrices. Not only hydrophobicity but also cation exchange, cation bridging, surface complexation, and hydrogen bonding can play an important role to retain pharmaceuticals on a solid matrix (Tolls, 2001). Sorption characteristics of pharmaceuticals are normally evaluated with closed experimental systems such as batch

or column tests in the laboratory for better control of parameters. Several selective compounds have been assessed to determine the sorption characteristics in solid matrixes including soil, manure, and sewage sludge in field and laboratory condition (Rabolle, 2000; Thiele, 2000; Loke et al., 2002; Das et al., 2004; Kay et al., 2004; Kim et al., 2004; Oppel et al., 2004; Ternes et al., 2004; Thiele-Bruhn et al., 2004; Kay et al., 2005b). The main parameters controlled when conducting experiments were soil moisture ratio, clay content with different soil type, and varied concentration.

3.6 Predicted Environmental Concentration (PEC) of Pharmaceuticals in Soil

Given that sufficient information can be provided, a prediction method is a useful tool to evaluate the risk of pharmaceuticals in the environment. However, there is often limited information for developing precise modeling for predicted environmental concentrations of pharmaceuticals and only a few studies have been conducted, mainly focusing on animal-origin pharmaceuticals.

Spaepen et. al. (1997) developed a uniform approach to estimate the PEC of animal-origin pharmaceuticals in soil (Spaepen et al., 1997). This study used the concentration of active ingredients in manure with different animal excretion practices and combined this with manure application in the field taking into account applied soil characteristics. A more defined calculation was also conducted to consider the fraction of animal excreta, the metabolite or transformed product of the active ingredient, and the degradation time of parent compounds in manure. Another study showed the distribution of applied growth promoters in different components of the environment such as soil,

surface water, groundwater, fish, and crops and also indicates the importance of biodegradability for more precise model development (Jorgensen et al., 1998).

In addition, four different scenarios were presented for three pathways of animal-origin pharmaceuticals for the assessment of environmental risks and the predicted value was compared with measured concentrations (Montforts et al., 1999; Castiglioni et al., 2004). The result of the comparison between the predicted value and the measured concentration indicated that sufficient information is necessary to estimate the environmental fate of human and animal-origin pharmaceuticals more accurately.

3.7 Conclusion

Long term exposure of pharmaceuticals in the environmental can have adverse effects to human and animal populations through several potential mechanisms. However, the lack of information on robust and available analytical methods for measuring these compounds in solid matrices is an impediment for a better understanding of the environmental risk. We have reviewed the methods that have been adapted for this application and described how these techniques can be used to further our understanding of occurrence, fate, and even removal of pharmaceuticals.

HPLC/MS/MS is the most widely used method to detect and separate pharmaceuticals in environmental samples and is combined with various forms of SPE for further cleanup or purification. While this method can easily achieve detection limits below the microgram per liter level, we often need nanogram per liter resolution. To enhance the signal/noise ratio (and lower the detection level) in complex environmental samples, the suppression of compounds that contribute matrix effects is still an issue to be addressed.

Further research should be conducted to collect more information about occurrence, fate, and transport mechanisms from the source to different parts of the environment and sufficient information can be used to develop more precise prediction tools for better risk assessment of pharmaceuticals in the environment.

Chapter 4

Quantification of Human and Veterinary Antibiotics in Water and Sediment Using SPE/LC/MS/MS Method

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4.1 Abstract

An analytical method was developed and tested for four different groups of veterinary antibiotics in both river water and sediment matrices. Solid phase extraction (SPE) was used to enrich and to cleanup the aqueous sample. Also, McIlvaine and ammonium hydroxide buffer solutions were used to extract the compounds from the sediment matrix. High performance liquid chromatography (HPLC) equipped with tandem mass spectrometry (MS/MS) was used to separate and quantify the samples. The range of recoveries (%) for tetracyclines (TCs), sulfonamides (SAs), macrolides (MLs), and ionophore polyethers (IPs) in the water matrix were 102.2-124.8, 76.6-124.3, 89.5-114.7, 82.7-117.5 with 1-13 (%) of relative standard deviation respectively with three different concentrations. For sediment, the percent recovery ranges were 32.8-114.8, 62.4-108.9, 53.4-128.4 and 51.3-105.4 for TCs, SAs, MLs and IPs, respectively. The relative standard deviation ranged from 16 - 27 (%) over three different concentrations. The limit of quantification (LOQ) was determined using two different methods and calculated to be at

the range of 0.01-0.004 μg/L and 0.3-2.5 μg/kg for TCs, SAs, and MLs in water and sediment, respectively. For IPs, the LOQ was 0.001-0.003 μg/L in river water and 0.4-3.6 μg/kg for sediment. The sediment concentration measured in an agriculture-influenced river was much higher than in the overlying water matrix indicating a high degree of sediment partitioning for these compounds.

Keywords: Sediment; Human and veterinary antibiotics; SPE-HPLC/MS/MS.

4.2 Introduction

Tetracyclines (TCs), sulfonamides (SAs), and macrolides (MLs) are three antibiotic groups that are commonly used in human and veterinary medicine. These drugs are used for therapeutic treatment of infectious disease in humans and for treating and protecting the health of animals (Bruhn, 2003). In addition, ionophore polyethers (IPs) are used to promote growth and feed efficiency in a range of animals.

The basic structure of TCs is a hydronaphthacene backbone containing four fused rings. Different TCs are characterized by various substitutions in the C6, C8, and C10 position of the backbone (Lock et al., 1999; Bruno et al., 2002). All SAs investigated have at least two nitrogen functional groups. The amide attached to the sulfur is deprotonated at pH > 5.5-7 and the amine attached to aromatic group is deprotonated at pH < 2.5. Thus, most sulfonamides are positively charged under acidic conditions and negatively charged under alkaline conditions (Haller et al., 2002). MLs are composed of large lactone rings substituted with hydroxyl, alkyl, and ketone groups. Neutral and amino sugars are bound to the nucleus by the substitution of hydroxyl groups (McArdell et al., 2003). IPs consist of a carboxylic polyether backbone that forms pseudo-macro

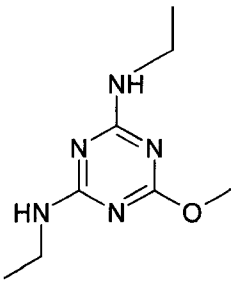
cyclic complexes with cations (Volmer et al., 1998). The chemical structures of the compounds discussed in this paper are shown in Figure 4.1-4.3.

According to the Animal Health Institute's 2002 Market Sales Report, animal health product sales in the United States for 2002 totaled $4.5 billion including $3.3 billion for pharmaceuticals and $557 million for feed additives (2002). In the United States, livestock producers use 24.5 million pounds of antimicrobials every year in the absence of disease for nontherapeutic purposes: approximately 10.3 million pounds in hogs, 10.5 million pounds in poultry, and 3.7 million pounds in cattle (Mellon et al., 2001).

The relatively high usage of veterinary antibiotics and their potential persistence in the environment has led to an interest the ultimate fate of these compounds. Few studies have been conducted on the occurrence of veterinary pharmaceutical compounds in waterways, particularly in the sediments (Diaz-Cruz et al., 2003).

Solid phase extraction (SPE) is the most common method to enrich the water matrix for subsequent analysis of veterinary pharmaceuticals. Various materials and sizes of reversed-phase octadecyl (C18), environmental+ (ENV+), and hydrophilic-lipophilic balance (HLB) materials have been reviewed for extraction of TCs, SAs, and MLs from aqueous media (Hirsch et al., 1998; Hirsch et al., 1999; Lindsey et al., 2001; Zhu et al., 2001; McArdell et al., 2003). Among the various techniques, the HLB cartridge shows the most robust recovery ratio and reproducibility for both polar and non-polar compounds. Recently, tandem SPE methods using a strong anion exchange cartridge (SAX) and HLB cartridge were studied for soil and pig slurry (Blackwell et al., 2004b; Jacobsen et al., 2004).

(A)



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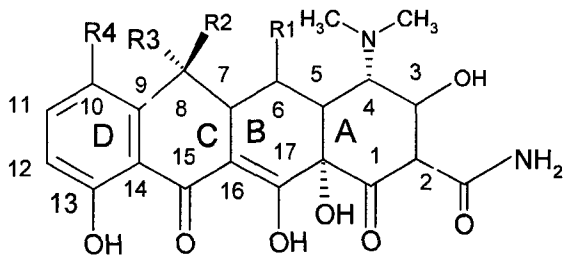
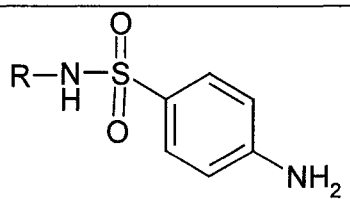


Compounds	R_1	R_2	R_3	R_4	pKa		
					pK_1	pK_2	pK_3
Oxytetracycline (OTC)	OH	OH	CH_3	H	3.3	7.3	9.1
Chlortetracycline (CTC)	H	OH	CH_3	Cl	3.3	7.4	9.3
Minocycline (MNC)	H	H	H	$(CH_3)_2N$	2.8	5.0	7.8
Demeclocycline (DMC)	H	OH	H	Cl			
Meclocycline (MCC)	OH	CH_2	-	Cl			
Tetracycline (TC)	H	OH	CH_3	H	3.3	7.7	9.7
Doxycycline (DXC)	OH	H	CH_3	H	3.5	7.7	9.5

(C)



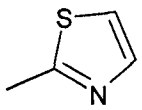
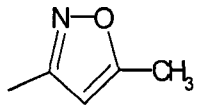
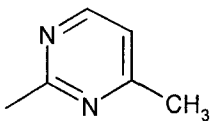
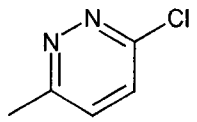
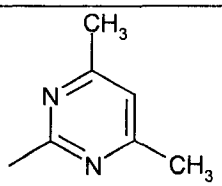
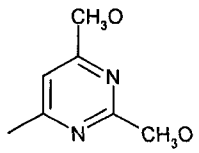
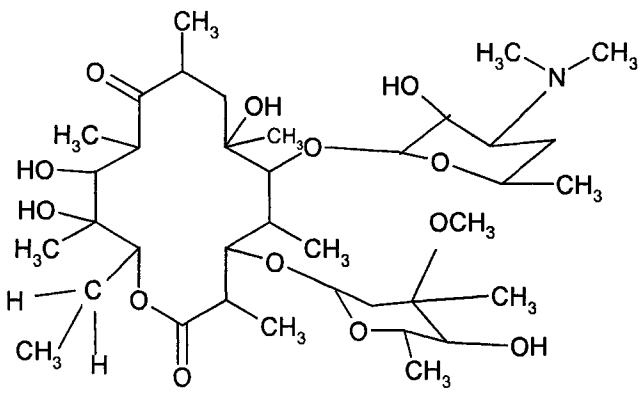
Name	R	Name	R	pKa
Sulfathiazole (STZ)		Sulfamethoxazole (SMX)		
Sulfamerazine (SMR)		Sulfachlorpyridazine SCP		pK1 : 5.4 – 7.5 pK2 : 2.5
Sulfamethazine (SMT)		Sulfadimethozine (SDM)		

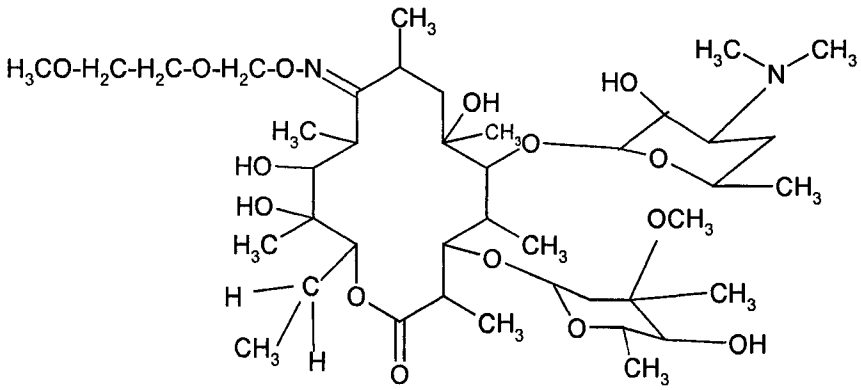
Figure 4.1 Chemical structure of (A) simatone: internal standard, (B) TCs and (C) SAs examined in this study.

(A)



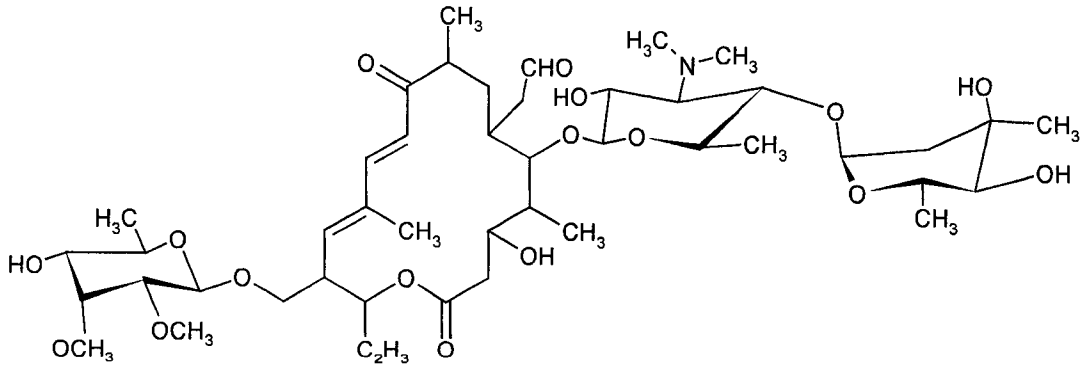
Erythromycin (ETM)

(B)



Roxithromycin (RTM)

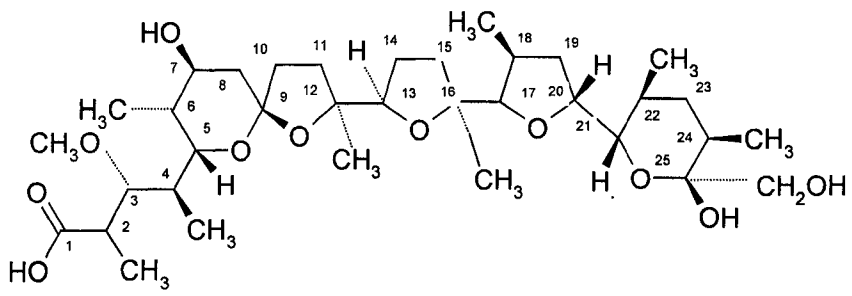
(C)



Tylosin (TYL)

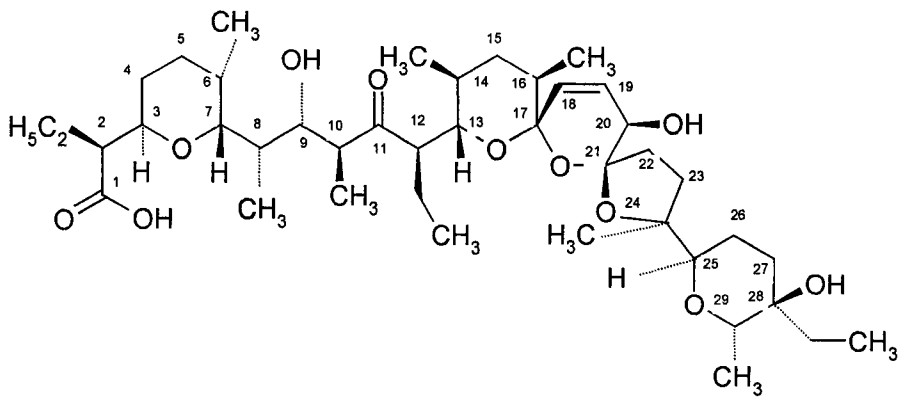
Figure 4.2 Structure of MLs compound examined in this study.

(A)



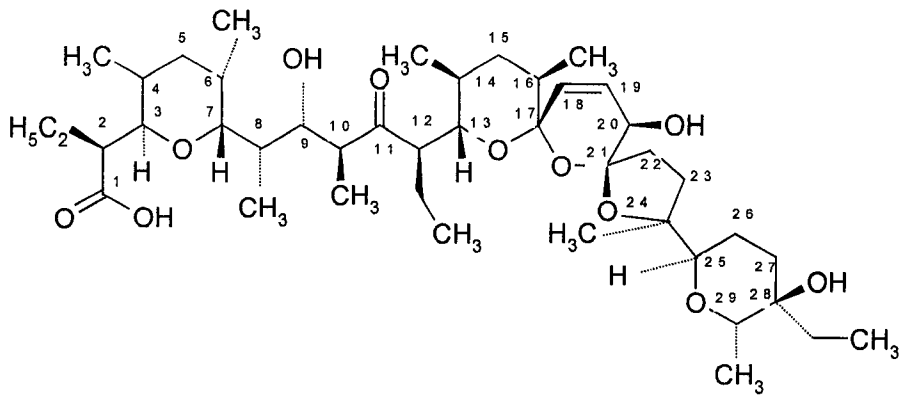
Monensin (MNS)

(B)



Salinomycin (SLM)

(C)



Narasin (NRS)

Figure 4.3 Chemical structures of IPs examined in this study.

These method has the advantage of removing natural organic matter (NOM) mainly composed of negatively charged humic and fulvic acids in the environmental sample. However, no comparison was made between a single cartridge and tandem cartridge for the SPE procedure and cost issues were not discussed.

The major challenge when analyzing sediment is the extraction of the antibiotic compounds from the solid phase to the liquid phase prior to further purification. Since most antibiotics are sensitive to strong acids and bases, a weakly acidic buffer solution has an advantage in this extraction (Bruhn, 2003). For example, a McIlvaine-EDTA solution was used to extract oxytetracycline in fish farm sediment (Jacobsen et al., 1987; Bjorklund et al., 1990). A citric acid buffer solution in combination with ethyl acetate was used to extract tetracyclines in egg, poultry, fish and tissues (Cooper et al., 1998). This method was also adapted to measure the concentration of tetracyclines and tylosin in fertilized soil (Hamscher et al., 2002). Both methods show a good recovery ratio for TCs although weakly acidic extractants are not suitable with macrolides and ionophore polyethers.

Few studies have been reported on the extraction of MLs and IPs in soil and sediment samples. Schlusener et al. used a liquid-liquid extraction method with ethyl acetate for MLs, IPs and tiamulin in liquid manure (Schlusener et al., 2003). However, the limit of quantification (LOQ) was too high (59.7 μg/kg for monensin and 68.1 μg/kg for tylosin) to measure these compounds at the parts per billion concentrations expected in environmental samples. Also, the liquid-liquid extraction method is time consuming and produces a hazardous organic solvent as the waste product. To address these issues, an ammonium hydroxide buffer solution was used in this study. Five MLs were extracted

in honey using 0.1 M phosphate buffer solution (pH 8.0) (Wang, 2004). This study shows acceptable recovery and low detection limits that might be adapted for environmental sample analysis. However, the use of phosphate buffer solution might cause degradation of the analytes during the evaporation step, especially the TCs (Lindsey et al., 2001). Three different antibiotic groups (TCs, SAs, and MLs) were simultaneously extracted in soil and pig slurry using McIlvaine buffer solution (pH 7.0) (Blackwell et al., 2004b) with recoveries ranging from 27-51 %, 68-85 %, and 47-61 % for oxytetracycline, sulfachloropyridazine, and tylosin in a clay soil. However, the limit of detection was again too high (18 μg/kg – 40 μg/kg for soil and 70 μg/kg – 140 μg/kg for pig slurry) to measure most environmental samples.

High performance liquid chromatography (HPLC) equipped with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) is the most widely used method to separate and quantify antibiotic samples. HPLC/MS or MS/MS with electrospray ionization (ESI) has been used in several types of water and food matrices (Hirsch et al., 1998; Hirsch et al., 1999; Lindsey et al., 2001; Zhu et al., 2001; Bruno et al., 2002; Loke et al., 2003; McArdell et al., 2003). Alternatively, metal chelate affinity chromatography HPLC (MCAC-HPLC) and HPLC-fast atom bombardment (FAB) have been adapted to measure tetracycline in animal products and sediment respectively (Cooper et al., 1998; Delepee et al., 2000). However, the MCAC-HPLC method needs an additional step to confirm the concentration with HPLC/MS and the HPLC-FAB method has shown a low recovery indicating that this method is not optimal for reproducibility. In addition, a LC/UV method was adapted to measure TC residual in honey (Vinas et al., 2004). This method can be adapted for TCs and SAs compounds that show good UV

spectrometry. However, MLs show poor UV spectrometry and also the poor chromophore characteristic of IPs needs post-column derivatization for UV detection (Volmer et al., 1998; Yang et al., 2004a).

The objective of the study described in this paper was to develop a sensitive and reliable analytical method for quantifying environmental concentrations of human and veterinary antibiotics in water and sediment matrices. Despite the fact that the occurrence of pharmaceuticals and veterinary antibiotics has been receiving increasing attention, little is known about the occurrence and fate of these compounds, primarily due to the analytical challenges. This paper describes the development and testing of an analytical method for quantifying selected TCs, SAs, MLs and IPs in water and sediment matrices.

4.3 Experimental

4.3.1 Materials

Nineteen pharmaceutical and veterinary antibiotics of four different groups: tetracyclines (TCs) (tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), minocycline (MNC), demeclocycline (DMC), meclocycline (MCC), doxycycline (DXC)), sulfonamides (SAs) (sulfathiazole (STZ), sulfamerazine (SMR), sulfamethazine (SMT), sulfachlorpyridazine (SCP), sulfamethoxazole (SMX), sulfadimethoxine (SDM)), macrolides (MLs) (erythromycin (ETM), roxythromycin (RTM), tylosin (TYL)), and the sodium salt of three ionophore polyethers (IPs) (90-95 % monensin (MNS) and 97 % narasin (NRS)) were purchased from Sigma-Aldrich Co. (St. Louis, MO). The third IP (96 % salinomycin (SLM)) was purchased from ICN (Aurora, OH). HPLC grade methanol (99.9 %), analytical grade formic acid (99 %), citric acid-monohydrate, sodium

phosphate-dibasic anhydrous, and disodium ethylene diaminetetraacetic acid (Na_2EDTA) were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Standard solutions of each of the four antibiotic groups with 100mg/L concentration were prepared in methanol and stored at 4 °C for one month. TCs can decompose rapidly under the influence of light and atmospheric oxygen forming degradation products (Liang et al., 1998). In order to prevent degradation of the standard solutions, amber bottles were used to avoid light penetration. Also, freshly prepared standard solutions (2 μg/L) were examined in the HPLC/MS/MS system prior to every experiment to check the degradation of standard solution. Working solutions, 5 mg/L and 0.5 mg/L, were prepared weekly by dilution of the standard solution in methanol. HLB (Hydrophillic-Lipophillic-Balance) solid phase extraction (SPE) cartridges (3 ml/60 mg) were purchased from Water Oasis Co. (Milford, MA). Milli-Q water (18.3 MΩ) from a Millipore (Billerica, CA) purification system was used when DI water was required.

4.3.2 Sample Collection

To test the optimized method, both water and sediment samples were collected along the Cache la Poudre River in Northern Colorado at May 2005. Water and sediment properties and descriptions of the five sampling sites are summarized in Table 4.1. Water samples were collected in amber glass bottles pre-rinsed with DI water at three different cross-sectional locations in the river at each site and aliquots of each sample was carefully mixed in one bottle. Collected water samples were kept in a cooler with ice until transportation to the lab. The top layer (0 – 5 cm) of the sediment sample was collected with a spatula at the same place where water samples were collected and also composted in one sampling jar. The collected water samples were filtered through 0.2 μm pore size

glass fiber filters and stored at 4 °C before each analysis. Sediment samples were air-dried in a dark room to prevent photo degradation. After being air-dried completely, the sample was passed through 2 mm and 0.75 μm pore size sieves. The 2 mm sieved sediment sample was used to analyze physicochemical properties of the sediment and the 0.75 μm sieved sediment sample was used to measure the antibiotic concentrations. Since most of studied compounds are assumed to be strongly sorbed in clay fraction, size of less than 0.75 μm was used for entire analysis (Boxall et al., 2002; Figueroa et al., 2004; Kim et al., 2004; Thiele-Bruhn et al., 2004).

4.3.3 Solid Phase Extraction

The optimized solid phase extraction (SPE) method including cartridge material, cartridge precondition, and elution solvent for the aqueous phase was adapted from previous studies of our research group for antibiotic classes of TCs, SAs, and MLs (Yang et al., 2004a; Yang et al., 2004b). To develop the optimum SPE condition for IPs, four different conditions were examined. All four different conditions were conducted using 3 mL/60 mg HLB cartridges. 3 ml/60 mg HLB cartridges were chosen to avoid the irreversible binding of tetracycline to silanol groups while maintaining durable capacity for both hydrophilic and hydrophobic compounds.

Once the optimum SPE condition was determined, 120 mL of sample was carefully measured into a flask. The HLB cartridge was pre-conditioned before loading the sample with 3 mL of MeOH and 3 mL of deionized (DI) water. MeOH and DI water were used to activate the cartridge and to remove impurities in the cartridge respectively. The sample was loaded under 40 (psi) of vacuum with a flow rate of approximately 2 mL/min. After loading the sample, the SPE cartridge was rinsed with 9 mL of water to

Table 4.1 Sampling site description with physical and chemical properties of water and sediment (DO: Dissolved Oxygen and ORP: Oxidation Reduction Potential).

	Water				Sediment					
	Temp. (°C)	pH	DO (mg/L)	ORP (mV)	pH	Organic Matter (%)	NO_3-N (ppm)	P	Fe	Texture Estimate
Site 1	10.2	7.5	7.5	319	7.1	0.4	6.0	1.7	21.1	Sand
Site 2	12.2	8.1	6.7	340	7.3	0.6	4.0	3.4	51.7	Sand
Site 3	11.8	7.4	5.9	339	7.2	0.4	4.4	47.4	72.5	Sand
Site 4	15.4	8.2	4.8	340	7.4	0.8	11.2	18.6	82.9	Sand
Site 5	15.7	8.4	4.7	337	7.5	0.5	15.3	32.6	68.7	Sand
Site 5	15.7	8.4	4.7	337	7.5	0.5	15.3	32.6	68.7	Sand
Site description	Site 1: Pristine area, no urban or agricultural activity is observed. Site 2: Entrance of urban area. Site 3: Urban area, two wastewater facilities are located Site 4: Agricultural influence area, Several feedlots and dairy are located. Site 5: Urban and agricultural influenced area.									

remove weakly bound impurities. 5 mL of methanol was used to extract the loaded sample into the 15 mL graduate cylinder containing 50 μL of the internal standard, simatone (0.24 mg/L stock solution), for quantification. Use of simatone as the internal standard was chosen based on a previous study and showed good response to our experimental conditions (Lindsey et al., 2001). The sample was concentrated with evaporation in a 50 °C water bath under a gentle nitrogen gas flow to prevent oxidation. The samples were concentrated to a volume of 50 μL and 70 μL of mobile phase solution (99.9 % water + 0.1 % formic acid, v/v) was added. The samples were transferred into amber vials equipped with 150 μl of glass inserts for HPLC-MS/MS analysis.

4.3.4 Sediment Sample Extraction

For sediment samples, two stages of analytical preparation were required. The first stage was to extract the compounds from the solid phase into the liquid phase. Different variables to optimize the extraction buffer solution for four groups of pharmaceutical and veterinary antibiotics were examined. Four different extraction buffer conditions of McIlvain buffer solution with varied citric acid concentration in buffer solution and buffer pH were compared for TCs and SAs. Also, three different pHs of the buffer solution were examined for optimizing the extraction buffer of MLs and IPs. Once the optimum conditions of the buffer solution were selected, the general procedure for extracting the pharmaceutical and veterinary antibiotics in sediment matrix was followed.

One g of sediment sample was weighed at an accuracy of 0.001 g and transferred into a 40 mL Teflon tube. 20 mL of McIlvain buffer solution or ammonium hydroxide buffer solution was added followed by 200 μL of 5 % Na_2EDTA (1mmol in solution). The sample was vigorously mixed in a parallel shaker (Model No-4626, Lab-line

instrument) for 20 minutes at 400 rpm. The sample was then centrifuged at 4000 rpm (IEC Clinical Centrifuge, International Equipment Co., Needham Hights, MA) for 15 minutes followed by filtration using 0.2 μm glass fiber filters. The filtered sample was decanted into another 40 mL vial and kept at 4 °C. Extraction was repeated in the same manner as described above and the supernatants were combined for the SPE cleanup procedure.

The second stage of the analytical preparation was for clean up and concentration of the sample. The SPE condition for this clean up and concentration step was the same as the aqueous sample except the addition of 0.5 M HCL was omitted.

4.3.5 High Performance Liquid Chromatography / Tandem Mass Spectrometry

The HPLC system was an HP 1100 Series Liquid Chromatograph (Agilent, Palo Alto, CA) equipped with an Agilent 1100 Series Thermostatted Auto Sampler and a variable wavelength UV detector. An XTerra MS C_{18} (Waters, Milliford, MA) 2.1×50 mm (2.5 μm pore size, end-capped) reversed-phase column was used to analyze the standards and samples. A C_{18} guard column (Phenomenex, Torrence, CA, USA) was used to filter any particulates from the sample.

A combination of three mobile phases was used depending on the antibiotic group being measured. Mobile phase A was composed of 99.9 % water and 0.1 % formic acid (v/v, pH 2.74), mobile phase B was 99.9 % of acetonitrile mixed with 0.1 % formic acid (v/v) and mobile phase C was pure MeOH. The column temperature was set to 15 °C for TC and SA measurement and the gradient for TCs was ramped from 96 % mobile phase A and 4 % of mobile phase B to 70 % mobile phase A and 30 % mobile phase B for 29

minutes and then 96 % mobile phase A and 4 % mobile phase B for 1 minute. The gradient for measurement of SAs was programmed for 21 minutes with the same conditions as for TCs. For MLs, the column temperature was set to 45 °C and the gradient was programmed to ramp from 80 % mobile phase A and 20 % mobile phase B to 65 % mobile phase A and 35 % mobile phase B for 14 minutes and then to the original condition for 1 minute. 10 minutes of post-run time was used to equilibrate the column. The flow rate for the three groups was set at 0.32 ml/min and the injection volume was 20 μL for TCs, SAs, and MLs.

For IPs, the column was maintained at 15 °C with a flow rate of 0.25 ml/min. The gradient was ramped from 50 % mobile phase A and 50 % mobile phase C to 10 % mobile phase A and 90 % mobile phase C in the first minute and held isocratic for 19 minutes. The injection volume was 20 μl and a 10-minute post-run period was allowed between each analysis to re-equilibrate the column. A ThermoFinnigan LCQ Duo ion trap mass spectrometer (ThermoQuest, Woburn, MA) equipped with a heated capillary interface and electrospray ionization (ESI) was used to perform the mass spectrometric analysis. 10 μM standard solutions of TCs, SAs, MLs, and IPs were made in DI water and injected using the LCQ Duo syringe pump at a flow rate of 5 μL/min to optimize the mass spectrometry parameters as needed. Nitrogen gas was used for drying and nebulizing. The spray voltage was set to 4.5 kV and the capillary voltage autotuned to 21 V. The capillary temperature was set to 165 °C and the instrument was operated in the positive ion mode. The sheath gas flow rate was optimized at 40 units (arb) and the auxiliary gas was turned off. The precursor ion and product ion optimized tandem mass spectrometry parameters are summarized in Table 4.2.

Table 4.2 Optimized HPLC tandem mass (MS/MS) parameters (bold face values were used to quantify the concentration of sample, simatone was detected with selective ion monitoring (SIM) mode).

Compounds	Precursor ion $[M+H]^+$ (*m/z*)	Fragment ions	Isolation Width	Collision Energy (%)
Simatone	198			
TC	445	**427**	2.0	26
CTC	479	**462**,444	2.0	32
OTC	461	**443**, 426	2.0	28
MNC	458	**441**	2.0	32
DMC	465	**448**	2.0	30
MCC	477	**460**	2.0	40
DXC	445	**428**	2.0	32
STZ	256	**156**	2.0	32
SMR	265	**156**, 190	2.0	36
SMT	279	156, **204**	2.0	38
SCP	285	**156**,108,92	2.0	32
SMX	254	156, **188**	2.0	36
SDM	311	**156**, 245	2.0	38
ETM-H_2O	716	522, **558**	3.0	26
RTM	837	558, **679**	3.0	26
TYL	916	**772**	3.0	30
Compounds	Precursor ion $[M+Na]^+$ (*m/z*)	Fragment Ions	Isolation Width	Collision Energy (%)
MNS	693	**675**	2.0	28
SLM	773	**755**,531,431	2.0	30
NRS	787	**769**,531,431	2.0	30

4.4 Results

4.4.1 Method Optimization for Ionophore Antibiotics in the Aqueous Phase

The main parameters used to optimize the extraction condition of IPs were sample pH and cartridge preconditioning additives between methanol and DI water. For the optimized extraction method development, a standard solution of three IPs (2 μg/L) was spiked in 120 mL of control river water from sampling site 1. Sample from sampling site

1 was assumed to be a control since this part of the river is pristine and no residual of compounds was found after analysis with the optimized method.

Sample pH adjustment was the first variable for optimizing extraction efficiency in the aqueous sample. Previous research adapted for extracting TCs, SAs, and MLs shows that sample pH is an important factor to increase the retention on the cartridge or to convert a degradation ion for quantification (Lindsey et al., 2001; Yang et al., 2004a; Yang et al., 2004b). Thus, two sample pH adjustment processes were examined.

As shown in Table 4.3, the observed recovery ratio of the sample that pH was adjusted to 2.5 using 40 % H_2SO_4 (v/v) is lower than any other sample with no pH adjustment. This result indicates that IPs are unstable in strongly acidic conditions. Since the measured pH of the samples for the five sampling sites ranged from 7.4 to 8.4 (data shown in Table 1) and the pKa value of the three IPs being measured is approximately 6.4, no pH adjustment was selected for the optimized method.

Table 4.3 Examined SPE conditions for optimizing three IPs in aqueous phase and comparison of observed recovery ratio (Average of triplicate (%) ± Relative standard deviation (%)).

	Sample pH			
	2.5	No adjustment		
		Cartridge preconditioning Additives		
		0.5M HCL	5% Na_2EDTA (w/v)	No Addictives
MNS	13.4 ± 2.4	39.7 ± 6.6	68.4 ± 1.4	107.8 ± 4.9
SLM	9.3 ± 8.2	10.6 ± 5.5	31.4 ± 8.3	108.7 ± 6.1
NRS	5.7 ± 6.6	8.2 ± 8.1	20.5 ± 5.0	78.2 ± 5.5

The second variable studied was the cartridge additives between MeOH and DI water using 3 mL of 0.5 M HCL, 5 % Na_2EDTA (w/v), and no additives. 0.5 M HCL and 5 % Na_2EDTA were chosen as additives to increase the hydrophobicity and to remove

metal residual in the cartridge, respectively. The lowest recovery ratio was observed among the three different conditions when 0.5 M HCL was added to the cartridge and the highest recovery ratio was measured when no additives were added. This result indicates that hydrophobicity is not the main mechanism in the cartridge to bind the IPs. Also, Na_2EDTA not only reacts with metals but also with IPs in the cartridge resulting in lower extraction efficiency. Thus, no sample pH adjustment and no addition between methanol and DI water in the cartridge were selected as the optimum extraction condition for IPs in the aqueous phase. The rest of procedure is explained in the experimental section.

4.4.2 Method Optimization for Human and Veterinary Antibiotics in Sediment

During analytical method development, the pH of the buffer solution used for sediment extraction was highly dependent on the pKa value of each compound. TCs have three pKa values (3.3/7.7/9.3) and SAs are characterized by two pKa values (2-3/5-11) (Bruhn, 2003). Specifically, TCs are relatively stable in acid, but not in bases. Based on the physicochemical properties of the two groups, a McIlvain buffer solution (pH 4.0) was chosen as the extraction buffer solution for TCs and SAs. Furthermore, the McIlvain buffer solution is widely used to extract tetracyclines in biological matrices (e.g. tissue, egg, milk, muscle) (Cooper et al., 1998; Cinquina et al., 2003).

Standard solutions of the four antibiotic groups (180 μg/kg) were spiked in 1 g of control sediment sample prior to extraction and the recovery ratio was determined. Again, control sediment samples from sampling site 1 were assumed to have no residual of the examined compounds due to the lack of agricultural and urban activity around or

upstream of the sampling site. This assumption was confirmed after method optimization found no background concentration of the investigated compounds.

Table 4.4 Summary of different buffer concentrations and pH conditions for optimizing extraction of TCs and SAs in sediment phase and comparison of observed recovery ratio (Average of triplicate (%) ± Relative standard deviation (%))

	Citric acid concentration in McIlvaine buffer (M)			
	0.01		0.1	
	McIlvaine buffer pH			
	4.0	2.5	4.0	5.5
TC	29.7 ± 8.6	69.7 ± 7.0	105.0 ± 4.9	104.3 ± 5.6
CTC	13.4 ± 4.4	19.8 ± 2.4	74.7 ± 3.5	57.4 ± 15.6
OTC	27.5 ± 9.4	109.1 ± 9.1	45.0 ± 3.9	36.4 ± 11.7
MNC	30.8 ± 9.1	47.9 ± 1.4	< 30	< 30
DMC	12.6 ± 4.6	113.2 ± 14.3	39.5 ± 5.7	ND
MCC	64.3 ± 9.2	13.2 ± 15.3	109.7 ± 17.8	32.2 ± 2.9
DXC	51.3 ± 2.3	ND	98.1 ± 4.2	42.3 ± 7.4
STZ	90.6 ± 3.7	78.1 ± 10.3	89.7 ± 2.0	79.9 ± 4.9
SMR	86.4 ± 5.3	77.3 ± 7.2	100.8 ± 12.8	77.0 ± 1.0
SMT	81.4 ± 4.4	80.2 ± 16.7	100.4 ± 1.3	79.4 ± 8.3
SCP	60.9 ± 5.8	62.4 ± 8.8	94.1 ± 3.1	59.6 ± 7.5
SMX	68.4 ± 3.4	86.0 ± 3.5	104.4 ± 2.9	86.2 ± 4.2
SDM	109.9 ± 7.8	68.5 ± 1.0	97.5 ± 3.9	77.2 ± 1.2

For further investigation, the citric acid concentration and pH of the McIlvaine buffer solution was varied and the recovery ratios are summarized in Table 4.4. The McIlvaine buffer solution was prepared according to United States Department of Agriculture (USDA) guide for measuring TCs in tissues (USDA, 2003). In general, TCs show the highest variability depending on the compound and the measured recovery ratio of SAs shows little variation with four different conditions. The first comparison was made with 0.01 and 0.1 M citric acid concentrations in McIlvaine buffer solution with the same pH (4.0). As the citric acid concentration is lowered, the extraction efficiency of the seven TCs decreased. The recovery ratio of four of the seven TCs was below 30%. This

result may indicate that the 0.01 M citric acid concentration is not strong enough to desorb the antibiotics from the sediment particles. The citric acid concentration was increased to 0.1 M in the McIlvaine buffer solution and the pH of the buffer solution was varied with formic acid (99 %) and NH_4OH (38 %). TC, CTC, MCC, and DXC show the highest recovery ratio at a pH 4.0 yet the recovery ratio of MNC was below 30 %. Since all seven TCs were recovered with an acceptable recovery ratio at pH 4.0, the optimized pH of the McIlvaine solution was determined to be pH 4.0.

In addition, Na_2EDTA was used to chelate metals that were present in samples before extraction. However, as the added amount of Na_2EDTA was increased, the recovery efficiency was decreased in sediment for TCs (data not shown). This result may indicate that excess amounts of Na_2EDTA chelate not only metals but also organic compounds affecting the recovery efficiency. Thus, the optimum amount of Na_2EDTA was chosen as 1 mmol in the 40 ml sediment extracting solution.

As mentioned before, no noticeable difference was observed in SA extraction with the four different conditions. Thus, pH 4.0 of McIlvaine solution was also used to extract SAs for experimental convenience.

Since little has been reported on extracting MLs and IPs from sediment samples, a wide range of buffer pH was examined and summarized in Table 4.5. For pH 3.75, 1 M of formic acid was titrated with NH_4OH and 1 M of NH_4OH was titrated with formic acid to make pH 5.5 and 10.0 buffer solutions. The recovery ratio of all MLs and IPs was below 30 % for pH 3.75 and 5.5 except ETM-H_2O. In contrast, the recovery ratio showed good extraction efficiency at pH 10.0 for all measured compounds. This result seems to indicate that the basic characteristic of the MLs (pKa: 7.7-8.9) and the neutral properties

of IPs (pKa: 6.4) require a high pH buffer solution to increase the extraction efficiency in the sediment matrix.

Table 4.5 Summary of different buffer pH conditions for optimizing extraction of MLs and IPs in sediment phase and comparison of observed recovery ratio (Average of triplicate (%) ± relative standard deviation (%)).

	Buffer pH		
	3.75	5.5	10.0
ETM-H_2O	37.3 ± 2.6	52.3 ± 11.1	109.1 ± 15.9
RTM	14.8 ± 4.9	12.5 ± 3.7	47.2 ± 2.5
TYL	19.3 ± 7.4	22.9 ± 5.8	104.0 ± 1.9
MNS	11.1 ± 7.0	25.0 ± 2.3	113.2 ± 11.0
SLM	4.4 ± 4.0	15.8 ± 5.8	80.7 ± 12.1
NRS	16.0 ± 6.1	9.7 ± 8.6	59.1 ± 3.1

4.4.3 HPLC Tandem Mass Analysis

The HPLC tandem mass spectrometry (MS/MS) method was used to quantify all 19 compounds. Once the precursor ion, $[M+H]^+$, for TCs, SAs, and MLs was determined, fragmentation was conducted via collision-induced dissociation (CID) in the ion trap. In the case of ETM, ETM-H_2O [M+H-H2O] was chosen as the precursor ion based on previous research (Hirsch et al., 1999; Yang et al., 2004a). Since the mobile phase was used in an acidic condition, the degraded form of ETM as ETM-H_2O would be expected. The sodium complex ion, $[M+Na]^+$, was chosen as the precursor ion for IPs followed by the fragmentation procedure in the ion trap. The product ion showing the highest signal was chosen for selective reaction monitoring (SRM) and quantification. Representative full scan tandem mass spectra of each group; CTC, SCP, and TYL are shown in Figure 4.4.

The product ion of all 19 compounds using the acidic mobile phase condition has been confirmed by several researchers (Hirsch et al., 1998; Lindsey et al., 2001; Zhu et al., 2001; Yang et al., 2004a; Yang et al., 2004b). For TCs, the common fragment ion was a neutral loss of 17 amu, $[M+H-NH_3]$, and 35 amu, $[M+H-NH_3-H_2O]$ except for the 18 amu loss of TC and OTC. The common fragment ion, *m/z* 156, representing the sulfanilyl ring, was seen for all 6 SAs. This common ion was used to quantify the concentration of each sulfonamide.

For macrolides, each compound has different cleavage pathways. Hirsch et al. (Hirsch et al., 1999) suggests that ETM loses one molecule of water at a pH of 7.0. Also, he confirmed that the degraded form of erythromycin exists in the aquatic environment. Thus, $[M+H-H_2O]$ (*m/z* 716) was used as the precursor ion. Two fragment pathways for ETM-H_2O, $[M\text{-desosamine-}2H_2O+H]^+$ (*m/z* 522) and $[M\text{-desosamine-}H_2O+H]^+$(*m/z* 558) were detected under our condition. $[M\text{-desosamine}+H]^+$ (m/z 679) was used to quantify the concentration of RTM. For TYL, $[M\text{-cladinose}+H]^+$ (m/z 772) was used to quantify the concentration of the sample.

Chromatograms showing the standard solution (2 μg/L) of the three IPs and full scan tandem mass spectra for the three compounds are presented in Figure 4.5 and 4.6.

For MNS, water loss,$[M+Na-H_2O]$, from the precursor ion was detected at the low collision energy, 28 %. For SLM and NRS, two major fragment pathways were found in addition to the neutral water loss at the collision energy of 30 %. Volmer *et al.* (1998) proposed that initial cleavage of the oxygen activated carbon-carbon bonds on either sides of the C (11) carbonyl function is involved in the pathway. β-Cleavage and subsequent hydrogen migration occurs at C (9) – C (10) and produces the first fragment

ion at *m/z* 531. The other fragment ion at *m/z* 431 originates from the C (12) – C (13) bond dissociation (Volmer et al., 1998).

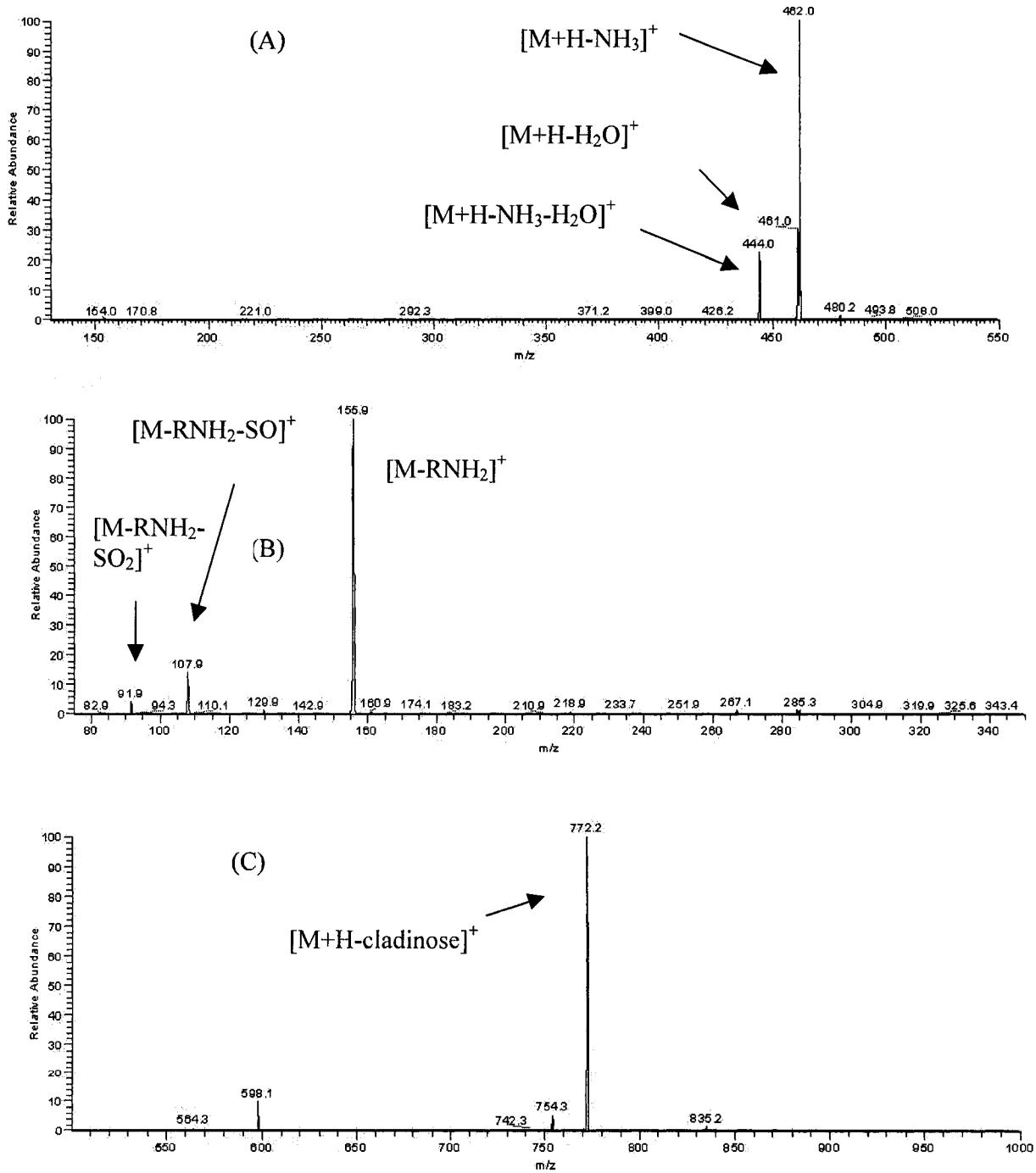


Figure 4.4 Full scan tandem mass spectra of: (A) CTC, (B) SCP, and (C) TYL

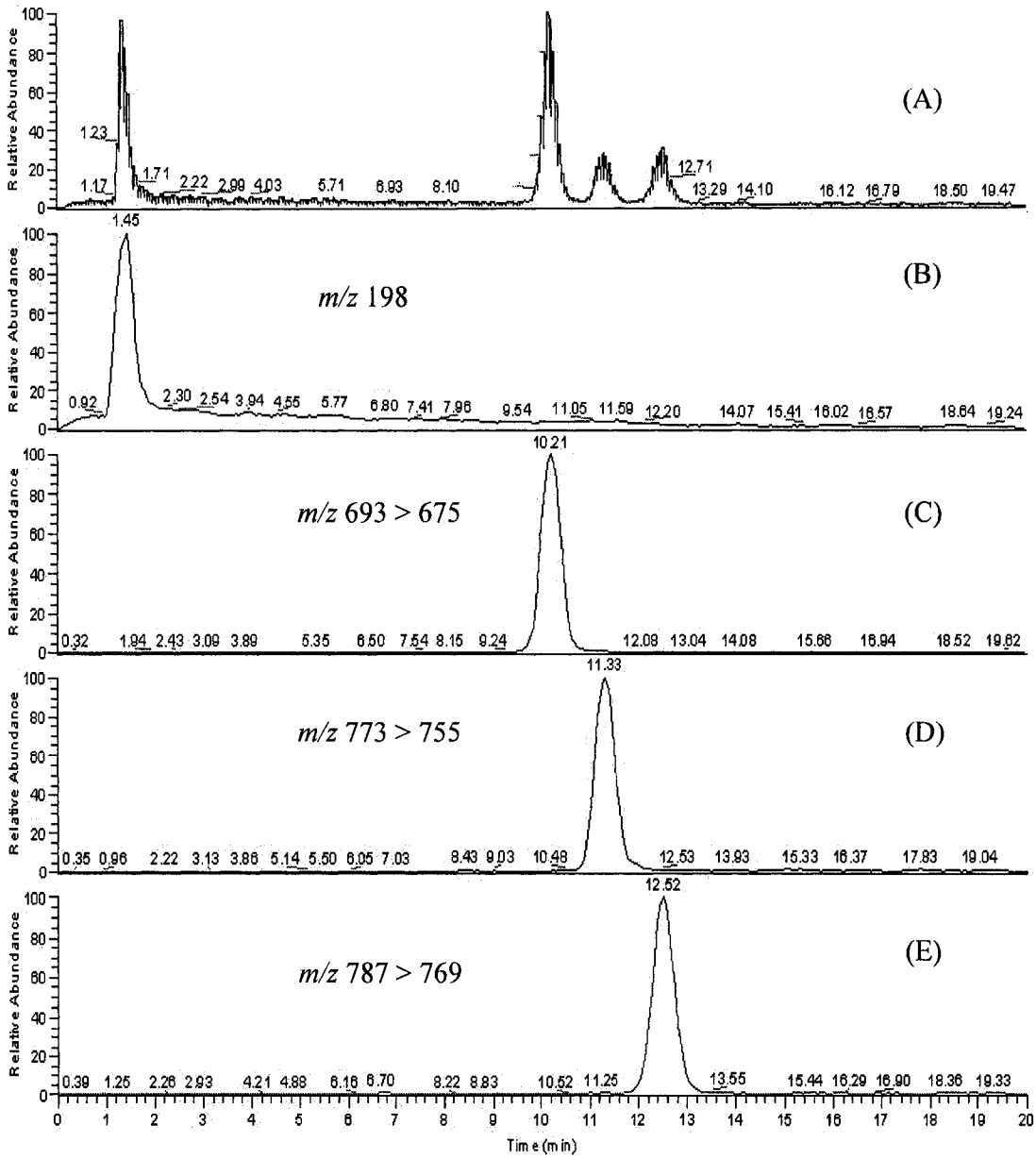


Figure 4.5 Reconstructed chromatogram showing standard solution (2µg/l) of three IPs: (A) total ion chromatogram (TIC), (B) simatone (internal standard), (C) MNS, (D) SLM, and (E) NRS

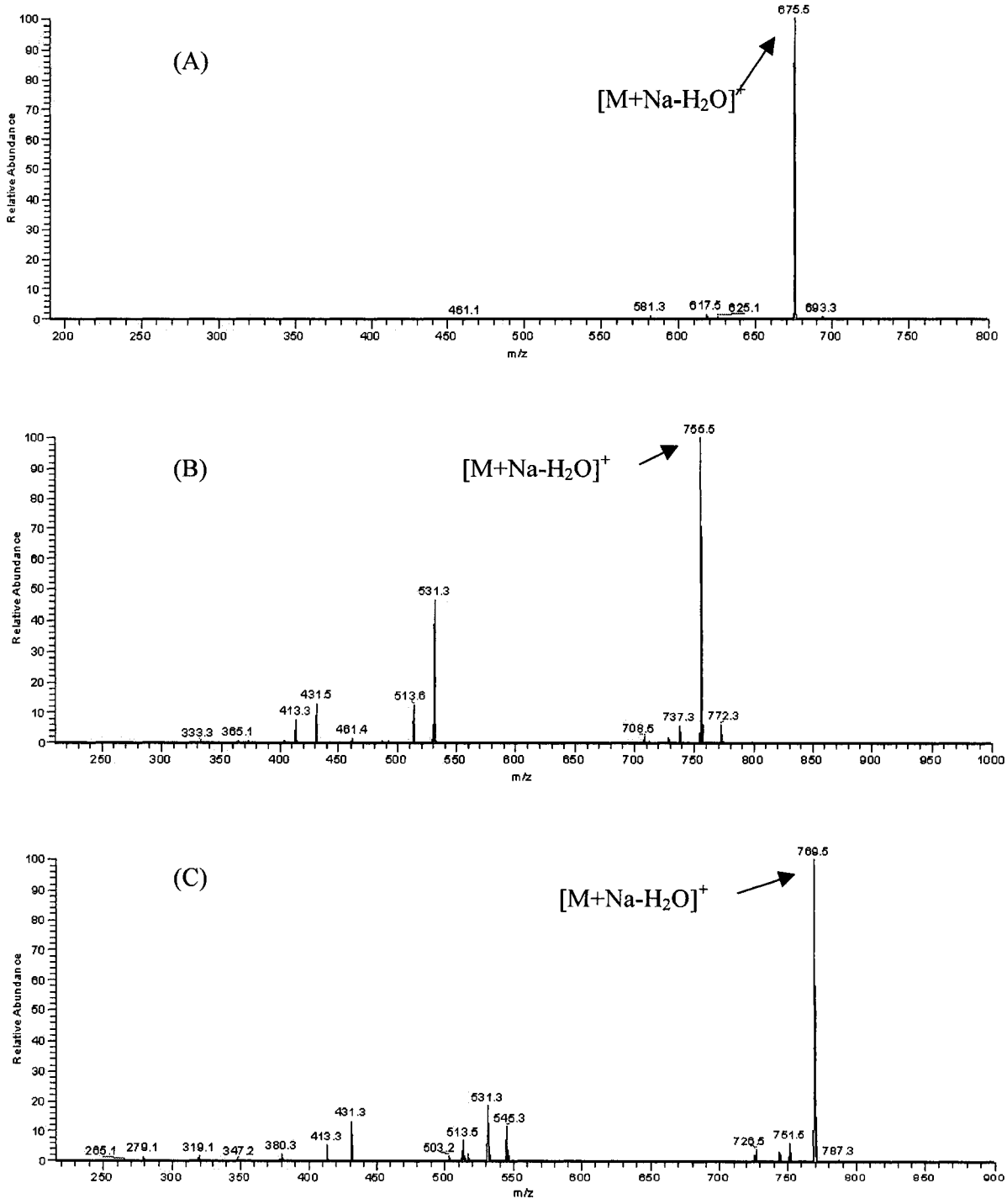


Figure 4.6 Full scan tandem mass spectra of: (A) MNS, (B) SLM, and (C) NRS

4.4.4 Recovery and Limit of Quantification (LOQ)

The recovery efficiency of the extraction process was studied by comparing spiked samples into the control river water or air-dried sediment samples before the SPE and spiked sample after SPE into the MeOH extractant. For the water recovery study, three different concentrations, 0.1 μg/L, 1 μg/L, and 5 μg/L were examined. The desired concentration was spiked in 120 mL of water from sample site 1, the pristine river water, and the procedure described in the experimental section was followed. Before using sample site 1 water as a control (no antibiotic compounds), experiments were conducted to confirm the absence of residual antibiotics in the sample. The recovery ratio was calculated as shown in Equation 4.1.

$$\text{Recovery Ratio} = \frac{\text{Detect Response of Spiked sample prior to SPE}}{\text{Detect Response of Spiked sample after SPE}} \times 100 \qquad \textit{Eq. 4.1}$$

The detect response was calculated using the area of the internal standard, simatone, and the area of each sample based on HPLC-MS/MS analysis. The calculated recovery for three different concentrations in water and sediment is shown in Table 4.6. Recoveries were determined for a sediment matrix at concentrations of 1 μg/kg, 30 μg/kg, and 90 μg/kg. Sediment from sample site 1 was examined before the experiment to verify the absence of residual antibiotics and the suitability of this sample as a control.

Table 4.6 Recovery study for water and sediment (N = 3 independent sample, average recovery is shown. Relative standard deviation ranged between 1 and 13 % for water and 16 and 27 % for sediment).

	Water			Sediment		
	0.1 µg/L	1 µg/L	5 µg/L	1 µg/kg	30 µg/kg	90 µg/kg
TC	106.6	109.6	104.2	82.2	90.5	91.2
CTC	104.2	124.8	107.3	90.6	69.3	64.9
OTC	100.2	102.6	106.3	43.9	53.3	40.5
MNC	< 30	< 30	< 30	< 30	< 30	< 30
DMC	124.5	127.3	109.5	106.2	44.1	32.8
MCC	112.3	106.0	104.9	107.7	100.7	114.8
DXC	109.7	122.8	103.9	101.1	74.8	78.3
STZ	77.8	101.9	92.2	108.9	106.1	78.1
SMR	100.0	103.2	91.9	98.8	102.1	77.3
SMT	108.4	109.5	96.6	92.3	105.5	80.2
SCP	104.1	87.9	87.1	100.1	111.7	62.4
SMX	124.3	92.5	76.6	97.5	107.9	86.0
SDM	78.3	99.0	85.8	87.6	104.8	68.5
ETM-H_2O	102.3	101.4	103.5	127.4	128.4	101.8
RTM	104.9	114.1	114.3	69.1	53.4	76.0
TYL	103.2	89.5	114.7	79.7	74.9	77.5
MNS	113.8	123.6	106.3	71.3	71.1	105.4
SLM	117.5	82.7	99.4	98.1	73.6	72.2
NRS	109.5	103.6	102.1	56.6	51.7	51.3

To study the loss of antibiotics during SPE with the sediment matrix, the desired concentration was spiked at three different stages of the procedure. First, the desired concentration was spiked into the sample prior to extraction and then the same recovery procedure described in the experimental section was followed. Second, the recovery was determined when spiking the desired concentration immediately before SPE and following the procedure described earlier. Finally, the desired concentration was spiked after SPE into the 5mL of methanol extractant. The results of this experiment showed less than a 5 % loss between the second and third stages indicating a slight loss of antibiotics due to SPE for the sediment matrix. Thus, the recovery for sediment was calculated as the difference between stages 1 and 3 in the experiment described above.

The range of percentage recoveries for TCs, SAs, MLs, and IPs in the water matrix was 102.2-124.8, 76.6-124.3, 89.5-114.7, and 82.7-117.5 with 1-13 % of relative standard deviation, respectively, with three different concentrations. For sediment, percent recovery ranged from 32.8-114.8, 62.4-108.9, 53.4-128.4, and 51.3-105.4 with a relative standard deviation range of 16-27 %. These values were calculated for TCs, SAs, MLs, and IPs individually with three different concentrations. The recovery ratio for TCs and MLs was slightly higher than 100 %. This result indicates that matrix effects can impact the recovery in surface water samples. A matrix effect of surface water can result in the enhancement of signal intensity showing higher than 100 % of recovery in the sample (Lindsey et al., 2001). Recovery of MNC shows less than 30 % for the three measured concentrations confirming the fact that the two amino groups of MNC increase the cation exchange at the low pH condition (Lindsey et al., 2001).

For MNS, a concentration of 90 μg/kg showed the highest recovery rate with 105.4±1.0 but significantly lower recoveries (71 %) were observed at lower concentrations. In contrast, the lowest concentration range of 1 μg/kg showed the highest recovery for SLM (98.1 %) with lower recoveries at higher concentrations. The recovery of NRS in a sediment matrix was lower than the other IPs. The lower recovery of NRS is likely due to the inability of ammonium hydroxide to hydrolyze the bond between this compound and sediment particles since NRS can easily bind to soil when an abundance of mono- or divalent cations are present.

Quantification limits have been determined by other researchers using several methods including the signal/noise ratio (Hamscher et al., 2002), selection of the second lowest point of the calibration curve(Hirsch et al., 1998; Hirsch et al., 1999), and a

statistical method using the student *t*-variate (Zhu et al., 2001). Two approaches were used in this study to determine the quantification limits for both water and sediment. First, the lowest point of the calibration curve with a signal/noise ratio (S/N) of greater than 3 was determined as a limit of quantification (LOQ). LOQ was 0.01 μg/L for TCs, SAs, and MLs and 0.001 μg/L for IPs with a S/N range of 3 – 10 depending on the compounds. For sediment, the LOQ was determined to be 1 μg/kg with a signal/noise ratio of greater than 3 for TCs, SAs, and MLs and 5 for IPs at this concentration. Different signal/noise ratios were used for LOQ determination due to variable instrument responses. Subsequently, 0.001 μg/L and 0.01 μg/L concentrations of antibiotics for water (depending on the compound groups) and 1 μg/kg for sediment were spiked in the control sample from sampling site 1 to verify the LOQ with the statistical method. Three independent experiments were conducted and the concentration of each sample was calculated based on the external calibration curve. The calculated standard deviation of each concentration was multiplied by the Student's *t*-variate for a one-sided *t*-test at the 95 % confidence interval to estimate the LOQ. Table 4.7 shows the LOQ calculated with both techniques.

Calibration curves were constructed for the range of 0.001 μg/L to 5 μg/L for water and 1 μg/kg to 90 μg/kg for sediment and all calibration curves were linear with r^2 > 0.99. In order to consider the matrix effect, calibration curves were constructed using river water and sediment instead of DI water. The extraction procedures described previously were used for these samples.

Table 4.7 Limit of quantification (LOQ) for four different antibiotic groups in water and sediment matrices. (The average concentration of each compound was calculated for TCs, SAs, and MLs. The concentration of the three individual compounds was calculated for IPs. LCP denotes the lowest calibration curve point).

	Water (N=3) (μg/L)		Sediment (N=3) (μg/kg)	
	LCP	Statistical Method	LCP	Statistical Method
TC	0.01	0.01	1.0	1.9
CTC	0.01	0.01	1.0	0.9
OTC	0.01	0.01	1.0	0.5
MNC	0.01	0.01	1.0	0.3
DMC	0.01	0.02	1.0	2.5
MCC	0.01	0.04	1.0	1.8
DXC	0.01	0.04	1.0	1.2
STZ	0.01	0.01	1.0	1.2
SMR	0.01	0.02	1.0	1.4
SMT	0.01	0.01	1.0	1.8
SCP	0.01	0.01	1.0	1.7
SMX	0.01	0.02	1.0	0.5
SDM	0.01	0.01	1.0	0.3
ETM-H_2O	0.01	0.01	1.0	1.7
RTM	0.01	0.02	1.0	0.5
TYL	0.01	0.03	1.0	1.1
MNS	0.001	0.001	1.0	0.4
SLM	0.001	0.002	1.0	3.6
NRS	0.001	0.003	1.0	0.7

4.5 Occurrence of Antibiotics in a Watershed

The measured concentrations of the antibiotics in water and sediment are summarized in Table 4.8. As expected, no studied antibiotics were found in sampling site 1, pristine region. Thirteen compounds were detected out of the 19 that were examined in the water matrix. For the TCs, MCC shows the highest concentration with 0.11 μg/L at sampling site 3. SMX was detected at sampling site 3 with the highest water concentration of 0.08 μg/L among SAs. Of the MLs, only ETM-H_2O was detected with

the highest concentration of 0.18 μg/L at sampling site 3. It was not surprising that the highest concentration of MCC and SMX that are assumed to be human influenced antibiotics was detected in sampling site 3 located in near wastewater facilities (Table 4.1). IPs were detected at sampling sites 2 - 5 with the highest concentration of NRS (0.038 μg/L) at sampling site 3. Although certain amount of NRS was found in site 2 and 3, the other two IPs, MNS and SLM, were only detected in sampling site 4 and 5. Those locations are regarded as heavily animal influenced area and our study result might indicate that MNS and SLM are agricultural originated.

The concentration of the four antibiotic groups in sediment shows significantly higher concentrations compared to the overlying water matrix. Even though RTM and TYL were not detected in the water matrix, 5.9 μg/kg of RTM and 2.6 μg/kg of TYL were detected at sampling sites 3 and 5 in the sediment. These results underscore the importance of developing the capability for measuring these compounds in a sediment matrix.

4.6 Conclusion

An analytical method using HPLC tandem mass spectrometry for different groups of human and veterinary antibiotics in river water and sediment was developed. The SPE condition was optimized depending on the physicochemical characteristics of compounds in river water. For sediment, two different buffer solutions were chosen to extract compounds from solid phase to liquid phase for further purification. The method was utilized to measure 19 antibiotic compounds in river water and sediment throughout a watershed and the results indicate that the concentration in sediment is much higher than river water. The high rate of partitioning of these compounds to the sediment

demonstrates the importance of including the sediment matrix in antibiotic fate and occurrence studies.

Table 4.8 Measured antibiotic concentrations of the four groups. The data represents the average of three independent sample concentrations and standard deviation. Values less than limit of quantifications are not shown. P1 to P5 represents the sampling sites

	Water (μg/L)					Sediment (μg/kg)				
	P1	P2	P3	P4	P5	P1	P2	P3	P4	P5
TC					0.02±0.01		9.0±0.4	32.8±0.3	4.5±0.4	21.3±0.3
CTC			0.01±0.01	0.04±0.01			9.6±0.2	11.6±0.4	24.9±0.3	30.8±0.3
OTC					0.01±0.00		5.6±0.4	8.3±0.9	6.6±0.5	5.9±0.3
MNC										
DMC							3.1±0.7	5.6±0.8	2.6±0.6	3.6±0.2
MCC		0.03±0.01	0.11±0.04	0.03±0.03	0.05±0.02		6.3±0.5	15.7±0.8	8.5±0.6	9.9±0.3
DXC			0.02±0.02	0.01±0.01	0.02±0.02		13.0±0.5	27.6±0.5	19.9±1.2	15.2±0.3
STZ				0.01±0.01			2.6±0.6	3.4±0.6	3.2±0.4	1.7±0.5
SMR			0.06±0.02	0.01±0.01						
SMT										
SCP								2.7±0.4	2.8±0.3	1.9±0.2
SMX			0.08±0.01	0.07±0.01	0.06±0.01		1.2±0.2	1.6±0.4	1.6±0.2	1.7±0.6
SDM				0.04±0.00	0.01±0.01		1.7±0.3	1.7±0.5	3.6±0.2	2.3±0.8
ETM-H_2O			0.18±0.03	0.02±0.01	0.03±0.00			12.9±0.3	5.2±0.7	11.9±0.9
RTM								5.9±0.4		1.4±0.5
TYL									2.4±0.4	2.6±0.1
MNS				0.011±0.001	0.009±0.003				14.6±0.2	2.9±0.3
SLM				0.006±0.001	0.007±0.001				3.7±0.4	2.3±0.3
NRS		0.025±0.001	0.038±0.003	0.032±0.001	0.035±0.005		2.5±0.4	3.3±0.3	5.3±0.3	5.2±0.2

Chapter 5

Occurrence of Ionophore Antibiotics in Water and Sediments of a Mixed-Landscape Watershed

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5.1 Abstract

Analytical methods for quantifying three ionophore antibiotics, monensin, salinomycin, and narasin, were developed for water and sediment matrices. Sample preparation was based on solid phase extraction (SPE) and HPLC tandem mass spectrometry (HPLC/MS/MS) was used to separate and detect the compounds. Recoveries ranged from 83 to 117% for water and from 51 to 105% for sediment in three different concentrations with less than 10% of relative standard deviation. The statistical detection limit was 0.001 – 0.003 μg/L for water and 0.4 – 3.6 μg /kg for sediment respectively. Ionophore antibiotics are only used to treat coccidiostats for broilers or turkeys, and to increase growth and feed efficiency for beef and dairy cattle. Since they are not used for human purposes, these compounds can act as markers for the transport of animal pharmaceuticals to the watershed. The occurrence of three ionophore compounds was determined at five sampling sites along the Cache la Poudre River in Northern Colorado representing pristine, urban, and agriculture landscapes. Statistical analysis demonstrates

that the measured concentration was significantly different among sampling sites in different sampling events for both water and sediment. In addition, significant differences were observed among different sampling times at each sampling site. Furthermore, all three ionophores were found in the sediments at much higher concentrations than in water indicating the importance of this matrix when determining environmental impacts.

Key words: Ionophore antibiotics; Sediment; HPLC/MS/MS; Pseudo partitioning coefficient

5.2 Introduction

Human and veterinary pharmaceutical compounds in the environment have received increased attention in recent years. These medicines are used for therapeutic treatment of infectious diseases in humans and for treating and protecting the health of animals (Bruhn, 2003). In addition, veterinary antibiotics are used to promote growth and feed efficiency in a range of animals. Rumensin, for example, is a common feed additive for beef cattle that contains the antibiotic monensin.

Human-used pharmaceutical compounds are found in sewage treatment plants (STPs) and released into the environment through this point-source mechanism. Several previous studies have reported human-used pharmaceuticals in surface water and groundwater (Richardson et al., 1985; Hirsch et al., 1999; Lindsey et al., 2001; McArdell et al., 2003; Yang et al., 2004a; Yang et al., 2004b). Among others, tetracyclines, sulfonamides, and macrolides are the most frequently detected classes with measured concentrations of less than 1 μg/L. Recently, a nationwide reconnaissance of the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants (OWCs) in water samples from a network of 139 streams across 30 states during 1999

and 2000 was conducted (Kolpin et al., 2002). They found OWCs in 80% of the streams sampled. In terms of antibiotics, trimethoprim, a potentiator often administrated with sulfonamides, was detected most frequently (27.4%) with a maximum concentration of 0.30 μg/L.

Meanwhile, veterinary pharmaceuticals (VPs) can be introduced into the environment in agricultural landscapes when manure and liquid waste containing residual VPs is land applied or when waste lagoons leak. Measured concentration of VPs in animal waste or manure from previous studies has ranged from 11 to 12400 μg/kg (Haller et al., 2002; Schlusener et al., 2003). Residuals of two antibiotics extensively used in livestock production were recently detected in soil fertilized with animal slurry (Hamscher et al., 2002). This study found an average concentration of 198.7 μg/kg of tetracycline and 4.6 – 7.3 μg/kg of chlortetracycline at a soil depth of 10-20 cm. The researchers concluded that when liquid manure is applied repeatedly, antibiotics could enter the environment in significant concentrations and accumulate persistent residues in the soil. Another study also measured the concentrations of chlortetracycline and tylosin in soil taken from manure-amended fields and found 0.6 – 15.5 μg/kg for chlortetracycline and 1.8 – 57.4 μg/kg for tylosin during the following 146 days period (Jacobsen et al., 2004). In addition, a recent study compared two different techniques (Enzyme-Linked Immunosorbent Assay and Liquid Chromatography-Mass Spectrometry) to determine the persistence of tetracyclines and concluded that tetracyclines are strongly sorbed on to soil particles leading to a low mobility (Aga et al., 2005).

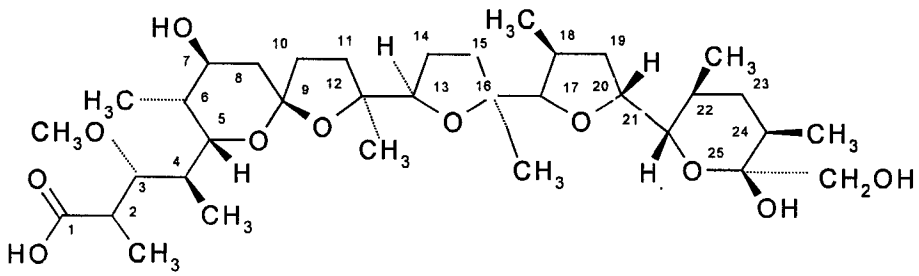
To evaluate the transfer of VPs from manure to soil and even surface water, the concentration of VPs was measured in three different environmental compartments. The study verified that certain VPs are highly stable in manure and have a strong potential to transfer from manure to soil and surface water (Christian et al., 2003; Liguoro et al., 2003).

Drugs used in aquaculture can also be transported directly into surface water or accumulate in the sediment (Jacobsen et al., 1988; Bjorklund et al., 1990). In these studies, a range of 0.3 - 16 μg/g oxytetracycline was found in the sediments downstream of a fish farm.

Monensin, salinomycin, and narasin (Figure 5.1) are ionophore antibiotics predominantly produced by *Streptomyces*. These antibiotics are used in veterinary applications as coccidiostats for broilers, turkey, and layers and as growth promoters for beef and dairy cattle (Harris et al., 1998; Volmer et al., 1998). An important aspect of the ionophore class of antibiotics is their exclusive use in veterinary applications.

Several methods have been developed to measure and quantify ionophore antibiotics in different matrices. High-performance thin layer chromatography was used for measurement of monensin in feed and food (Bertini et al., 2003). Also, enzyme-linked immunosorbent assay (ELISA) methods have been developed but these techniques only detected one compound and the cross-reactivity with metabolites was poorly understood lessening the utility of the method (Rosen, 2001). High performance liquid chromatography mass spectrometry (HPLC-MS) or HPLC-tandem MS (MS/MS) techniques have been shown to be the most sensitive and powerful methods for identifying and quantifying ionophore antibiotics in different matrices. HPLC-MS/MS

(A)



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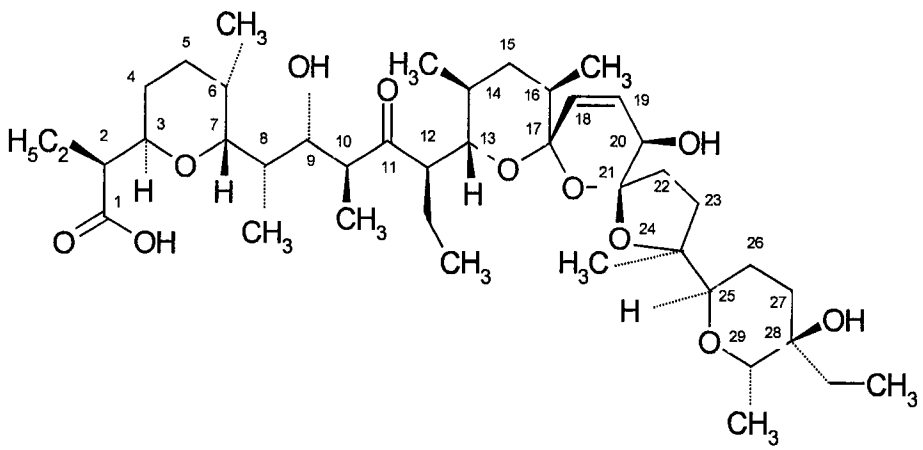

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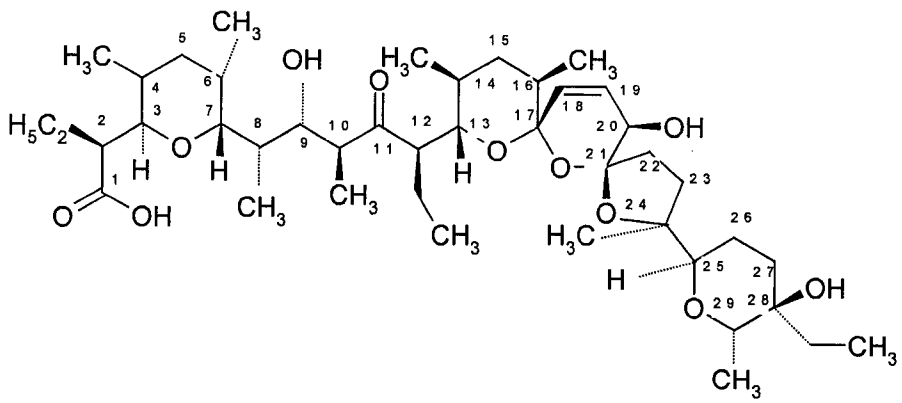

Figure 5.1 Chemical structure of ionophore antibiotics that were examined in this study: (A) Monensin, (B) Salinomycin, (C) Narasin

has also been adapted for measurement of feed samples (Volmer et al., 1998; Turnipseed et al., 2001; Hormazabul et al., 2002), liver and eggs (Rosen, 2001; Matabudul et al., 2002), and animal tissue (Matabudul et al., 2001).

Another separation technique, HPLC-atmospheric pressure chemical ionization, was used to quantify monensin and salinomycin in liquid manure (Schlusener et al., 2003). These researchers found 11μg/kg of salinomycin in manure samples with an average recovery of 119%.

Human and veterinary pharmaceutical compounds have been found in groundwater, surface water, waste lagoon water and effluent water from wastewater treatment plants. Since previous research has shown that ionophore antibiotics are hydrophobic compared to other pharmaceuticals, significant concentrations in sediments would be expected (Tolls, 2001). However, limited research on the occurrence of antibiotics in river sediments has been reported.

The objective of this study was to simultaneously determine the occurrence of the ionophore antibiotics, monensin, salinomycin, and narasin in the surface water and sediment of a river exposed to pristine, urban and agricultural landscapes. Since ionophore antibiotics are only used in veterinary and animal feed applications, their presence would verify the existence of a farm-to-stream transport mechanism. A secondary objective was to understand the partitioning of antibiotics into the sediments to provide an understanding of the importance of quantifying compounds in this matrix.

5.3 Materials and Method

5.3.1 Materials

The ionophore antibiotics, monensin (90-95%) and narasin (97%) as a sodium salt were purchased from Sigma-Aldrich Co. (St. Louis, MO). Salinomycin sodium salt (96%) was purchased from ICN (Aurora, OH). HPLC grade methanol (99.9%) and analytical grade formic acid (99%) were obtained from Sigma-Aldrich Co. (St. Louis, MO). 50 mg/L of each standard solution was prepared in methanol and stored at 4°C before use. Working solutions, 5 mg/L and 0.5 mg/L, were prepared weekly by dilution of the standard solution in methanol. The solid phase extraction (SPE) cartridges had a capacity of 3cc/60 mg of HLB (hydrophillic-lipophillic-balanced) and were purchased from Waters Oasis Co. (Milford, MA). Milli-Q water from a Millipore (Billerica, MA) purification system was used when DI water was required.

5.3.2 Sample Collection and Site Description

Both water and sediment samples were collected in four different time periods from May 2003 to February 2005 along the Cache la Poudre River in Northern Colorado. Each sampling date had different stream flows measured at four different USGS gauging stations. The collected samples for both water and sediment during May 2003 represent the high flow, due to snowmelt and runoff. In contrast, the samples collected during April 2004 represent low flow conditions right before the runoff season begins. Collected samples in August 2004 and February 2005 correspond to summer and winter time periods, respectively. A description of each gauging station and the monthly stream flow for each sampling date is summarized in Table 5.1.

Table 5.1 USGS station location and monthly mean stream flow in four sampling dates.

USGS Station Number	Coordinates (NAD 27)		Drainage area (Km^2)	Monthly mean stream flow (cms)			
	Latitude	Longitude		May 2003	April 2004	August 2004	February 2005
06751150	40°52'42"	105°20'15"	919	9.2	0.5	1.8	0.7
06751490	40°47'15"	105°15'06"	1396	7.2	0.3	0.8	0.8
06752260	40°35'21"	105°04'09"	2919	7.2	1.1	2.3	0.2
06752280	40°33'07"	105°00'39"	3225	3.5	0.3	1.3	0.5

A map of the watershed sampling sites and the characteristics of these sites is shown in Figure 5.2. Sampling sites were chosen to represent pristine, urban, and agricultural areas. The Poudre River originates in Rocky Mountain National Park and flows through mountainous terrain before entering Fort Collins, CO and sample site 1 was chosen above any urban or agricultural activity. Sample points 2 and 3 are located in Fort Collins and represent water quality influenced by an urban landscape.

Sample point 3 was located downstream of one of two wastewater treatment plants in Fort Collins. Sample point 4 was chosen to be downstream of Fort Collins but upstream of the next urban area, Greeley, CO. Significant agricultural activity occurs between sample points 3 and 4 including several concentrated animal feeding operations (CAFOs) and multiple lower density cattle operations. Sample point 5 is downstream of Greeley and represents urban and agricultural influences. Greeley is home to several CAFOs near town and is home to a beef processing plant that processes up to 3000 animals per day. Water samples were collected in polyethylene bottles pre-rinsed with DI water at three different cross-sectional locations in the river at and aliquots of each sample were carefully mixed in one bottle. Collected water samples were kept in a cooler until transportation to the lab and contained in amber glass bottle to prevent any sorption

to polyethylene bottle during storage. The top layer (0 – 5 cm) of the sediment sample was collected with a spatula at the same place where water samples were collected and also composted in one sampling jar.

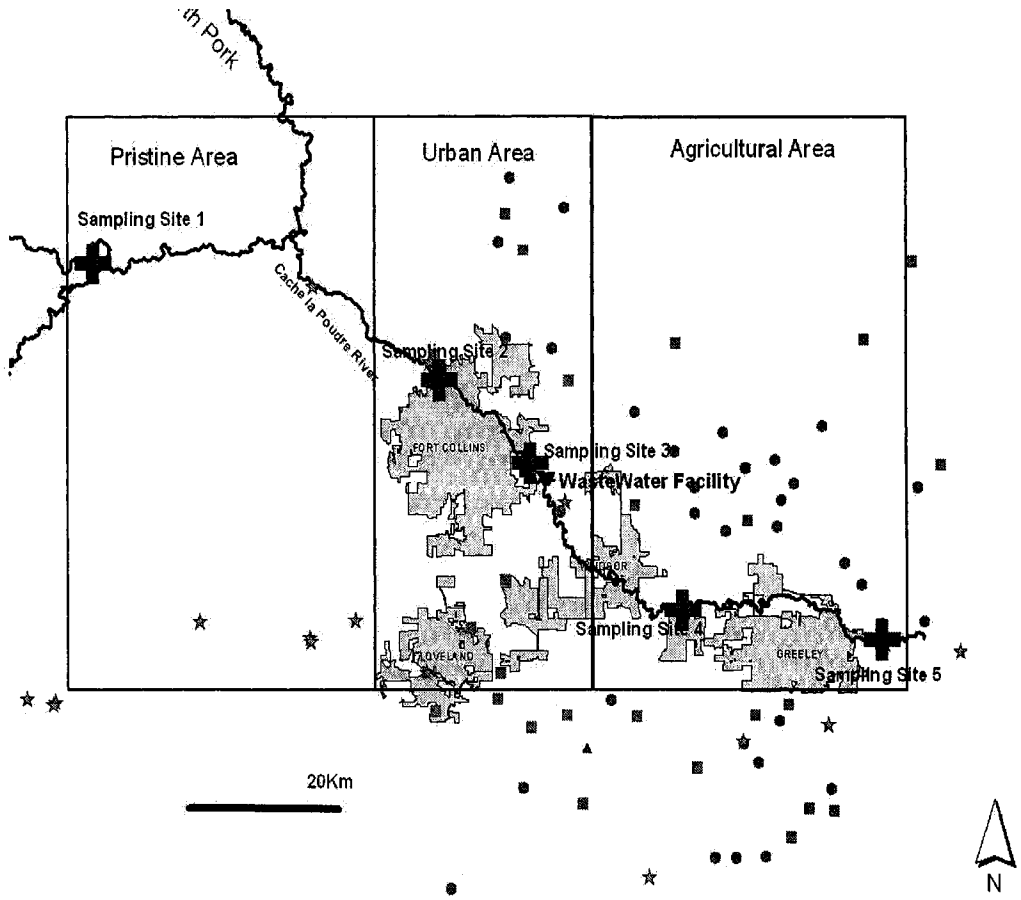

Figure 5.2 Sampling sites for water and sediment in the Cache la Poudre watershed: Pristine, urban and agricultural landscapes are indicated (+: sampling sites, ★: USGS gage station, ●: feedlots, ■: dairy, ▲: ranch).

After transporting the sample to the lab, each water sample was filtered through a 0.2μm glass fiber filter and stored at 4°C until extraction, typically less than 7 days. Sediment samples were air dried in the dark to prevent photo-degradation of the sorbed antibiotic compounds and passed through 2 mm and 75 μm sieves. Sediment samples

sieved through a 75 μm particle size, the silt-clay fraction, were used for analysis because the silt-clay fraction is assumed to sorb most of the pharmaceuticals (Thiele-Bruhn et al., 2004).

Water quality parameters and physical characteristics of the sediment passed through a 2 mm sieve were analyzed at the Soils Analysis Lab (Colorado State University, Fort Collins, CO). The nitrate nitrogen, phosphorus, and iron concentrations were measured using the ammonium bicarbonate DTPA extraction method (Soltanpour et al., 1977).

The physical and chemical properties of the water and sediment in August 2004 and February 2005 at sampling site 3 and 4 are summarized in Table 5.2. Sampling sites 3 and 4 represent urban and agricultural influenced regions respectively and August 2004 represents summer conditions and February 2005 winter conditions. Fairly constant pH values were measured at different times and places for both water and sediment. The fraction of organic matter associated with the sediment ranged from 0.2% to 0.8% through sampling sites and the highest fraction of organic matter, 0.8%, was measured at sampling site 4 in August 2004.

5.3.3 Sample Extraction

After filtration, the water samples were further cleaned up with SPE. One hundred twenty mL of the water sample was passed through a 3cc/60 mg HLB cartridge that was conditioned with 3 mL of methanol and 3mL of water prior to sample addition. Methanol was used to activate the HLB cartridge and water was used to remove any impurities in the cartridge. The sample was loaded under 40 (psi) of vacuum with a flow rate of approximately 2 mL/min. After loading the sample, the SPE cartridge was rinsed 3 times

Table 5.2 Physical and chemical properties of water and sediment.

			pH	E.C.	Ca	HCO_3	SO_4	NO_3-N	TDS
				µmhos/cm	.....	.....	mg/L	.....	.....
	August	Site 3	7.8	450.0	47.9	150.0	81.0	11.2	433.0
Water	2004	Site 4	8.3	619.0	69.4	117.0	232.0	6.3	600.0
	February	Site 3	7.5	698.0	40.4	87.8	59.1	28.7	501.0
	2005	Site 4	8.1	1430.0	132.0	252.0	474.0	3.8	1115.0

			pH	E.C.	% OM	NO_3-N	P	Fe	Texture Estimate
				mmhos/cm		.....	mg/L	.....	.....
	August	Site 3	7.2	0.7	0.4	4.4	47.4	72.5	Sand
Sediment	2004	Site 4	7.4	2.9	0.8	11.2	18.6	82.9	Sand
	February	Site 3	6.5	1.2	0.7	16.1	63.6	83.1	Sand
	2005	Site 4	7.3	2.0	0.2	2.4	19.9	60.0	Sand

with 3 mL aliquots of water to remove the impurities and two volumes of 2.5 mL of methanol were used to extract the loaded sample into the vial that contained 50 μL of the internal standard simatone (0.24mg/L). After extraction, the sample was concentrated at 50°C under a gentle nitrogen gas flow to prevent oxidation. The samples were concentrated to a volume of 50 μL and then 70 μL of mobile phase solution (99.9% water + 0.1% formic acid, v/v) was added. The samples were transferred into amber vials equipped with inserts for HPLC-MS/MS analysis.

The first stage of analysis for sediment was to extract the antibiotics from the solid phase to the liquid phase. One g (d.w.) of sediment was weighed with an accuracy of 0.001 g and added to a 40 mL Teflon tube. Twenty mL of an ammonium hydroxide buffer solution (1M, pH 10.0) was added to the tube and shaken (Model No-4626, Lab-line instrument) for 20 minutes at 400 (rpm). Then, the sample was centrifuged (IEC Clinical Centrifuge, International Equipment Co., Needham Hights, MA) for 10 minutes at 4000 (rpm). The separated liquid phase sample was passed through a 0.2 μm glass fiber filter and decanted into another 40 mL glass vial. The filtered sample was kept at 4°C until SPE clean up. The remaining solid sample was re-extracted once with the same procedure described above and supernatant was combined to make 40 mL of total volume. After extraction, SPE and concentration procedures were conducted in the same manner described for aqueous samples.

For the recovery study, three different concentrations, 0.01 μg/L, 0.5 μg/L, and 2 μg/L for water and 1 μg/kg, 30 μg/kg, and 90 μg/kg for sediment, were examined. The desired concentration with a constant volume of 50 μL was spiked in 120 mL of water or 1 g of sediment from sampling site 1, assumed to be pristine and therefore devoid of

pharmaceutical residues. The procedure described above was followed for water and sediment, respectively. Sample from sampling site 1 was examined before the experiment to verify the absence of residual antibiotics and the suitability of this sample as a control. The measured detect response was compared with the detect response of a sample spiked at the desired concentration into 5mL of methanol extractant. Detect response was calculated using the area of the internal standard, simatone, and the area of each sample based on HPLC-MS/MS analysis. Simatone was used as an internal standard because it eluted within the same chromatographic timeframe as the analytes and responded well to positive electro-spray ionization (Lindsey et al., 2001). Also, there is no noticeable matrix effect with simatone and since river water and sediment are assumed to have matrix effects, this was a critical factor for choosing the internal standard.

5.3.4 High Performance Liquid Chromatography / Tandem Mass Spectrometry

The HPLC system consisted of a HP 1100 Series Liquid Chromatograph (Agilent, Palo Alto, CA) equipped with an Agilent 1100 Series Thermostatted Auto Sampler and a variable wavelength UV detector. An XTerra MS C_{18} (Waters, Milliford, MA) 2.1×50 mm (2.5 μm pore size, end-capped) reversed-phase column was used to analyze the standards and samples. A C_{18} guard column (Phenomenex, Torrence, CA, USA) was used to filter any particulates from the sample. The column was maintained at 15°C with a flow rate of 0.25 mL/min. Mobile phase A was composed of a mixture of HPLC grade water and formic acid (99.9%+0.1% v/v). Mobile phase B was HPLC grade methanol (100%). The gradient was ramped from 50% mobile phase A and 50% mobile phase B to 10% mobile phase A and 90% mobile phase B in the first minute and held isocratic for 19

minutes. The injection volume was 20 μL. A 10-minute post-run period was allowed between each analysis to re-equilibrate the column.

A ThermoFinnigan LCQ Duo ion trap mass spectrometer (ThermoQuest, Woburn, MA) equipped with a heated capillary interface and electrospray ionization (ESI) was used to perform the mass spectrometric analysis. A standard solution of three polyether compounds (10 μM) was infused using the LCQ Duo syringe pump at a flow rate of 5 μL/min to optimize the mass spectrometry parameters as needed. Nitrogen gas was used for drying and nebulizing. Spray voltage was set to 4.5 kV and the capillary voltage autotuned to 21 V. Capillary temperature was set to 165°C and the instrument was operated in the positive ion mode. Sheath gas flow rate was optimized at 40 units (arb) and the auxiliary gas was turned off. The precursor mass and product ion optimized tandem mass spectrometry parameters are summarized in Table 5.3.

Table 5.3 Retention time and HPLC tandem mass parameters.

Compound	Precursor Mass $[M+Na]^+$ (*m/z*)	Product Ions (*m/z*) (% abundance)	Retention time (min)	Collision Energy (%)
Monensin	693	675(100)	10.2	28
Salinomycin	773	755(100),531(50),431(15)	11.3	30
Narasin	787	769(100),531(20),431(15)	12.4	30

5.3.5 Statistical Analysis

Friedman's test (MINITAB, 2000), an extension of the sign test to more than two populations, was evaluated to examine temporal and spatial effects on the measured concentration of three ionophore antibiotics in both water and sediment matrices. Statistical results were evaluated based on the approximate P-value of 0.05 obtained from

Minitab. Since sampling site 1 shows no residual of the three ionophore antibiotics, the statistical evaluation was omitted for this site.

5.4 Results

5.4.1 Quality Assurance: Recovery, Limit of Quantification and SPE Breakthrough

The result of recovery study for water and sediment matrix is shown in Table 5.4. Three experiments were conducted with each concentration and the mean and relative standard deviations of the recoveries for each concentration were calculated.

For monensin and narasin in water matrix, recoveries for the full concentration range are slightly over 100% with less than 10% standard deviation. The recovery of salinomycin showed greater variability ranging from a low of 82.7% at 0.5 μg/L to a high of 117.5% at 0.01 μg/L.

Table 5.4 Average recovery of three ionophore antibiotics for water and sediment.

	Water (N=3)			Sediment (N=3)		
	0.01 μg/L	0.5 μg/L	2.0 μg/L	1 μg/kg	30 μg/kg	90 μg/kg
Monensin	113.8±9.0	123.6±5.7	106.3±6.3	71.3±0.9	71.1±5.3	105.4±1.0
Salinomycin	117.5±4.5	82.7±4.8	99.4±1.9	98.1±2.7	73.6±2.4	72.2±1.2
Narasin	109.5±9.9	103.6±6.4	102.1±5.5	56.6±8.5	51.7±4.8	51.3±3.0

In sediment for monensin, a concentration of 90 μg/kg showed the highest recovery rate with 105.4±1.0% but lower recoveries were observed (71%) at lower concentrations. In contrast, the lowest concentration range of 1 μg/kg showed the highest recovery for salinomycin (98%) with lower recoveries at higher concentrations. The recovery of narasin ranged from 51% to 57% depending on spiked concentration in a

sediment matrix was lower than the other ionophore antibiotics. The lower recovery of narasin is likely due to the inability of ammonium hydroxide to hydrolyze the bond between this compound and sediment particles since narasin can easily bind to soil when an abundance of mono- or divalent cations are present (Blanchflower et al., 1996; Volmer et al., 1998).

Limits of quantification (LOQ) have been determined by other researchers using several methods including signal/noise ratio (Hamscher et al., 2002), selection of the second lowest point of the calibration curve (Hirsch et al., 1998; Hirsch et al., 1999), and a statistical method using the student *t*-variate (Zhu et al., 2001). Two approaches were used in this study to determine the limits of quantification for both water and sediment. First, the lowest point of the calibration curve with a signal/noise ratio of greater than 10 was determined as 0.001 µg/L for water and 1 µg/kg with a signal/noise ratio of greater than 10 for sediment. Subsequently, 0.001 µg/L and 1 µg/kg concentrations of antibiotics were spiked to water and sediment respectively to verify the detection limit with the statistical method. The calculated standard deviation was multiplied by the Student's *t*-variate for a one-sided *t*-test at the 95% confidence interval to estimate the detection limit. Table 5.5 shows the limits of quantification calculated with both techniques.

Table 5.5 Limits of quantification of three ionophore antibiotics for water and sediment.

	Water (N=3) (µg/L)		Sediment (N=3) (µg/kg)	
	Signal/Noise (10)	Statistical Method	Signal/Noise (10)	Statistical Method
Monensin	0.001	0.001	1.0	0.4
Salinomycin	0.001	0.002	1.0	3.6
Narasin	0.001	0.003	1.0	0.7

The LOQ determined in this study was comparable to that found in the literature (Hamscher et al., 2002; Loffler et al., 2003b; Hamscher et al., 2005). Calibration curves were constructed with 6 points at a range of 0.001 μg/L to 5 μg/L for water and 1 μg/kg to 90 μg/kg for sediment and all six calibration curves were linear with $r^2 > 0.99$.

Finally, an experiment of breakthrough on HLB cartridges was conducted by extracting aliquots of 20, 40, and 60 mL of sediment extracts from negative samples spiked with 10 μg/L, 100 μg/kg in sediment, in two stacked HLB cartridges. After loading the sample, each cartridge was separated and extracted with MeOH to compare the concentration between the top and bottom cartridge. No breakthrough was observed in 20 and 40 mL of extracts and 1.6%of concentration was measured in the bottom cartridge compared to the top when 60 mL of sediment extract was loaded. Thus, 40 mL of sediment extract was used for the entire experiment.

5.4.2 HPLC-MS/MS Analysis

Sodium salt ions, $[M+Na]^+$, at 693, 773, and 787 (*m/z*) for monensin, salinomycin, and narasin were the precursor ions and HPLC tandem mass spectrometry was performed. As shown in Figure 5.3, good separation was obtained within 20 minutes for the three-ionophore antibiotics including the internal standard. Fragmentation was produced via collision-induced dissociation (CID) in the ion trap and the product ion producing the highest signal was used for quantification. For monensin, only neutral water loss, $[M+Na-H_2O]$, from the precursor ion was detected at the low collision energy, 28%. For salinomycin and narasin, two major fragment pathways were found in addition to the neutral water loss at the collision energy of 30% (Figure 5.4).

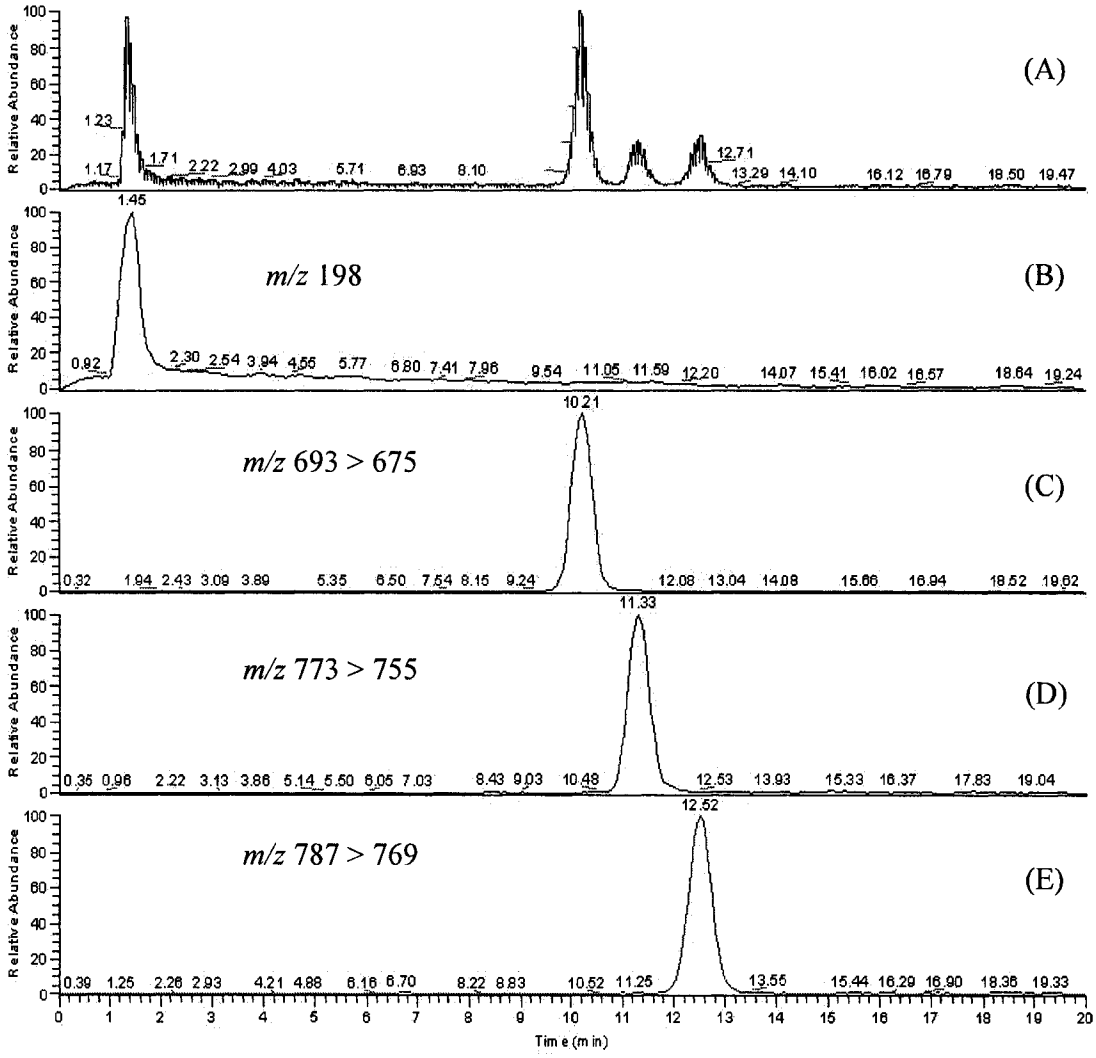

Figure 5.3 Reconstructed chromatogram showing standard solution (2μg/L) of three ionophore antibiotics: (A) total ion chromatogram (TIC), (B) simatone (internal standard), (C) Monensin, (D) Salinomycin, and (E) Narasin.

Volmer *et al.* (1998) proposed that initial cleavage of the oxygen activated carbon-carbon bonds on either sides of the C(11) carbonyl function is involved in the pathway. β-cleavage and subsequent hydrogen migration occurs at C (9) – C (10) and produces the first fragment ion at *m/z* 531. The other fragment ion at *m/z* 431 originates from the C (12) – C (13) bond dissociation.

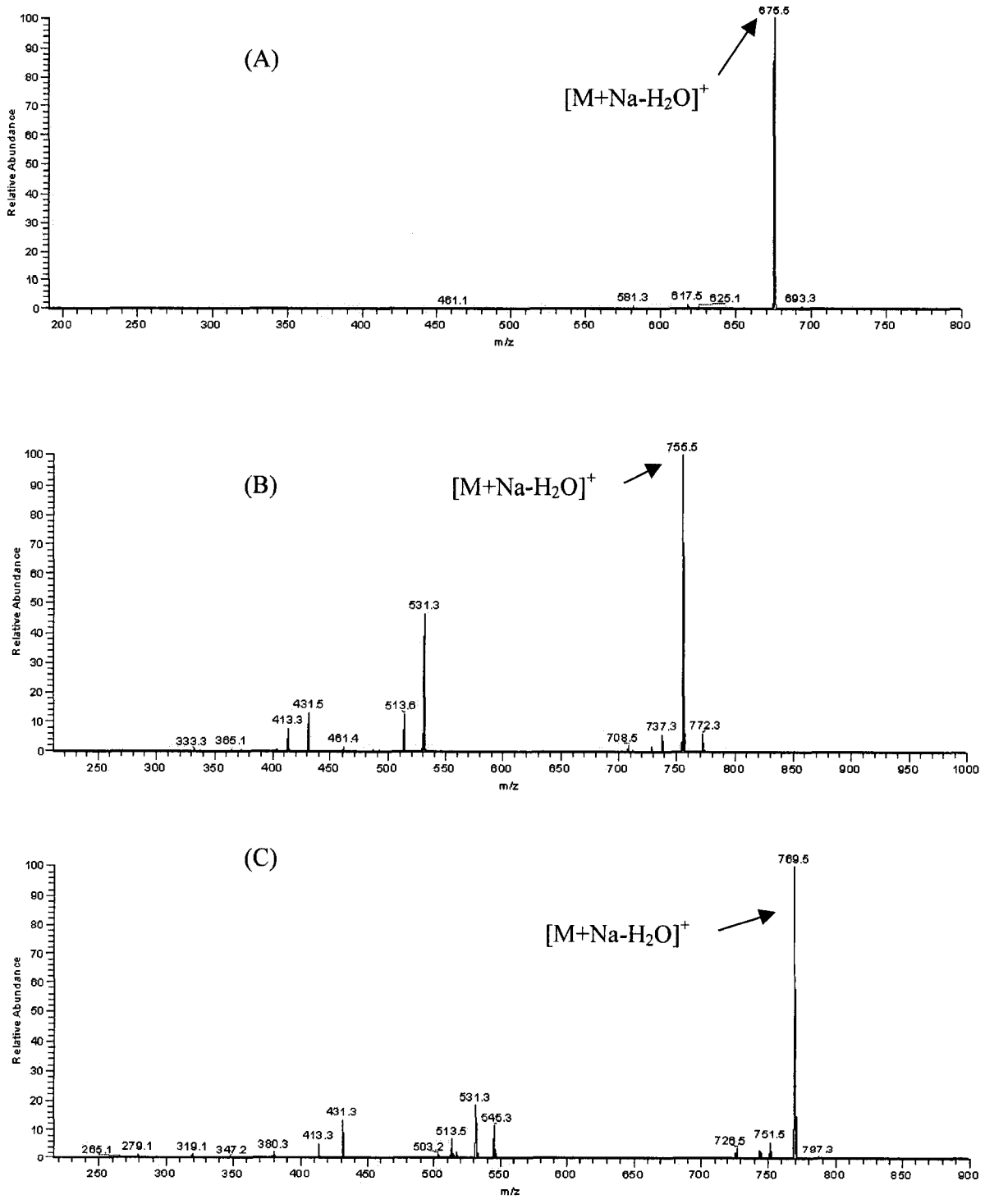

Figure 5.4 Full scan tandem mass spectra of: (A) Monensin, (B) Salinomycin, and (C) Narasin.

HPLC/MS selective ion monitoring (SIM) results were compared with HPLC-MS/MS (data not shown). While the HPLC/MS SIM method showed a large degree of chemical noise as well as great potential for ambiguity when assigning chromatographic peaks, HPLC-MS/MS derived the clean base line of each compound without any noise and interference. Also, HPLC-MS/MS enhances the specificity of compounds and provides lower quantitation limits due to the lower levels of noise. Based on the HPLC-MS/MS spectra, the most dominant ions, *m/z* 675, 755, 769 for monensin, salinomycin, and narasin respectively, were chosen to analyze and quantify the samples.

5.4.3 Ionophore Antibiotics in the Watershed

The occurrence of ionophore polyether antibiotics in the Cache la Poudre watershed for both water and sediment at different sampling dates is shown graphically in Figure 5.5. Each concentration was corrected by the recovery ratio shown in Table 5.4. None of the antibiotics were observed at sample site 1 in either water or sediment verifying this part of the watershed as pristine.

According to the Food and Drug Administration (FDA) green book (Food and Drug Administration), monensin is used for chicken and broilers for improving feed efficiency and the prevention of coccidiosis with a dose limit of 90- 110 grams per ton of feed. Also, it is used in cattle and dairy feedlots for increasing the rate of weight gain and preventing coccidiosis due to *E. bovis and E. zuernii* with a dose limit of 5 – 400 grams per ton of feed depending on the species. Salinomycin has similar uses and dose limits to monensin.

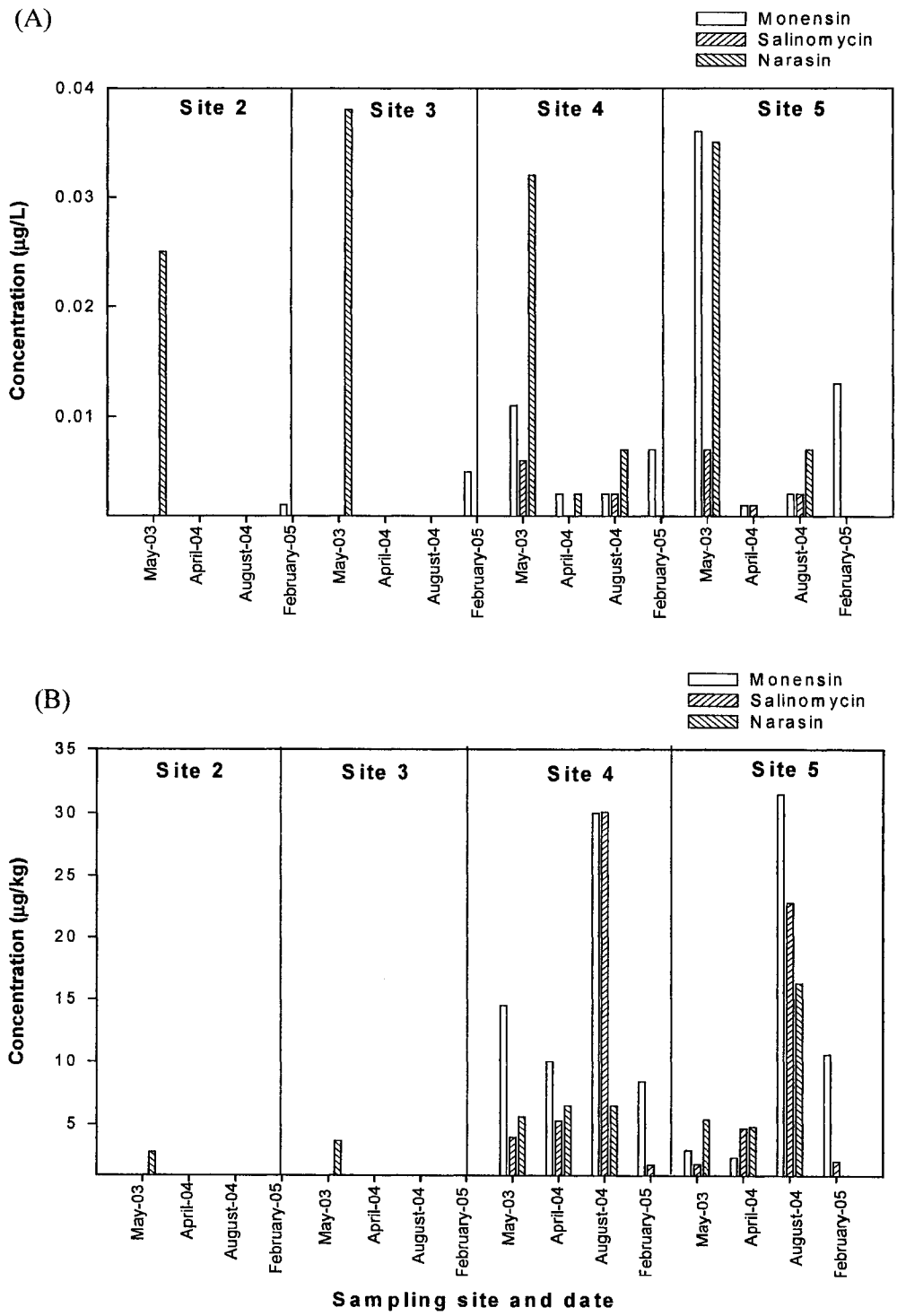

Figure 5.5 Occurrence of ionophore antibiotics in (A) water and (B) sediment.

Narasin is also used to aid in the prevention of coccidiosis in broiler chickens with a dose limit of 54 to 72 grams per ton of feed. Also, this medicine is used for increasing the rate of weight gain and improving feed efficiency for finishing swine.

In the water matrix, the measured concentration of monensin and salinomycin was significantly different among sampling sites during four sampling events except in February 2005 for salinomycin (Table 5.6). Monensin and salinomycin were mainly found at sample sites 4 and 5, the region of the watershed that is considered to be agriculture-influenced with the highest concentration of 0.036 μg/L and 0.007 μg/L respectively at sample site 5 in May 2005. Even though the highest concentration of narasin was detected at sampling site 3 with 0.038 μg/L in May 2003, narasin was detected frequently at sampling sites 4 and 5. The finding of narasin at sampling site 2 and 3 in May 2003 may be explained by limited small-scale agricultural activity upstream of the urban area. Upon further study, several small chicken farms were identified in this part of the watershed possibly explaining the presence of this agriculture-only antibiotic.

Furthermore, there were significant differences in concentration of the three ionophore antibiotics during different sampling times at each sampling sites except monensin at sampling site 5 and salinomycin at sampling site 2 (Table 5.7). Focused on sampling site 4 and 5, the highest concentration of the three ionophore antibiotics was measured in May 2003. As shown in Table 5.1, the highest average flow was recorded in May 2003 during the snow runoff period. Since most of flow in Poudre River comes from runoff composed of snowmelt and agricultural irrigation, the result of higher concentrations during high flow condition may indicate that ionophore antibiotics are contributed from runoff.

Table 5.6 Spatial statistical evaluation result of three ionophore antibiotics.

	May 2003		April 2004		August 04		February 2005	
	Friedman S value	P-value	Friedman S value	P-value	Friedman S value	P-value	Friedman S value	P-value
			Water					
Monensin	8.1	0.04*	8.8	0.03*	9.0	0.03*	9.0	0.03*
Salinomycin	8.8	0.03*	8.3	0.04*	8.1	0.04*	1.3	0.73
Narasin	7.8	0.05	9.0	0.03*	8.5	0.04*	0.0	1.00
			Sediment					
Monensin	3.0	0.08	3.0	0.08	3.0	0.08	3.0	0.08
Salinomycin	3.0	0.08	0.3	0.56	3.0	0.08	0.3	0.56
Narasin	2.0	0.16	3.0	0.08	3.0	0.08	0.0	1.00

* Denotes a significant difference among different sampling sites

Table 5.7 Temporal statistical evaluation result of three ionophore antibiotics.

	Site 2		Site 3		Site 4		Site 5	
	Friedman S value	P-value	Friedman S value	P-value	Friedman S value	P-value	Friedman S value	P-value
			Water					
Monensin	9.0	0.03*	9.0	0.03*	8.4	0.04*	8.0	0.05
Salinomycin	4.7	0.19	3.0	0.39	8.4	0.04*	8.2	0.04*
Narasin	8.1	0.04*	9.0	0.03*	9.0	0.03*	9.0	0.03*
			Sediment					
Monensin					9.0	0.03*	8.2	0.04*
Salinomycin					9.0	0.03*	8.2	0.04*
Narasin					8.2	0.04*	9.0	0.03*

* Denotes a significant difference among different sampling dates

For sediment, statistics were evaluated only at sampling sites 4 and 5 since no residuals were found at sampling sites 2 and 3 except narasin in May 2003. Even though no significant difference was observed among sampling sites 4 and 5 during each sampling events (Table 5.6), the residual of three ionophore antibiotics was detected at sampling sites 4 and 5 in the sediment. In addition, the detected concentration of three

ionophore antibiotics was significantly different among each sampling event at sampling site 4 and 5 (Table 5.7). The measured concentration in August 2004 shows the highest concentration for monensin and narasin at sampling site 5 with 31.5 μg/kg and 16.3 μg/kg respectively and 30.1 μg/kg for salinomycin at sampling site 4. This result can be explained that interconnected irrigation ditches might contribute the input of three ionophores to river during runoff and the sediment contributes to saturate after runoff season.

Since ionophore antibiotics are only used in animals, they act as a marker for contamination from agricultural sources. Therefore, it's not surprising that three ionophore antibiotics were frequently found only at sampling sites 4 and 5 where agricultural activity is the greatest. In addition, a significant difference of measured concentration among different sampling events indicates that flow conditions and seasonal variability may affect the concentration of the three ionophore antibiotics.

Another important finding of this study is that much greater concentrations of three ionophore antibiotics were found in the sediment compared to the overlying water matrix. A pseudo- partitioning concept is introduced in this study to understand the importance of the sediment matrix for ionophore antibiotics. Average pseudo partitioning-coefficients were calculated using sediment concentrations with overlaying water concentrations (Table 5.8). Even though this partition coefficient is only dependent on specific sampling events and sampling sites with a non-equilibrium state in the river, this value can help understand the partitioning characteristics of ionophore antibiotics in the environment.

Table 5.8 Calculated pseudo-partitioning coefficient (L/kg).

	May 2003	April 2004	August 2004	February 2005
Monensin	680	2261	10248	988
Salinomycin	446	4956	8320	1960
Narasin	165		1663	

For the higher flow condition, May 2003, the concentration of monensin in the sediment is approximately three orders of magnitude greater than in the river at sample sites 4 and 5. Salinomycin was approximately 500 times greater in the sediment than the water column and narasin 100 times greater in sediment than water. Higher sediment partitioning was observed at the low stream flow conditions and the highest value was calculated in August 2004. The pseudo-partitioning coefficient is ranged from 680 to 10248 L/kg for monensin, from 446 to 8320 L/kg for salinomycin, and finally from 165 to 1663 L/kg for narasin.

Higher partitioning observed at the low flow condition compared to the high flow condition may be explained by the ionophore antibiotics sorbing not only to sediment particles but also to suspended solids. Since there is a higher concentration of suspended solids during high flow events, a greater fraction of the compounds would be present in this form. In contrast, these solids will settle to a greater extent during the low flow affecting the calculated ratio of sediment to dissolved concentrations. Future work will attempt to quantify the impact of suspended solids on the measurement of antibiotics in rivers.

5.5 Conclusion

An analytical method to measure three ionophore antibiotics in river water and sediment was developed and applied to the Cache la Poudre River in Northern Colorado.

Three ionophore antibiotics are only used as veterinary antibiotics to promote growth for beef and to treat coccidiosis in poultry. Thus, occurrence of ionophore antibiotics in the watershed can be considered agriculture-influenced. However, more research should be conducted to verify the pathway of veterinary antibiotics to the watershed.

Also, our study indicates that antibiotics can significantly accumulate in the sediment potentially impacting the stream benthic biota. Therefore, when studying the occurrence of antibiotics in the environment, it is imperative to include the sediments in the analysis. To date, there has been little documented research of the occurrence of veterinary or human antibiotics in river sediments.

Chapter 6

Temporal and Spatial Trends in Occurrence of Human and Veterinary Antibiotics in a Mixed Watershed with Long Term Monitoring

Submitted to Environmental Science and Technology

6.1 Abstract

The occurrence of 15 antibiotics belonging to three different groups, tetracyclines (TCs), sulfonamides (SAs), and macrolides (MLs), mainly used to prevent or treat illness for humans and also to control disease or to promote the growth for animals was studied in aqueous and sediment matrices. The result of spatial and temporal statistical analysis revealed that measured concentrations of individual antibiotics were significantly different depending on sampling location and time periods for aqueous and sediment samples. High concentrations of human-used antibiotics were detected downstream of a wastewater reclamation facility and animal-used antibiotics were mainly found in a region with significant agricultural activity. Generally, the winter season showed the highest concentrations of antibiotics for both water and sediment samples indicating that low flow conditions and cold-water temperatures might enhance the persistence of these compounds. Furthermore, a pseudo-partitioning coefficient (P-PC) was introduced to

provide a better understanding of the partitioning of antibiotics into the sediment. Different P-PC values were found depending on the sorption characteristics of the individual antibiotics. Sediment samples showed a greater detection frequency and a much higher concentration compared to aqueous samples taken at the same site. Since microorganism antibiotic resistance can develop in sediments, the importance of analyzing this matrix is underscored.

6.2 Introduction

According to the Animal Health Institute's 2002 Market Sales Report, animal health product sales in the United States for 2002 totaled $4.5 billion included $3.3 billion for pharmaceuticals and $557 million for feed additives (2002). Mellon et al. (Mellon et al., 2001) also estimated that livestock producers in the United States use 24.6 million pounds of antimicrobials every year in the absence of disease for nontherapeutic purposes: approximately 10.3 million pounds in hogs, 10.5 million pounds in poultry, and 3.7 million pounds in cattle. The excretion ratio will vary depending on the compound but it can be as high as 80 – 90% of the parent compound being excreted via urine and faeces (Heberer, 2002a; Bound et al., 2004). For example, the excretion ratio of roxythromycin and erythromycin is 30% and 5 – 10%, respectively (McArdell et al., 2003). As a result, antibiotics consumed by humans and animals can be introduced into different environmental compartments depending on the physicochemical properties of the compounds such as water solubility, octanol/water partitioning coefficient, and the acid dissociation constant.

For human-used antibiotics, wastewater treatment plants (WWTPs) are the major source of release to the environment due to the partial removal efficiency in the treatment

process. The occurrence of antibiotics in wastewater effluent or surface water and groundwater influenced by WWTPs has been reported to be as high as 1μg/L although the concentrations vary considerably (Hirsch et al., 1999; Lindsey et al., 2001; Heberer, 2002b; Kolpin et al., 2002; Miao et al., 2002; McArdell et al., 2003; Gobel et al., 2004; Bendz et al., 2005). A recent study also verified the human impact from WWTP effluents to streams and proposed that bacterial culture tests can be replaced by measuring certain persistent pharmaceuticals as indicators of human fecal contamination (Glassmeyer et al., 2005).

Animal used antibiotics enter the environment mostly through manure and waste lagoon water application to fields as fertilizer. Accidental overflow or leakage from storage lagoons or tanks also likely contribute to the release of these compounds to the environment (Hamscher et al., 2002; Jacobsen et al., 2004). Understanding the antibiotic occurrence in the different environmental compartments (e.g. surface water, sediments and soil) could be used to evaluate the transport and ultimate fate of animal used antibiotics via surface runoff or leaching through sub-surface (Christian et al., 2003; Liguoro et al., 2003). Another pathway of antibiotic release to the environment is aquaculture and the occurrence of antibiotic residuals has been reported from these operations (Jacobsen et al., 1988; Bjorklund et al., 1990; Hektoen et al., 1995; Smith et al., 1996; Carson et al., 2002; Lalumera et al., 2004).

Initial research to determine occurrence of human and animal used antibiotics has been focused on developing analytical methods and only water grab samples and short term monitoring is conducted. Longer term monitoring combined with temporal and spatial trend analysis is necessary to better understand the effects of watershed conditions

(e.g. water temperature, flow etc) and to determine the origin of antibiotic residuals in the watershed. In particular, the sediment matrix has not been studied to a large extent and if one of environmental end-points of concern is microbial antibiotic resistance, the occurrence of these compounds in the benthic zone of streams is important data to collect.

A major objective of this study was to monitor the residuals of commonly used human and animal antibiotics (Table 6.1) in both water and sediment matrices based on previously published analytical methods for water (Yang et al., 2004a; Yang et al., 2004b) and a modified analytical method from animal tissue extraction for sediment (2003). Spatial and temporal trends were examined to determine statistically significant differences in location and season. A secondary objective was to understand the partitioning of antibiotics into the sediments relative to the concentration of the overlying water. These results will help determine the fate of these compounds and contribute to the design of sampling plans in the future.

6.3 Experimental Method

6.3.1 Materials

Fifteen reference compounds (Table 6.1) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). HPLC grade methanol (99.9%), analytical grade formic acid (99%), citric acid-monohydrate, sodium phosphate-dibasic anhydrous, and disodium ethylene diaminetetraacetic acid (Na2EDTA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Stock solutions (100μg/mL) of six tetracyclines (TCs), six sulfonamides (SAs), and three macrolides (MLs) were prepared in methanol every month

Table 6.1 Properties and primary usage of investigated compounds in this study.

Compounds	Acronym	CAS number[a]	pKa[b]	Log Kow[b]	Water Solubility (mg l^{-1})[b]	Primary Usage[c]
Tetracycline	TC	60-54-6	3.3/ 7.7/ 9.3	-1.3 – 0.05	230 – 52000	Human
Chlortetracycline	CTC	64-72-2				Horse, Sheep, Swine
Oxytetracycline	OTC	79-57-2				Cattle, Beef
Demeclocycline	DMC	127-33-3				Human
Meclocycline	MCC	2013-58-3				Human, Cattle, Beef, Sheep, Swine, Turkey
Doxycycline	DXC	564-25-0				Human
Sulfathiazol	STZ	72-14-0	2 – 3/ 4.5 – 10.6	-0.1 – 1.7	7.5 – 1500	Swine
Sulfamerazine	SMR	127-79-7				Human
Sulfamethazine	SMT	57-68-1				Human
Sulfachloropyridazine	SCP	80-32-0				Cattle, Beef
Sulfamethoxazole	SMX	723-46-6				Cattle, Calves
Sulfadimethoxine	SDM	112-11-2				Human
						Cattle, Beef, Chicken, Turkey
Erythromycin-H_2O	ETM-H_2O	114-07-8	7.7 – 8.9	1.6 – 3.1	0.45 - 15	Human
						Cattle, Beef, Chicken, Turkey
Roxithromycin	RTM	80214-83-1				Human
Tylosin	TYL	1401-69-0				Swine

[a] reference from (Glassmeyer et al., 2005), [b] reference from (Thiele-Bruhn, 2003), [c] reference from FDA (Food and Drug Administration)

and stored at 4°C. Two working solutions, 5μg/mL and 0.5μg/mL were prepared immediately before the experiment by dilution of the stock solution.

Solid phase extraction (SPE) cartridges, 3ml/60mg of HLB (Hydrophillic-Lipophillic-Balance), were purchased from Water Oasis Co. (Milford, MA, USA). Milli-Q water (18.3MΩ) from a Millipore (Billerica, CA, USA) purification system was used when DI water was required.

6.3.2 Sample Collection and Site Description

Four different sampling events were conducted from May 2003 to February 2005 at five sampling sites representing pristine, urban and agricultural influenced areas along the Cache La Poudre River of northern Colorado. The Cache La Poudre River originates in Rocky Mountain National Park and flows through mountain, urban, and agricultural areas. Sampling site 1 represents a pristine region in the mountains above urban or agricultural activity. Sampling sites 2 and 3 were picked in Fort Collins, CO to represent an urban and domestic wastewater influenced areas. Sampling sites 4 and 5 were located in an area with significant agricultural activity including several concentrated animal feeding operations (CAFOs). Also, dairies and small horse and cattle breeding operations were distributed in this area. Figure 6.1 shows characteristic of each sampling sites including CAFOs, dairies, and ranches distributed around this area.

To examine the possible effects of snow runoff and high water flow in the watershed, samples were collected during two dates in late spring of 2003 and 2004. Another two sampling events in August 2004 and February 2005 were regarded as summer and winter conditions, respectively. Monthly average stream flow information

for each of the sampling months was obtained from four USGS (United States Geological Survey) stream flow gages located in the watershed (Table 6.2).

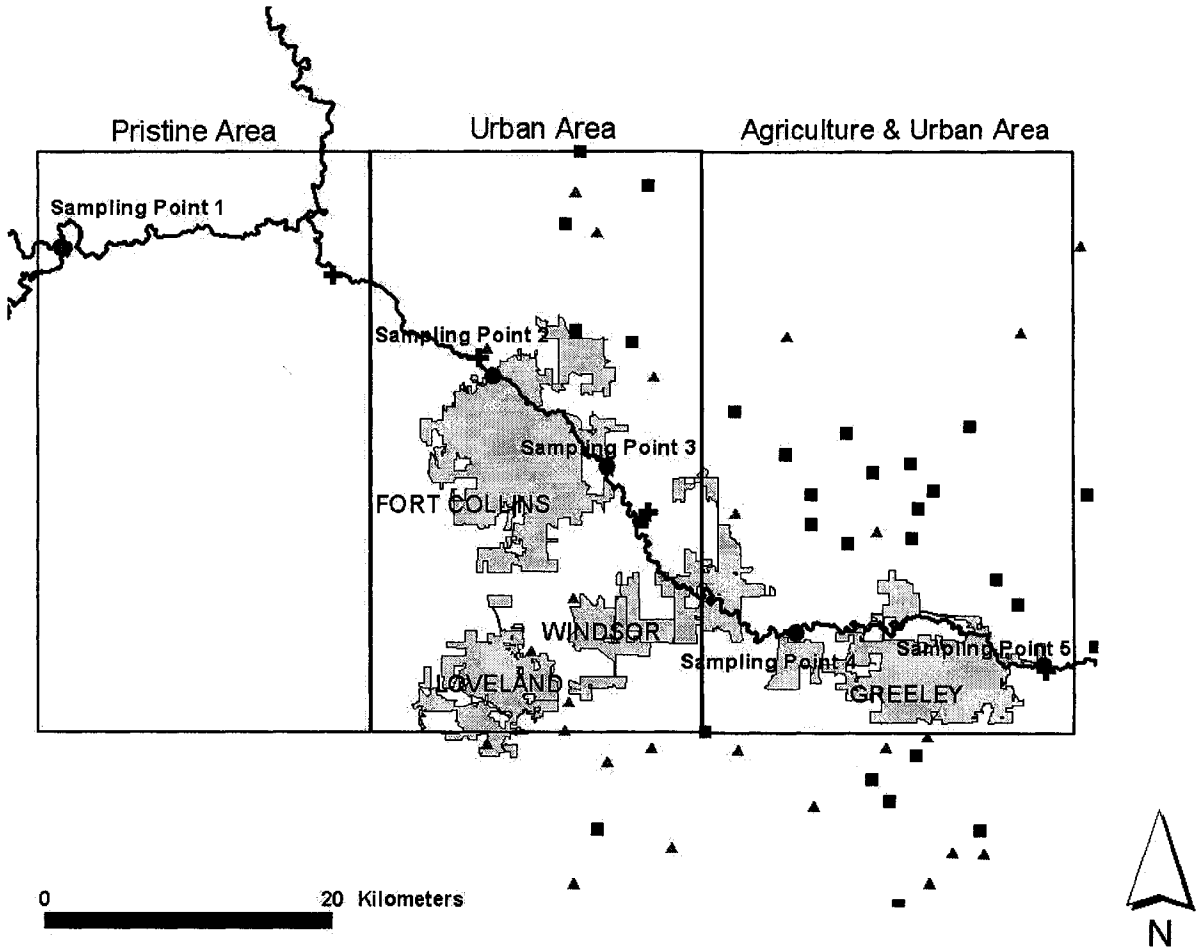

Figure 6.1 Sampling sites for water and sediment in the Cache la Poudre watershed (● represents 5 sampling points, ✚ represents stream gages, ■ represents feedlots, and ▲ represents dairies respectively)

Three replicate samples were collected across a stream point for both water and sediment. Sampling techniques from previous studies (8, 28) were used to ensure to ensure representative samples and the top part of sediment (0-3cm) was collected using a spatula. Water and sediment samples were kept in a cooler during sampling events and

were immediately transported to the lab and stored at 4°C. Water samples were filtered using 0.2μm glass fiber filters (Waters, Milford, MA, USA) and kept at 4°C until analysis, usually within one week. Sediment samples were completely air dried in the dark to prevent any loss of compounds by photo-degradation. Then, each sediment sample was passed through 2mm and 75μm sieves. The physical properties of the sediments were determined at the Soils Analysis Lab (Colorado State University, Fort Collins, CO) and the concentration of antibiotics was measured using the completely sieved fraction. Water quality and physical characteristics of the sediment are summarized in Table 6.3.

Table 6.2 Locations of USGS stations and monthly mean stream flow for four sampling dates

USGS Station Number	Coordinates (NAD 27)		Monthly mean stream flow (cms)			
	Latitude	Longitude	May 2003	April 2004	August 2004	February 2005
06751150	40°52'42"	105°20'15"	9.2	0.5	1.8	0.7
06751490	40°47'15"	105°15'06"	7.2	0.3	0.8	0.8
06752260	40°35'21"	105°04'09"	7.2	1.1	2.3	0.2
06752280	40°33'07"	105°00'39"	3.5	0.3	1.3	0.5

Table 6.3 Physicochemical properties of water and sediment

		pH	E.C.	Ca	HCO_3	SO_4	NO_3-N	TDS
			μmhos/cm	.....	.....	mg/L	.....	.....
Water	Site 1	7.7	88.4	10.0	43.3	4.2	0.1	69.0
	Site 2	8.0	561.0	61.0	176.0	107.0	0.1	307.0
	Site 3	7.5	698.0	40.4	87.8	59.1	28.7	501.0
	Site 4	8.1	1430.0	132.0	252.0	474.0	3.8	1115.0
	Site 5	8.0	1430.0	130.0	275.0	462.0	6.4	1137.0
		pH	E.C.	% OM	NO3-N	P	Fe	Texture Estimate
			mmhos/cm		.....	mg/L	.....	.....
Sediment	Site 1	6.7	0.2	0.3	1.3	4.0	30.5	Sand
	Site 2	7.0	0.8	0.7	1.2	6.2	40.5	Sand
	Site 3	6.5	1.2	0.7	16.1	63.6	83.1	Sand
	Site 4	7.3	2.0	0.2	2.4	19.9	60.0	Sand
	Site 5	7.3	3.5	2.3	23.5	77.0	151.0	Sand

6.3.3 Sample Preparation

Aqueous samples for TCs, SAs, and MLs were analyzed based on previously described analytical methods (Lindsey et al., 2001; Yang et al., 2004a; Yang et al., 2004b). Briefly, the sample pH was adjusted to the range of 2.0 – 2.5 for TCs and SAs and 5.0 for MLs to increase the hydrophobicity in the SPE cartridge. The cartridge was pre-conditioned with 3mL methanol and 3mL HCl (0.5N) followed by 3mL water. 5mL of methanol was used to extract the compounds. Simatone was added as an internal standard (50μL of 240μg/L) in 15mL conical graduate vials prior to extraction. The extracted sample was transferred to a 50°C water bath equipped with nitrogen gas for concentration. The sample was concentrated until 50μL remained and 70μL of mobile phase A (99.9% water + 0.1% formic acid, v/v, pH 2.75) was added into vials. Then, the sample was transferred into amber vials fitted with auto sampler inserts for HPLC-MS/MS analysis

For sediment samples, two stages of extraction were conducted. The first stage is intended to pre-extract the antibiotics sorbed on the solid phase into the liquid phase. McIlvaine buffer solution (pH 4.0) prepared according to USDA (United States Department of Agriculture) guidelines (USDA, 2003) was used for pre-extracting TCs and SAs. Ammonium hydroxide buffer solution (pH 10.0) titrated with formic acid was used to pre-extract MLs. One g of sediment sample was carefully weighed and transferred into 40mL vials. Twenty mL of McIlvaine buffer or ammonium hydroxide buffer solution was added followed by 200 μL of 5% Na2EDTA (1mmol in solution). The sample was vigorously mixed in the parallel shaker (Model No-4626, Lab-line instrument, Dubuque, IA, USA) for 20 minutes at 400 (rpm). The sample was then centrifuged at 4000 (rpm) (IEC Clinical Centrifuge, International Equipment Co.,

Needham Hights, MA, USA) for 15 minutes followed by filtration using 0.2μm glass fiber filters. The filtered sample was decanted to another 40mL vial and kept at 4°C. Extraction was repeated again in the same manner as described above and the supernatants were combined for an SPE cleanup procedure. The second stage of the sample preparation procedure was to cleanup and concentrates the sample. The SPE conditions were the same as for aqueous samples except the 0.5N HCl addition was omitted due to lower extraction efficiency during optimization process.

6.3.4 High Performance Liquid Chromatography / Tandem Mass Spectrometry

High performance liquid chromatography (HPLC) was conducted with an HP 1100 series Liquid Chromatograph (Agilent, Palo Alto, CA) equipped with an Agilent 1100 Series Thermostatted Auto Sampler and a variable wavelength UV detector. An XTerra MS C_{18} (Waters, Milliford, MA) 2.1×50mm (2.5μm pore size, end-capped) reversed-phase column was installed with a C_{18} guard column (Phenomenex, Torrence, CA, USA) to filter any particles from the sample. Mobile phase A consisted of 99.9% water and 0.1% formic acid (pH 2.74) and mobile phase B was 99.9% acetonitrile mixed with 0.1% formic acid. Column temperature was set to 15°C when measuring TCs and SAs. The gradient for TCs was ramped from 96% mobile phase A and 4% of mobile phase B to 70% mobile phase A and 30% mobile phase B for 29 minutes and back to 96% mobile phase A and 4% mobile phase B for 1 minute. The gradient for measuring SAs was programmed for 21 minutes with same conditions as TCs.

HPLC conditions for MLs were different than TCs and SAs. The column temperature was set to 45 °C and the gradient was programmed to ramp from 80% mobile

phase A and 20% mobile phase B to 65% mobile phase A and 35% mobile phase B for 14 minutes and back to the original condition for 1 minute. 10 minutes of post-run time was used to equilibrate the column between each analysis for all three groups. The injection volume was 20μL for TCs, SAs and MLs.

Table 6.4 HPLC tandem mass parameters.

Compound	Precursor Ions $[M+H]^+$ (*m/z*)	Fragment Ions (*m/z*)[a]	Isolation Width	Collision Energy (%)
Simatone[b]	198			
TC	445	**427**	2.0	26
CTC	479	**462**,444	2.0	32
OTC	461	**443**, 426	2.0	28
DMC	465	**448**	2.0	30
MCC	477	**460**	2.0	40
DXC	445	**428**	2.0	32
STZ	256	**156**	2.0	32
SMR	265	**156**,189	2.0	36
SMT	279	156, **204**	2.0	38
SCP	285	**156**	2.0	32
SMX	254	156,**188**	2.0	36
SDM	311	**156**,245	2.0	38
ETM-H_2O	716	522,**558**	3.0	26
RTM	837	558,**679**	3.0	26
TYL	916	**772**	3.0	30

[a] Bold was used to quantify the concentration of sample, [b] internal standard.

A ThermoFinnigan LCQ Duo ion trap mass spectrometer (ThermoQuest, Woburn, MA) equipped with a heated capillary interface and electrospray ionization (ESI) was used to perform the mass spectrometric analysis. TC, STZ, and ETM were used as representative compounds of each class and a standard solution of each compound (10μM) was infused using the LCQ Duo syringe pump at a flow rate of 5 μL/min to optimize the mass spectrometry parameters as needed. Capillary temperature was set to 165°C and the instrument was operated in the positive ion mode. Sheath gas

flow rate was optimized at 40 units (arb) and the auxiliary gas was turned off. Nitrogen gas was used for drying and nebulizing. Spray voltage was set to 4.5 kV and the capillary voltage was autotuned to 21 V. The precursor mass and product ion optimized tandem mass spectrometry parameters are summarized in Table 6.4.

6.3.5 Statistical Analysis

To examine spatial and temporal effects of the measured concentrations of the fifteen compounds, Friedman's test (MINITAB, 2000), an extension of the sign test to more than two populations, was utilized. The significance of results was determined based on the approximate P-value of 0.05 obtained from Friedman's test. In addition, the Pearson product moment correlation coefficient was used to determine if the concentration of different antibiotics are related. Statistical results were also evaluated based on a significance level of 0.05. Temporal and spatial statistical analysis was evaluated if more than 50% of compounds were detected during different sampling events and locations and missing values were recorded as one half of the limit of quantification for water and sediment.

6.4 Results

6.4.1 Quality Assurance: Recovery and Limit of Quantification

A recovery study for both water and sediment matrices was conducted with 3 different antibiotic groups. Sampling site 1 was validated before the experiment to be suitable as a control matrix since no antibiotic residuals were found at this site. The recovery ratio was calculated by comparing the Detect Response (DR) between samples spiked with three different concentrations in 120mL or 1g of sample prior to SPE and samples spiked with

the equivalent concentration in a 5mL final methanol extract. DR was calculated based on the area of the internal standard, simatone, and the area of the target compounds. Simatone, adapted from Lindsey et al. (Lindsey et al., 2001) as an internal standard shows good responsibility in positive ion mode and was effective in previous experiments with similar HPLC/MS/MS conditions (Yang et al., 2004a; Yang et al., 2004b). The calculated recoveries for the three different concentrations in water and sediment are shown in Table 5. The recovery (%) range of TCs, SAs, and MLs in the aqueous matrix is 100 – 127, 76 – 124, and 89 – 114, respectively, and 40 – 114, 62 – 111, and 53 – 128 for sediment.

Matrix effects may cause the slightly higher than 100% recovery ratio in both aqueous and sediment samples (Lindsey et al., 2001). The standard addition method or using an adequate internal standard can reduce the matrix effects for analyzing complex samples (Zwiener et al., 2004a).

Several approaches have been used in previous research to calculate the detection limit or limit of quantification (LOQ). Hirsch et al. (Hirsch et al., 1998; Hirsch et al., 1999) used the second lowest calibration point of the linear correlation and Hamscher et al. (Hamscher et al., 2002) used the signal/noise ratio (less than 6) as the LOQ. Zhu et al. (Zhu et al., 2001) adapted a statistical method using student's *t*-variate. This statistical method multiplied the calculated standard deviation by the Student's *t*-variate for a one-sided t-test at the 95% confidence interval to estimate the LOQ. Both methods were used in this study to determine the LOQ.

Table 6.5 Recovery and limit of quantification (LOQ) study of water and sediment

	Water Recovery (%)			LOQ (µg/L)		Sediment Recovery (%)			LOQ (µg/kg)	
	0.1 µg/L	1 µg/L	5 µg/L	S/N (3)	Statistical Method	1 µg/kg	30 µg/kg	90 µg/kg	S/N (3)	Statistical Method
TC	106	109	104			82	90	91		
CTC	104	124	107			90	69	64		
OTC	100	102	106	0.01	0.02	43	53	40	1.0	2.3
DMC	124	127	109			106	44	32		
MCC	112	106	104			107	100	114		
DXC	109	122	103			101	74	78		
STZ	77	101	92			108	106	78		
SMR	100	103	91			98	102	77		
SMT	108	109	96	0.01	0.01	92	105	80	1.0	1.8
SCP	104	87	87			100	111	62		
SMX	124	92	76			97	107	86		
SDM	78	99	85			87	104	68		
ETM-H_2O	102	101	103			127	128	101		
RTM	104	114	114	0.01	0.01	69	53	76	1.0	0.6
TYL	103	89	114			79	74	77		

(N = 3 independent sample, Relative standard deviation (RSD) ranged between 1 and 11% for water and 13 and 31% for sediment).

Once the lowest calibration point with the signal/noise of greater than 3 was chosen as the detection limit for water and sediment matrix, 0.01 μg/L and 1μg/kg for water and sediment were spiked in the control sample from sampling site 1. Three independent analyses were conducted to calculate the concentration of each sample based on the external calibration curve. The standard deviation was calculated and the student *t*-variate is applied to measure the LOQ with statistical method (Table 6.5). Calibration curves were constructed for the range of 0.01μg/L to 5 μg/L for water and 1 μg/kg to 90 μg/kg for sediment and all six calibration curves for the three antibiotics groups were linear with $r^2 > 0.99$.

6.5 Occurrence of Antibiotics in the Watershed

6.5.1 Measured Concentration of Antibiotics in Water and Sediment

The measured concentration of 15 antibiotics at 5 sampling sites along the Cache la Poudre River for both water and sediment at four different time periods is summarized in Table 6.6. The concentration of all measured compounds was corrected by the recovery ratio shown in Table 6.5. None of the antibiotic residuals was found at sampling site 1 in either aqueous or sediment matrices confirming the fact that sampling site 1 is suitable as the control site. The detection frequency of the 6 TCs in water was over 30% for all compounds among 60 measurements. MCC, a human-used compound (Table 6.1), shows the highest detection frequency of 47% followed by a 45% detection frequency for CTC (Table 6). The calculated mean values of the individual 6 TCs ranged from 0.02 – 0.18

Table 6.6 Summary of measured concentration in both water and sediment

Compounds	Water Frequency of detection (%)	Detected Concentration (μg/L)				Sediment Frequency of detection (%)	Detected Concentration (μg/kg)			
		Mean[a]	Standard deviation	Maximum	Minimum		Mean[a]	Standard deviation	Maximum	Minimum
TC	42	0.02	0.01	0.03	0.01	80	17.9	24.3	102.7	1.1
CTC	45	0.08	0.07	0.21	0.01	80	10.8	9.2	30.8	1.1
OTC	37	0.18	0.40	1.21	0.01	80	14.8	13.9	56.1	2.4
DMC	30	0.03	0.01	0.05	0.02	80	6.9	5.5	23.6	2.1
MCC	47	0.03	0.03	0.10	0.01	80	24.3	21.5	72.0	4.3
DXC	33	0.02	0.01	0.05	0.01	65	15.7	10.1	38.9	2.2
STZ	33	0.01	0.01	0.03	0.01	70	3.3	2.3	5.4	1.3
SMR	30	0.02	0.02	0.06	0.01	15	4.8	2.3	6.8	2.3
SMT	10	0.02	0.00	0.02	0.02	25	4.7	5.2	13.7	1.0
SCP	5	0.03	NM	0.03	0.03	25	2.7	0.5	3.2	1.9
SMX	60	0.11	0.09	0.32	0.04	25	1.6	0.3	1.9	1.2
SDM	33	0.02	0.02	0.04	0.01	30	3.8	2.1	6.8	1.7
ETM-H_2O	65	0.12	0.13	0.45	0.02	75	10.0	7.6	25.6	1.3
RTM	0	NM	NM	ND	ND	30	2.1	1.9	5.9	1.1
TYL	5	0.05	NM	0.05	0.05	53	3.0	2.8	9.3	1.1

[a] Averaged value of all measurements in different time and locations.

μg/L and all measured concentration were less than 1μg/L except OTC with a maximum concentration of 1.21μg/L in February 2005 at sample site 4.

Among the 6 SAs, SMX was detected most frequently (60%) with the highest average concentration of 0.11μg/L (Table 6). The measured concentration of the other 5 SAs was close to the LOQ and SCP was only detected in February 2005 at sampling site 2.

Dehydrated-ETM was the most frequently detected macrolide in water with the highest concentration of 0.45μg/L and average concentration of 0.12μg/L. In contrast, none of the RTM and only 5% of the TYL samples had concentrations above the LOQ. This result agrees with a previous study showing the highest concentration and detection frequency of ETM-H2O followed by TYL and no detectable RTM among 3 compounds in U.S streams (Kolpin et al., 2002; Glassmeyer et al., 2005).

For sediment, TCs were measured at the highest frequency and mean concentration followed by MLs. The lowest detection frequency and average concentration were found for SAs. This result might be expected since SAs have the lowest Koc and are least hydrophobic of the compounds studied (Tolls, 2001; Thiele-Bruhn, 2003). TCs are known to have strong sorption affinity to soil particles or soil organic matter (Tolls, 2001; Bruhn, 2003). All TCs were detected in the sediment from sampling site 2 through sampling site 5 except DXC and the calculated mean concentration ranged from 6.9 – 24.3μg/kg. STZ was detected most frequently in the sediment matrix among the 6 SAs and the highest mean concentration was SMR (4.8μg/kg). While no RTM was detected in the aqueous matrix, 30% of the samples in the sediment matrix were found to have residuals with a mean concentration of 2.1μg/kg.

6.5.2 Spatial and Temporal Statistical Analysis

To examine concentration variance of investigated compounds in more detail, spatial and temporal statistical analyses using Friedman's test were evaluated. Since sampling site 1 showed no detectable compounds, statistical analysis was evaluated from sampling site 2 to sampling site 5 for all compounds in both water and sediment matrices. The purpose of spatial analysis is to determine whether examined compounds are due primarily to human or agricultural influences depending on different characteristics of the sampling points (Figure 6.1). For instance, CTC is only used for animals (Table 6.1) and showed a significantly different concentration at 2 different time periods of sampling (Table 6.7).

As illustrated in Figure 6.1, sampling site 4 is a heavily agricultural influenced region and not surprisingly the highest concentration was observed at this location in May 2003 and August 2004. This result suggests that the measured concentration of CTC at sampling site 4 is originating from an agricultural source. Another example of origin that can be derived from the analysis is SMR and SMX. These compounds are assumed to be human derived (Table 6.1) and they are found at significantly higher concentrations at sampling site 3, just downstream of a wastewater reclamation facility. The highest concentrations of SMR (0.06μg/L) in May 2003 and SMX (0.32μg/L) in August 2004 were measured at sampling site 3. This result agrees with a previous study reporting SMX as one of the 35 most frequently detected chemicals coming from wastewater treatment plants (Glassmeyer et al., 2005). For ETM-H2O in the water matrix, a significant difference was observed through sampling sites at all 4 sampling events. Since ETM-H2O is used for both human and animals (Table 6.1), this compound can be contributed from either wastewater treatment or distributed animal farms. However, the

highest concentration was measured at sampling site 3 where it is assumed to be human influenced.

Table 6.7 Spatial Statistical Analysis of Selected Compounds at Different Time Periods for Water and Sediment.

	May 2003		April 2004		August 04		February 2005	
	Friedman S value	P-value	Friedman S value	P-value	Friedman S value	P-value	Friedman S value	P-value
	Water							
TC	NE[a]	NE	NE	NE	7.5	0.06	6.1	0.17
CTC	9.0	0.03*	NE	NE	9.0	0.03*	7.0	0.07
OTC	NE	NE	7.2	0.07	NE	NE	8.4	0.04*
DMC	NE	NE	7.7	0.05	8.0	0.05	5.7	0.13
MCC	7.3	0.06	NE	NE	7.3	0.06	6.8	0.08
DXC	6.0	0.11	NE	NE	NE	NE	9.0	0.03*
STZ	NE	NE	8.8	0.03*	NE	NE	8.3	0.04*
SMR	8.3	0.04*	9.0	0.03*	8.3	0.04*	9.0	0.03*
SMT	NE	NE	NE	NE	NE	NE	8.8	0.03*
SCP	NE	NE	NE	NE	NE	NE	NE	NE
SMX	8.8	0.03*	9.0	0.03*	9.0	0.03*	9.0	0.03*
SDM	8.3	0.04*	NE	NE	7.3	0.06	8.3	0.04*
ETM-H_2O	8.8	0.03*	9.0	0.03*	8.4	0.04*	8.8	0.03*
RTM	NE	NE	NE	NE	NE	NE	NE	NE
TYL	NE	NE	NE	NE	NE	NE	NE	NE
	Sediment							
TC	9.0	0.03*	8.2	0.04*	9.0	0.03*	9.0	0.03*
CTC	9.0	0.03*	8.2	0.04*	9.0	0.03*	9.0	0.03*
OTC	9.0	0.03*	8.2	0.04*	8.2	0.04*	9.0	0.03*
DMC	7.0	0.07	7.7	0.05	2.6	0.46	9.0	0.03*
MCC	9.0	0.03*	9.0	0.03*	8.2	0.04*	9.0	0.03*
DXC	9.0	0.03*	9.0	0.03*	9.0	0.03*	9.0	0.03*
STZ	5.4	0.15	8.2	0.04*	9.0	0.03*	6.7	0.08
SMR	NE	NE	NE	NE	NE	NE	8.2	0.04*
SMT	NE	NE	9.0	0.03*	9.0	0.03*	NE	NE
SCP	8.2	0.04*	NE	NE	NE	NE	NE	NE
SMX	5.4	0.15	NE	NE	NE	NE	NE	NE
SDM	7.8	0.05	NE	NE	NE	NE	8.8	0.03*
ETM-H_2O	8.2	0.04*	9.0	0.03*	8.8	0.03*	8.8	0.03*
RTM	9.0	0.03*	NE	NE	NE	NE	7.0	0.07
TYL	8.1	0.04*	8.3	0.04*	9.0	0.03*	5.0	0.17

* denotes a significant difference among different sampling sites. [a] NE = Not Evaluated

This result might indicate that the majority of ETM-H2O residuals detected in water samples are contributed from human rather than animal sources. Neither RTM nor TYL was evaluated with statistical analysis due to lack of detected concentration for water samples.

For sediment, 13 out of the 15 compounds showed significant differences in concentrations at least once at different sampling locations within sampling periods (Table 6.7) and a similar trend was observed with water samples. Only animal-used compounds, CTC, STZ, SCP, SDM, and TYL were measured at significantly higher concentrations at either sampling point 4 or 5 where is assumed to be highly agricultural influenced region. The highest concentration of human derived antibiotics was measured at either sampling site 3 or 5, both regions under the influence of wastewater treatment plants. This result agrees with previous studies that the major pathway for release of human-used antibiotics is wastewater treatment facilities (Hirsch et al., 1999; Kolpin et al., 2002; McArdell et al., 2003; Stamatelatou et al., 2003; Gobel et al., 2004; Kolpin et al., 2004; Glassmeyer et al., 2005). For TC, OTC, and ETM-H2O, used by both human and animals, the highest concentration varied between sampling sites and the origin of the antibiotics was not apparent. However, the highest concentration of TC was measured at either sampling site 3 or 5 and three out of four sampling events for OTC at these sites. Also, ETM-H2O showed the highest concentration at sampling site 3 on three of four sampling events. These results suggest that the release of these 3 antibiotics is more influenced by human than animal sources.

The result of temporal statistical analysis shows that the concentration of antibiotics is significantly different from season to season for both water and sediment

depending on individual antibiotics (Table 6.8). Among seasons showing statistically significant differences in concentration of antibiotics between different regions, February 2005 showed the highest concentration compared to other sampling periods for both

Table 6.8 Temporal statistical analysis of selected compounds at different sampling sites for water and sediment.

	Site 2		Site 3		Site 4		Site 5	
	Friedman S value	P-value	Friedman S value	P-value	Friedman S value	P-value	Friedman S value	P-value
	Water							
TC	8.1	0.04*	8.3	0.04*	NE	NE	7.3	0.06
CTC	NE	NE	8.3	0.04*	8.8	0.03*	NE	NE
OTC	NE	NE	4.9	0.18	9.0	0.03*	8.3	0.04*
DMC	NE	NE	7.6	0.06	NE	NE	5.6	0.13
MCC	8.6	0.04*	7.3	0.06	7.2	0.07	8.3	0.04*
DXC	9.0	0.03*	8.5	0.04*	NE	NE	NE	NE
STZ	8.3	0.04*	NE	NE	5.9	0.12	NE	NE
SMR	NE	NE	9.0	0.03*	5.4	0.15	NE	NE
SMT	NE	NE	NE	NE	NE	NE	NE	NE
SCP	NE	NE	NE	NE	NE	NE	NE	NE
SMX	NE	NE	9.0	0.03*	6.7	0.08	8.8	0.03*
SDM	8.8	0.03*	4.8	0.19	NE	NE	6.0	0.11
ETM-H_2O	8.8	0.03*	8.2	0.04*	8.8	0.03*	9.0	0.03*
RTM	NE	NE	NE	NE	NE	NE	NE	NE
TYL	NE	NE	NE	NE	NE	NE	NE	NE
	Sediment							
TC	9.0	0.03*	9.0	0.03*	9.0	0.03*	8.2	0.04*
CTC	8.2	0.04*	9.0	0.03*	9.0	0.03*	9.0	0.03*
OTC	9.0	0.03*	8.8	0.03*	9.0	0.03*	9.0	0.03*
DMC	9.0	0.03*	6.9	0.07	8.2	0.04*	9.0	0.03*
MCC	9.0	0.03*	9.0	0.03*	8.2	0.04*	9.0	0.03*
DXC	8.2	0.04*	9.0	0.03*	9.0	0.03*	9.0	0.03*
STZ	6.6	0.09	8.2	0.04*	8.8	0.03*	9.0	0.03*
SMR	NE	NE	NE	NE	NE	NE	NE	NE
SMT	NE	NE	9.0	0.03*	NE	NE	NE	NE
SCP	NE	NE	8.1	0.04*	NE	NE	NE	NE
SMX	NE	NE	8.1	0.04*	NE	NE	NE	NE
SDM	9.0	0.03*	9.0	0.03*	9.0	0.03*	9.0	0.03*
ETM-H_2O	7.8	0.05	8.2	0.04*	8.2	0.04*	8.2	0.04*
RTM	NE	NE	9.0	0.03*	NE	NE	8.3	0.04*
TYL	5.9	0.12	9.0	0.03*	8.2	0.04*	9.0	0.03*

* denotes a significant difference among different sampling periods. [a] NE = Not Evaluated

water and sediment matrices except MLs in sediment showed the highest frequency in August 2004. The highest concentration of 3 classes of antibiotics in water samples was measured 13/19 times during February 2005 and 22/42 times in sediment samples during the same period.

One of the factors contributing to higher concentrations of antibiotics in the river during February 2005 was the low flow. Previous research has shown that the concentration of different organic compounds including antibiotics varied with flow with the highest concentration and frequency observed during low-flow conditions (40%) compared to high (8.7%) and medium flow (8.7%) conditions (Kolpin et al., 2004). Another factor contributing to higher concentrations during this period might be the cold-water temperature. Since the average water temperature during February 2005 was 5°C, microbial activity was inhibited relative to periods of warmer water. Since biodegradation is an important natural attenuation mechanism, the decay of these compounds would be less than during warmer water periods (Ingerslev et al., 2000; Ingerslev et al., 2001; Vaclavik et al., 2004).

To address the runoff effect on the measured concentration of antibiotics, concentrations were compared between May 2003 and April 2004. While no significant differences were observed in water samples for animal-used antibiotics (Table 6.1) between the two time periods at all sampling locations, statistical analysis showed that the measured concentration of MCC and SMR in water samples was significantly different with the highest concentration of the two antibiotics detected at sampling sites 3 and 5. Both MCC and SMR are considered human-used medicines and the result showing the highest concentrations in urban regions during runoff season indicates that water

released from wastewater treatment facilities still dominate the aqueous phase contribution.

Meanwhile, two veterinary antibiotics, CTC and SDM, showed significantly higher concentration in collected sediment samples from agriculture-influenced regions, sampling sites 4 and 5, during May 2003. In contrast to the aqueous samples, significantly higher concentrations of CTC were detected in the sediment sample collected in May 2003. Since CTC is known to have strong sorption characteristics to the solid matrix (Tolls, 2001; Thiele-Bruhn, 2003), this result may be due to the sorbed compound accumulating at higher flow rates. In addition, a recent study indicated that residuals of SAs were transported from the field to the watershed via runoff after manure was applied as fertilizer (Burkhardt et al., 2005). The application of manure as fertilizer could coincide with the higher flows experienced during the runoff period.

In the case of TYL, a significantly higher concentration was measured in sediment samples collected during August 2004 at all sampling sites. Considering the higher water temperature (around 20°C) at this time of the year, this result was unexpected. One explanation is that the release of this compound is increased during the later summer months, possibly due to the return flow from irrigation ditches. In fact, a previous study has documented that pesticides in sediment from irrigation channels and drains showed a higher concentration than the nearby river (Muller et al., 2000). The researchers concluded that this increased load of pesticides in irrigation channels and drains could be transported to the watershed and impact the downstream environment (Muller et al., 2000). In addition, the average monthly flow of the river during August 2004 is higher than other low flow periods (Table 6.2). This information might support

input of irrigation flow to main watershed. No concentration comparison was made between irrigation ditches and the main watershed of our studied region in the present study but additional research is underway to quantify the impact of irrigation ditches on antibiotic transport.

6.6 Pseudo-Partitioning Coefficient (P-PC) Calculation

To understand the relative importance of sediment and aqueous antibiotic concentrations, a pseudo-partitioning coefficient (P-PC) is introduced and calculated as the ratio of the measured concentration in sediment to the concentration in overlying river (Table 6.9). Since the river and sediment are not at equilibrium, this value cannot be regarded as true partitioning coefficient. However, calculated P-PC values can be a valuable indicator of the sorption characteristics of individual compounds. Since microbial antibiotic resistance most likely originates in the benthic sediments it is important be able to characterize the sorption characteristics in an actual aquatic system. Previous research has determined partitioning coefficients of certain veterinary pharmaceuticals including tetracycline, oxytetracycline, sulfathiazole, sulfamethazine, and tylosin. The values varied depending on soil type and pH and ranged from 1140 – 1620 (TC), 290 – 1030 (OTC), 4.9 (STZ), 0.6 – 3.1 (SMT), and 8.3 – 128 (TYL) L/kg (Tolls, 2001).

Our results also showed that TCs were the most strongly sorbed compound group and TYL was calculated within the range of literature values. In contrast, SAs showed much higher partitioning compared to Toll's review, most likely due to the low concentration of water samples.

Significantly higher concentrations of antibiotics were detected in the sediment matrix compared to water. These findings indicate that antibiotics can accumulate in

sediment and be released to the water in the future depending on benthic environmental changes. Thus, it's necessary to study both water and sediment matrix to identify the occurrence and fate of antibiotics. Furthermore, the pseudo-partitioning coefficient found to be a useful tool to summarize the impact of all environmental variables that contribute to sorption of antibiotics to sediment. The P-PC value determined in our study was within the range of literature values for several hydrophobic compounds. Further study will be aimed at understanding the differences between lab determined equilibrium PCs and those determined with field data.

Table 6.9 Pseudo-partitioning coefficient (P-PC) of selected compounds

Compounds	Mean (L/kg) [a]	Reference (L/kg) [b]
TC	1051	1140 – 1620
CTC	305	
OTC	1267	78 – 3020
DMC	423	
MCC	1848	
DXC	1018	
STZ	378	3 - 5
SMR	517	
SMT		1 – 3
SCP	97	1 – 2
SMX	20	
SDM	402	
ETM-H_2O	211	
RTM		
TYL	91	8 - 128

[a] Mean was calculated based on detected concentration for both water and sediment, [b] reference from (Tolls, 2001; Thiele-Bruhn, 2003) and missing value is either has not been reported or found.

Chapter 7

Simulated Rainfall Study for Transport of Veterinary Antibiotics – Mass Balance Analysis

Submitted to Chemosphere

7.1 Abstract

Occurrence of human- and animal-associated antibiotics has been reported in various environmental compartments. Yet, there is a lack of information verifying the transport mechanisms from source to environment, particularly the transport of veterinary antibiotics as a non-point source pollutant. A rainfall simulation study was conducted to address surface runoff as a possible transport mechanism of veterinary antibiotics introduced in the field and mass balance was calculated with supplementary surface and depth soil measurement. Seven veterinary antibiotics commonly used in agriculture for therapeutic and non-therapeutic (growth-promotion) purposes were examined in this study, including tetracycline (TC), chlortetracycline (CTC), sulfathiazole (STZ), sulfamethazine (SMZ), erythromycin (ETM), tylosin (TYL), and monensin (MNS). Runoff in aqueous and sediment phases was collected every five minutes during the rainfall simulation, and additional surface (0 – 2 cm) and depth (2 – 30 cm) soil samples

were collected after rainfall simulation for mass balance analysis. Quantification of antibiotic concentration in all collected samples was based on solid phase extraction (SPE) followed by measurement with high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). MNS showed the highest concentration in runoff aqueous samples, while ETM showed the highest concentration in runoff sediment samples. The highest concentration of each applied antibiotic in surface soil samples occurred at different locations. This result might indicate the mobility of these compounds in surface soil varies due to different physicochemical properties among the antibiotics. Further, the analysis results showed that all of the subject antibiotics had penetrated into the subsurface; yet, no residuals were found for STZ, suggesting this compound might have penetrated even deeper into the soil. These results indicate that aqueous or sediment erosion control might reduce the transport of veterinary antibiotics in the environment.

Keywords: Veterinary antibiotics; HPLC/MS/MS; Rainfall simulation; Transport; Mass balance

7.2 Introduction

Human- and animal- associated antibiotics are regarded as micro-pollutants, and there has been increased concern about the adverse effects of released antibiotics in the environment. A significant concern is that antibiotics introduced to the environment can result in bacterial resistance and eventual antibiotic ineffectiveness in human health management (Guardabassi et al., 1998; Chee-Sanford et al., 2001; Ohlsen et al., 2003; Schwartz et al., 2003; Sengelov et al., 2003). Consequently, researchers have investigated the occurrence of human- and animal-sourced antibiotics in several different

environmental compartments through different transport mechanisms. Previous studies reported the presence – less than 1 μg L^{-1} – of human-derived antibiotics in the effluent of municipal wastewater treatment plants (WWTPs) and in nearby watersheds receiving that effluent (Farre et al., 2001; Kolpin et al., 2002; Miao et al., 2002; McArdell et al., 2003; Vanderford et al., 2003; Gobel et al., 2004; Glassmeyer et al., 2005). In contrast to human-applied antibiotics directly discharged to the environment through this point source, veterinary antibiotics (VAs) are primarily introduced to the environment as a non-point source. Although accidental leakage or leaching from animal waste storage can be a source, VAs generally are introduced to agricultural fields in manure applied as fertilizer.

VAs have been detected in soil at high concentrations and can persist for long periods of time (Hamscher et al., 2002), increasing the chance of transport into other environments. Campagnolo et al. (Campagnolo et al., 2002) measured five groups of antibiotics - including 12 individual VAs - in animal waste, surface water, and ground water resources near large-scale swine and poultry feeding operations. Researchers found the greatest VA concentrations in lagoons – 1,000 μg L^{-1} of chlortetracycline and 400 μg L^{-1} of sulfamethazine – followed by lesser levels found in surface water and even lesser levels in ground-water. Although specific transport mechanisms were not discussed, the results of this study verify that VAs could be transported from animal waste to surface water and even to ground water. Other researchers measured 29 individual VAs in manure, soil, and surface water and described the possible transport of VAs from manure to surface water (Christian et al., 2003).

It is likely that VAs introduced to the environment are transported to other compartments, and surface runoff or leaching to subsurface soils after large rainfall

events are possible transport pathways. Experimental rainfall simulation has been widely used to evaluate the transport of pesticides (Truman et al., 1998; Ma et al., 1999; Wesenbeeck et al., 2001; Li et al., 2002; Muller et al., 2004); studies applying this method show the mobility of pesticides varied depending on their persistence and sorption characteristics in soil.

There are few reported studies examining the transport of VAs. Only recently, Kay et al. (Kay et al., 2004, 2005c) assessed the transport of VAs in soil amended with slurry, measuring concentrations of VAs in a drainflow and in runoff aqueous and sediment samples. This work highlighted the importance of preferential flow and overland flow in the transport of VAs from soil to other environmental compartments. Other researchers measured surface runoff of sulfonamides from soil treated with liquid manure and concluded not only that applied manure could enhance runoff volume but also that the pH of the manure induced interaction between the manure and sulfanomides, increasing mobility of the sulfonamides (Burkhardt et al., 2005). However, one of the shortcomings of previous studies is that there was no mass calculation of VAs remaining in surface and subsurface soil, leaving an incomplete understanding of transport of VAs.

The objective of this study was to measure the concentration of seven different VAs in runoff aqueous and sediment samples to evaluate rainfall effects on transport of VAs. The study targeted measurement of VA mass not only in runoff but also in surface and subsurface soils to better identify possible transport pathways of VAs.

7.3 Materials and Methods

7.3.1 Chemicals

The seven subject VAs, identified in Table 7.1, were purchased from Sigma-Aldrich Co. (St. Louis, MO); monensin was available as a sodium additive (90-95%). Organic solvents used were all HPLC grade (99.9%); formic acid was analytical grade (99%). Citric acid-monohydrate, sodium phosphate-dibasic anhydrous, and disodium ethylene diaminetetraacetic acid (Na_2EDTA) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Simatone (1000 mg L^{-1} in methanol) used as an internal standard was purchased from Absolute Standards (Hamden, Connecticut, USA). The HLB (Hydrophillic-Lipophillic-Balance) solid phase extraction (SPE) cartridges (3 mL/60 mg) were purchased from Water Oasis Co. (Milford, MA). Milli-Q water (18.3 MΩ) from a Millipore (Billerica, CA) purification system was used when deionized (DI) water was required.

A standard solution of the seven subject antibiotics (100 mg L^{-1}) was prepared each month in methanol and stored at 4 °C (Liang et al., 1998) during the experimental period. Working solutions, 5 mg L^{-1} and 0.5 mg L^{-1}, were prepared weekly by diluting the standard solution in methanol.

7.3.2 Site Description and Rainfall Simulation Method

The rainfall simulation study was conducted at the Agricultural Research, Development, and Educational Center (ARDEC) at Colorado State University, Fort Collins, CO, USA. Soil texture was sandy clay loam and physicochemical properties of the surface soil (0 to 2 cm) are summarized in Table 7.2. Soil water content determined

Table 7.1 Summary of the physicochemical properties of seven subject veterinary antibiotics.

Class	Compounds	Acronym	M.W (g)	Usage[a]	Log K_{ow}	K_d[e]	K_{oc}[e]	Reference
Tetracyclines	Tetracycline	TC	444.5	Cattle and swine	-1.3[c]	1140-1620		(Sithole et al., 1987)
(TCs)	Chlortetracycline	CTC	478.9	Cattle and beef	-0.6[c]	22-164973		(Sassman et al., 2005)
Sulfonamides	Sulfamethazine	SMT	278.3	Cattle and beef	0.28[d]	1-3	82-208	(Thiele-Bruhn, 2003)
(SAs)	Sulfathiazole	STZ	255.3	Swine	-0.43[d]	3	97	
Macrolides	Tylosin	TYL	916.1	Swine	1.63[c]	8-128	553-7990	(Rabolle, 2000)
(MLs)	Erythromycin	ETM-H_2O[b]	733.9	Cattle, beef Feedlot, chicken	3.06[d]			
Ionophores (IPs)	Monensin	MNS	692.9	Cattle, beef	5.4-8.5			(Thiele-Bruhn, 2003)

[a] Reference from U.S. Food and Drug Administration (2005).
[b] Metabolite of Erythromycin was analyzed in this study according to previous study (Hirsch et al., 1999).
[c] Reference from (Thiele-Bruhn, 2005).
[d] Reference from (Adams et al., 2002).
[e] Values vary depending on soil properties, pH, and % organic carbon.

prior to rainfall simulation with the gravimetric method averaged 3.1% in topsoil (0 to 2 cm) and 18.9% at a depth of 2 to 15 cm. Measured soil density of the topsoil was 1.1 g mL^{-1}.

Three plots were arranged along the slope (~ 2%) in the row direction, about 3 m apart. The surface area of each plot was 6 m^2 (2 m × 3 m, W×L), and the three simulation plots were separated with metal barriers to a depth of 20 cm. Two half-beds were constructed in each plot, and a gutter was installed at the down slope of each plot to collect runoff and soil erosion. A schematic diagram of plot design is shown in Figure 7.1.

Table 7.2 Physicochemical properties of surface soil in studied area.

Contents[a] (%)	pH[b]	EC[c] (dS m^{-1})	OM[d] (%)	CEC[e] (cmoles kg^{-1})	$CaCO_3$[f] (g kg^{-1})
Sand: 55 Silt: 16 Clay: 29	7.9	1.19	18	24.2	44

[a] Analytical method adapted from (Gee et al., 2002).
[b] (Thomas, 1996).
[c] (Rhoades, 1996).
[d] (Nelson et al., 1996).
[e] (Summer et al., 1996).
[f] (Loeppert et al., 1996).

The prepared stock solution of the seven subject antibiotics was diluted in a backpack sprayer containing deionized water to yield a final concentration of 1 mg L^{-1}. Application of antibiotics to each plot was implemented using a backpack sprayer with four nozzles (#8005 with screen) on a 1.5-m boom operated at 207 kPa (30 psi). Targeted application rate was 209 mg/plot to mimic the estimated amount of antibiotics present in liquid manure applied to corn at the typical rate of 2.2 g-N/m^2 (Davis et al., 2002). Rainfall simulation was started one hour after antibiotic application to each plot.

A rainfall simulator equipped with oscillating nozzles was located three meters above the surface of each plot to simulate natural rainfall. Rainfall intensity was programmed to correspond to historical 15-minute rainfall data in the cities of Fort Collins (1975-2002) and Byers (1971-2002) from March through August and reflect the climate of northeastern Colorado. Duration of the rainfall period (60 min) allowed 60 mm of precipitation, to reflect the most extreme rainfall event and worst-case scenario. Ground water with pH = 8.0 and EC = 0.10 dS m^{-1} was used as simulation rain throughout the study.

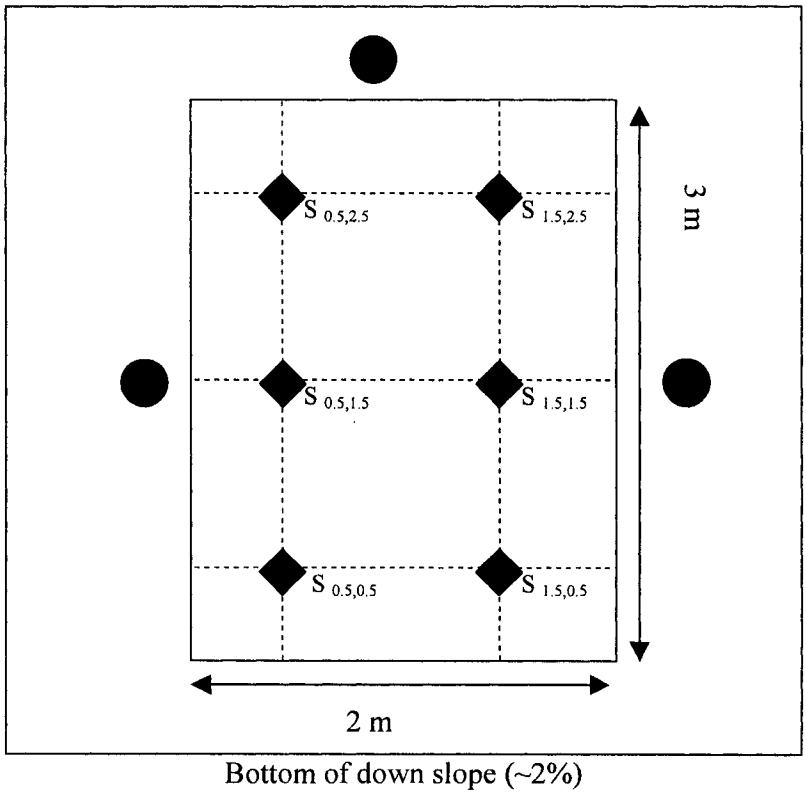

Figure 7.1Rainfall simulation plot: ♦denotes sampling locations for surface and subsurface soil after rainfall simulation and • denotes filter paper locations. S_{ij} represents the coordinates of X-Y direction from the bottom. Diagram not to scale.

7.3.3 Sample Collection and Pre-Treatment

Surface runoff and eroded sediment were collected at the down slope of each plot every five minutes in 1-L sampling bottles pre-rinsed with deionized water. To identify the rate of runoff, an additional one-liter of sample was collected and weighed after transfer to the lab. After the collected bottles were held for 24 hours at room temperature to separate aqueous and sediment phases gravimetrically, sample bottles were placed in an oven at 105 °C for 24 hours and weighed again.

Three filter papers (12.5-cm diameter, Baxter Grade 360) placed outside of each plot as illustrated in Figure 7.1 were collected immediately after rainfall simulation and wrapped with aluminum foil. They were transferred to the lab and held at 4 °C until antibiotic analysis.

Runoff samples collected for antibiotic analysis were kept in a cooler with ice during rainfall simulation and immediately transferred to the lab for pre-treatment. Each 1-L sample collected at five-minute intervals was evenly decanted into 50-mL centrifuge tubes and centrifuged at 3500 rpm for 30 min to separate aqueous and solid phases. The separated aqueous phase was filtered through a 0.2-μm glass fiber filter to another 1-L amber glass bottle and held at 4 °C until analysis. Post-centrifuge solid phases were transferred to sampling bags and held in a dark room to prevent possible photo-degradation until completely air-dried.

After rainfall simulation, surface soil samples (0 to 2 cm) and depth soil samples (2 - 10, 10 – 20, 20 – 30 cm) were collected at the six locations of each plot shown in Figure 7.1 for subsequent mass balance analysis. Collected soil samples also were held in a dark room to dry completely.

7.3.4 Extraction and Analysis of Antibiotics

Filter papers collected from each plot were placed in separate 250-mL flasks, then 40 mL of methanol was added to each flask. After shaking flasks vigorously for 20 minutes at 400 rpm (Model No-4626, Lab-line instrument), a 5-mL aliquot from each flask was transferred to a 15-mL graduated cylinder containing internal standard simatone (0.1 mg L^{-1}). Prepared samples were concentrated in a water bath (50 °C), with gentle nitrogen flow to prevent oxidation of antibiotics, until 50 μL remained, after which 70 μL of mobile phase A (99.9 % deionized water + 0.1 % formic acid) was added for HPLC/tandem mass spectrometry (HPLC/MS/MS) analysis.

The procedure for extracting the subject antibiotics in aqueous and sediment samples is detailed elsewhere (Kim et al., 2006). Solid phase extraction (SPE) with a 3 mL/60 mg HLB (Hydrophilic-Lipophilic-Balanced) cartridge was used for cleanup and enriching the samples. This cartridge is durable over a wide range of pH levels and contains no silanol groups that might bind irreversibly with tetracyclines (TCs). Two different buffer solutions – McIlvaine buffer solution (pH 4.0) for TC, CTC, STZ, and SMT and ammonium hydroxide buffer solution (1M, pH 10.0, titrated with formic acid) for ETM-H_2O, TYL, MNS – were used to extract antibiotics from solid to liquid phase.

High performance liquid chromatography (HPLC) was utilized to separate extracted antibiotics. The HPLC system (HP 1100, Agilent, Palo Alto, CA) consisted of an Agilent 1100 Series Thermostatted Auto Sampler and a variable-wavelength UV detector. The analytical column was a reverse-phase Xterra MS C18 (Waters, Milliford, MA), 2.1 × 50 mm (diameter × length, 2.5-μm pore size, end-capped). A C_{18} guard

Table 7.3 Optimized HPLC/MS/MS parameters

Compounds	Precursor Ions ($m\ z^{-1}$)	Isolation Width	Collision Energy (%)	Fragment Ions ($m\ z^{-1}$)	Mobile Phase Composition [a] (%)	Flow ($mL\ min^{-1}$)	Column Temperature (°C)
TC	445	2.0	26	**427 $[M+H-H_2O]^+$**	A: 96 + B: 4 (0 min) A: 70 + B: 30 (29 min) A: 96 + B: 4 (30 min)	0.32	15
CTC	479	2.0	32	**462 $[M+H-NH_3]^+$**			
STZ	256	2.0	32	**156**			
SMT	279	2.0	38	**204**			
ETM-H_2O	716	3.0	26	**558 $[M+H\text{-desosamine-}H_2O]^+$**	A: 80 + B: 20 (0 min) A: 65 + B: 35 (13 min) A: 96 + B: 4 (14 min)	0.32	45
TYL	916	3.0	30	**772 $[M+H\text{-cladinose}]^+$**			
MNS	693	2.0	28	**675 $[M+Na-H_2O]^+$**	A: 50 + C: 50 (0 min) A: 10 + C: 90 (19 min) A: 50 + B: 50 (20 min)	0.25	15
Optimized MS/MS	Nitrogen gas for drying and nebuilizing, Spray voltage: 4.5V Capillary voltage: 21V, Capillary temperature: 165 °C						

[a] Mobile phase A: 99.9% water + 0.1% formic acid, Mobile phase B: 99.9% acetonitrile + 0.1% formic acid, Mobile phase C: 100% methanol

column (Phenomenex, Torrence, CA, USA) was installed to filter any particulates from the sample.

For tandem mass spectrometry (MS/MS) analysis, a ThermoFinnigan LCQ Duo ion trap mass spectrometer (ThermoQuest, Woburn, MA) equipped with a heated capillary interface and electrospray ionization (ESI) was used. Optimization of MS/MS parameters was conducted with a LCQ Duo syringe pump containing 10 μM of each antibiotic standard solution at a flow rate of 5μL min^{-1}. Optimized HPLC/MS/MS parameters, including precursor and fragment ions, are summarized in Table 7.3. Positive mode was used for all antibiotics, and ten minutes of post-run was allowed at the end of each analysis to achieve column equilibrium.

For quality assurance (QA) purposes, recovery and limit of quantification (LOQ) summarized in Table 7.4 was adapted from a previous study that evaluated river water and sediment (Kim et al., 2006). Concentrations were measured in a negative sample spiked with three different concentrations of each antibiotic.

Table 7.4 Summary of antibiotic recovery and limit of quantification (LOQ)

	(a) Water		(b) Sediment	
	Recovery (%)[a]	**LOQ (μg L^{-1})**	**Recovery (%)[a]**	**LOQ (μg kg^{-1})**
TC	107	0.006	88	1.9
CTC	112	0.009	75	0.9
STZ	91	0.002	98	1.2
SMT	105	0.005	93	1.8
ETM-H_2O	102	0.008	119	1.7
TYL	102	0.009	77	1.1
MNS	115	0.001	83	0.4

[a] Average value of three standard spikes: 0.1, 1, and 5 μg L^{-1} for water and 1, 30, and 90 μg kg^{-1} for sediment. Relative standard deviation (RSD) for water and sediment was 3 to 13% and 3 to 24%, respectively.

7.3.5 Statistical Analysis

To evaluate the significance of variation of measured concentrations in surface runoff aqueous and sediment samples from the three plots, one-way analysis of variance (ANOVA) at the probability level of $p < 0.05$ was applied. For normality and variance homogeneity analysis, the Shapiro-Wilk test and Levene's test were applied with a Minitab software program (MINITAB, 2000). When differences were found to be significant, Tukey's and Hsu's MCB (Multiple Comparisons with the Best) method were applied in post-statistical analysis to compare the difference of individual means.

7.4 Results and Discussion

Simulated rainfall intensity and runoff rate in both aqueous and sediment phases were averaged for three plots and are presented in Figure 7.2. Rainfall intensity was quickly increased during the first 20 minutes to obtain a maximum of 115 mm h^{-1} then gradually decreased until the end of the experiment. The pattern of runoff rate in aqueous and solid phases was similar to rainfall intensity, except the highest runoff rate for both phases was observed five minutes after maximum rainfall intensity was reached.

Calculated actual application rate based on the measured concentration of the subject antibiotics on filter papers located outside of each plot was varied, depending on antibiotics and plot (Table 7.5). Although anticipated application rate was 209 mg $plot^{-1}$ based on pre-calibrated backpack sprayer application rate, more antibiotics were recovered on filter papers for all subject antibiotics except ETM-H_2O. This variance might be caused by unintended excess application from the backpack sprayer.

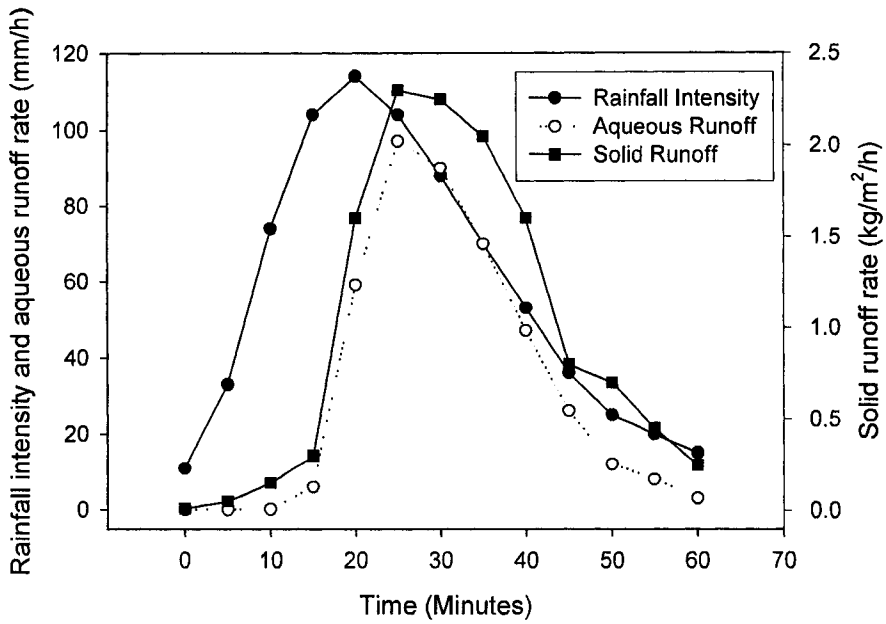

Figure 7.2 Rainfall intensity and runoff rate in both aqueous and solid phases. Values shown are the average of three plots.

Table 7.5 Measured application rate of subject antibiotics in each plot

	Application Rates (mean ± standard deviation)[a] mg plot^{-1}				
Antibiotic	**Plot 1**	**Plot 2**	**Plot 3**	**Plot mean**	**% Difference[b]**
TC	316 ± 32	296 ± 23	313 ± 16	308 ± 10	32
CTC	245 ± 51	219 ± 15	247 ± 28	237 ± 16	12
STZ	274 ± 46	251 ± 48	278 ± 28	267 ± 15	22
SMT	258 ± 59	224 ± 3	258 ± 30	247 ± 20	15
ETM-H_2O	206 ± 55	175 ± 34	209 ± 30	197 ± 19	-6
TYL	277 ± 65	241 ± 32	275 ± 32	264 ± 20	21
MNS	282 ± 39	252 ± 34	280 ± 17	271 ± 17	23

[a]Average of three filter papers placed outside each plot.
[b] Difference was calculated between plot mean and intended application rate (209mg plot^{-1}).

Measured concentrations of antibiotics in runoff aqueous and sediment matrices of three plots are averaged and summarized in Table 7.6. The highest concentration of all antibiotics in runoff aqueous and sediment samples occurred within the first two

sampling events (at five and ten minutes), except for STZ (5.71 μg kg^{-1} at 15 minutes in runoff sediment). TC and CTC showed measurable concentration in runoff aqueous samples until 25 and 45 minutes, respectively, after which detected concentration for both VAs was less than the LOQ until the end of the experiment.

Table 7.6 Average antibiotic concentration in runoff aqueous and sediment phases

	Aqueous (μg L^{-1}) [a]						
Tim	**TC**	**CTC**	**STZ**	**SMT**	**ETM-H_2O**	**TYL**	**MNS**
5	0.12 ± 0.02	0.09 ± 0.02	1.32 ± 0.33	3.45 ± 0.28	0.51 ± 0.10	0.17 ± 0.07	1.87 ± 0.76
10	0.09 ± 0.05	0.07 ± 0.01	0.44 ± 0.13	0.57 ± 0.23	0.67 ± 0.26	0.27 ± 0.28	2.75 ± 0.49
15	0.05 ± 0.03	0.08 ± 0.01	0.42 ± 0.16	0.86 ± 0.53	0.39 ± 0.15	0.24 ± 0.12	2.23 ± 0.63
20	0.02 ± 0.01	0.05 ± 0.00	0.29 ± 0.07	0.48 ± 0.17	0.30 ± 0.10	0.11 ± 0.06	1.81 ± 0.11
25	0.01 ± 0.01	0.04 ± 0.02	0.23 ± 0.00	0.34 ± 0.04	0.12 ± 0.06	0.06 ± 0.06	1.21 ± 0.22
30	ND	0.03 ± 0.01	0.19 ± 0.01	0.24 ± 0.05	0.10 ± 0.07	0.04 ± 0.04	0.87 ± 0.15
35	ND	0.02 ± 0.01	0.20 ± 0.01	0.23 ± 0.05	0.08 ± 0.07	0.03 ± 0.05	0.86 ± 0.48
40	ND	0.02 ± 0.01	0.18 ± 0.02	0.14 ± 0.01	0.07 ± 0.03	0.03 ± 0.04	0.83 ± 0.15
45	ND	0.01 ± 0.01	0.18 ± 0.02	0.17 ± 0.03	0.07 ± 0.04	0.02 ± 0.03	0.44 ± 0.17
50	ND	ND	0.19 ± 0.02	0.17 ± 0.04	0.09 ± 0.08	0.01 ± 0.02	0.45 ± 0.08
55	ND	ND	0.18 ± 0.02	0.17 ± 0.05	0.07 ± 0.06	0.01 ± 0.02	0.55 ± 0.09
60	ND	ND	0.18 ± 0.02	0.17 ± 0.01	0.07 ± 0.05	0.05 ± 0.08	0.51 ± 0.02
	Sediment (μg kg^{-1})						
5	4.6 ± 1.7	6.0 ± 2.6	3.0 ± 0.9	ND	30.5 ± 22.2	5.6 ± 3.4	56.9 ± 17.9
10	3.7 ± 0.6	6.2 ± 3.9	1.5 ± 0.3	ND	52.9 ± 22.9	11.8 ± 8.8	35.7 ± 24.2
15	2.4 ± 0.7	ND	5.7 ± 1.2	ND	31.0 ± 14.1	9.8 ± 6.6	13.9± 4.1
20	ND	ND	3.1 ± 1.0	ND	26.8 ± 9.7	8.9 ± 5.6	6.7 ± 3.0
25	ND	ND	2.9 ± 1.2	ND	13.4 ± 8.4	8.3 ± 6.9	3.0 ± 2.4
30	ND	ND	1.6 ± 0.9	ND	9.8 ± 3.3	7.2 ± 6.9	2.3 ± 1.4
35	ND	ND	3.4 ± 2.8	ND	10.4 ± 10.5	8.7 ± 6.4	3.7 ± 1.9
40	ND	ND	1.3 ± 1.6	ND	7.4 ± 5.4	8.0 ± 4.7	1.1 ± 1.1
45	ND	ND	ND	ND	8.1 ± 6.5	6.9 ± 5.1	ND
50	ND	ND	ND	ND	6.1 ± 3.7	7.1 ± 4.3	1.1 ± 0.5
55	ND	ND	ND	ND	5.5 ± 3.6	8.3 ± 4.6	ND
60	ND	ND	ND	ND	2.5 ± 1.4	5.8 ± 2.9	ND

[a] More significant numbers are shown due to lower concentration, ND denotes non-detect or below detection limit.

Similarly, observed concentration was either non-detectable or less than LOQ after the first three and two sampling events for TC and CTC in runoff sediment samples.

While STZ and SMT were detected at all sampling events in runoff aqueous samples, no STZ was detected after 40 minutes in runoff sediment samples, and no SMT was detected in any runoff sediment sample. In comparison, ETM-H_2O and TYL could be measured at all sampling events in both runoff aqueous and sediment phases, and MNS was measured in all runoff sediment samples except at those collected at 45, 55, and 60 minutes.

Statistical analysis revealed that the measured concentration of each antibiotic in both runoff aqueous and sediment samples was not significantly different among plots, indicating that the designed experiment was consistent across plots. However, there were significant differences among antibiotics within each plot. The highest concentration was observed for MNS in a runoff aqueous sample and for ETM-H_2O in a runoff sediment sample during the rainfall simulation period at plot 1 ($p < 0.05$). In contrast, the lowest concentration was observed for CTC in a runoff aqueous sample and for SMT in a runoff sediment sample. Similarly, plot 2 also showed significant difference ($p < 0.05$) among the measured concentration of antibiotics in runoff aqueous and sediment samples. Two antibiotics, SMT and MNS, showed higher concentration in aqueous samples compared to the other antibiotics, and TC, CTC, and SMT showed much lower concentration in sediment samples whereas ETM-H_2O, TYL, and MNS were measured at higher concentrations in plot 2. In plot 3, MNS and ETM-H_2O again showed much higher concentration ($p < 0.05$) in runoff aqueous samples and sediment samples, respectively, than other antibiotics. In general, the highest loss was observed in MNS in runoff

aqueous samples and in ETM-H_2O in runoff sediment samples, while the least loss was measured for TC and CTC in runoff aqueous samples and for STZ and SMT in runoff sediment samples.

Measuring the concentration of the subject antibiotics in surface soil (0 to 2 cm) was done to understand mobility of antibiotics at the soil surface. As shown in Figure 7.3, the most persistent antibiotic in surface soil was ETM-H_2O, and the least persistent was STZ among the detected antibiotics. Each plot showed a similar trend of antibiotic mobility after rainfall simulation, but different behavior was observed among antibiotics. In general, the lowest concentration was measured at the bottom of plots for all antibiotics except for CTC, which showed its highest concentration at the left corner of the bottom edge, and for STZ at the right corner of the bottom edge. TC and CTC showed the highest concentration at the middle of the plot, and STZ showed lower concentrations throughout the entire plot. In comparison, ETM-H_2O and TYL were evenly distributed from top to bottom, but the highest concentration was measured at the top for ETM-H_2O and at the middle of the plot for TYL. For MNS, concentration was highest at the top of the plot and gradually decreased toward the bottom of the plot.

Since the subject antibiotics were exposed for only a short period of time (one hour) prior to rainfall simulation, biotic degradation and photo-degradation were minimized. However, different physicochemical properties of the subject antibiotics and other possible abiotic dissipation, including sorption to solid particles and leaching to subsurface, could explain the differences.

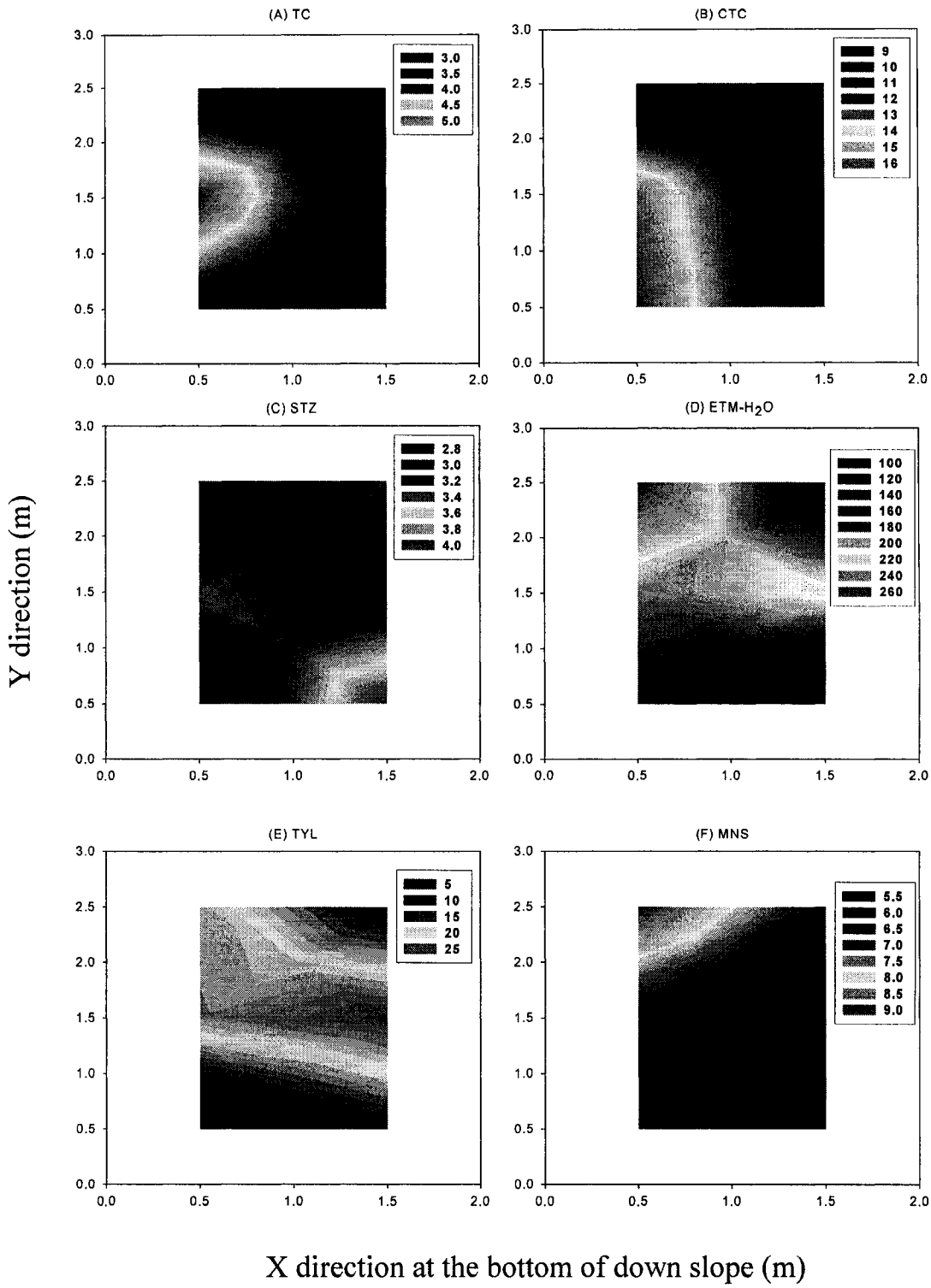

Figure 7.3 Contour map of averaged antibiotic concentration in the surface soil across 3 plots, with different colors representing different concentration (μg/kg).

7.5 Mass Balance Analysis

To track the residuals of applied antibiotics after rainfall simulation in more detail, mass balance analysis was conducted with additional soil depth samples (2 to 30 cm). In this analysis, six samples collected from each plot (Figure 7.1) represented 1 m^2 respectively. Measured soil density at the surface was considered in the mass balance calculation. Since density of depth soil samples was not available, density of topsoil was used for depth soil mass balance calculation. Table 7.7 shows the average mass of each antibiotic from the three plots and the calculated absolute and relative mass. The range of absolute mass recovery was from 0.0 to 90.2 %, and calculations revealed ETM-H_2O exhibited the greatest absolute mass and SMT showed the least absolute mass.

Relative mass calculation identifies the different locations of antibiotic presence. Most of the antibiotics remained within the plot area, and the level in runoff aqueous and sediment samples was relatively minimal. Residuals of all applied antibiotics, except SMT, were detected in surface and depth soil samples after rainfall simulation. In the case of SMT, more than 99 % of its mass was lost via runoff in the aqueous phase. Calculated relative mass in the soil gradually increased with depth for TC, CTC, and STZ, which showed the greatest concentration in 20 to 30-cm depth soil samples. TC and CTC are well documented in binding strongly with solid particles (Rabolle, 2000; Loke et al., 2002; Figueroa et al., 2004; Jones et al., 2005) finding residuals of those antibiotics in depth soil samples is contradictary to their sorption characteristics. However, this result could be explained by colloids acting as a transport carrier. In fact, a previous study

Table 7.7 Average mass of antibiotics in various phases and calculated absolute and relative mass

			TC	CTC	STZ	SMT	ETM-H_2O	TYL	MNS
Plot Mean Mass (mg plot^{-1})[a]	Runoff[d]	Aqueous	0.001 (29) a	0.006 (10) a	0.045 (8) bc	0.059 (19) c	0.026 (24) ab	0.011 (49) a	0.219 (9) d
		Sediment	0.001 (58) a	0.001 (55) a	0.013 (40) a	0.000 (13) a	0.081 (40) b	0.050 (68) ab	0.024 (39) a
	Surface Soil (cm) Depth Soil (cm)	0 to 2	0.5 (4) a	1.5 (3) b	0.3 (8) a	0.0 (ND) c	23.1 (1) d	2.2 (4) e	0.8 (4) f
		2 to 10	14.8 (10) a	40.9 (2) b	6.7 (16) c	0.0 (ND) d	74.5 (1) e	15.3 (3) a	27.3 (4) f
		10 to 20	20.3 (2) a	52.4 (4) b	11.9 (17) c	0.0 (ND) d	48.8 (5) b	10.7 (26) c	33.9 (3) e
		20 to 30	21.3 (6) a	55.6 (2) b	20.8 (24) a	0.0 (ND) c	30.9 (4) d	9.7 (13) e	31.7 (2) d
		Sum	**56.8**	**150.2**	**39.7**	**0.1**	**177.5**	**37.9**	**93.9**
Absolute Mass (%)[b]			18.4	63.4	14.9	0.0	90.2	14.4	34.6
Relative Mass (%)[c]	Runoff[d]	Aqueous	0.002	0.004	0.113	99.391	0.015	0.029	0.233
		Sediment	0.003	0.000	0.033	0.609	0.045	0.133	0.025
	Surface Soil (cm) Depth Soil (cm)	0 – 2	0.8	1.0	0.8	0.0	13.0	5.7	0.8
		2 – 10	26.0	27.2	16.8	0.0	42.0	40.2	29.0
		10 – 20	35.7	34.9	30.0	0.0	27.5	28.3	36.1
		20 – 30	37.5	37.0	52.3	0.0	17.4	25.6	33.7

[a] Average of measured antibiotics from each plot and relative standard deviation (RSD) is in parenthesis. Antibiotics with a common letter are not significantly different by Tukey's method ($p < 0.05$) within each raw.[b] Absolute mass is the ratio between sum of mass in all phases and that recovered in target filter papers (Table 5).[c] Relative mass is the ratio between sum of the mass and recovered mass in each phase.[d] More significant values are shown to display the lower mass and ratio. ND denotes not determined due to no detection.

showed that a strongly sorbed pesticide could be transported into subsoil via a colloid-facilitated transport mechanism (Jonge et al., 1998; Worrall et al., 1999; Villholth et al., 2000; Grolimund et al., 2005). In contrast, the sorption capability of STZ and SMT is much less than that of TC and CTC (Thiele, 2000; Boxall et al., 2002; Thiele-Bruhn et al., 2004), leading to the expectation that the highest mass of STZ was calculated to be at the very bottom of the soil profile. Further, the fact that no mass was detected in a depth-soil column (0 to 30 cm) for SMT could be explained by the fact that SMT had penetrated deeper than the 30-cm depth. For ETM-H_2O, TYL, and MNS, the percentage of relative mass varied within the soil depth column; the highest relative mass was calculated at a soil depth of 2 to 10 cm for ETM-H_2O and TYL and at 10 to 20 cm for MNS.

7.6 Conclusion

A rainfall simulation study was conducted to evaluate the different behaviors after heavy rainfall of veterinary antibiotics applied in the field. Significantly different behavior was observed in runoff aqueous and sediment phases, depending on the different physicochemical properties of the subject antibiotics. Among the antibiotics studied, ETM-H_2O and TYL showed higher mass loss in runoff sediment samples than in runoff aqueous samples while the opposite behavior were observed in the other antibiotics except TC. The measured concentration of subject antibiotics in surface soil (0 to 2 cm) after rainfall simulation was significantly different among antibiotics. Differences in sorption characteristics and persistence among antibiotics might be responsible for this varied presence in surface soil.

Further, mass balance analysis was conducted to calculate antibiotic residuals in each plot and recovered absolute mass in runoff after rainfall simulation. None of the subject antibiotics was fully recovered after rainfall simulation, suggesting that the applied antibiotics might have been degraded or transported outside of the sampling depth. The result of relative mass calculation revealed that SMT was lost primarily via surface aqueous runoff; more-strongly sorbed antibiotics, TC and CTC, might have been transported to subsurface levels via a colloid-facilitated mechanism.

This study demonstrated that different control practices should be implemented, depending on the demonstrated behaviors of introduced veterinary antibiotics after severe rainfall, to reduce contamination of other environmental compartments. Both surface soil and subsurface soil can be exposed, even with strongly sorbed antibiotics.

Chapter 8

Dissolved and Colloidal Fraction Transport of Antibiotics in Soil Columns

Submitted to Environmental Science and Technology

8.1 Abstract

Veterinary antibiotics leached from contaminated manure applied as fertilizer could be transported to surface water as runoff or infiltrated to groundwater after significant rainfall. Transport of veterinary antibiotics is influenced by different physicochemical properties and sorption characteristics. The main objective of this study was to evaluate the transport behavior of 10 antibiotics belonging to 4 different groups. Among other groups, tetracyclines (TCs) assumed to be strongly sorbed compounds were found to have no residuals in the leachate and mainly remained sorbed to the fixed column soil (range between 34 – 81% of initially applied mass). In contrast, one of the sulfonamides (SAs), sulfamethazin (SMT) was almost completely recovered in the leachate (74 – 83%). Although the recovered mass of only 4 compounds, tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), and salinomycin (SLM) showed a significant difference in the soil between biotic and abiotic treatment, a generally lower mass was recovered in abiotic leachate and soil columns indicating that microorganism

activity is a minor attenuation mechanism. Another finding of this study was that colloidal transport could be a major mechanism for antibiotic transport. Colloidal transport was observed most strongly for macrolides (MLs) and this information should be considered when transport of antibiotics is studied.

8.2 Introduction

Human and animal-derived antibiotics have been found in several different environmental compartments due to the widespread use of these compounds (Mellon et al., 2001). The antibiotics are only partially metabolized and the parent compounds along with the metabolites can be released to the environment. (Heberer, 2002a; Diaz-Cruz et al., 2003; Kay et al., 2005a). Consequently, there is an increased concern that released antibiotics can lead to the development of antibiotic resistant microorganisms via either horizontal or vertical resistance gene transfer mechanisms (Chee-Sanford et al., 2001; Ohlsen et al., 2003; Sengelov et al., 2003; Rooklidge, 2004).

Among several suggested transport pathways of antibiotics from source to the environment, the major release pathway of human-used antibiotics has been assumed to be wastewater treatment plants (WWTPs) due to incomplete treatment efficiency. Several previous studies have reported the residuals of released antibiotics in the effluent of a WWTP or streams near the WWTP (Hirsch et al., 1999; Heberer, 2002b; Miao et al., 2002; Barreiro et al., 2003; McArdell et al., 2003; Stamatelatou et al., 2003; Gobel et al., 2004). In addition, veterinary antibiotics can be introduced in soil when manure containing residual of antibiotics is applied in the field as fertilizer (Hamscher et al., 2002; Christian et al., 2003; Liguoro et al., 2003; Blackwell et al., 2004; Jacobsen et al., 2004) and after rainfall, the introduced antibiotics can be transported to surface water as

runoff (Burkhardt et al., 2005; Pedersen et al., 2005; Davis et al., 2006) or leach to the sub-surface. As a result, field and laboratory studies have been conducted to verify the fate and transport of released veterinary antibiotics. Sorption experiments were carried out to examine the partitioning behavior of veterinary antibiotics in solids (e.g. soil or manure) with various conditions (Thiele-Bruhn et al., 2004; Kolz et al., 2005; Mackay et al., 2005; Sassman et al., 2005) and these studies concluded that several mechanisms including hydrophobicity, cation exchange, surface bridging, and hydrogen bonding are involved in the sorption process. Indeed, pH might be the most important parameter impacting sorption and desorption of veterinary antibiotics in soil (Sassman et al., 2005).

Lysimeter and column leaching experiments are alternative methods to observe the fate and transport of veterinary antibiotics in a soil environment. In general, less sorptive antibiotics tend to leach into the sub-surface causing contamination in groundwater and strongly sorptive antibiotics have the affinity to bind with soil particles. With this hypothesis, Rabolle et.al. (Rabolle, 2000) investigated the leaching behavior of four antibiotics and documented that the most strongly sorbed compound, oxytetracycline, was not recovered in the leachate from the soil column. Tylosin was only observed in the top portion of the soil columns and the least sorptive two compounds, metronidazole and olaquindox, were almost completely recovered in the leachate. Another study also evaluated the leaching behavior of three veterinary antibiotics and similar results showed that the more strongly sorbed compounds are less likely to leach to the groundwater (Kay et al., 2005b, a). One of the deficiencies of previous leaching experiments has been that the role of microorganisms was not considered. Although, microorganism activity is not the major dissipation mechanism in most soil column

systems, the biotic effect should not be ignored. Thus, the first objective of our study was to evaluate the leaching behavior of selected antibiotics in the leachate and soil column with inhibition of microorganism activity as a variable.

Table 8.1 Properties of studied substances.

Substances	Acronym	CAS number[a]	Log Kow[b]	Kd (L/kg)[f]	Degradation (texture/%/time(d))[g]
Tetracycline	TC	60-54-6	-1.2	1140 - 1620	Manure/65/84 Soil+manure/100/14 Manure/50/55-105
Chlortetracycline	CTC	64-72-2	-0.6[c]	22 - 164973	Manure/24/84 Soil+manure/88/30
Oxytetracycline	OTC	79-57-2	-1.2	78 - 3020	Soil+manure/0/180 Sediment/50/44
Sulfathiazol	STZ	72-14-0	0.1	3 - 5	NA
Sulfamethazine	SMT	57-68-1	0.9	1 - 3	NA
Erythromycin-H_2O	ETM-H_2O	114-07-8	3.1	NA	Soil+manure/25/30
Tylosin	TYL	1401-69-0	3.5[d]	8 - 128	Slurry+sand/50/4 Manure/50/>3
Monensin	MNS	22373-78-0	5.4 – 8.5[e]	NA	Manure/30-40/70
Salinomycin	SLM	53003-10-4		NA	NA
Narasin	NRS	55134-13-9		NA	NA

[a] reference from (Glassmeyer et al., 2005), [b] reference from (Tolls, 2001) if stated otherwise, [c] reference from (Thiele-Bruhn, 2005), [d] reference from (Jacobsen et al., 2004), [e] reference from (Thiele-Bruhn, 2003); value for polyether group is shown and individual substance value can not be found, [f] reference from (Tolls, 2001; Thiele-Bruhn, 2003; Sassman et al., 2005); values are ranged depending on different soil texture and pH; detailed information can be found in references, [g] reference from (Thiele-Bruhn, 2003), NA: information is not available.

The second objective of this study was to examine whether mobile colloids might be able to facilitate the transport of antibiotics in the subsurface even though the compounds are particularly hydrophobic. In fact, the colloidal-facilitated transport mechanism of other organics including pesticides plays an important role in transport of

these compounds to groundwater (Chaplain et al., 1992; Jonge et al., 1998; Worrall et al., 1999; Sprague et al., 2000; Grolimund et al., 2005). However, colloidal related transport of antibiotics has not been studied and only one of the recent studies proposed that partitioning of tylosin in colloidal matter of manure was as strong as the solid (Kolz et al., 2005). Thus, the result of our study evaluating the effect of colloids as carriers to leach into the sub-surface will increase the understanding of fate and transport of antibiotics.

8.3 Experimental Method

8.3.1 Materials

All HPLC grade organic solvents and chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). Analytical grade chemicals included formic acid (88%, HCOOH, F.W. 46.03), citric acid-monohydrate ($C_6H_8O_7 \bullet H_2O$, F.W. 210.14), sodium phosphate-dibasic anhydrous (Na_2HPO_4, F.W. 141.96), disodium ethylene diamine tetra-acetic acid (Na_2EDTA, F.W. 372.24), sodium chloride (NaCl, F.W. 58.44), and calcium chloride dehydrate ($CaCl_2 \bullet 2H_2O$, F.W. 147.02). Test substances shown in Table 1 were purchased from Sigma-Aldrich Co. (St. Louis, MO) and stock solutions (100μg/mL) were prepared in methanol every month and stored at 4°C. Solid phase extraction (SPE) cartridges, 3ml/60mg of HLB (Hydrophillic-Lipophillic-Balanced), were purchased from Water Oasis Co. (Milford, MA). Milli-Q water (18.3MΩ) from a Millipore (Billerica, CA) purification system was used when DI water was required.

8.3.2 Column System

The soil was collected from the surface (0-10 cm) of a test plot at the Agricultural Research, Development, and Educational Center (ARDEC) at Colorado State University located north of Fort Collins, CO. The collected soil was air dried in the dark at room temperature to prevent losses due to photolysis and sieved (<2mm) to remove any debris. The main texture of soil was sandy clay loam and the properties are summarized in Table 8.2.

Table 8.2 Properties of soil used in column study

Soil texture (%)	pH	CEC (cmoles/kg)	EC (mmhos/cm)	OM (%)	NO3-N (ppm)	P [a]	Bulk density g/ml
Sand: 55 Silt: 16 Clay: 29	7.6	24.2	1.1	2.1	16.3	4.0	1.1

[a] AB-DTPA extraction method was used

The general procedure of the experiment followed OECD guidelines (OECD, 2000). Small portions of soil were manually packed into glass columns (35cm × 5 cm, W × I.D) under gentle vibration to obtain uniform filling up to a height of approximately 30cm. A glass wool plug (pore size of 8μm) and 50g of acid washed sand were placed at the bottom of columns to prevent any leakage of soil and a glass sinter disk was positioned at the top of the columns to ensure equal distribution of the artificial rain (0.01M $CaCl_2$). Before test substances were applied to the columns, pre-wetting was conducted with artificial rain from the bottom of the columns to avoid air bubbles and channelling in the soil columns (Rabolle, 2000). After pre-wetting, artificial rain was drained with gravity and equilibrated for 24h.

The amount of test substances (65μg) applied on top of the soil columns was calculated according to OECD guidelines and prepared in water by dilution from stock solution (100μg/ml). To feed continuous artificial rain, a peristaltic pump was used and the flow rate was set to 0.11ml/min corresponding to a rate of 84mm over a 24hr period. During the study period, all columns were covered with aluminium foil at room temperature and 500ml amber bottles were placed to collect the leachate.

8.3.3 Column Experiments

Two sets of column experiments were conducted during the study period and the experimental setup is summarized for each in Table 8.3.

Table 8.3 Column experimental setup

	Column ID	Test substances	Treatment[a]	Measured matrix
First Set	Column 1,2	TC, CTC, OTC STZ, SMT	Biotic	Dissolved Phase (<0.2μm) and Soil
	Column 3,4	ETM-H2O, TYL, MNS, SLM, NRS	Biotic	
	Column 5,6	TC, CTC, OTC STZ, SMT	Abiotic	
	Column 7,8	ETM-H2O, TYL, MNS, SLM, NRS	Abiotic	
	Column 9,10	TC, CTC, OTC STZ, SMT ETM-H2O, TYL, MNS, SLM, NRS	Abiotic	
Second Set	Column 1,2	TC, CTC, OTC STZ, SMT ETM-H2O, TYL, MNS, SLM, NRS	Biotic	Colloidal Phase (0.2μm < PS[b] >1.2μm and 1.2μm < PS >2.7μm)
	Column 3,4	TC, CTC, OTC STZ, SMT ETM-H2O, TYL, MNS, SLM, NRS	Abiotic	

[a] Biotic and abiotic treatment indicate with NaN_3 and without NaN_3 in feeding solution respectively; [b] PS: pore size

The first set of experiments was focused on the mobility of test substances in the dissolved phase and sorption to soil with and without microbial activity. Abiotic conditions were achieved by adding NaN_3 (10mg/l) as an inhibitor. The second set of experiments studied the mobility of test substances in the colloidal phase. Dissolved and colloidal fractions were differentiated with various sizes of filters (0.2, 1.2, and 2.7μm).

After the first set of experiments, the columns were allowed to drain completely for 24h and the core of the soil column was collected with an auger (I.D. 4cm). The collected soil was sectioned in 3cm intervals and kept at -20°C until analysis. Applied test substances were combined for experimental convenience.

8.3.4 Extraction of test substances

The concentration of test substances in the dissolved and colloidal fractions was analyzed according to procedures described previously (Lindsey et al., 2001; Yang et al., 2004a; Yang et al., 2004b). To cleanup the sample, solid phase extraction (SPE) was conducted using HLB (hydrophilic-lipophillic-balanced, 3ml/60mg) cartridges. Sectioned soil samples were carefully homogenized manually and a sub-sample (1g) was used for analysis. The procedure of soil extraction was modified from USDA (United States Department of Agriculture) guidelines (USDA, 2003). Two different buffer solutions, McIlvaine buffer solution (pH 4.0) for TC, CTC, OTC, STZ, SMT and 1M of ammonium hydroxide buffer solution (pH 10.0) titrated with formic acid for ETM-H_2O, TYL, MNS, SLM, NRS, were applied to extract test substances from the solid to liquid phase. After 20ml of buffer solution and 200 μL of 5% Na_2EDTA (1mmol in solution) to chelate metals was added, the sample was mixed for 20 minutes at 400 (rpm) (Model No-4626, Lab-line instrument, Needham Hights, MA), followed by 10 minutes of centrifuging at

4000 (rpm) (IEC Clinical Centrifuge, International Equipment Co., Needham Hights, MA). Finally, the liquid phase was filtered with 0.2μm glass fiber filters and decanted into another 40mL vial and kept at 4°C. Extraction was repeated once in the same manner as described above and supernatants were combined to make a total volume of 40ml for the SPE cleanup procedure. The SPE conditions were the same as for aqueous samples. Adequate concentration of the internal standard, simatone (50μL of 0.24mg/L), was used to quantify the test substances. Recoveries and limits of quantification are summarized in Table 8.4. All measured concentrations in the experiment were corrected for recoveries.

Table 8.4 Recovery and limit of quantification (LOQ) study for water and soil

	Recovery (%)[a]		Limit of quantification (μg/L or μg/kg)[b]	
	Water	Soil	Water	Soil
TC	106	88	0.02	2.3
CTC	112	74	0.02	2.3
OTC	103	45	0.02	2.3
STZ	90	97	0.01	1.8
SMT	104	92	0.01	1.8
ETM-H_2O	102	119	0.01	0.6
TYL	102	77	0.01	0.6
MNS	115	82	0.001	0.4
SLM	100	81	0.002	3.6
NRS	105	53	0.003	0.7

[a] Average of three different concentration (0.1,1,5μg/L for water and 1,30,90μg/kg for sediment). Relative standard deviation (RSD) ranged between 1 and 11% for water and 13 and 31% for sediment. [b] Statistical calculation based on reference from (Zhu et al., 2001).

8.3.5 Separation and Detection of Test Substance

To separate the test substances, a HP 1100 high performance liquid chromatograph (HPLC) equipped with an auto sampler and variable UV detector was used. The

analytical column was an XTerra MS C_{18} (Waters, Milliford, MA)(2.1×50mm, 2.5μm pore size, end-capped) with a C_{18} guard column (Phenomenex, Torrence, CA, USA) installed to filter any particles from the sample. For detection of test substances, a ThermoFinnigan LCQ Duo ion trap mass spectrometer (ThermoQuest, Woburn, MA) was used and the instrument was operated in the positive ion mode with electrospray ionization (ESI) to perform the mass spectrometric analysis. Detailed information of the optimized HPLC/MS/MS parameters is shown in Table 8.5.

Table 8.5 Optimized parameters of high performance liquid chromatography (HPLC) and mass spectrometry (MS).

	Optimized HPLC Condition		
	Column temp.(°C)	Flow rate (mL/min)	Mobile phase condition[a]
TC, CTC, OTC	15	0.32	A: 96% + B: 4% (0 min)
STZ, SMT			A: 70% + B: 30% (29 min)
			A: 96% + B: 4% (30 min)
ETM-H2O, TYL	45	0.32	A: 80% + B: 20% (0 min)
			A: 65% + B: 35% (13 min)
			A: 96% + B: 4% (14 min)
MNS, SLM, NRS	15	0.25	A: 50% + C: 50% (0 min)
			A: 10% + C: 90% (19 min)
			A: 50% + B: 50% (20 min)
Optimized MS condition	Nitrogen gas for drying and nebulizing Spray voltage: 4.5V Capillary voltage: 21V Capillary temperature: 165°C		

[a] Mobile phase A: 99.9% water + 0.1% formic acid, Mobile phase B: 99.9% acetonitrile + 0.1% formic acid, and Mobile phase C: 100% methanol.

8.3.6 Statistical Analysis

The significance of difference was evaluated with one way analysis of variance (ANOVA) at the level of $p < 0.05$ (Kay et al., 2005a). Shapiro-Wilk tests for normality and Levene's tests for variance homogeneity were also evaluated with Minitab

(MINITAB, 2000). As a post-statistical analysis to compare the difference of individual means, Tukey's and HSU's MCB method was used.

8.4 Results and Discussions

8.4.1 Tracer Test

A tracer test was conducted to evaluate the hydraulic characteristics of the column system with sodium chloride (1g/L) as the reference compound. The application pattern was continuous flow from the feed reservoir at the same flow rate as the test substances application (0.11mm/min). As shown in Figure 8.1, the ten columns were consistent with each other and an estimated average retention time was 54 – 56 hrs. A tracer test of the second set of columns was examined in the same manner and the same result was obtained (data is not shown).

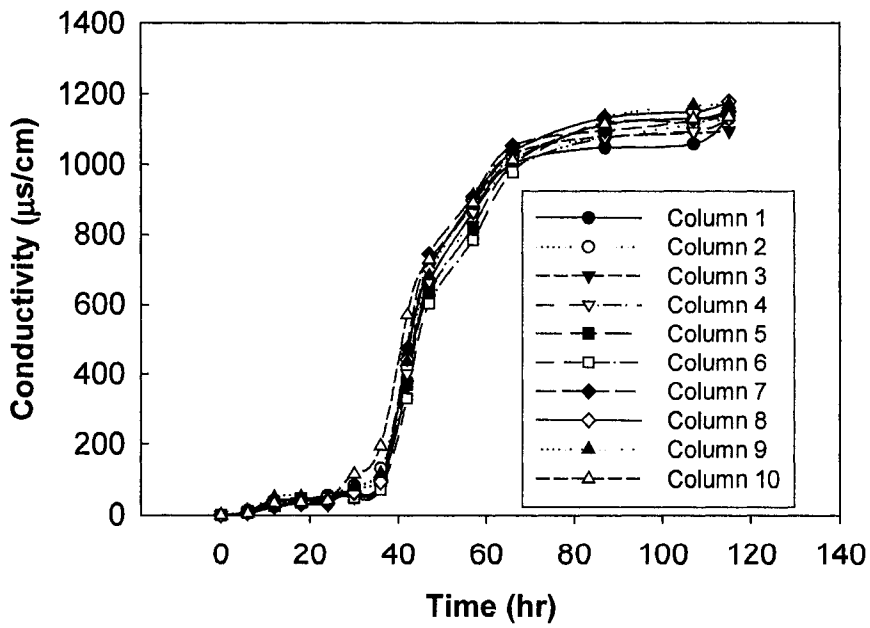

Figure 8.1 Result of tracer test for columns

8.4.2 Mobility of Test Substances in Dissolved Phase and Sorption to Soil

In the first set of experiments, the mobility of 10 substances in the dissolved phase and sorption characteristics in soil were evaluated with a disturbed and repacked soil column leaching system. The adapted column system was originally designed for screening tests to evaluate potential leaching behavior of different substances (Worrall et al., 1999; Kay et al., 2005a). As shown in Figure 8.2, the travel time of leachate in the dissolved phase varied significantly depending on the class of veterinary antibiotic. While none of the 3 tetracyclines (TCs) or TYL was detected in the dissolved phase of the leachate during the study period (45days), the sulfonamides (SAs) showed a relatively high mobility, followed by the MNS, SLM, NRS, and ETM under both biotic and abiotic conditions.

The recovered mass of SMT shown in Figure 8.2 had the highest peak at 6 days after the test substance application and it followed a decreasing trend until the end of the experiment for both treatments. A similar trend was observed for STZ in the dissolved phase of leachate but ETM-H_2O and NRS showed much different profiles. In the case of NRS, the initial peak was observed 3 days after application of the test substance in the soil column and two additional peaks were measured 7 and 17 days after the initial peak. The highest recovered mass was observed in the second peak. This result may indicate that a pH or redox change caused increase mobility of NRS. Previous studies have shown that sorption of acidic and basic compounds can be decreased and mobility increased as pH changes (Jonge et al., 1998; Kay et al., 2005a).

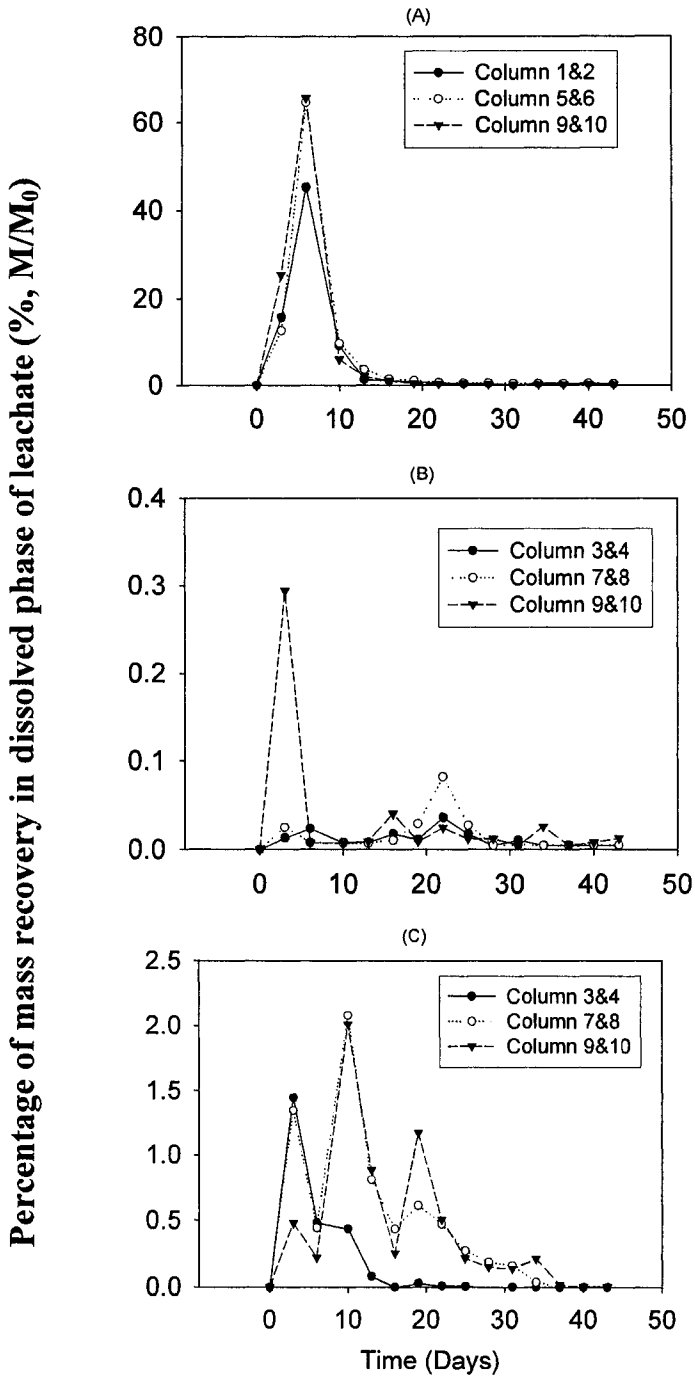

Figure 8.2 Mass recovery profile of representative three studied substances in the dissolved phase of leachate during the study period (A) SMT, (B) ETM-H_2O, and (C) NRS; Condition of each column is summarized in Table 8.3; Note that scale of y-axis is different.

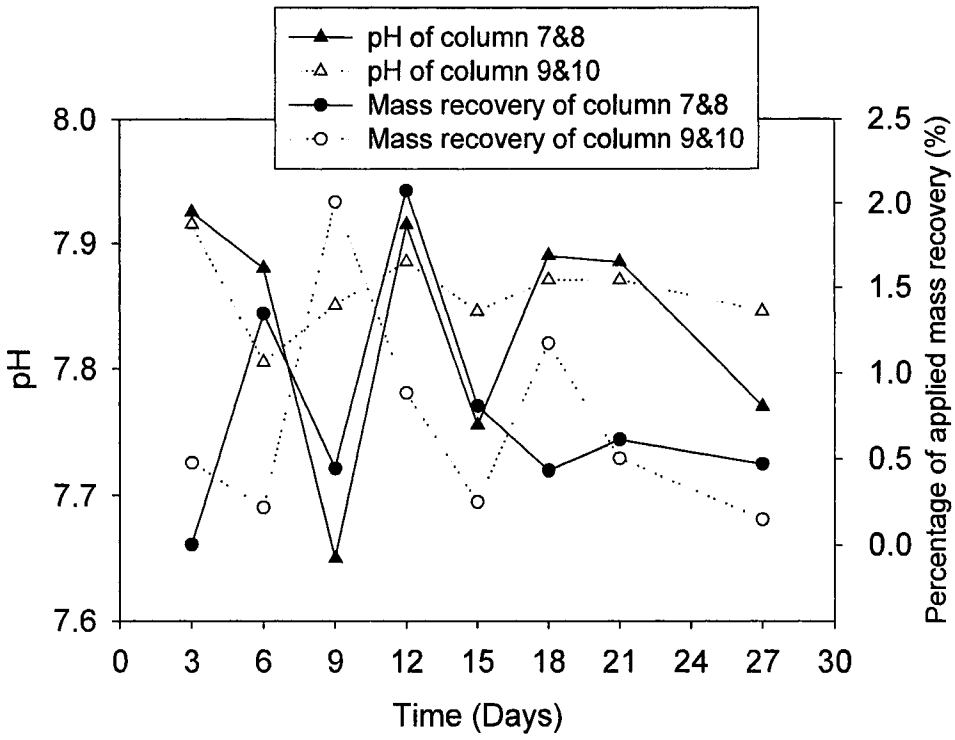

Figure 8.3 Measured pH and percentage of applied mass recovery in leachate for NRS; Column condition is shown in Table 8.3.

As shown in Figure 8.3, the pH value varied between the range of 7.6 – 8.1 during the first 30 days of the experiment in the leachate and the recovered mass is lower as pH is decreased except during the first high peak. Although the effect of pH was hard to judge in leachate of NRS due to the low mass recovery, this result showed agreement with previous studies that pH might play on important role in sorption and mobility of test substances.

The results of statistical analysis reveal that the recovered mass of test substances in the dissolved phase of leachate was significantly different ($p = 0.04$) with biotic treatment (without NaN_3 addition). In contrast, no significant difference was observed with abiotic treatment (with NaN_3 addition) among test substances. Furthermore, the

difference between biotic and abiotic treatment for the recovered mass of all individual substances was not statistically significant although lower mass recovery was observed in biotic treatment for all substances. This result indicates that biodegradation might affect the attenuation of test substances but it is not the major leachate removal mechanism. In fact, Ingerslev et. al. conducted a screening test for 12 SAs and reported that all evaluated compounds were not readily biodegradable (Ingerslev et al., 2000). Also, the calculated half-life of OTC resulting from a biodegradation experiment in sediment ranged from 31-145 days (Ingerslev et al., 2001).

Since measuring the solid residual of test substances in the columns can provide information on sorption characteristics, the soil was sectioned after the first experiment. The percentages of mass recovery of selected substances from each group are shown in Figure 8.4. While none of the TCs was observed in the dissolved phase of leachate, the most abundant mass was detected in soil for TCs. Residuals of CTC, TYL, and NRS were found in all depths of soil and CTC was evenly distributed from top to bottom of the soil. For TYL and NRS, the highest mass was detected at the top of soil column (0 – 3cm) and generally similar amounts of mass were present throughout. However, SMT was only detected at 3 sampling points through the soil column.

This result can be expected due to different physicochemical properties of test substances in each group (Table 8.1). In general, TCs has a high K_d value resulting in greater sorption and SAs have the lowest K_d value. The different sorption results agree with the high rate of mass recovery in leachate for SAs and none for TCs.

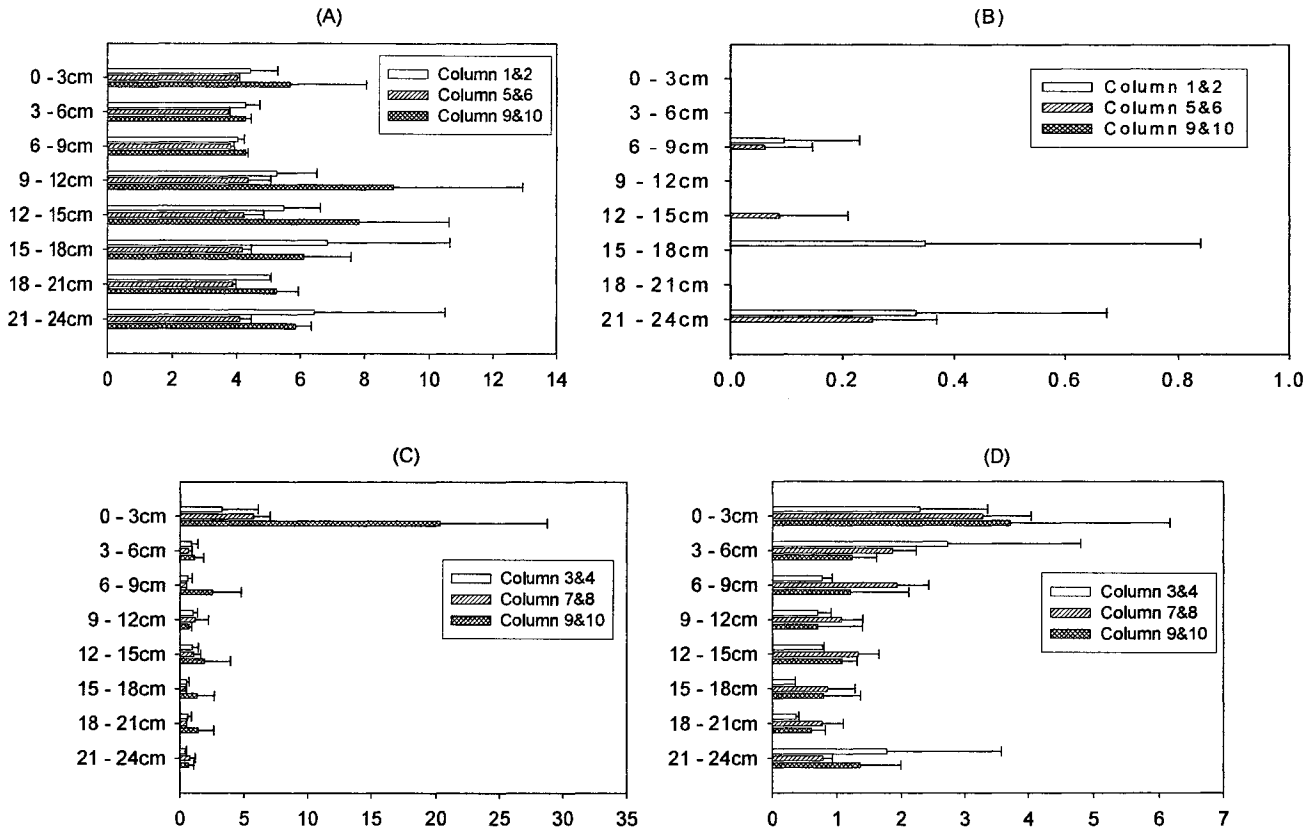

Figure 8.4 Percentage of mass recovery for four studied substances in soil column after study periods (A) CTC, (B) SMT, (C) TYL, and (D) NRS; Condition of each columns is summarized in Table 8.3; Note that scale of x-axis is different

Since TYL has a high K_{ow} and a relatively high K_d value (Table 8.1) and most of the recovered mass was observed in the top part of the soil (Figure 8.4), hydrophobicity might have been the primary sorption mechanism. In comparison, the low K_{ow} and high K_d value (Table 8.1) of TCs indicate that hydrophobicity is not the major sorption mechanism of these substances in soil. Other studies investigated the sorption of TCs with varied pH, clay content, and cation exchange capacity (CEC) conditions and showed greater sorption of TCs at lower pH and higher ionic strength. The conclusion was that sorption behavior of TCs is pH dependent and cation exchange might be responsible for sorption of TCs in soil (Figueroa et al., 2004; Sassman et al., 2005). Although no information is available for sorption of polyethers (PEs), the high K_{ow} (Table 8.1) might be able to explain the sorption characteristic of PEs in soil.

Among the studied substances, the measured mass of TC ($p<0.001$), CTC ($p=0.01$), OTC ($p=0.01$), and SLM ($p=0.02$) showed significant differences in soil column residuals between biotic and abiotic treatment (Table 8.3). The difference was not significant for other substances.

8.4.3 Effect of Mobile Colloid in Transport of Test Substances

The second set of experiments was focused on the transport behavior of test substances in the colloidal phase. Colloid-facilitated transport can increase the mobility of strongly sorbed organic compounds and several studies have reported the enhanced transport of pesticides due to this mechanism (Chaplain et al., 1992; Jonge et al., 1998; Worrall et al., 1999; Sprague et al., 2000; Villholth et al., 2000; Grolimund et al., 2005). In addition, a recent sorption study of TYL onto manure showed that the sorption of TYL on colloidal materials (<1.2μm) is as high as on particulates (<2mm) (Kolz et al., 2005). The

objective of the second experiment was to examine whether colloid-facilitated transport is an important mobility mechanism for the test substances we evaluated. Since no significant difference was observed when separating or combining all test substances in a column (Figures 8.2 and 8.4), all 10 substances were applied in the soil columns for this set of experiments. Again, sodium azide (NaN_3) was used to study the impact of biotic and abiotic conditions.

Two different filter paper sizes (1.2 and 2.7 μm) were used to differentiate particle size. The extraction procedure was the same as used with the dissolved phase and the mass recovery of each particle size was calculated with subtraction from mass recovery of 2.7 μm to 1.2 μm and 1.2 μm to 0.2 μm, respectively.

As shown in Figure 8.5, colloidal-facilitated transport occurred for all tested substances. Among different groups, SAs were measured to have the highest mass recovery in the colloidal form followed by MLs and PEs. TCs were detected the least in the colloidal phase. The recovered colloidal mass result of SAs and TCs indicates that the strongest sorbing compounds (most hydrophobic) are the least likely to be transported in the colloidal form. Although exact sorption mechanisms were not verified in our study, Thiele-Bruhn et al (Thiele-Bruhn et al., 2004) showed that the polar functional group of SAs has a high affinity to interact with the surface of clay particles or soil organic matter (SOM) initially but the weak binding forces (van der Waals or hydrolysis) can lead to release. This information might be applied to our result because most of mass for SAs was recovered at the first 2 times of sampling events and drastically decreased after the second sampling event (Figure 8.5).

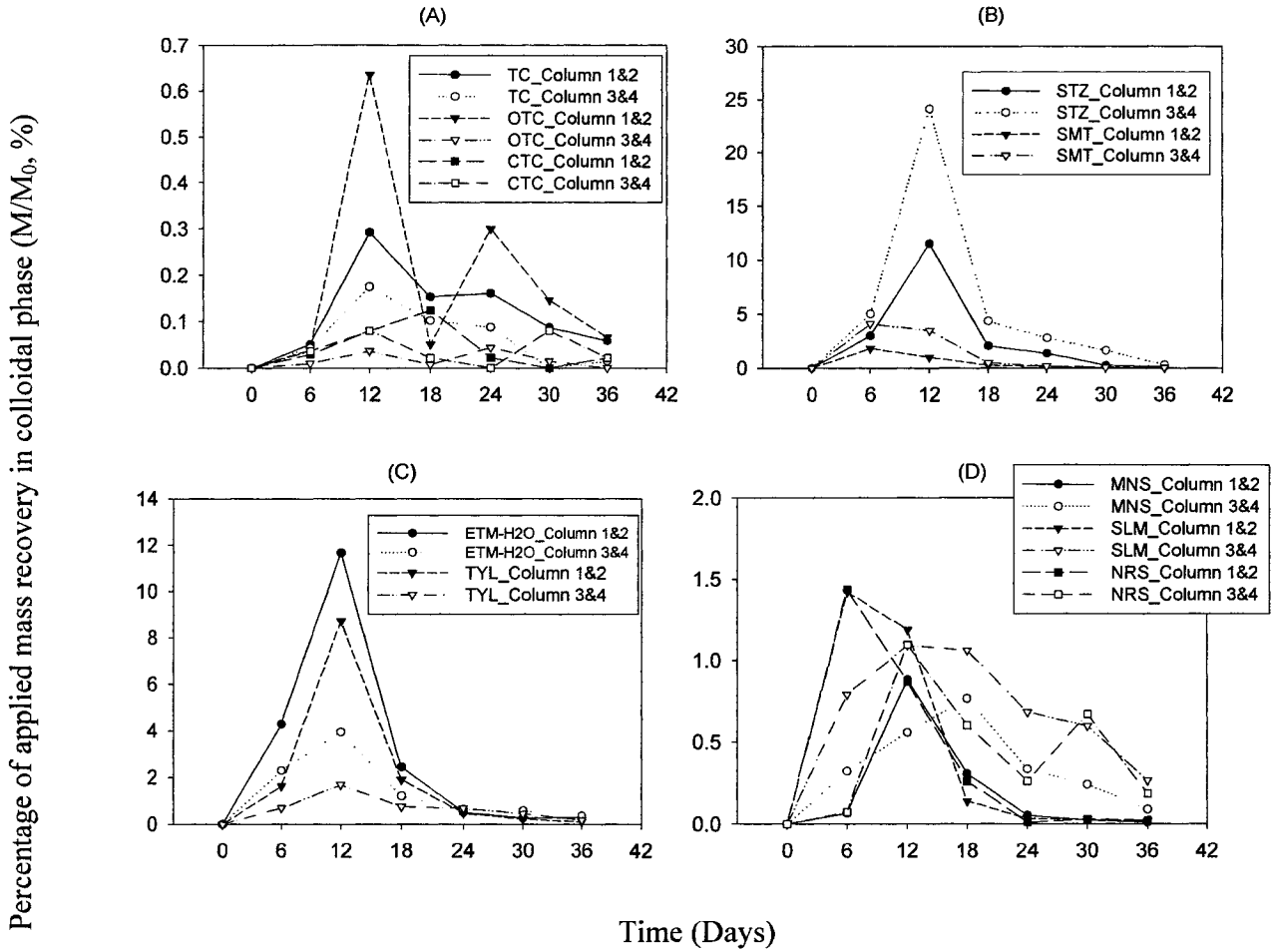

Figure 8.5 Percentage of applied mass recovery in colloidal phase at the size of 1.2μm < particle size < 2.7μm (A) TCs, (B) SAs, (C) MLs, and (D) PEs; Condition of each columns is summarized in Table 8.3; Note that scale of y-axis is different

Between the two different treatments (biotic and abiotic), STZ showed the highest mass recovery in both treatments followed by ETM-H_2O in biotic treatment and SMT in abiotic treatment (Table 8.6). Statistical analysis comparing the significance of different treatments revealed that there were no significant differences ($p > 0.05$) for all studied compounds indicating that the bioavailability of organic compounds is minimal after partitioning to a colloidal phase.

8.4.4 Comparison of Transport Behavior

To compare transport behavior of tested substances, the percentage of applied mass recovery in different fractions is summarized in Table 8.6. The range of total mass recovery was 17.5 – 83.0(%) for biotic treatment and 27.8 – 97.0(%) for abiotic treatment. Incomplete absolute mass recovery might be caused by either transformation of parent compounds to other metabolites or strong sorption to soil particles that cannot be extracted.

Diverse transport behavior was observed across the 10 compounds studied. While most of the applied mass was recovered in soil matrix for the three TCs and none of the residuals were observed in the dissolved phase of the leachate, transport of SMT in the dissolved phase and colloidal-facilitated transport of STZ was apparent. For ETM-H_2O and TYL, colloid associated mobility was the most dominant transport mechanism in biotic treatment and sorption to soil was mainly observed in abiotic treatment. Although the reason that majority of recovered mass was measured in different fractions for ETM-H_2O and TYL is not clear, our result clearly showed that microorganism activity is minimal once compounds are sorbed to colloid fraction.

Table 8.6 Summary of mass recovery in various matrix

(A) Percentage of mass recovery in biotic treatment (%)											
Matrix	PS (μm)	TC	CTC	OTC	STZ	SMT	ETM-H_2O	TYL	MNS	SLM	NRS
Dissolved	< 0.2	0.0	0.0	0.0	11.4	73.9	0.2	0.0	0.2	3.3	2.3
Colloid	0.2<>1.2	1.1	0.2	0.3	16.4	3.6	21.7	14.5	1.6	1.3	0.2
	1.2<>2.7	0.2	1.3	0.3	6.3	4.7	0.9	0.9	0.2	0.2	0.2
Soil		33.6	56.7	47.2	5.0	0.8	17.2	10.9	15.6	48.4	18.8
Sum		34.8	58.1	47.8	39.1	83.0	40.0	26.4	17.5	53.1	21.4
(B) Percentage of mass recovery in abiotic treatment (%)											
Matrix	PS (μm)	TC	CTC	OTC	STZ	SMT	ETM-H2O	TYL	MNS	SLM	NRS
Dissolved	< 0.2	0.0	0.0	0.0	30.5	83.4	0.5	0.0	1.3	4.4	6.1
Colloid	0.2<>1.2	0.8	0.2	0.5	39.1	8.0	9.2	4.4	2.3	5.2	1.1
	1.2<>2.7	0.3	0.0	0.0	23.4	5.6	1.1	0.5	0.2	0.3	0.3
Soil		54.7	80.8	62.5	2.1	0.0	49.5	39.8	18.8	26.6	20.3
Sum		55.8	80.9	63.0	96.1	97.0	60.3	44.7	22.5	36.4	27.8

Generally, the least amount of total mass was recovered for PEs except for SLM in biotic treatment column. Among recovered mass, the majority remained in the soil (15.6 – 48.4% for biotic and 18.8 – 26.6% for abiotic) and similar amount of mass was recovered in dissolved (0.2 – 3.3% for biotic and 1.3 – 6.1% for abiotic) and colloidal phase (0.2 – 14.5% for biotic and 0.2 – 5.2% for abiotic).

The main objective of the study was to characterize the different transport behavior of antibiotic compounds in biotic and abiotic soil environments. The results of the study clearly show that the fate and transport of antibiotics in soil columns vary significantly depending on the chemical structure (polarity), physicochemical characteristics (K_{ow} and K_d etc) and bulk parameters such as pH and ionic strength. Also, fractionation experiments suggest that colloidal-facilitated mobility was important for all compounds and the dominant transport mechanism for TYL, ETM-H_2O and STZ.

Chapter 9

Concentration and Environmental Loading of Veterinary Antibiotics in Agricultural Irrigation Ditches

Submitted to Environmental Science and Technology

9.1 Abstract

The concentration of veterinary antibiotics in aqueous and sediment matrices was measured in agricultural irrigation ditches bordering several animal-feeding operations (AFOs) and then compared to measured antibiotic levels in the watershed. Analytical determination in aqueous samples was based on solid phase extraction (SPE) methodology. For sediment samples, appropriate buffer solutions were used to extract residuals. Separation and detection of extracted veterinary antibiotics were performed with high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS). In general, higher concentrations of antibiotic were observed in the aqueous phase of irrigation ditches than in aqueous watershed samples, while higher concentrations were measured in river sediment than in irrigation ditch sediment samples. There was a high calculated correlation between precipitation and measured concentration in aqueous samples from the irrigation ditches for five of the ten targeted

veterinary antibiotics, indicating that surface runoff could be an important transport mechanism of veterinary antibiotics from field to environment. Further, environmental loading calculation based on measured concentrations in aqueous samples and flow information clearly showed that much greater mass was present at the irrigation ditches than at the river. This result suggests the likelihood that veterinary antibiotics can be transported via irrigation ditches to the watershed. The transport via surface runoff and likely environmental loading via irrigation ditches identified in this study helps identify the pathway of veterinary antibiotics residuals in the environment.

9.2 Introduction

Pharmaceutical antibiotics are used to prevent and treat disease in humans and to prevent illness and promote growth and weight in livestock (Diaz-Cruz et al., 2003; Wiegel et al., 2004). It has been estimated that three million pounds of antibiotics, or 9% of total consumption in the U.S., are involved in human treatment annually, and more than 29 million pounds, or 84% of total consumption, are used in therapeutic and non-therapeutic animal application (Mellon et al., 2001).

Concern grows over the occurrence of antibiotics in the environment because most antibiotics are excreted either as the parent compound or as a slightly modified form generally conjugated to polar molecules that can be cleaved easily under certain environmental conditions (Heberer, 2002a; Diaz-Cruz et al., 2003). Depending on physicochemical properties such as water solubility, octanol/water partitioning coefficient, dissociation coefficient (pKa), and Henry's Law constant, pharmaceutical antibiotics can persist in soil or sediment and mobilize to ultimately reach the ground water (Tolls, 2001; Bruhn, 2003).

Occurrence of antibiotics in surface water was first reported in the late 1980's; subsequently, several researchers have found residuals of different classes of antibiotics in surface water, wastewater, and even ground water at sub-microgram concentrations (Hirsch et al., 1999; Lindsey et al., 2001; Zhu et al., 2001). In 1999 and 2000, the first nationwide reconnaissance reported the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants (OWCs) in U.S. streams (Kolpin et al., 2002); the OWCs identified included pharmaceutical antibiotics originating from industrial, residential, and agricultural application. Based on such findings, an increasing level of research has focused on the pathways through which antibiotics enter the environment. Municipal sewage treatment plants (STPs) are a primary point source of human-use pharmaceutical antibiotics that reach the environment, prompting research on concentrations of antibiotics in STP influent and effluent (Hirsch et al., 1999; McArdell et al., 2003; Gobel et al., 2004; Rooklidge, 2004).

Meanwhile, animal-associated antibiotics can enter the environment through the application of manure and slurries on agricultural fields as fertilizers and even the leaking of animal-waste handling equipment (Hamscher et al., 2002; Kolpin et al., 2002; Kay et al., 2005b). After manure and slurries are applied in the field, veterinary antibiotics can be found in both aqueous and sediment phases. High levels of veterinary antibiotics have been reported in lagoons, liquid manures, and fertilized agricultural fields. As much as 4.0 mg/kg of tetracycline has been measured in liquid manure, a much higher concentration than found in an aqueous matrix (Campagnolo et al., 2002; Haller et al., 2002; Hamscher et al., 2002; Liguoro et al., 2003; Schlusener et al., 2003; Blackwell et al., 2004b; Jacobsen et al., 2004).

While the measured concentration of antibiotics in the environment (e.g. surface water, ground water, soil, and sediment) was several orders of magnitude lower than levels that can be toxic or directly detrimental to ecological systems, accumulated trace amounts of antibiotics can result in bacterial resistance. Tetracycline-resistant bacteria were identified in lagoons and ground water underlying large swine production operations (Chee-Sanford et al., 2001). In addition, resistance to three different antibiotic groups (tetracycline, macrolides, and streptomycin) was observed in bacteria found in soil treated with pig manure (Sengelov et al., 2003).

This study targeted the concentration of veterinary antibiotics in irrigation ditches bordering several animal-feeding operations. In addition, environmental loading calculations based on flow information and measured concentrations were made at irrigation ditches and in-river. Through these concentration measurements and calculated environmental loading indicators, the current investigators aimed to achieve a better understanding of the potential intermediate pathways of veterinary antibiotics from the animal farm to surface water.

9.3 Methods

9.3.1 Materials

Ten veterinary antibiotics were targeted in this study; tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), sulfathiazole (STZ), sulfamethazine (SMT), erythromycin (ETM), tylosin (TYL), monensin (MNS), and narasin (NRS) were sourced from Sigma Aldrich Co. (St. Louis, MO), and salinomycin (SLM) was obtained from ICN (Aurora, OH). A stock solution of 100 mg/L was prepared in HPLC grade methanol (99.9%) for

each compound and stored at 4°C until use. HLB (Hydrophillic-Lipophillic-Balanced) cartridges used for solid phase extraction (SPE) had 3mL/60mg capacity and were obtained from Water Oasis Co. (Milford, MA). High purity DI water – 18.3 mmho/cm – was used throughout the study. Properties of the selected compounds are summarized in Table 9.1.

Table 9.1 Properties of study-targeted compounds

Compound	Acronym	CAS Number	pKa[a]	Log K_{ow}[a]	Water Solubility (mg/L)[a]
Tetracycline	TC	60-54-6			
Chlortetracycline	CTC	64-72-2	3.3/ 7.7/ 9.3	-1.3 to 0.05	230 to 52000
Oxytetracycline	OTC	79-57-2			
Sulfathiazol	STZ	72-14-0	2 – 3/ 4.5 – 10.6	-0.1 to 1.7	7.5 to 1500
Sulfamethazine	SMT	57-68-1			
Erythromycin-H_2O	ETM-H_2O	114-07-8	7.7 to 8.9	1.6 to 3.1	0.45 to 15
Tylosin	TYL	1401-69-0			
Monensin	MNS	22373-78-0			
Salinomycin	SLM	53003-10-4	6.4	NA	NA
Narasin	NRS	55134-13-9			

[a] reference from (Thiele-Bruhn, 2003); NA: No information is available.

9.3.2 Hydrology of Cache La Poudre River

The studied area, the Cache la Poudre River basin in northern Colorado, USA, represents one of the most highly managed and complex hydrologic systems in the western United States. The Cache la Poudre River, or Poudre River, flows north and east through the Roosevelt National Forest as it tumbles down the slopes of the Colorado Front Range and meanders through the City of Fort Collins, Colorado. From its headwaters to the confluence with the South Platte River east of Greeley, Colorado, the Poudre River drops 7,000 feet. In the mountainous areas upstream of Fort Collins, native flow occurs throughout the year on the river's main stem. Significant diversions on the main stem

begin approximately 62 river miles upstream of the river's mouth, and return flows from agricultural irrigation and municipal/industrial wastewater treatment plants are an integral part of the water supply in the lower basin during the storage season, which generally lasts from October through April, and the irrigation season, which generally lasts from May to September. In the reach of the Poudre River from Fort Collins to the City of Greeley, returns from agricultural irrigation contribute approximately 4.5 m^3/s to the river's main stem, some of which is in the form of seepage, discharge from various drains, and influent from natural tributaries.

9.3.3 Sample Collection and Pretreatment

Samples from agricultural irrigation ditches and the Poudre River were collected at three different time periods (August 2004 and July/August 2005). Four sampling points were located along with the Larimer and Weld Canal, which connects with the Eaton Canal, and three sampling points were selected along the Cache la Poudre Canal, locally known as the Greeley #2 Ditch. An additional two collection points were chosen in the Graham Seep Ditch, another river tributary. The Larimer and Weld Canal, located 47 miles upstream from the mouth of the Poudre River, and the Greeley #2 Canal, located 33 miles upstream from the river's mouth, are major river diversions during irrigation season, normally from June to September. Several feedlots and dairy farms, including a small breeding operation, are located between these two ditches. Sampling sites along agricultural irrigation ditches and at watershed points are shown in Figure 9.1; average flow of the three ditches and the main Poudre River is summarized in Table 9.2.

Aqueous samples were collected in sampling bottles previously rinsed with DI water. The sample was collected vertically at three different depths at each sampling

point. The collected sample was carefully mixed in one bottle and kept in a cooler before transporting to the lab, where it was filtered through a 0.2-μm glass fiber filter and stored at 4 °C until analysis. Sediment samples were collected at only three ditch locations due to limited depth and at two river locations downstream of the ditches. Collected sediment samples were completely air dried at 20 °C in a dark room to prevent any possible photolysis loss.

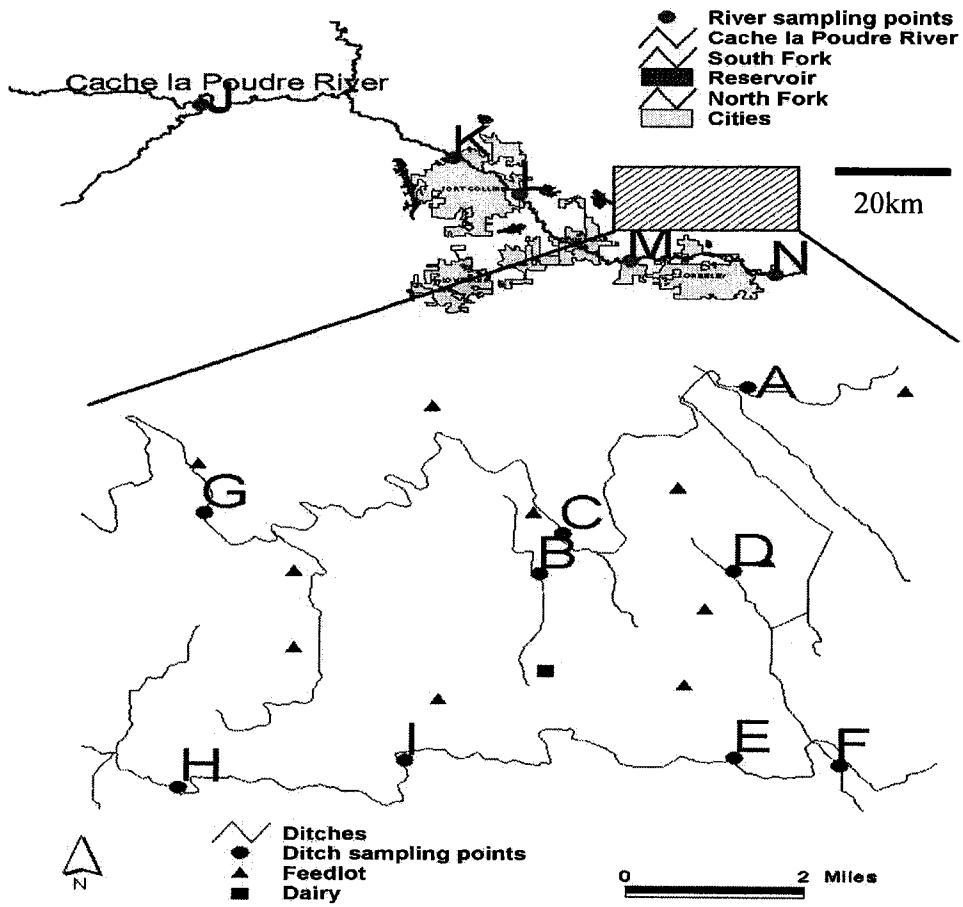

Figure 9.1 Map of studied area: upper figure shows the watershed area from which aqueous and sediment river samples were collected; lower figure shows ditch sampling locations

Table 9.2 Mean stream flow at sampling sites

Sampling Site	Site Name or Description	Average Monthly Mean Stream Flow (m^3/s)[a]
A, C, G	Eaton Canal	6.8
B	Local Ditch	NA
D, F	Graham Seep Ditch	0.1
E, H, I	Greeley #2 Canal	2.8
J	Pristine Area (no urban or agricultural activity)	NA
K	Urban Area	NA
L	Urban Area (near wastewater facility)	NA
M	Agricultural Area	1.4
N	Urban and agricultural combined area	1.4

[a] Average monthly mean stream flow at canal or ditch site was calculated for August 2004 and July/August 2005. Monthly mean stream flow of river sites was adapted from U.S. Geological Survey (USGS). NA: No information is available.

Air-dried sediment samples were sieved at 2 mm and 75 μm. The 2-mm-sieve fraction was used for physicochemical property determinations, and the 75-μm-sieve fraction was used to extract antibiotic residuals since the clay portion is known to sorb the antibiotics (Kim et al., 2004).

Table 9.3 Physical and chemical properties of (a) water and (b) sediment at sampling sites in ditches and river

	(a) Water (mg/L unless noted)					
Sampling Site	pH (units)	Ca	HCO_3	SO_4	NO_3-N	TDS[a]
A	7.6	32.2	76.9	89.1	0.2	228.0
F	8.2	120.0	246.0	468.0	6.5	773.0
I	8.5	65.1	161.0	204.0	0.6	521.0
L	7.8	47.9	150.0	80.7	11.2	433.0
M	8.3	69.4	117.0	232.0	6.3	600.0
	(b) Sediment (mg/L unless noted)					
	pH (units)	EC (mmhos/cm)	OM (%)	NO_3-N	P	Fe
D	7.7	1.5	1.8	8.8	20.5	14.4
M	7.4	2.9	0.8	11.2	18.6	82.9
N	7.5	1.8	0.5	15.3	32.6	68.7

[a] TDS: Total Dissolved Solids.

Physicochemical properties of collected samples were determined at the Soil, Water, and Plant Testing Laboratory at Colorado State University (Fort Collins, Colorado) and are summarized in Table 9.3.

9.3.4 Analytical Method

The methodology for extraction and cleanup of the collected samples is shown in Figure 9.2. The general procedure for aqueous sample preparation was adapted from a previous study (Lindsey et al., 2001; Yang et al., 2004a); the extraction procedure for MNS, SLM, and NRS was slightly modified to eliminate pH adjustment of samples prior to the SPE process. The procedure for extracting antibiotics in sediment was modified from a USDA (United States Department of Agriculture) guideline (USDA, 2003); two different buffer solutions were used to extract antibiotics from solid to liquid phase – McIlvaine buffer solution (pH 4.0) for TC, CTC, OTC, STZ, and SMT; and ammonium hydroxide buffer solution (pH 10.0) titrated with formic acid for ETM-H2O, TYL, MNS, SLM, and NRS – all followed by SPE.

The HPLC system (HP 1100 Series Liquid Chromatograph, Agilent, Palo Alto, CA) consisted of an Agilent 1100 Series Thermostatted Auto Sampler and a variable-wavelength UV detector. The analytical column was an XTerra MS C_{18} (Waters, Milliford, MA), 2.1 × 50 mm (diameter x length, 2.5-μm pore size, end-capped). A reversed-phase C_{18} guard column (Phenomenex, Torrence, CA) was installed to filter any particulates from the sample.

Mobile phase A was composed of 99.9% water and 0.1% formic acid, mobile phase B was 99.9% acetonitrile mixed with 0.1% formic acid, and mobile phase C was pure MeOH. Once the mobile phase condition was set, the composition of the mobile

phase was gradually increased or decreased with time, and no isocratic condition was applied except for MNS, SNM, and NRS determinations. Injection volume was 20 μL for all compounds; a ten-minute column equilibrium period was included after each sample run.

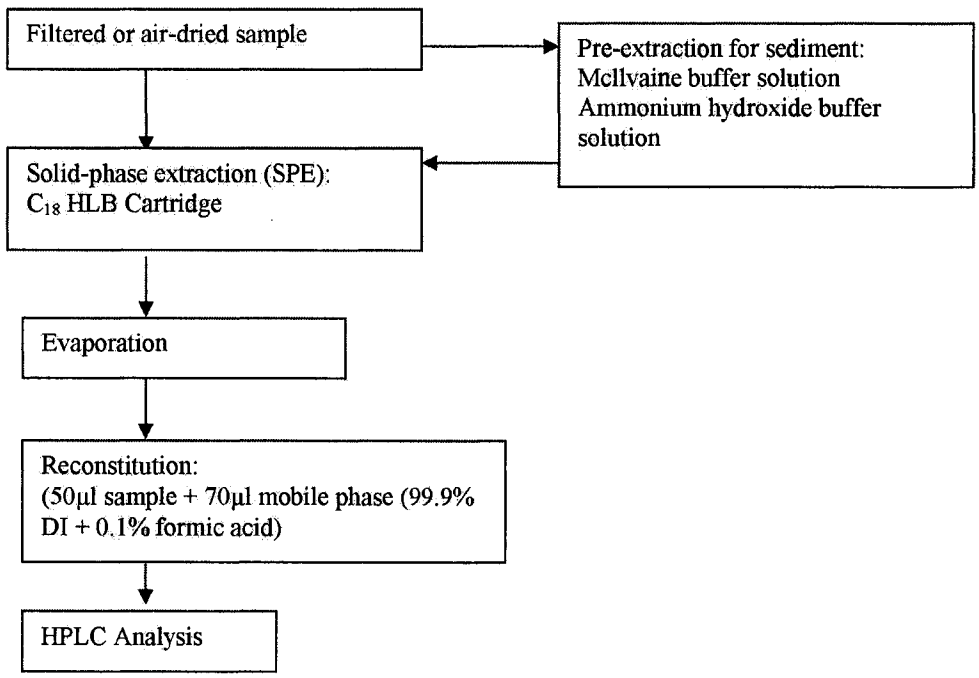

Figure 9.2 Extraction Procedure for Samples from River and Ditches.

A ThermoFinnigan LCQ Duo ion trap mass spectrometer (ThermoQuest, Woburn, MA) equipped with a heated capillary interface and electrospray ionization (ESI) was used for mass spectrometric determinations. A 10μM standard solution of each compound was made in DI water and injected using the LCQ Duo syringe pump at a flow rate of 5 μL/min to optimize the mass spectrometry parameters as needed. HPLC conditions for specific compounds and optimized mass spectrometry parameters are summarized in Table 9.4.

Table 9.4 (a) Compound-specific HPLC conditions and (b) optimized mass spectrometry parameters

		TC, CTC, OTC, STZ, and SMT	ETM-H_2O and TYL	MNS, SLM, NRS
(a)	Flow Rate (mL/min)	0.32	0.32	0.25
	Mobile Phase Composition (%); Time	A: 4 + B: 96; 1 min	A: 20 + B: 80; 1 min	A: 50 + C: 50; 1 min
		A: 30 + B: 70; 29 min	A: 35 + B: 65; 13 min	A: 10 + C: 90; 14 min
		A: 4 + B: 96; 1 min	A: 20 + B: 80; 1 min	A: 50 + C: 50; 1 min
	Column Temp. (°C)	15	45	15
	Wavelength (nm)	360 for TC and CTC; 260 for STZ and SMT	No measurable wavelength	
(b)	N_2 gas: drying and nebulizing			
	Spray voltage: 4.5kV			
	Capillary voltage: 21V			
	Capillary temperature: 165°C			
	Sheath gas flow rate: 40 units (arb)			
	Auxiliary gas: off			

Positive ion mode was utilized for all compounds. Once the precursor ion was identified – $[M+H]^+$ for seven compounds and $[M+Na]^+$ for MNS, SNM, and NRS – fragment ions were produced with different collision energy. It should be noted that ETM-H_2O was used as a precursor ion based on a previous study (Hirsch et al., 1999). The dehydrated form of ETM can be achieved using an acidic mobile condition. The precursor and product ion for each compound, and tandem mass spectrometry parameters, are identified in Table 9.5.

Table 9.5 Precursor and fragment ions associated with collision energy and isolation width

Compound	Precursor Ion $[M+H]^+$ (m/z)	Isolation Width	Collision Energy (%)	Fragment Ion *(m/z)*
Simatone[a]	198			
TC	445	2.0	26	427 $[M+H-H2O]^+$
CTC	479	2.0	32	462 $[M+H-NH3]^+$
OTC	461	2.0	28	443 $[M+H-H2O]^+$
STZ	256	2.0	32	156
SMT	279	2.0	38	204
ETM-H_2O	716	3.0	26	558 $[M\text{-desosamine-}H_2O+H]^+$
TYL	916	3.0	30	772 $[M\text{-cladinose}+H]^+$
Compound	Precursor Ion $[M+Na]^+$ (m/z)	Isolation Width	Collision Energy (%)	Fragment Ion *(m/z)*
MNS	693	2.0	28	675 $[M+Na-H2O]^+$
SLM	773	2.0	30	755 $[M+Na-H2O]^+$
NRS	787	2.0	30	769 $[M+Na-H2O]^+$

[a] Internal standard. Simatone was quantified with selective ion monitoring (SIM).

9.3.5 Recovery and Limit of Quantification (LOQ)

To validate recovery and limit of quantification (LOQ), the sample from river sampling site J was chosen as control sample for both water and sediment samples; this particular sample was chosen because of the site's pristine condition and the confirmation of no residual antibiotics. Average recovery in three aliquots spiked at different concentrations

ranged from 91 to 115% for water samples and 46 to 119% for sediment samples. The LOQ was calculated based on documented statistical methodology (Zhu et al., 2001); results are presented in Table 9.6. An external calibration curve constructed to calculate concentration demonstrated linearity of $r^2 > 0.97$ for all compounds from 0.01 to 5 μg/L.

Table 9.6 Summary of analysis recovery and limit of quantification (LOQ)

	(a) Water		(b) Sediment	
	Recovery (%)[a]	LOQ (μg/L)	Recovery (%)[a]	LOQ (μg/kg)
TC	107	0.006	88	1.9
CTC	112	0.009	75	0.9
OTC	102	0.007	46	0.5
STZ	91	0.002	98	1.2
SMT	105	0.005	93	1.8
ETM-H_2O	102	0.008	119	1.7
TYL	102	0.009	77	1.1
MNS	115	0.001	83	0.4
SLM	100	0.002	81	3.6
NRS	105	0.003	53	0.7

[a] Average value of three spike concentrations, 0.1, 1, and 5 μg/L, for water and 1, 30, and 90 μg/kg for sediment. Relative standard deviation (RSD) for water was 3 to 13% and 3 to 24% for sediment.

9.4 Results and Discussion

9.4.1 Concentration of Veterinary Antibiotics in Irrigation Ditches and River

The measured concentration of ten veterinary antibiotics at the irrigation ditches and the Cache la Poudre River in aqueous and sediment samples is summarized in Tables 9.7 and 9.8, respectively. Higher concentrations of all compounds were observed in ditch aqueous samples compared to river aqueous samples. Compounds TC, CTC, ETM-H_2O, MNS, SLM, and NRS showed the highest concentrations at sampling site A located downstream in the Eaton Canal. Specifically, the highest concentration of TC and CTC was measured

at sampling sites A, E, and F, the last sampling points of each irrigation ditch. Measured CTC concentration at sampling points G, B, C, and A in the Eaton Canal were 0.08μg/L, 0.11μg/L, 0.03μg/L, and 0.22μg/L, respectively, while CTC concentrations at sampling sites H, I, and E in the Greeley #2 Canal were 0.05μg/L, 0.02μg/L, and 0.06μg/L respectively. These measurements show the concentration at sampling site A equaled the combined concentrations at sampling sites G, B, and C, while the concentration at sampling site E was 86% of the combined levels measured at sampling sites H and I, indicating CTC was being added to the irrigation ditches as water flowed through animal feeding operations, despite slightly lower concentration at sampling sites C and I.

Although STZ was rarely found along the irrigation ditches, the highest STZ concentration was detected at sampling site B (0.05μg/L). In contrast, SMT was detected at all irrigation ditch sampling sites, and concentrations were higher than river sample levels at all locations.

Another recent study also verified that runoff is one of the transport mechanisms for veterinary antibiotics and that amended manure can increase the transport efficiency of veterinary antibiotics (Burkhardt et al., 2005). In the current study, fairly consistent levels of MNS, SLM, and NRS were observed in the irrigation ditches and river samples within the agricultural area (sampling sites M and N). However, no detectable levels were found upstream of heavily agricultural influenced region, indicating irrigation ditches affected transport of these three compounds.

Table 9.7 Summary of measured antibiotic concentration in water (values are the calculated mean of two individual measurements at three different sampling events, N = 6)

	Measured concentration (μg/L) [a]									
	TC	CTC	OTC	STZ	SMT	ETM-H_2O	TYL	MNS	SLM	NRS
G	0.02	0.08	0.03	0.00	0.08	0.02	0.01	0.002	0.003	0.003
B	0.05	0.11	0.11	0.05	0.05	0.09	0.07	0.005	0.003	0.005
C	0.01	0.03	0.04	0.01	0.15	0.03	0.03	0.003	0.002	0.004
A	0.12	0.22	0.10	0.01	0.08	0.53	0.06	0.006	0.005	0.010
H	0.01	0.05	0.03	0.00	0.12	0.02	0.01	0.003	0.003	0.003
I	0.00	0.02	0.07	0.00	0.10	0.06	0.01	0.005	0.003	0.004
E	0.03	0.06	0.05	0.01	0.02	0.02	0.02	0.005	0.003	0.004
D	0.02	0.02	0.22	0.01	0.02	0.02	0.03	0.004	0.003	0.007
F	0.03	0.06	0.06	0.01	0.07	0.05	0.04	0.006	0.002	0.003
J	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000	0.000	0.000
K	0.01	0.01	0.03	0.00	0.00	0.12	0.05	0.000	0.000	0.000
L	0.02	0.04	0.03	0.03	0.02	0.15	0.05	0.000	0.000	0.000
M	0.01	0.02	0.16	0.00	0.02	0.03	0.04	0.002	0.003	0.003
N	0.01	0.01	0.06	0.00	0.01	0.08	0.03	0.003	0.002	0.003

[a] More significant figures are shown for MNS, SLM, and NRS due to low concentration.

Table 9.8 Summary of measured concentration in sediment (values are the calculated mean of two individual measurement at three different sampling events, N = 6)

	Measured concentration (μg/kg)									
	TC	CTC	OTC [a]	STZ	SMT	ETM-H_2O	TYL	MNS	SLM	NRS
B	35.3	31.2	155.2	1.4	<LOQ	18.1	20.3	4.7	7.3	5.8
D	6.8	7.3	20.4	<LOQ	1.1	19.8	11.7	6.1	6.1	6.2
F	11.8	4.0	63.5	<LOQ	<LOQ	7.9	7.3	2.2	5.7	6.6
M	28.4	7.8	91.4	4.7	9.1	30.7	19.6	9.4	10.6	8.5
N	27.9	7.9	110.9	<LOQ	2.7	59.0	37.1	10.8	8.9	6.9

[a] Only two sampling events, July and August 2005, were averaged.

Only three sites were appropriate for sediment sampling. Along the river, only sampling sites M and N located below the irrigation ditches were sampled. In contrast to aqueous samples, generally higher concentration was observed in river sediment samples than in ditch sediment samples. The exception was the three tetracycline compounds TC,

CTC, and OTC, which showed highest concentrations in ditch sampling site B (Table 9.8). This contradiction in observed concentrations in sediment and aqueous samples can be explained by higher flow energy in the ditches that might minimize the sorption of antibiotics to sediment, compared to a more static flow in the river that would favor sorption of antibiotics in the sediment.

9.4.2 Correlation Analysis

Several mechanisms might be involved in transporting veterinary antibiotics from the field to the watershed. Preferential flow has been identified as a transport mechanism (Kay et al., 2004), and surface runoff might serve as an important transport mechanism. Previous studies showed transport of applied veterinary antibiotics from field to watershed via surface runoff (Burkhardt et al., 2005; Kay et al., 2005c; Pedersen et al., 2005). Although mass losses of veterinary antibiotics via surface runoff was usually less than 1%, surface runoff after severe rainfall still could affect significant transport.

Consequently, this study included estimation of the correlation coefficient between average monthly precipitation and measured concentration of target antibiotics in aqueous samples at selected irrigation ditches to assess the impact of surface runoff on residual concentration. Average monthly precipitation in Greeley, located in Northern Colorado, was obtained from the Western Region Climate Center (WRCC, Reno, Nevada, USA) and is shown in Figure 9.3. During the study periods, the greatest rainfall – 104 mm – was recorded in June 2005; generally, more precipitation events are documented during the irrigation season from April to September. The average monthly precipitation at three sampling events was 36 mm for August 2004, 14 mm for July 2005, and 19 mm for August 2005.

Calculated correlation coefficients are shown in Table 9.9. Although limited data were used in the calculations, five of the ten antibiotics – TC, CTC, OTC, TYL, and MNS – showed high correlation (> 0.95) between recorded precipitation and measured concentration in the irrigation ditches. Compounds SMT and NRS showed moderate correlation, and a negative correlation was calculated for STZ and ETM-H_2O. While the exact cause of the negative correlation could not be identified, it is evident the highest average concentration of STZ and ETM-H_2O was measured during the lowest and second-lowest precipitation periods. Overall results, however, clearly show that runoff might be significant in the transport of veterinary antibiotics from field to environment.

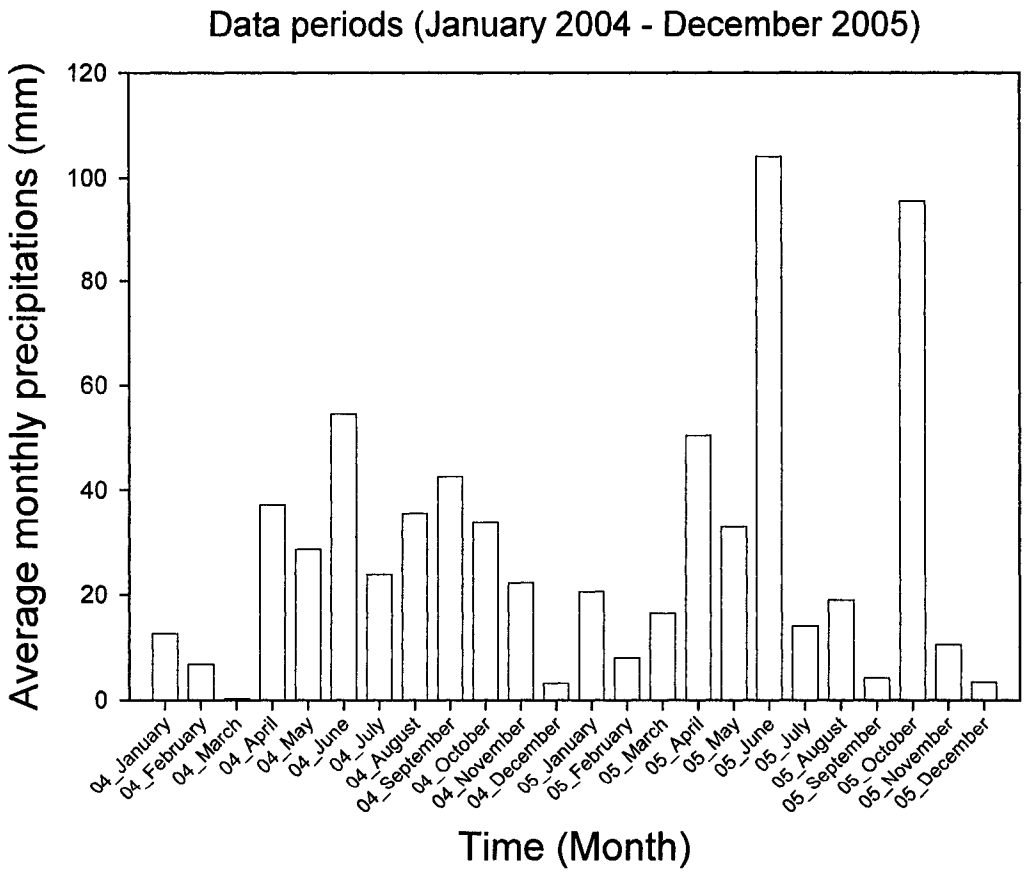

Figure 9.3 Average Monthly Precipitation in Greeley (Northern Colorado).

Table 9.9 Calculated correlation coefficient between average monthly precipitation and measured concentration in irrigation ditches

Compounds	TC	OTC	CTC[a]	STZ	SMT	ETM-H_2O	TYL	MNS	SLM	NRS
Correlation Coefficient	0.99	0.97	1.00	-0.92	0.85	-0.01	1.00	0.96	0.13	0.63

[a] Only two sampling events were evaluated.

9.4.3 Environmental Loading at Irrigation Ditches and Watershed

Environmental loading in grams of compound was calculated based on measured concentration in aqueous samples at each site (Table 9.7) and flow information (Table 9.2) and is summarized in Table 9.10.

Table 9.10 Summary of calculated environmental loading (g/yr).

Sampling Site	TC	CTC	OTC	STZ	SMT	ETM-H_2O	TYL	MNS	SLM	NRS
G[a]	542	1446	542	0	1446	362	181	36	54	54
B	904	1989	1989	904	904	1627	1266	90	54	90
C	181	542	723	181	2712	542	542	54	36	72
A	2170	3977	1808	181	1446	9584	1085	108	90	181
H	151	376	226	0	903	151	75	23	23	23
I	75	151	527	0	753	452	75	38	23	30
E	151	452	376	75	151	151	151	38	23	30
D	3	6	71	3	6	6	10	1	1	2
F	10	19	19	3	22	16	13	2	1	1
Avg.	**465**	**995**	**698**	**150**	**927**	**1432**	**378**	**43**	**34**	**54**
M	36	72	579	0	72	108	145	7	11	11
N	36	36	217	0	36	289	108	11	7	11
Avg.	**36**	**54**	**398**	**0**	**54**	**199**	**127**	**9**	**9**	**11**

[a] Location is ordered according to flow direction, from upstream to downstream of irrigation ditches

Because sampling sites M and N are located downstream of the study irrigation ditches, only those sites were included for comparison of environmental loading between

irrigation ditches and river. The results of environmental loading calculation clearly show that much higher mass was estimated along the irrigation ditches, compared to the river. Calculated masses at all irrigation ditches for eight of the ten compounds were greater than river-site concentrations, except for sampling sites D and F due to low-flow conditions. In addition, the ratio of average calculated ditch site mass (N = 9) and average calculated river-site mass (N = 2) ranged from 2 to 18, depending on the compound. These results suggest that irrigation ditches might act as intermediate transport pathways of veterinary antibiotics from soil, where they might be exposed by surface runoff or seepage flow, to the watershed.

Chapter 10

Degradation Kinetics of Three Veterinary Antibiotics in High and Low Level Manure Management

Submitted to Journal of Agricultural Food and Chemistry

10.1 Abstract

Two typical animal waste management practices, composting and stockpiling, were evaluated for their effect on the degradation of three veterinary antibiotics (VAs), chlortetracycline (CTC), Tylosin (TYL), and monensin (MNS). The VAs were applied to manure plots subject to composting or stockpiling, and core samples were collected over a period of time. Selected buffer solutions were used to extract the VAs , and analysis for concentration was conducted with solid phase extraction (SPE) followed by high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) technique. The VAs demonstrated rapid dissipation within ten days followed by a gradual decrease in concentration until the end of the experimental period (141days). All three VAs degraded more rapidly in the composting samples than in the stockpiling samples, particularly between 20 and 60 days of the observation period. Degradation of the three VAs generally followed a first-order kinetic model, and a fitted model with a calculated rate constant was determined for each treatment. TYL in composting showed

the fastest degradation, with a calculated rate constant of 0.91 (day^{-1}); the slowest degradation was exhibited by MNS in stockpiling, with rate constant of 0.17 (day^{-1}). Calculated correlation coefficients ranged from 0.89 to 0.96, indicating a strong correlation between measured concentrations and fitted values in this study. Although TYL in composting treatment showed the only complete elimination during the test period, this study suggests that composting can reduce animal waste contaminants prior to field application as fertilizer.

KEYWORDS: Veterinary antibiotics, Degradation, Composting, Stockpiling, Animal waste treatment

10.2 Introduction

Veterinary antibiotics (VAs) are used in animals for therapeutic treatment and for non-therapeutic, growth-promotion purposes. According to recent reports (Mellon et.al., 2001, and (Mellon et al., 2001), the estimated annual VA application for non-therapeutic purpose in the U.S is 25 million pounds – 77% of the total consumption of human and animal antibiotics. Given this enormous quantity of VAs and the fact that 50% of these antibiotics can be excreted as parent compounds or metabolites, there is a high chance of finding residuals of VAs in the environment (Kim et al., 2005).

The majority of excreted VAs is found in manure, and a considerable amount can be introduced to soil, groundwater, and surface water by spreading contaminated manure as fertilizer, through accidental leakage from storage, and overflowing as runoff. Studies measuring residuals of several VAs in different environmental compartments have found relatively higher concentrations detected in animal waste (Campagnolo et al., 2002; Haller et al., 2002; Christian et al., 2003; Liguoro et al., 2003; Schlusener et al., 2003;

Blackwell et al., 2004b; Kolz et al., 2005a) than in soil (Hamscher et al., 2002; Christian et al., 2003; Liguoro et al., 2003; Blackwell et al., 2004b; Jacobsen et al., 2004; Aga et al., 2005; Halling-Sorensen et al., 2005) and in surface water (Zhu et al., 2001; Campagnolo et al., 2002; Christian et al., 2003).

Currently, two techniques for managing animal waste on site are composting and stockpiling. The primary mechanism involved in composting is the enhanced aerobic decomposition of organic matter by microorganisms, using adequate moisture and temperature (Liang et al., 2003). A recent study reported the effect of composting on the dissipation of endocrine disruptor chemicals (EDCs) 17β-estradiol and testosterone in poultry manure. This report concluded that 84% and 90% of the quantity of 17β-estradiol and testosterone, respectively, diminished over a period of 139 days (Hakk et al., 2005). While this study indicated that composting could not eliminate the EDCs completely, this technique is efficient in removing organics.

The stockpiling technique involves piling the manure without any treatment, in an anaerobic state. Although composting can generate hazardous gas (ammonia, methane, and carbon dioxide) and might require more labor and time than stockpiling, composting is a more effective method than stockpiling in destroying pathogens, decreasing bulk density, and enriching nutrients.

With manure regarded as a main source of VA residuals in the environment, more information about the fate of VAs in manure is needed to identify animal waste treatment options. The purpose of this study was to determine the fate of excreted VAs during composting and stockpiling and to provide a possible means of reducing VA residuals prior to manure application in field as fertilizer.

10.3 Materials and methods

10.3.1 Chemicals and Veterinary Antibiotics

The target veterinary antibiotics (VAs) chlortetracycline, tylosin, and monensin (90-95%, sodium salt), and HPLC-grade methanol solvent (99.9%) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Other chemicals used – analytical grade formic acid (99%), ammonium hydroxide (29% by weight), citric acid-monohydrate, sodium phosphate-dibasic anhydrous, and disodium ethylene diaminetetraacetic acid (Na_2EDTA) – were purchased from Fisher Scientific (Fair Lawn, NJ). Each VA stock solution (100 μg/mL) was prepared in methanol monthly and stored at 4°C. Working solutions of 5 μg/mL and 0.5 μg/mL, diluted from stock solution, were prepared fresh immediately prior touse. The 3ml/60mg-capacity HLB (Hydrophillic-Lipophillic-Balance) cartridges used for solid phase extraction (SPE) were purchased from Water Oasis Co. (Milford, MA); the Milli-Q water (18.3mmho/cm) purification system from Millipore (Billerica, CA) was used when DI water was required.

10.3.2 Study Plots

Horse manure was chosen for the study medium because horses are not given antibiotics as growth promoters. Horse manure was collected from the Colorado State University Equine Center and either composted or stockpiled in three replicate piles of 5.4 m^3. Two control piles, one for composting and one for stockpiling, were set up and not spiked with antibiotics. After establishing the treatment plots, baseline samples from each of the eight plots were analyzed by the Soil, Water and Plant Testing Lab (Colorado State University, Fort Collins, CO) for physicochemical properties. As shown in Table 10.1, average

moisture content was 31.7 (%) for composting piles and 28.1 (%) for stockpiling plots. The calculated average C/N ratio was 13 and 15 for composting and stockpiling plots, respectively.

Table 10.1 Physicochemical properties of horse manure in each plot.

Plot [a]	Moisture (%)	pH	EC (mmhos/cm)	C (%)	N (%)	NH_4-N (mg/kg)	NO_3-N (mg/kg)	K (mg/kg)
Control	31.1	8.2	3.8	7.5	0.6	3.9	207.8	14000.0
C-1	30.8	8.3	4.3	11.5	0.8	21.5	167.7	14685.0
C-2	32.1	8.2	4.0	7.6	0.6	14.5	206.7	13770.0
C-3	33.0	8.2	4.1	8.9	0.7	8.2	207.2	14495.0
Control	30.6	8.4	5.5	13.2	0.9	312.6	191.7	18780.0
S-1	29.4	8.3	4.9	10.4	0.7	291.6	291.2	14590.0
S-2	29.3	8.5	4.8	12.5	0.9	411.4	291.6	18305.0
S-3	23.2	8.3	4.2	12.8	0.8	378.7	252.3	16500.0

[a] C and S denote the composting and stockpiling respectively.

The six treatment plots were spiked at approximately 328 μg/kg with CTC and TYL dissolved in water and sprayed onto the piles while mixing and with MNS ground in a blender and sprinkled over the piles while mixing and watering. Composted manure was amended with 1.5 m^3 of leaves and alfalfa.

The stockpiles initially heated to temperatures greater than 52 °C by the third week of the experiment. These temperatures dropped to less than 40 °C by the fourth week and decreased steadily throughout the remainder of the study. The composted piles heated up more gradually, reaching temperatures greater than 50 °C during the fourth week of the study and maintained temperatures at or greater than 40 °C for at least two more weeks. Plots of both treatments had dropped to temperatures less than 10 °C by the eighth week of the experiment. Because of the small size of the compost piles, watering and turning was needed only three times during the study (day 4, day 17, and day 38).

10.3.3 Sample Collection and Preparation

Samples were collected in ten locations from each pile at each sampling event using a coring device. The multiple samples collected from each pile were composited and slurried in sterile distilled water to form a homogenous mixture for subsequent antibiotic extraction. Sampling was performed three times per week during the first eight weeks of the study, and a final sample was taken at day 141 of the study.

10.3.4 Analysis of Veterinary Antibiotics

Analysis was performed on samples collected from eight selected sampling events. Prepared slurry samples contained in 50mL sterilized centrifuge tubes were thoroughly mixed immediately before analysis, then a 1-g aliquot from each tube was transferred to a 40mL Teflon tube. Two different buffer solutions were used to extract the target VAs from solid phase to liquid phase: McIlvaine buffer solution (pH 4.0) reflecting USDA (U.S. Department of Agriculture) guidelines (USDA, 2003) was used for CTC, and 1M ammonium hydroxide buffer solution (pH 10.0) titrated with formic acid was used for TYL and MNS. For CTC extraction only, 200μL of Na_2EDTA (5% w/v, 1mmol in solution) was added to complex the metal in solution. After adding 20 mL of the appropriate buffer, samples were shaken vigorously (Model No-4626, Lab-line instrument) for 20 minutes at 400 rpm. Shaken samples were centrifuged (IEC Clinical Centrifuge, International Equipment Co., Needham Hights, MA) for ten minutes at 4000 rpm to separate liquid and solid phases. The supernant from each sample was filtered through a 0.2-μm glass fiber filter and decanted into another 40mL glass vial. The filtered sample was kept at 4°C until SPE clean up.

The remaining solid sample was re-extracted with the same procedure described above to yield 40 mL of total volume. SPE and concentration procedures were conducted as described in a previous report (Kim et al., 2006).

Average measured moisture content of the slurries was 71%. After this determination, all measured concentrations were determined as dry weight.

10.3.5 High Performance Liquid Chromatography/Tandem Mass Spectrometry (HPLC/MS/MS)

High performance liquid chromatography (HPLC) with an HP 1100 Series Liquid Chromatograph (Agilent, Palo Alto, CA) was used to separate the VAs. The analytical column was an XTerra MS C_{18} column (Waters, Milliford, MA), 2.1×50mm (inner diameter×length, 2.5-μm pore size, end capped) equipped with a C_{18} guard column (Phenomenex, Torrence, CA, USA) to filter any particulates from the sample. Optimized HPLC conditions are summarized in Table 10.2. Injection volume was 20μL for all VAs. Ten minutes of post-run time was allowed to equilibrate the column between each analysis.

A ThermoFinnigan LCQ Duo Ion Trap Mass Spectrometer (ThermoQuest, Woburn, MA) equipped with a heated capillary interface and electrospray ionization (ESI) was used for mass spectrometric (MS) analysis. Optimization of MS was performed with the LCQ Duo syringe pump at a flow rate of 5 μL/min by infusing a standard solution of each compound (10 μM). Optimized MS parameters are summarized in Table 10.2.

Table 10.2 Compound-specific optimized HPLC parameters and optimized MS parameters

	Optimized HPLC Conditions		
	Column temp.(°C)	**Flow rate (mL/min)**	**Mobile phase conditions[a]**
CTC	15	0.32	A: 96% + B: 4% (0 min)→ A: 70% + B: 30% (29 min)→ A: 96% + B: 4% (30 min)
TYL	45	0.32	A: 80% + B: 20% (0 min)→ A: 65% + B: 35% (13 min)→ A: 96% + B: 4% (14 min)
MNS	15	0.25	A: 50% + C: 50% (0 min)→ A: 10% + C: 90% (19 min)→ A: 50% + B: 50% (20 min)
Optimized MS Conditions	Nitrogen gas for drying and nebuilizing Spray voltage: 4.5V Capillary voltage: 21V Capillary temperature: 165°C		

[a] Mobile phase A: 99.9% water + 0.1% formic acid;
Mobile phase B: 99.9% acetonitrile + 0.1% formic acid;
Mobile phase C: 100% methanol.

10.3.6 Quality Assurance

Recovery and limit of quantification (LOQ) determinations were conducted with raw horse manure analyzed prior to treatment to verify the absence of residual antibiotics. To determine analysis recovery, measured sample detection response was compared to the detection response of the same spike concentration in 5mL of methanol extractant. Detection response was calculated using the curve area of the internal standard, simatone, and the curve area of each sample, as determined by HPLC-MS/MS analysis. Simatone was used as an internal standard because it eluted within the same chromatographic timeframe as the analytes and responded well to positive electrospray ionization (Lindsey et al., 2001). Further, there is no noticeable matrix effect with simatone, a criterion

critical for this complex study matrix. Two different concentrations, 30 μg/kg and 90 μg/kg, were examined; recovery ranged from 94% to 98%, depending on the VA.

Limit of quantification was assessed in the statistical manner described in a previous report (Zhu et al., 2001). Calculated LOQ varied from 0.7 to 1.2 μg/kg. A summary of recovery and LOQ is shown in Table 10.3. A linear calibration curve was constructed from 1 to 180 μg/kg with $r > 0.99$.

Table 10.3 Summary of analysis recovery and limit of quantification (LOQ)

Compound	Recovery[a]		LOQ[b]
	30 μg/kg	90 μg/kg	μg/kg
CTC	98.9	94.6	1.2
TYL	92.4	93.1	1.0
MNS	94.2	96.1	0.7

[a] Average of duplicated experiments.
[b] Calculated standard deviation of three individual samples spiked with 1 μg/kg in control samples was multiplied by the one sided Student-t variate at 95% confidence intervals.

10.4 Results and Discussion

10.4.1 Degradation Profile of Antibiotics with Time

Initial recovery concentration of the target VAs in three treatment replicates averaged 317 μg/kg and 315 μμg/kg for CTC in composting and stockpiling, respectively; 180 μg/kg and 228 μg/kg for TYL; and 249 μg/kg and 243 μg/kg for MNS. Although recovered concentration at time 0 for TYL was much lower than the spike concentration of 328 μg/kg, initial measurement of the other two target compounds was close to the spike concentration in both management treatments. Variance of the three replicate

measurements at time 0 ranged from 3 to 30%, with the greatest variance observed for TYL in stockpiling.

Degradation of the VAs exhibited a similar trend with time. As shown in Figure 10.1, all three VAs rapidly dissipated within the first ten days and gradually decreased until the end of the observation period (141days). The percentage of degradation at the end of experiment in the two management treatments, composting and stockpiling, was 99.3 and 98.8, respectively, for CTC; 100 and 98.8 for TYL; and 94.7 and 90.7 for MNS. While elimination of all three VAs was incomplete in five of the six treatment plots by the end of the experiment, TYL in the composting plot was completely depleted at 27 days. MNS showed the slowest degradation rate and the highest residuals, 13.3 μg/kg and 22.5 μg/kg (dry weight) in the composting and stockpiling treatment plots, respectively, at the end of experiment. Rapid degradation was observed for TYL in both treatments, compared to the other two compounds; moderate degradation of CTC was observed.

While initial degradation (up to ten days) and final concentrations of the three VAs were similar between the two waste management treatments, composting showed more rapid degradation of all three VAs from 20 to 60 days of the observation period. This result suggests that composting management might be more efficient in the dissipation of VA residuals when contaminated animal waste is stored for a short period of time.

10.4.2 First-Order Kinetic Model

The degradation of the three VAs in composting and stockpiling management was fitted with a simple first-order kinetic model (Eq. 10.1) commonly used to frame the dissipation of environmentally related contaminants.

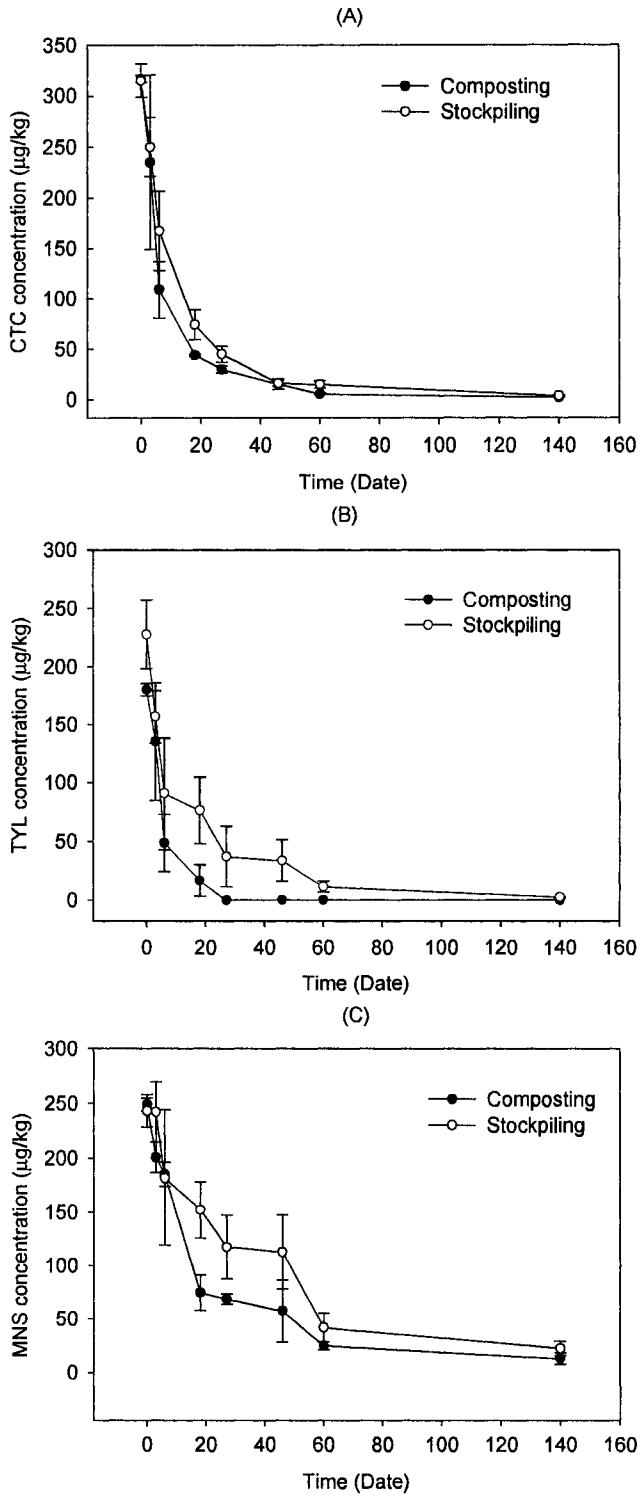

Figure 10.1 Measured concentration of CTC (A), TYL (B), and MNS (C) in high and low level manure management samples with time (error bar reflects standard deviation of three plots)

$$\frac{dC}{dt} = -kC \quad \text{(Eq. 10.1)}$$

where C is the measured concentration (μg/kg) at time t (day) and k is the pseudo rate constant (day^{-1}).

The first-order rate constant, k, represents the contribution of chemical and biological reactions in the dissipation of compounds; a higher k value signals faster degradation. Integrating both sides of the first-order kinetic model shown in Eq.1 yields the degradation kinetic model shown in Eq. 10.2.

$$C_t = C_0 e^{-kt} \quad \text{(Eq. 10.2)}$$

where C_0 is the initial concentration at time 0 and C_t is the concentration at time t.

The fitted first-order kinetic model for the three VAs in both treatments is shown in Figure 10.2; the calculated rate constant, k, and correlation coefficient are presented in Table 10.4.

Table 10.4 Calculated parameters for fitting the degradation of three VAs in composting and stockpiling

Antibiotic/Waste Management	Rate constant, k (day^{-1})	Correlation coefficient, r	$t_{1/2}$ (day)
CTC Composting	0.034	0.89	20.3
CTC Stockpiling	0.031	0.94	22.4
TYL Composting	0.091	0.96	7.6
TYL Stockpiling	0.030	0.91	23.5
MNS Composting	0.021	0.93	33.8
MNS Stockpiling	0.017	0.96	39.8

In the degradation of CTC, a slightly higher rate constant was calculated for composting, 0.034, than stockpiling, 0.031, indicating slightly more rapid degradation in composting. However, a higher correlation coefficient was calculated between the

experimental data and the fitted model for stockpiling. This result suggests that the degradation rate at each sampling event was more variable in composting than stockpiling, causing less accuracy in the composting fitted model (Wang et al., 2006).

TYL degradation in composting showed the highest calculated rate constant, 0.091, and a much lower calculated rate constant for stockpiling. Previous investigation of the degradation of TYL in manure reported that TYL depleted fairly fast in anaerobic conditions and even faster in aerobic conditions (Kolz et al., 2005a). The majority, 90%, of initially applied TYL dissipated in less than five days after the beginning of aerated composting management in the present study, an observation that supports these previously reported results.

Of the three VAs, the lowest rate constant was calculated for MNS in both management treatments. As seen with the other two compounds, composting showed more rapid degradation than stockpiling management; both treatments yielded a correlation coefficient showing the fitted model closely reflected the experimental data. Time for half life of the three VAs was calculated according to follow equation:

$$t_{50\%} = -\frac{0.693}{k} \quad \text{(Eq. 10.3)}$$

Calculations showed that TYL in composting exhibited the shortest half-life, 7.6 (days), while MNS in stockpiling showed the longest, 39.8 (days). Previous investigation documented a 25-58 (days) half-life for CTC and 49-76 (days) for TYL in soil (Halling-Sorensen et al., 2005), a much longer half-life than that observed in the present study.

Another investigation comparing the half-life of CTC, TYL, and MNS reported a much shorter half-life in manure-amended and manure-free soil (Carlson et al., 2006); in that study, the calculated half-life in manure-amended soil was 24, 4.5, and 3.3 (days) for

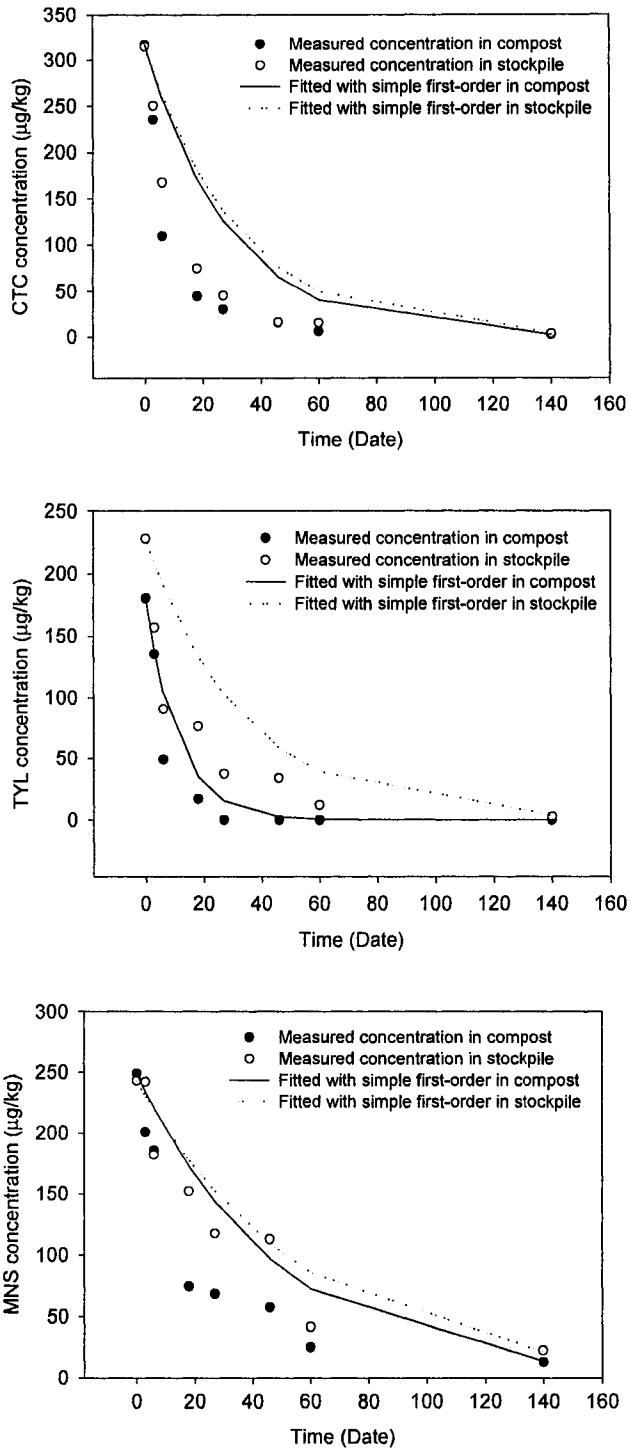

Figure 10.2 Fitted first-order model for CTC (A), TYL (B), and MNS (C) in composting and stockpiling

CTC, TYL, and MNS, respectively, and 21, 6.1, and 3.8 (days) in manure-free soil. The persistence order, CTC > TYL > MNS, in the latter study also differed from that observed in the present study, MNS > CTC > TYL. The difference might be explained by the different microorganisms present in soil and manure that would control the degradation rate for different antibiotics.

10.5 Conclusions

Dissipation of veterinary antibiotic residuals with commonly used animal waste management practices, composting and stockpiling, was evaluated. Traditional analysis of results showed a 92-to-99% recovery that supported sub-microgram concentration measurement.

Composting showed more efficient degradation of the target VAs than stockpiling. Calculated half-life of the target VAs ranged from 8 to 34 (days) for composting and 20 to 40 (days) for stockpiling, depending on the antibiotic compound. Although incomplete elimination was observed in most of the treatment plots during the experimental period (141 days), at least 95% of the applied antibiotics had degraded over this time. The results of this study show that composting can efficiently reduce the residuals of veterinary antibiotics in manure prior to field application and might be useful in assessing environmental contamination risk in future investigations.

CHAPTER 11

Conclusions

The first objective of this study was to evaluate the presence of antibiotics originating from human and animal source in both aqueous and sediment matrices in the Cache la Poudre watershed. To manage this task, a suitable and reproducible analytical method was developed. A total of 19 antibiotics in 4 groups with different physiochemical properties were studied including tetracyclines (TCs), sulfonamides (SAs), macrolides (MLs), and ionophores (IPs). With these characteristics varying with antibiotic group, an optimized solid phase extraction (SPE) method was utilized to measure antibiotic residuals in aqueous matrix. Also, adequate buffer solutions, McIlvaine buffer solution (pH 4.0) and ammonium hydroxide buffer solution (pH 10.0) were selected to extract antibiotics in sediment followed by SPE for cleanup and concentration. For separation and quantification of extracted antibiotics, a high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) technique was adapted for all studied antibiotics.

The result of recovery and limit of quantification (LOQ) studies for quality assurance (QA) purposes revealed that the developed analytical methods for both aqueous and sediment matrices were suitable to measure environmentally relevant levels of antibiotic concentrations. One of the important findings of surveying measured

concentrations of antibiotics in aqueous and sediment matrices was that much higher concentrations were measured in sediment highlighting the importance of this matrix evaluating environmental risks.

Furthermore, temporal and spatial variance of the concentration of studied antibiotics was assessed with long term monitoring. Across different sampling periods, significantly higher concentrations were measured during the winter months in both the aqueous and sediment matrices. This result could indicate that low flow conditions and cold-water temperatures might contribute to dilution effects and inhibition of microorganism activity to cause higher concentration of antibiotics. In addition, spatial analysis verified that wastewater treatment plants (WWTP) and animal feeding operations (AFOs) are major contributors of human and animal originated antibiotics in the watershed. In general, significantly higher concentrations were observed near WWTPs and AFOs for human and animal used antibiotics respectively in both matrices. Once the presence of antibiotics was confirmed in the watershed, transport mechanisms and pathways of antibiotics were studied. Due to lack of information regarding transport mechanism of veterinary antibiotics, this study was focused on evaluation of transport behavior of introduced veterinary antibiotics in the field. The assumed transport mechanism of veterinary antibiotics is through surface runoff or leaching into the subsurface after rainfall. Based on this assumption, rainfall simulation and column leaching experiments were conducted followed by mass balance calculations for better understanding of the transport behavior of veterinary antibiotics. The result of the rainfall simulation experiment indicated that surface runoff in aqueous and sediment could transport veterinary antibiotics to other environmental compartments. Depending on

different characteristics of studied veterinary antibiotics, variable transport behavior was observed. In general, strongly sorbed veterinary antibiotics were lost via sediment associated runoff while less sorptive veterinary antibiotics were transported via aqueous runoff. However, the amount of total loss during rainfall simulation was minimal and remaining veterinary antibiotics in the plot were found between a soil depth range of 0 to 30 cm. Even strongly sorbed veterinary antibiotics were detected in deeper soil and this result could be interpreted that colloids might act as carriers of sorbed compounds into the groundwater.

Column studies also confirmed that veterinary antibiotics could leach into subsurface in a short time period for less sorptive veterinary antibiotics and even strongly sorbed veterinary antibiotics could be transported into deeper soil due to colloid-facilitated transport mechanisms. Consequently, there is an increased chance that veterinary antibiotics can contaminate groundwater and possibly threaten human health when groundwater is used as a drinking water source. In addition to surface runoff and penetrating into subsurface as transport mechanisms of veterinary antibiotics in the environment, irrigation ditches interconnected with agricultural fields and the watershed might play an important role in movement of antibiotics in the environment. The measured concentration of veterinary antibiotics in local irrigation ditches surrounded by several AFOs during the irrigation season was higher than the adjunct watershed and the calculated mass loading in irrigation ditches was as high as 18 times greater than river concentrations. The result of this study clearly showed that irrigation ditches might act as an important potential transport pathway of veterinary antibiotics from source to watershed.

The last objective of this research was to investigate animal waste management to reduce the release of veterinary antibiotics to the environment. To assess this task, two commonly used animal waste management practices, stock piling and composting, were evaluated regarding dissipation of veterinary antibiotics over time. Between the two approaches, composting showed more efficient reduction of veterinary antibiotics than stockpiling. In addition, the observed half-life was shorter with composting management than stockpiling for the veterinary antibiotics studied. Although, incomplete elimination was observed for most veterinary antibiotics with the two management approaches during the experimental periods, the majority of applied antibiotics (> 95%) were degraded. The result of this study suggests composting treatment of veterinary antibiotics in animal waste for reducing residuals prior to application in the field.

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Appendices

Table A1-1. Parameters for constructing calibration curve of studied antibiotics in aqueous and sediment matrices

Antibiotics	Acronym	Aqueous [b]			Sediment [c]		
		Slope	Interception	R^2	Slope	Interception	R^2
Tetracycline	TC	2.07	0.15	0.99	0.01	0.01	0.97
Chlortetracycline	CTC	1.80	0.16	0.99	0.01	0.05	0.97
Oxytetracycline	OTC	2.45	0.13	0.99	0.01	0.04	0.99
Minocycline [a]	MNC				ND		
Demeclocycline	DMC	1.32	0.17	0.99	0.01	0.03	0.98
Meclocycline	MCC	1.39	0.43	0.99	0.01	0.05	0.98
Doxycycline	DXC	1.62	0.47	0.99	0.01	0.13	0.97
Sulfathiazole	STZ	0.16	0.03	0.99	0.002	0.005	0.99
Sulfamerazine	SMR	0.15	0.02	0.99	0.002	0.001	0.98
Sulfamethazine	SMT	0.41	0.05	0.99	0.004	0.008	0.98
Sulfachlorpyridazine	SCP	0.14	0.02	0.99	0.001	0.001	0.98
Sulfamethoxazole	SMX	0.17	0.02	0.99	0.002	0.001	0.97
Sulfadimethoxine	SDM	0.59	0.07	0.99	0.005	0.012	0.98
Erythromycin	ETM	1.64	0.01	0.99	0.004	0.006	0.99
Roxithromycin	RTM	2.51	0.04	0.99	0.025	0.006	0.99
Tylosin	TYL	2.25	0.27	0.99	0.011	0.004	0.99
Monensin	MNS	71.39	1.01	0.99	0.44	0.99	0.99
Salinomycin	SLM	18.24	0.41	0.99	0.17	0.21	0.99
Narasin	NRS	20.60	0.33	0.99	0.21	1.55	0.99

[a] ND denotes Not Determined due to low recovery (< 30%), [b] Linearity was calculated from 0.01 to 5 µg/L, [c] Linearity was calculated from 1 to 180 µg/kg.

Table A2-1. Measured concentration of tetracyclines in aqueous at various time and sampling locations

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
TC			0.02	0.01			0.01	0.04				0.03	0.02	0.03	0.04	0.01
			0.01	0.03				0.03				0.01	0.02	0.02	0.01	0.01
			0.01	0.03			0.01	0.03				0.02	0.03	0.01	0.01	
Avg.			**0.01**	**0.02**			**0.01**	**0.03**				**0.02**	**0.02**	**0.02**	**0.02**	**0.01**
Std.			0.01	0.01			0.00	0.01				0.01	0.01	0.01	0.02	0.00
CTC				0.06	0.02	0.02	0.01	0.14	0.05		0.08	0.24				0.12
				0.05	0.01	0.03	0.01	0.07	0.03		0.09	0.18				0.19
				0.04	0.01	0.02	0.01	0.10	0.04		0.04	0.21				0.17
Avg.				**0.05**	**0.01**	**0.02**	**0.01**	**0.10**	**0.04**		**0.07**	**0.21**				**0.16**
Std.				0.01	0.01	0.01	0.00	0.04	0.01		0.03	0.03				0.04
OTC				0.01		0.02		0.01		0.01		1.78	0.01	0.01		0.48
						0.01	0.01	0.01		0.01		0.73				0.38
				0.01		0.01	0.02			0.01		1.12	0.01	0.01		0.25
Avg.				**0.01**		**0.01**	**0.02**	**0.01**		**0.01**		**1.21**	**0.01**	**0.01**		**0.37**
Std.				0.00		0.01	0.01	0.00		0.00		0.53	0.00	0.00		0.12

Table A2-1. Continued

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
DMC						0.03	0.02	0.03				0.05		0.02	0.02	0.02
						0.01	0.04	0.05				0.04		0.02		0.02
						0.02	0.03	0.04				0.05			0.02	
Avg.						**0.02**	**0.03**	**0.04**				**0.05**		**0.02**	**0.02**	**0.02**
Std.						0.01	0.01	0.01				0.01		0.00	0.00	0.00
MCC	0.03		0.01	0.03	0.07		0.01	0.01	0.06		0.02		0.05			0.01
	0.03			0.03	0.15		0.01		0.01		0.04	0.01	0.07			
	0.04		0.01	0.02	0.09			0.01	0.04		0.03	0.01	0.03			0.01
Avg.	**0.03**		**0.01**	**0.03**	**0.10**		**0.01**	**0.01**	**0.04**		**0.03**	**0.01**	**0.05**			**0.01**
Std.	0.01		0.00	0.01	0.04		0.00	0.00	0.03		0.01	0.00	0.02			0.00
DXC			0.04	0.01	0.02	0.01		0.05	0.02				0.04			
			0.05	0.01	0.03	0.03		0.04	0.01				0.01			
			0.03	0.01	0.02			0.05	0.01				0.01			
Avg.			**0.04**	**0.01**	**0.02**	**0.02**		**0.05**	**0.01**				**0.02**			
Std.			0.01	0.00	0.01	0.01		0.01	0.01				0.02			

Table A2-2. Measured concentration of tetracyclines in sediment at various time and sampling locations

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
TC	9.3	3.9	1.0	11.0	33.0	8.7	6.8	101.2	4.8	7.8	17.1	3.7	21.6	10.6	20.7	24.9
	9.1	3.7	1.1	11.3	33.0	8.8	6.9	103.8	4.6	9.4	16.4	3.9	21.2	10.6	20.8	24.6
	8.6	3.3	1.2	10.7	32.5	8.6	6.5	103.1	4.0	8.0	16.5	3.9	21.0	9.5	22.0	24.8
Avg.	**9.0**	**3.6**	**1.1**	**11.0**	**32.8**	**8.7**	**6.7**	**102.7**	**4.5**	**8.4**	**16.7**	**3.8**	**21.3**	**10.2**	**21.1**	**24.8**
Std.	0.4	0.3	0.1	0.3	0.3	0.1	0.2	1.3	0.4	0.9	0.3	0.1	0.3	0.6	0.7	0.2
CTC	9.9	2.9	1.2	9.4	12.0	3.3	4.4	19.2	25.2	4.6	5.2	15.2	30.6	3.7	2.9	21.5
	9.5	2.8	1.2	9.2	11.6	3.2	4.1	19.0	24.6	4.6	5.3	16.3	30.8	3.4	3.2	22.8
	9.5	3.3	1.0	10.1	11.2	2.8	5.1	19.8	24.9	4.7	5.5	15.6	31.1	4.3	2.5	21.6
Avg.	**9.6**	**3.0**	**1.1**	**9.6**	**11.6**	**3.1**	**4.6**	**19.3**	**24.9**	**4.6**	**5.3**	**15.7**	**30.8**	**3.8**	**2.9**	**21.9**
Std.	0.2	0.3	0.1	0.5	0.4	0.3	0.5	0.4	0.3	0.1	0.2	0.6	0.3	0.5	0.3	0.7
OTC	5.1	2.6	19.5	7.3	8.2	6.8	9.4	56.2	6.2	8.0	13.0	18.1	5.6	23.7	10.5	35.7
	5.8	1.8	18.8	7.6	7.5	7.5	10.4	56.0	6.5	6.8	13.2	19.7	5.9	23.1	8.7	35.4
	5.9	2.8	19.1	8.4	9.3	7.6	9.9	56.2	7.1	7.4	13.9	18.9	6.1	24.1	8.7	35.2
Avg.	**5.6**	**2.4**	**19.1**	**7.8**	**8.3**	**7.3**	**9.9**	**56.1**	**6.6**	**7.4**	**13.3**	**18.9**	**5.9**	**23.6**	**9.3**	**35.4**
Std.	0.4	0.5	0.4	0.6	0.9	0.4	0.5	0.1	0.5	0.6	0.5	0.8	0.3	0.5	1.0	0.3

Table A2-2. Continued

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
DMC	2.5	2.3	6.2	7.2	6.3	7.1	5.5	14.8	2.7	2.3	4.3	10.4	3.7	7.4	6.9	23.6
	3.9	1.5	4.2	5.9	4.8	6.6	6.7	15.0	3.1	1.8	7.1	8.9	3.7	6.5	6.4	23.7
	3.0	2.6	4.7	6.3	5.8	6.7	5.8	14.4	2.0	2.2	4.8	9.3	3.4	6.7	6.4	23.6
Avg.	**3.1**	**2.1**	**5.0**	**6.5**	**5.6**	**6.8**	**6.0**	**14.7**	**2.6**	**2.1**	**5.4**	**9.5**	**3.6**	**6.9**	**6.5**	**23.6**
Std.	0.7	0.6	1.0	0.7	0.8	0.3	0.6	0.3	0.6	0.3	1.5	0.8	0.2	0.5	0.3	0.0
MCC	6.0	28.8	7.3	38.2	16.2	21.3	6.5	66.7	9.1	27.3	4.5	27.4	10.0	41.4	3.6	71.6
	6.1	29.7	8.3	37.9	14.7	21.6	4.6	68.1	8.1	29.4	7.1	25.4	10.2	41.4	4.2	72.2
	6.9	30.0	7.9	38.9	16.1	21.8	5.9	67.9	8.2	28.5	5.6	26.0	9.6	42.1	5.0	72.3
Avg.	**6.3**	**29.5**	**7.8**	**38.3**	**15.7**	**21.6**	**5.7**	**67.6**	**8.5**	**28.4**	**5.7**	**26.3**	**9.9**	**41.6**	**4.3**	**72.0**
Std.	0.5	0.6	0.5	0.5	0.8	0.3	1.0	0.8	0.6	1.1	1.3	1.0	0.3	0.4	0.7	0.4
DXC	12.6	5.4		13.8	27.8	10.2	1.2	38.5	21.3	6.4		12.4	15.1	14.8		25.9
	13.5	5.1		13.0	27.0	10.7	3.1	39.3	19.5	6.1		11.5	15.4	15.0		24.9
	12.8	4.8		13.1	27.9	9.7	2.5	38.9	18.9	6.3		11.7	15.0	14.6		26.0
Avg.	**13.0**	**5.1**		**13.3**	**27.6**	**10.2**	**2.2**	**38.9**	**19.9**	**6.3**		**11.9**	**15.2**	**14.8**		**25.6**
Std.	0.5	0.3		0.4	0.5	0.5	1.0	0.4	1.2	0.2		0.5	0.2	0.2		0.6

Table A2-3. Measured concentration of sulfonamides in aqueous at various time and sampling locations

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
STZ			0.01	0.04		0.01			0.01	0.02		0.01				0.01
			0.01	0.03		0.01			0.01	0.01		0				0.01
			0.00	0.03		0.01			0.00	0.01		0.01				0.01
Avg.			**0.01**	**0.03**		**0.01**			**0.01**	**0.01**		**0.01**				**0.01**
Std.			0.01	0.01		0.00			0.01	0.01		0.01				0.00
SMR				0.03	0.06	0.03			0.01	0.01	0.01	0.01			0.00	
				0.03	0.07	0.02			0.00	0.01	0.02	0.01			0.01	
				0.03	0.06	0.02			0.01	0.01	0.02	0.01			0.01	
Avg.				**0.03**	**0.06**	**0.02**			**0.01**	**0.01**	**0.02**	**0.01**			**0.01**	
Std.				0.00	0.01	0.01			0.01	0.00	0.01	0.00			0.01	
SMT				0.01				0.02								
				0.03				0.03								
				0.02				0.02								
Avg.				**0.02**				**0.02**								
Std.				0.01				0.01								

Table A2-3. Continued

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
SCP				0.04												
				0.03												
				0.03												
Avg.				**0.03**												
Std.				0.01												
SMX					0.09	0.05	0.3	0.22	0.07	0.08	0.06	0.04	0.05	0.12	0.05	0.15
					0.08	0.04	0.34	0.23	0.06	0.07	0.08	0.05	0.06	0.08	0.05	0.17
					0.08	0.06	0.32	0.24	0.07	0.07	0.07	0.04	0.07	0.1	0.05	0.13
Avg.					**0.08**	**0.05**	**0.32**	**0.23**	**0.07**	**0.07**	**0.07**	**0.04**	**0.06**	**0.10**	**0.05**	**0.15**
Std.					0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.00	0.02
SDM			0.03	0.05			0.01	0.01	0.04				0.01		0.01	
			0.04	0.04			0.01	0	0.04				0.00		0.00	
			0.03	0.04			0.00	0.01	0.04				0.01		0.01	
Avg.			**0.03**	**0.04**			**0.01**	**0.01**	**0.04**				**0.01**		**0.01**	
Std.			0.01	0.01			0.01	0.01	0.00				0.01		0.01	

Table A2-4. Measured concentration of sulfonamides in sediment at various time and sampling locations

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
STZ	2.9	2.4		3.2	4.1	4.2	1.7	2.8	2.8	3.6	4.7	3.9	1.4	4.5		3.8
	3.0	2.6		3.8	3.3	4.9	1.3	2.1	3.5	3.7	6.2	3.8	1.4	4.6		4.1
	2.0	3.0		2.8	3.0	4.0	1.0	3.1	3.4	3.8	5.3	3.8	2.2	4.9		3.9
Avg.	**2.6**	**2.7**		**3.3**	**3.5**	**4.4**	**1.3**	**2.7**	**3.2**	**3.7**	**5.4**	**3.8**	**1.7**	**4.7**		**3.9**
Std.	0.6	0.3		0.5	0.6	0.5	0.4	0.5	0.4	0.1	0.7	0.1	0.5	0.2		0.2
SMR								2.9				6.4				5.7
								2.3				4.3				7.4
								1.8				4.8				7.2
Avg.								**2.3**				**5.2**				**6.8**
Std.								0.6				1.1				0.9
SMT		1.8				1.0	2.8				13.4				3.2	
		1.4				1.0	2.9				13.8				3.9	
		1.9				1.1	3.4				13.8				5.1	
Avg.		**1.7**				**1.0**	**3.0**				**13.7**				**4.1**	
Std.		0.3				0.1	0.3				0.2				1.0	

Table A2-4 Continued

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
SCP				3.4	2.3	3.0			2.8				1.7			
				3.0	2.8	2.7			2.3				2.1			
				3.3	3.0	3.4			2.9				1.9			
Avg.				**3.2**	**2.7**	**3.0**			**2.7**				**1.9**			
Std.				0.2	0.4	0.4			0.3				0.2			
SMX	1.0				1.2			2.6	1.4				1.1			
	1.4				1.9			2.0	1.7				1.8			
	1.3				1.6			1.1	1.7				2.3			
Avg.	**1.2**				**1.6**			**1.9**	**1.6**				**1.7**			
Std.	0.2				0.4			0.8	0.2				0.6			
SDM	1.4			6.8	1.2			6.9	3.7			2.4	1.4			5.4
	1.7			5.8	2.1			6.6	3.4			2.2	2.9			4.8
	2.0			6.8	1.8			6.8	3.8			2.2	2.8			5.0
Avg.	**1.7**			**6.5**	**1.7**			**6.8**	**3.6**			**2.3**	**2.4**			**5.1**
Std.	0.3			0.6	0.5			0.1	0.2			0.1	0.8			0.3

Table A2-5. Measured concentration of macrolides in aqueous at various time and sampling locations

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
ETM			0.03	0.03	0.17	0.46	0.23	0.31	0.02	0.06	0.01	0.09	0.03		0.02	0.14
			0.04	0.01	0.18	0.43	0.25	0.24	0.03	0.05	0.03	0.06	0.03		0.02	0.24
			0.03	0.02	0.19	0.45	0.24	0.25	0.02	0.07	0.02	0.08	0.03		0.02	0.21
Avg.			**0.03**	**0.02**	**0.18**	**0.45**	**0.24**	**0.27**	**0.02**	**0.06**	**0.02**	**0.08**	**0.03**		**0.02**	**0.20**
Std.			0.01	0.01	0.01	0.02	0.01	0.04	0.01	0.01	0.01	0.02	0.00		0.00	0.05
RTM																
Avg.																
Std.																
TYL							0.05									
							0.06									
							0.05									
Avg.							**0.05**									
Std.							0.01									

Table A2-6. Measured concentration of macrolides in sediment at various time and sampling locations

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
ETM	1.5	11.6	2.4	12.9	25.0	8.5	33.2	5.9	5.2	17.1	2.4	12.0	7.4	9.7	2.7	
	1.4	11.0	1.1	13.2	25.9	8.3	17.3	5.0	3.3	16.2	4.8	11.0	7.2	9.8	7.3	
	1.1	10.2	0.6	12.6	25.8	7.9	25.3	4.6	4.2	16.8	3.6	12.8	7.4	10.2	5.0	
Avg.	**1.3**	**10.9**	**1.4**	**12.9**	**25.6**	**8.2**	**25.3**	**5.2**	**4.2**	**16.7**	**3.6**	**11.9**	**7.3**	**9.9**	**5.0**	
Std.	0.2	0.7	0.9	0.3	0.5	0.3	7.9	0.7	1.0	0.4	1.2	0.9	0.1	0.3	2.3	
RTM	1.0			5.6			1.9				1.6	1.3			1.3	
	1.0			5.7			0.9				1.4	1.0			1.3	
	1.4			6.3			1.4				1.5	1.9			1.3	
Avg.	**1.1**			**5.9**			**1.4**				**1.5**	**1.4**			**1.3**	
Std.	0.2			0.4			0.5				0.1	0.5			0.0	
TYL		2.3				4.8	1.5	2.8	1.0	8.8	1.7	2.7	1.3	6.9	0.0	
		2.1	2.3			4.8	1.3	2.5	1.4	9.9	1.3	2.6	1.0	7.5	0.0	
		2.1	0.6			4.9	1.4	2.1	1.1	9.2	1.5	2.7	1.1	7.3	0.0	
Avg.		**2.2**	**1.5**			**4.8**	**1.4**	**2.5**	**1.2**	**9.3**	**1.5**	**2.7**	**1.1**	**7.2**	**0.0**	
Std.		0.1	1.2			0.1	0.1	0.4	0.2	0.6	0.2	0.1	0.2	0.3	0.0	

Table A2-7. Measured concentration of ionophores in aqueous at various time and sampling locations

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
MNS				0.003				0.004	0.011	0.002	0.004	0.008	0.008	0.002	0.004	0.011
				0.002				0.006	0.012	0.004	0.002	0.007	0.010	0.001	0.002	0.012
				0.002				0.005	0.011	0.003	0.003	0.007	0.090	0.003	0.003	0.015
Avg.				**0.002**				**0.005**	**0.011**	**0.003**	**0.003**	**0.007**	**0.036**	**0.002**	**0.003**	**0.013**
Std.				0.001				0.001	0.001	0.001	0.001	0.001	0.047	0.001	0.001	0.002
SLM				0.001			0.001	0.001	0.007	0.001	0.003		0.007	0.003	0.002	0.001
			0.001	0.001					0.006	0	0.004	0.001	0.008	0.002	0.003	0.001
								0.00	0.006	0.001	0.003	0.001	0.007	0.002	0.004	
Avg.			**0.001**	**0.001**			**0.001**	**0.001**	**0.006**	**0.001**	**0.003**	**0.001**	**0.007**	**0.002**	**0.003**	**0.001**
Std.			0.000	0.000			0.000	0.000	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.000
NRS	0.026		0.001		0.035				0.033	0.002	0.008		0.041		0.006	
	0.024				0.041				0.031	0.003	0.007		0.031		0.007	
	0.025				0.038				0.032	0.003	0.007		0.033		0.007	
Avg.	**0.025**		**0.001**		**0.038**				**0.032**	**0.003**	**0.007**		**0.035**		**0.007**	
Std.	0.001		0.000		0.003				0.001	0.001	0.001		0.005		0.001	

Table A2-8. Measured concentration of ionophores in sediment at various time and sampling locations

	Site 2				Site 3				Site 4				Site 5			
	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05	May 03	Apr 04	Aug 04	Feb 05
MNS									14.7	9.1	30.2	7.2	2.7	3.1	32.2	11.9
									14.4	10.4	29.4	8.9	3.4	2.4	31.5	9.4
									14.3	10.5	30.5	9.0	3.0	1.7	30.7	10.5
Avg.									**14.5**	**10.0**	**30.0**	**8.4**	**3.0**	**2.4**	**31.5**	**10.6**
Std.									0.2	0.7	0.4	0.8	0.3	0.6	0.6	1.0
SLM									3.8	5.0	30.2	2.6	1.7	5.1	22.1	1.2
									4.6	5.5	29.5	1.9	2.3	4.1	23.1	3.5
									3.8	5.2	30.7	1.0	1.6	4.9	23.2	1.5
Avg.									**4.0**	**5.3**	**30.1**	**1.8**	**1.9**	**4.7**	**22.8**	**2.1**
Std.									0.4	0.2	0.5	0.6	0.3	0.4	0.5	1.0
NRS	2.2				3.8				5.4	6.1	6.2		5.4	4.5	16.6	
	3.2				3.2				6.0	6.9	6.5		5.6	5.1	15.7	
	3.1				4.1				5.4	6.3	6.7		5.2	4.8	16.6	
Avg.	**2.8**				**3.7**				**5.6**	**6.5**	**6.5**		**5.4**	**4.8**	**16.3**	
Std.	0.4				0.4				0.3	0.3	0.2		0.2	0.2	0.4	

Table A3-1. Measured concentration of studied antibiotics in runoff aqueous and sediment at plot 1

Time	TC		CTC		STZ		SMT		ETM		TYL		MNS	
(min)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)
5	0.14	4.57	0.07	6.92	1.16	3.11	3.15	1.04	0.49	16.00	0.14	4.35	2.23	38.68
10	0.14	4.19	0.09	10.31	0.54	1.84	0.44	1.07	0.96	35.78	0.58	5.38	2.29	15.26
15	0.09	3.16	0.09	0.00	0.40	4.51	0.79	1.03	0.56	40.68	0.30	7.83	2.42	12.22
20	0.03	0.00	0.05	0.00	0.24	3.74	0.39	0.00	0.41	17.17	0.08	4.00	1.89	3.77
25	0.02	0.00	0.06	0.00	0.23	3.44	0.35	0.00	0.05	9.51	0.02	2.93	1.11	2.33
30	0.00	0.00	0.02	0.00	0.19	1.02	0.20	0.00	0.02	9.66	0.00	2.25	1.00	1.08
35	0.00	0.00	0.00	0.00	0.18	2.33	0.22	0.00	0.03	4.69	0.01	3.72	0.31	1.09
40	0.00	0.00	0.00	0.00	0.16	0.02	0.13	0.00	0.06	4.95	0.01	8.73	0.80	0.55
45	0.00	0.00	0.00	0.00	0.18	0.00	0.20	0.71	0.02	6.35	0.01	4.01	0.31	0.77
50	0.00	0.00	0.00	0.00	0.21	0.00	0.20	0.79	0.03	7.22	0.01	7.69	0.45	0.84
55	0.00	0.00	0.00	0.00	0.20	0.00	0.23	0.00	0.02	5.35	0.00	10.52	0.45	0.65
60	0.00	0.00	0.00	0.00	0.20	0.00	0.19	0.64	0.02	4.03	0.00	8.53	0.52	0.30

Table A3-2. Measured concentration of studied antibiotics in runoff aqueous and sediment at plot 2

Time	TC		CTC		STZ		SMT		ETM		TYL		MNS	
(min)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)
5	0.13	2.96	0.11	3.04	1.70	2.02	3.69	0.00	0.42	19.35	0.12	9.35	0.99	57.64
10	0.06	3.00	0.08	2.49	0.48	1.25	0.84	0.00	0.58	44.12	0.05	21.85	3.27	29.49
15	0.03	1.84	0.07	0.00	0.59	5.63	1.42	0.00	0.34	37.46	0.10	17.11	1.53	18.53
20	0.01	0.00	0.05	0.00	0.36	1.96	0.67	0.00	0.21	36.51	0.07	15.08	1.68	9.51
25	0.00	0.00	0.04	0.00	0.23	3.68	0.37	0.00	0.13	7.72	0.04	16.08	1.06	3.92
30	0.00	0.00	0.04	0.00	0.20	2.64	0.29	0.00	0.16	6.49	0.02	15.10	0.71	3.90
35	0.00	0.00	0.03	0.00	0.20	6.50	0.29	0.00	0.05	3.92	0.00	15.92	1.23	4.85
40	0.00	0.00	0.02	0.00	0.20	3.16	0.13	0.00	0.06	3.68	0.00	12.24	1.00	2.77
45	0.00	0.00	0.00	0.00	0.20	0.05	0.13	0.00	0.08	2.66	0.00	12.73	0.39	0.00
50	0.00	0.00	0.00	0.00	0.19	0.02	0.13	0.00	0.07	2.03	0.00	10.98	0.37	0.00
55	0.00	0.00	0.00	0.00	0.19	0.00	0.13	0.00	0.07	1.93	0.00	11.29	0.63	0.00
60	0.00	0.00	0.00	0.00	0.17	0.00	0.17	0.00	0.07	1.49	0.00	6.09	0.48	0.00

Table A3-3. Measured concentration of studied antibiotics in runoff aqueous and sediment at plot 3

Time	TC		CTC		STZ		SMT		ETM		TYL		MNS	
(min)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)	Aqueous (μg/L)	Sediment (μg/kg)
5	0.10	6.32	0.09	8.03	1.11	3.80	3.52	1.48	0.62	56.09	0.25	2.98	2.37	74.44
10	0.06	3.99	0.06	5.92	0.30	1.35	0.45	0.49	0.48	78.88	0.17	8.06	2.70	62.40
15	0.04	2.13	0.09	1.84	0.26	6.99	0.36	0.00	0.27	14.86	0.33	4.29	2.75	10.83
20	0.02	1.04	0.05	0.00	0.25	3.60	0.37	0.00	0.26	26.67	0.18	7.70	1.86	5.11
25	0.00	1.08	0.03	0.00	0.22	1.43	0.30	0.00	0.18	23.00	0.12	5.99	1.46	6.95
30	0.00	0.00	0.03	0.00	0.19	1.07	0.22	0.00	0.11	13.15	0.09	4.30	0.89	2.81
35	0.00	0.00	0.03	0.00	0.21	1.21	0.18	0.00	0.15	22.48	0.09	6.50	1.04	2.43
40	0.00	0.00	0.03	0.00	0.18	0.85	0.15	0.00	0.11	13.60	0.07	3.01	0.70	1.86
45	0.00	0.00	0.02	0.00	0.16	0.00	0.17	0.00	0.10	15.30	0.06	3.81	0.63	0.00
50	0.00	0.00	0.00	0.00	0.17	0.00	0.18	0.00	0.18	9.10	0.04	2.54	0.52	0.00
55	0.00	0.00	0.00	0.00	0.16	0.00	0.16	0.50	0.13	9.09	0.04	3.06	0.57	0.00
60	0.00	0.00	0.00	0.00	0.16	0.00	0.16	0.00	0.11	1.97	0.15	2.68	0.53	0.00

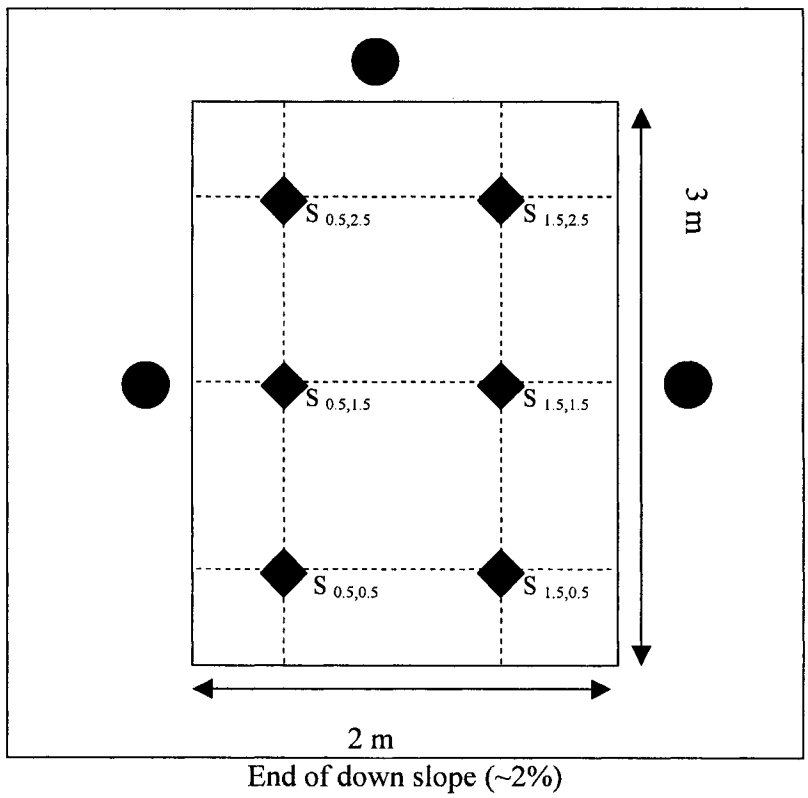

Figure A3-1. Plan view of rainfall simulation plot. ♦ denotes sampling locations for surface and depth soil after rainfall simulation and • denotes filter paper locations. S_{ij} represents the coordinates of X-Y direction from the bottom. Scale is not considered.

Table A3-4. Measured concentration of filter papers located at outside of each plot

	TC	CTC	STZ	SMT	ERY	TYL	MNS
				(mg/L)			
Plot1	17.91	15.53	16.32	16.67	13.63	17.90	16.67
	15.82	10.76	11.64	11.38	8.16	13.10	12.79
	14.70	11.24	14.11	11.52	9.88	11.50	13.81
Avg.	**16.14**	**12.51**	**14.02**	**13.19**	**10.56**	**14.17**	**14.42**
Std.	1.63	2.63	2.34	3.01	2.80	3.33	2.01
Plot2	16.07	11.05	10.24	11.29	7.13	14.06	11.08
	15.57	10.47	13.03	11.47	9.19	12.14	14.50
	13.82	12.01	15.18	11.57	10.57	10.85	13.12
Avg.	**15.15**	**11.18**	**12.82**	**11.44**	**8.96**	**12.35**	**12.90**
Std.	1.18	0.78	2.48	0.14	1.73	1.62	1.72
Plot3	15.87	13.77	15.75	14.12	12.10	14.38	14.90
	16.87	13.15	13.98	14.03	10.90	15.50	14.73
	15.26	11.00	12.87	11.45	9.02	12.30	13.30
Avg.	**16.00**	**12.64**	**14.20**	**13.20**	**10.67**	**14.06**	**14.31**
Std.	0.81	1.45	1.45	1.51	1.55	1.62	0.88

Table A3-5. Measured concentration of depth soil samples at plot 1

Coordinates	Depth	TC	CTC	STZ	SMT	ETM	TYL	MNS
	(cm)				(μg/kg)			
$S_{0.5,2.5}$	0 – 2	3.98	9.85			274.41	23.35	9.17
		4.35	9.28			270.68	22.59	9.57
	Avg.	**4.16**	**9.56**	**0.00**	**0.00**	**272.54**	**22.97**	**9.37**
	2 - 10	2.95	6.91			20.74	1.67	6.71
		4.02	7.52			21.50	-0.19	7.64
	Avg.	**3.48**	**7.22**	**0.00**	**0.00**	**21.12**	**0.74**	**7.18**
	10 - 20	1.95	6.03			9.90	-1.00	5.45
		4.31	6.77			18.37	2.22	4.04
	Avg.	**3.13**	**6.40**	**0.00**	**0.00**	**14.13**	**0.61**	**4.74**
	20 - 30	1.69	8.90	5.88		3.16	0.86	3.36
		3.18	8.44	6.49		0.82	0.66	5.59
	Avg.	**2.43**	**8.67**	**6.19**	**0.00**	**1.99**	**0.76**	**4.47**
$S_{1.5,2.5}$	0 – 2	2.24	6.54	2.37		190.04	12.92	5.91
		3.42	7.98	3.27		190.82	9.90	7.57
	Avg.	**2.83**	**7.26**	**2.82**	**0.00**	**190.43**	**11.41**	**6.74**
	2 - 10	1.14	6.30	2.14		10.05	2.51	5.89
		2.13	7.51	2.58		9.39	1.34	4.02
	Avg.	**1.64**	**6.91**	**2.36**	**0.00**	**9.72**	**1.93**	**4.96**
	10 - 20	2.91	7.89	2.73		8.00	0.92	5.78
		4.48	8.53	3.80		3.16	0.88	4.80
	Avg.	**3.69**	**8.21**	**3.27**	**0.00**	**5.58**	**0.90**	**5.29**
	20 - 30	3.36	9.34	2.19		3.65	0.89	5.19
		2.55	8.25	3.12		3.03	0.00	5.73
	Avg.	**2.96**	**8.80**	**2.66**	**0.00**	**3.34**	**0.44**	**5.46**

Table A3-5. Continued

Coordinates	Depth (cm)	TC	CTC	STZ	SMT (μg/kg)	ETM	TYL	MNS
$S_{0.5, 1.5}$	0 – 2	4.90	16.19	4.34		209.19	24.58	5.97
		5.01	14.41	3.46		222.62	24.72	6.40
	Avg.	**4.95**	**15.30**	**3.90**	**0.00**	**215.90**	**24.65**	**6.19**
	2 - 10	3.12	6.59	0.95		30.86	3.39	6.34
		2.94	7.38	1.82		26.30	1.20	5.41
	Avg.	**3.03**	**6.99**	**1.39**	**0.00**	**28.58**	**2.29**	**5.88**
	10 - 20	1.75	7.54	2.72		4.35	1.60	4.43
		3.38	7.56	3.68		4.86	0.86	6.57
	Avg.	**2.56**	**7.55**	**3.20**	**0.00**	**4.61**	**1.23**	**5.50**
	20 - 30	4.06	8.68	5.04		2.63	0.08	4.38
		4.32	8.12	1.90		2.02	0.00	4.59
	Avg.	**4.19**	**8.40**	**3.47**	**0.00**	**2.33**	**0.04**	**4.48**
$S_{1.5, 1.5}$	0 – 2	0.64	8.65	4.13		151.28	27.51	6.49
		3.17	7.57	3.89		162.92	26.15	5.07
	Avg.	**1.90**	**8.11**	**4.01**	**0.00**	**157.10**	**26.83**	**5.78**
	2 - 10	4.60	9.11			7.66	7.77	4.59
		3.84	10.12			8.27	5.52	3.64
	Avg.	**4.22**	**9.61**	**0.00**	**0.00**	**7.97**	**6.64**	**4.12**
	10 - 20	3.34	7.86			2.35	2.26	6.42
		2.01	8.11			3.04	2.35	6.16
	Avg.	**2.67**	**7.99**	**0.00**	**0.00**	**2.70**	**2.31**	**6.29**
	20 - 30	1.23	8.97	4.25		5.99	2.16	4.32
		2.99	8.23	6.11		5.25	0.88	3.69
	Avg.	**2.11**	**8.60**	**5.18**	**0.00**	**5.62**	**1.52**	**4.01**

Table A3-5. Continued

Coordinates	Depth (cm)	TC	CTC	STZ	SMT (μg/kg)	ETM	TYL	MNS
$S_{0.5, 0.5}$	0 – 2	4.96	16.10			88.82	4.61	4.96
		2.89	16.95			88.25	4.98	3.86
	Avg.	**3.92**	**16.53**	**0.00**	**0.00**	**88.54**	**4.80**	**4.41**
	2 - 10	3.90	6.98	2.26		3.74	5.36	5.91
		2.05	7.58	2.56		2.97	3.33	3.64
	Avg.	**2.97**	**7.28**	**2.41**	**0.00**	**3.35**	**4.34**	**4.78**
	10 - 20	4.05	7.62	3.91		10.57	-0.74	3.59
		1.99	7.44	3.65		8.62	3.63	3.48
	Avg.	**3.02**	**7.53**	**3.78**	**0.00**	**9.59**	**1.45**	**3.53**
	20 - 30	4.61	9.31	2.52		2.63	3.88	5.98
		3.94	7.73	3.57		3.99	4.73	6.04
	Avg.	**4.28**	**8.52**	**3.05**	**0.00**	**3.31**	**4.30**	**6.01**
$S_{1.5, 0.5}$	0 – 2	3.26	7.43	3.79		136.96	12.26	4.23
		2.54	8.34	2.39		119.55	11.39	6.30
	Avg.	**2.90**	**7.89**	**3.09**	**0.00**	**128.25**	**11.83**	**5.26**
	2 - 10	3.14	7.87			10.32	0.31	4.89
		2.73	8.34			16.35	1.32	4.74
	Avg.	**2.94**	**8.10**	**0.00**	**0.00**	**13.33**	**0.82**	**4.81**
	10 - 20	2.70	7.78			5.68	0.94	4.54
		4.87	8.12			5.01	2.75	4.06
	Avg.	**3.79**	**7.95**	**0.00**	**0.00**	**5.34**	**1.84**	**4.30**
	20 - 30	4.42	9.64	4.05		11.30	2.25	3.19
		4.70	7.19	2.81		11.06	1.49	5.11
	Avg.	**4.56**	**8.41**	**3.43**	**0.00**	**11.18**	**1.87**	**4.15**

Table A3-6. Measured concentration of depth soil samples at plot 2

Coordinates	Depth (cm)	TC	CTC	STZ	SMT (μg/kg)	ETM	TYL	MNS
$S_{0.5,2.5}$	0 – 2	2.90	7.68			271.34	20.68	8.09
		3.27	8.46			263.15	23.73	7.78
	Avg.	**3.09**	**8.07**	**0.00**	**0.00**	**267.24**	**22.21**	**7.94**
	2 - 10	2.59	7.97			27.39	1.52	6.40
		4.86	5.85			19.52	1.05	5.28
	Avg.	**3.72**	**6.91**	**0.00**	**0.00**	**23.45**	**1.28**	**5.84**
	10 - 20	3.22	8.66			16.73	0.49	3.37
		0.95	6.35			14.15	1.15	5.76
	Avg.	**2.09**	**7.51**	**0.00**	**0.00**	**15.44**	**0.82**	**4.57**
	20 - 30	4.64	7.87	2.44		2.38	1.72	5.33
		3.28	10.01	1.73		1.62	0.41	5.24
	Avg.	**3.96**	**8.94**	**2.08**	**0.00**	**2.00**	**1.06**	**5.28**
$S_{1.5,2.5}$	0 – 2	5.38	9.56	3.01		189.63	9.99	5.64
		4.52	7.80	2.17		190.91	13.49	6.08
	Avg.	**4.95**	**8.68**	**2.59**	**0.00**	**190.27**	**11.74**	**5.86**
	2 - 10	2.01	7.21	2.10		12.71	2.68	6.20
		0.94	7.36	3.24		8.94	2.08	5.38
	Avg.	**1.48**	**7.28**	**2.67**	**0.00**	**10.82**	**2.38**	**5.79**
	10 - 20	4.12	7.39	5.37		2.69	2.61	6.44
		1.76	7.21	2.39		6.23	2.04	4.44
	Avg.	**2.94**	**7.30**	**3.88**	**0.00**	**4.46**	**2.33**	**5.44**
	20 - 30	2.02	9.84	1.35		4.06	0.50	3.55
		4.27	6.24	4.53		1.94	0.00	4.76
	Avg.	**3.15**	**8.04**	**2.94**	**0.00**	**3.00**	**0.25**	**4.16**

Table A3-6. Continued

Coordinates	Depth (cm)	TC	CTC	STZ	SMT (μg/kg)	ETM	TYL	MNS
$S_{0.5, 1.5}$	0 – 2	4.87	14.79	5.18		215.68	23.72	7.32
		5.07	15.68	4.38		220.45	16.88	6.59
	Avg.	**4.97**	**15.24**	**4.78**	**0.00**	**218.07**	**20.30**	**6.95**
	2 - 10	2.52	6.42	3.68		31.85	2.10	5.66
		2.10	7.70	1.57		26.91	1.96	4.87
	Avg.	**2.31**	**7.06**	**2.62**	**0.00**	**29.38**	**2.03**	**5.26**
	10 - 20	4.94	8.84	4.04		5.96	0.00	5.65
		3.65	9.89	4.91		6.54	0.54	5.00
	Avg.	**4.30**	**9.37**	**4.47**	**0.00**	**6.25**	**0.27**	**5.33**
	20 - 30	3.43	7.47	3.60		2.64	-0.15	2.93
		5.35	6.47	0.22		3.13	1.98	5.82
	Avg.	**4.39**	**6.97**	**1.91**	**0.00**	**2.89**	**0.92**	**4.38**
$S_{1.5, 1.5}$	0 – 2	3.58	8.25	2.04		158.18	25.93	5.03
		2.34	9.26	4.09		160.22	21.59	4.71
	Avg.	**2.96**	**8.75**	**3.06**	**0.00**	**159.20**	**23.76**	**4.87**
	2 - 10	4.27	10.98			4.33	4.72	3.53
		4.48	10.20			8.10	6.09	6.01
	Avg.	**4.38**	**10.59**	**0.00**	**0.00**	**6.21**	**5.40**	**4.77**
	10 - 20	2.10	7.52			4.88	2.69	4.88
		3.04	9.86			0.99	1.90	6.67
	Avg.	**2.57**	**8.69**	**0.00**	**0.00**	**2.93**	**2.30**	**5.78**
	20 - 30	2.08	9.74	3.72		5.66	2.50	4.34
		1.71	9.27	5.81		6.70	2.76	7.30
	Avg.	**1.89**	**9.50**	**4.76**	**0.00**	**6.18**	**2.63**	**5.82**

Table A3-6. Continued

Coordinates	Depth (cm)	TC	CTC	STZ	SMT (μg/kg)	ETM	TYL	MNS
$S_{0.5, 0.5}$	0 – 2	2.92	18.87			90.45	3.96	3.49
		4.18	17.44			88.19	4.08	4.09
	Avg.	**3.55**	**18.15**	**0.00**	**0.00**	**89.32**	**4.02**	**3.79**
	2 - 10	4.30	7.18	3.86		4.75	6.40	5.33
		1.55	7.67	2.42		5.90	5.93	5.03
	Avg.	**2.93**	**7.42**	**3.14**	**0.00**	**5.32**	**6.17**	**5.18**
	10 - 20	2.65	6.33	4.53		9.13	2.73	4.44
		3.87	8.07	4.53		13.89	0.62	4.67
	Avg.	**3.26**	**7.20**	**4.53**	**0.00**	**11.51**	**1.67**	**4.55**
	20 - 30	3.58	8.91	1.88		2.66	4.17	5.26
		1.63	9.80	2.03		4.17	3.18	5.54
	Avg.	**2.61**	**9.35**	**1.95**	**0.00**	**3.42**	**3.68**	**5.40**
$S_{1.5, 0.5}$	0 – 2	2.73	7.13	4.37		127.35	11.26	6.47
		2.62	9.25	4.79		144.03	12.46	4.54
	Avg.	**2.68**	**8.19**	**4.58**	**0.00**	**135.69**	**11.86**	**5.51**
	2 - 10	2.96	8.50			12.27	0.61	5.96
		1.62	7.85			5.96	0.33	4.02
	Avg.	**2.29**	**8.18**	**0.00**	**0.00**	**9.12**	**0.47**	**4.99**
	10 - 20	4.10	8.19			5.03	1.97	6.73
		2.39	7.15			5.72	1.55	4.56
	Avg.	**3.24**	**7.67**	**0.00**	**0.00**	**5.38**	**1.76**	**5.64**
	20 - 30	2.18	7.70	1.91		8.80	2.23	4.45
		4.48	5.07	1.51		10.76	1.73	4.06
	Avg.	**3.33**	**6.39**	**1.71**	**0.00**	**9.78**	**1.98**	**4.25**

Table A3-7. Measured concentration of depth soil samples at plot 3

Coordinates	Depth (cm)	TC	CTC	STZ	SMT (μg/kg)	ETM	TYL	MNS
$S_{0.5,2.5}$	0 – 2	3.74	10.48			266.55	23.01	7.41
		3.13	9.89			257.11	22.30	8.23
	Avg.	**3.43**	**10.18**	**0.00**	**0.00**	**261.83**	**22.66**	**7.82**
	2 - 10	2.23	6.83			15.57	0.26	6.21
		4.48	8.54			26.03	3.05	6.04
	Avg.	**3.36**	**7.69**	**0.00**	**0.00**	**20.80**	**1.65**	**6.12**
	10 - 20	2.25	8.47			19.59	0.89	5.38
		3.55	6.68			10.88	2.74	5.10
	Avg.	**2.90**	**7.58**	**0.00**	**0.00**	**15.24**	**1.82**	**5.24**
	20 - 30	3.65	7.26	3.69		1.52	0.88	4.79
		2.21	8.07	4.14		1.06	0.57	6.44
	Avg.	**2.93**	**7.67**	**3.92**	**0.00**	**1.29**	**0.72**	**5.61**
$S_{1.5,2.5}$	0 – 2	2.94	7.36	3.22		184.36	12.46	6.37
		3.80	7.77	3.46		182.33	12.86	5.18
	Avg.	**3.37**	**7.56**	**3.34**	**0.00**	**183.35**	**12.66**	**5.77**
	2 - 10	0.74	7.21	1.42		11.92	2.98	5.35
		2.13	8.40	3.27		12.55	1.26	5.61
	Avg.	**1.43**	**7.81**	**2.35**	**0.00**	**12.24**	**2.12**	**5.48**
	10 - 20	2.80	7.19	2.92		5.22	2.26	6.17
		3.26	8.44	3.53		4.90	2.20	4.31
	Avg.	**3.03**	**7.81**	**3.23**	**0.00**	**5.06**	**2.23**	**5.24**
	20 - 30	2.70	8.12	4.21		4.10	1.65	4.99
		3.05	10.21	0.34		3.56	1.99	3.83
	Avg.	**2.88**	**9.17**	**2.27**	**0.00**	**3.83**	**1.82**	**4.41**

Table A3-7. Continued

Coordinates	Depth (cm)	TC	CTC	STZ	SMT (µg/kg)	ETM	TYL	MNS
$S_{0.5, 1.5}$	0 – 2	4.82	16.80	3.19		218.40	19.53	6.29
		6.43	16.48	3.81		223.98	21.80	7.31
	Avg.	**5.63**	**16.64**	**3.50**	**0.00**	**221.19**	**20.66**	**6.80**
	2 - 10	2.00	6.95	2.62		31.50	2.31	5.55
		0.50	6.57	3.13		34.16	2.17	7.83
	Avg.	**1.25**	**6.76**	**2.88**	**0.00**	**32.83**	**2.24**	**6.69**
	10 - 20	3.39	8.89	3.01		4.78	2.22	6.37
		3.35	8.19	2.14		6.06	1.11	4.61
	Avg.	**3.37**	**8.54**	**2.57**	**0.00**	**5.42**	**1.67**	**5.49**
	20 - 30	3.70	7.78	2.30		3.96	1.25	2.76
		5.20	8.62	1.67		3.28	0.00	6.23
	Avg.	**4.45**	**8.20**	**1.98**	**0.00**	**3.62**	**0.63**	**4.49**
$S_{1.5, 1.5}$	0 – 2	3.03	9.56	3.37		156.07	24.00	5.37
		2.60	7.49	1.65		159.56	26.87	4.16
	Avg.	**2.82**	**8.52**	**2.51**	**0.00**	**157.82**	**25.44**	**4.77**
	2 - 10	0.97	9.11			8.60	5.15	3.69
		6.38	8.58			6.34	5.53	5.20
	Avg.	**3.67**	**8.84**	**0.00**	**0.00**	**7.47**	**5.34**	**4.44**
	10 - 20	2.28	8.76			3.60	1.59	3.84
		4.20	7.62			3.79	1.72	5.36
	Avg.	**3.24**	**8.19**	**0.00**	**0.00**	**3.70**	**1.66**	**4.60**
	20 - 30	1.21	10.06	3.60		5.66	0.97	3.45
		2.68	7.93	4.63		5.82	4.62	3.76
	Avg.	**1.94**	**8.99**	**4.11**	**0.00**	**5.74**	**2.79**	**3.60**

Table A3-7. Continued

Coordinates	Depth (cm)	TC	CTC	STZ	SMT (μg/kg)	ETM	TYL	MNS
$S_{0.5,0.5}$	0 – 2	3.81	15.34			84.68	4.50	6.90
		4.76	16.40			80.75	4.25	4.81
	Avg.	**4.28**	**15.87**	**0.00**	**0.00**	**82.71**	**4.37**	**5.86**
	2 - 10	3.42	8.06	2.52		4.74	6.55	2.50
		2.40	5.34	3.29		4.65	5.27	3.28
	Avg.	**2.91**	**6.70**	**2.90**	**0.00**	**4.70**	**5.91**	**2.89**
	10 - 20	2.70	9.53	3.18		13.46	2.55	6.57
		2.15	7.33	4.00		8.36	2.77	4.47
	Avg.	**2.43**	**8.43**	**3.59**	**0.00**	**10.91**	**2.66**	**5.52**
	20 - 30	3.56	8.05	3.80		3.67	2.46	4.66
		2.08	11.31	1.91		2.78	1.23	7.03
	Avg.	**2.82**	**9.68**	**2.85**	**0.00**	**3.23**	**1.85**	**5.84**
$S_{1.5,0.5}$	0 – 2	3.56	8.82	4.52		140.12	14.69	3.40
		1.89	10.05	2.66		129.23	10.96	5.01
	Avg.	**2.72**	**9.43**	**3.59**	**0.00**	**134.68**	**12.83**	**4.21**
	2 - 10	1.85	7.75			10.50	0.40	4.04
		2.80	8.07			4.71	0.19	3.49
	Avg.	**2.32**	**7.91**	**0.00**	**0.00**	**7.61**	**0.29**	**3.76**
	10 - 20	2.51	7.99			4.99	4.55	3.82
		3.64	9.87			4.56	0.74	7.04
	Avg.	**3.08**	**8.93**	**0.00**	**0.00**	**4.78**	**2.65**	**5.43**
	20 - 30	2.84	8.12	1.92		11.33	0.55	4.42
		3.55	6.27	2.57		12.03	0.16	4.58
	Avg.	**3.19**	**7.20**	**2.25**	**0.00**	**11.68**	**0.36**	**4.50**

Table A4-1. Measured conductivity of established 10 columns to calculate retention time

Time (hr)	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10
	Conductivity (μS/cm)									
0	0	0	0	0	0	0	0	0	0	0
6	12	7	10	6	10	7	5	9	11	5
12	41	26	23	24	37	26	24	36	50	33
18	42	34	35	33	47	36	31	39	51	35
24	56	35	42	37	48	48	32	42	46	40
30	63	82	52	57	62	49	62	64	82	115
36	85	131	88	88	90	72	98	93	116	194
42	379	419	449	401	370	330	474	443	435	570
47	661	627	708	665	635	603	743	700	681	727
57	865	861	885	862	820	784	908	898	911	890
66	1002	1017	1028	990	1000	978	1053	1035	1036	1011
87	1047	1076	1079	1076	1112	1118	1095	1133	1134	1114
107	1058	1114	1089	1093	1131	1118	1126	1149	1166	1129
115	1130	1129	1095	1128	1141	1158	1150	1178	1170	1135

Table A4-2. Measured concentration of STZ and SMT in column leached effluent

	STZ (μg/L)														
Dates	**0**	**3**	**6**	**10**	**13**	**16**	**19**	**22**	**25**	**28**	**31**	**34**	**37**	**40**	**43**
Column 1	0.00	0.50	14.05	1.48	0.40	0.31	0.26	0.22	0.18	0.19	0.14	0.15	0.16	0.17	0.14
Column 2	0.00	2.59	4.11	0.93	0.24	0.24	0.23	0.18	0.17	0.17	0.14	0.14	0.15	0.14	0.13
Column 5	0.00	2.88	32.26	1.15	1.35	0.62	0.45	0.35	0.35	0.32	0.18	0.18	0.17	0.18	0.16
Column 6	0.00	0.48	20.25	4.85	1.03	0.35	0.47	0.35	0.25	0.24	0.17	0.16	0.15	0.16	0.15
Column 9	0.00	0.42	26.66	2.91	0.80	0.45	0.18	0.23	0.23	0.19	0.14	0.13	0.14	0.14	0.14
Column 10	0.00	10.86	25.81	2.54	0.79	0.40	0.36	0.19	0.23	0.21	0.13	0.17	0.15	0.14	0.14
	SMT (μg/L)														
Column 1	0.00	6.96	74.69	13.53	2.34	1.57	1.31	1.04	0.41	0.63	0.14	0.77	0.68	0.62	0.52
Column 2	0.00	36.09	49.03	11.56	1.41	1.48	1.06	0.58	0.63	0.31	0.14	0.35	0.44	0.60	0.43
Column 5	0.00	27.57	109.79	11.74	5.84	2.63	1.63	0.87	0.83	0.89	0.65	0.64	0.65	0.75	0.41
Column 6	0.00	7.31	66.88	15.15	4.14	1.28	1.41	0.87	0.60	0.53	0.58	0.48	0.37	0.49	0.45
Column 9	0.00	9.36	107.27	9.10	2.73	1.58	0.31	0.60	0.55	0.28	0.22	0.20	0.20	0.30	0.26
Column 10	0.00	59.92	72.33	7.28	3.01	1.32	1.00	0.30	0.59	0.37	0.13	0.24	0.29	0.33	0.21

Table A4-3. Measured concentration of ETM and MNS in column leached effluent

	ETM (µg/L)														
Dates	**0**	**3**	**6**	**10**	**13**	**16**	**19**	**22**	**25**	**28**	**31**	**34**	**37**	**40**	**43**
Column 3	0.00	0.01	0.05	0.01	0.00	0.01	0.01	0.06	0.03	0.01	0.02	0.01	0.01	0.01	0.01
Column 4	0.00	0.02	0.02	0.01	0.02	0.04	0.02	0.04	0.02	0.01	0.01	0.01	0.01	0.01	0.01
Column 7	0.00	0.01	0.01	0.01	0.01	0.02	0.06	0.18	0.04	0.01	0.01	0.01	0.01	0.01	0.01
Column 8	0.00	0.05	0.01	0.01	0.01	0.01	0.02	0.05	0.04	0.01	0.01	0.01	0.01	0.01	0.01
Column 9	0.00	0.10	0.01	0.01	0.02	0.06	0.01	0.02	0.02	0.02	0.01	0.02	0.01	0.01	0.01
Column 10	0.00	0.71	0.01	0.01	0.01	0.05	0.01	0.05	0.02	0.02	0.01	0.05	0.01	0.01	0.03
	MNS (µg/L)														
Column 3	0.00	0.18	0.18	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Column 4	0.00	0.00	0.06	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Column 7	0.00	0.14	0.19	1.19	0.22	0.04	0.02	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Column 8	0.00	0.17	0.23	0.75	0.27	0.05	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Column 9	0.00	0.04	0.06	0.70	0.28	0.04	0.03	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Column 10	0.00	0.47	0.75	0.97	0.21	0.05	0.04	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table A4-4. Measured concentration of SLM and NRS in column leached effluent

	SLM (µg/L)														
Dates	**0**	**3**	**6**	**10**	**13**	**16**	**19**	**22**	**25**	**28**	**31**	**34**	**37**	**40**	**43**
Column 3	0.00	3.90	1.49	0.59	0.03	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Column 4	0.00	1.68	0.82	0.37	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Column 7	0.00	2.55	1.61	3.95	0.52	0.38	0.07	0.17	0.11	0.13	0.00	0.01	0.00	0.00	0.00
Column 8	0.00	3.84	1.85	1.77	0.28	0.28	0.25	0.15	0.16	0.04	0.08	0.00	0.00	0.00	0.00
Column 9	0.00	2.40	1.27	1.83	0.43	0.18	0.45	0.19	0.10	0.12	0.04	0.04	0.00	0.00	0.00
Column 10	0.00	1.58	0.89	1.35	0.36	0.13	0.29	0.07	0.06	0.04	0.00	0.06	0.00	0.00	0.00
	NRS (µg/L)														
Column 3	0.00	3.47	1.16	0.88	0.07	0.00	0.06	0.02	0.02	0.00	0.01	0.00	0.00	0.00	0.00
Column 4	0.00	0.49	0.16	0.32	0.15	0.00	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Column 7	0.00	1.19	0.45	3.60	1.29	0.60	0.49	0.74	0.34	0.42	0.03	0.06	0.00	0.00	0.00
Column 8	0.00	2.50	0.76	2.08	0.92	0.58	1.18	0.54	0.39	0.08	0.40	0.05	0.00	0.00	0.00
Column 9	0.00	0.62	0.25	3.23	1.44	0.43	2.08	1.15	0.20	0.31	0.21	0.18	0.03	0.01	0.01
Column 10	0.00	0.69	0.34	2.26	0.97	0.25	1.12	0.22	0.39	0.09	0.16	0.39	0.01	0.00	0.00

Table A4-5. Measured concentration of TC, OTC, and CTC in depth soil samples of each column

				TC (μg/kg)				
Depth (cm)	**0 - 3**	**3 - 6**	**6 - 9**	**9 - 12**	**12 - 15**	**15 - 18**	**18 - 21**	**21 - 24**
Column 1	25.7	22.1	31.4	20.4	20.5	11.6	23.0	19.6
Column 2	40.0	25.9	25.6	56.6	49.5	24.1	32.7	45.3
Column 5	39.1	22.1	11.6	18.9	20.2	16.3	11.6	11.6
Column 6	27.4	18.1	18.5	30.1	20.5	21.9	22.5	15.6
Column 9	11.6	18.1	22.1	99.1	93.0	61.9	42.0	52.5
Column 10	121.7	35.6	30.1	40.1	44.2	36.6	27.2	38.2
				OTC (μg/kg)				
Column 1	19.5	18.7	18.0	18.2	17.1	32.7	21.5	15.1
Column 2	12.0	9.7	13.8	29.3	28.0	18.1	17.3	58.4
Column 5	12.5	13.9	15.4	13.4	11.5	13.0	13.1	17.4
Column 6	7.5	11.5	12.2	17.3	12.3	16.9	12.9	17.2
Column 9	34.9	10.7	13.3	73.1	39.0	28.6	20.0	48.2
Column 10	29.8	21.7	16.6	26.7	27.6	25.9	18.6	29.1
				CTC (μg/kg)				
Column1	31.1	37.3	33.9	35.5	37.9	77.5	40.7	29.1
Column2	40.9	32.2	31.6	49.9	51.1	34.0	41.2	75.7
Column5	32.2	30.0	30.3	31.3	37.8	35.6	30.9	31.5
Column6	33.0	30.6	31.6	39.5	30.9	32.3	32.0	35.4
Column9	32.6	33.7	35.2	95.5	79.6	58.1	38.8	50.4
Column10	59.9	35.7	34.4	49.1	47.6	41.3	46.6	44.7

Table A4-6. Measured concentration of STZ and SMT in depth soil samples of each column

				STZ (μg/kg)				
Depth (cm)	**0 - 3**	**3 - 6**	**6 - 9**	**9 - 12**	**12 - 15**	**15 - 18**	**18 - 21**	**21 - 24**
Column 1	2.9	3.1	3.0	2.7	2.7	8.6	3.0	3.0
Column 2	2.7	3.8	3.1	22.6	3.5	3.3	4.2	6.7
Column 5	3.2	4.6	2.7	2.7	3.7	3.4	6.6	4.6
Column 6	3.2	2.7	2.7	3.5	3.4	2.8	3.4	3.6
Column 9	3.7	3.5	2.7	4.4	4.1	2.7	2.7	3.5
Column 10	3.0	3.1	2.7	3.3	2.7	3.0	3.4	3.8
				SMT (μg/kg)				
Column 1	0.0	0.0	1.6	0.0	0.0	5.6	0.0	0.7
Column 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.7
Column 5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.7
Column 6	0.0	0.0	1.0	0.0	1.4	0.0	0.0	1.4
Column 9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Column 10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table A4-7. Measured concentration of ETM and TYL in depth soil samples of each column

				ETM (µg/kg)				
Depth (cm)	**0 - 3**	**3 - 6**	**6 - 9**	**9 - 12**	**12 - 15**	**15 - 18**	**18 - 21**	**21 - 24**
Column 3	81.5	21.1	15.8	23.1	8.1	12.6	5.2	9.9
Column 4	31.8	10.2	7.1	57.2	17.1	10.8	8.6	5.2
Column 7	43.5	13.2	6.7	9.3	12.2	11.0	3.8	3.1
Column 8	32.4	49.7	5.1	9.5	13.8	11.5	6.1	2.7
Column 9	157.2	7.4	6.0	5.1	25.2	4.6	6.1	6.2
Column 10	240.4	8.4	50.3	52.2	82.4	133.1	116.9	32.3
				TYL (µg/kg)				
Column 3	42.7	10.0	6.7	10.1	4.9	3.2	3.4	4.0
Column 4	10.2	4.1	2.6	6.2	10.3	5.3	6.5	2.9
Column 7	53.8	3.7	4.0	15.6	5.5	3.2	4.3	8.6
Column 8	38.9	6.8	3.8	3.3	11.7	3.9	4.1	3.9
Column 9	116.3	5.0	33.2	4.3	3.8	3.1	4.4	2.8
Column 10	213.5	13.3	7.3	6.8	27.0	18.4	18.4	7.8

Table A4-8. Measured concentration of MNS, SLM and NRS in depth soil samples of each column

				MNS (µg/kg)				
Depth (cm)	**0 - 3**	**3 - 6**	**6 - 9**	**9 - 12**	**12 - 15**	**15 - 18**	**18 - 21**	**21 - 24**
Column 3	33.1	19.5	7.3	10.5	17.7	25.4	10.5	6.2
Column 4	6.3	7.1	4.3	5.3	11.2	16.8	13.3	14.3
Column 7	14.1	16.6	15.2	14.5	16.2	18.8	21.1	19.9
Column 8	9.6	11.8	14.9	16.0	22.1	21.8	19.7	24.4
Column 9	27.8	17.9	5.5	14.5	12.4	20.8	23.2	34.2
Column 10	18.8	7.6	6.6	8.3	7.8	15.5	7.9	12.6
				SLM (µg/kg)				
Column 3	33.5	22.1	11.9	36.4	59.1	26.2	34.5	33.1
Column 4	39.2	23.9	30.1	39.8	56.5	79.0	46.7	59.6
Column 7	26.7	38.6	49.1	17.2	22.4	43.1	44.2	36.1
Column 8	25.4	22.5	36.9	22.7	26.5	56.2	31.1	65.0
Column 9	25.2	28.7	5.57	16.3	21.8	25.5	43.6	55.0
Column 10	11.2	16.1	10.5	10.0	30.3	24.2	18.4	11.2
				NRS (µg/kg)				
Column 3	24.7	34.1	5.3	4.5	6.2	2.9	2.7	24.8
Column 4	12.5	10.2	7.1	6.9	6.4	2.9	3.2	4.1
Column 7	31.0	17.3	12.8	6.8	9.0	4.5	4.4	5.6
Column 8	22.3	13.0	18.5	10.6	12.6	9.4	8.1	7.2
Column 9	44.2	12.2	15.0	9.7	7.4	9.7	6.1	7.5
Column 10	16.1	7.9	4.7	1.7	10.1	3.2	3.7	14.7

Table A4-9. Measured concentration of TC, OTC and CTC in colloid phase of column leached effluent

Particle Size								
		TC (μg/L)						
	Dates	**0**	**6**	**12**	**18**	**24**	**30**	**36**
0.2 μm – 1.2 μm	Column 1	0.00	0.07	0.42	0.19	0.00	0.10	0.07
	Column 2	0.00	0.00	0.42	0.19	0.03	0.02	0.01
	Column 3	0.00	0.07	0.37	0.19	0.07	0.00	0.03
	Column 4	0.00	0.04	0.19	0.07	0.00	0.00	0.00
1.2 μm – 2.7 μm	Column 1	0.00	0.04	0.07	0.39	0.07	0.00	0.01
	Column 2	0.00	0.03	0.46	0.17	0.17	0.00	0.00
	Column 3	0.00	0.10	0.52	0.08	0.10	0.00	0.00
	Column 4	0.00	0.01	0.28	0.09	0.08	0.00	0.00
		OTC (μg/L)						
0.2 μm – 1.2 μm	Column 1	0.00	0.08	0.14	0.09	0.00	0.00	0.03
	Column 2	0.00	0.07	0.07	0.18	0.07	0.00	0.00
	Column 3	0.00	0.08	0.25	0.06	0.00	0.01	0.02
	Column 4	0.00	0.07	0.05	0.01	0.03	0.00	0.01
1.2 μm – 2.7 μm	Column 1	0.00	0.05	0.03	0.02	0.00	0.00	0.00
	Column 2	0.00	0.07	0.07	0.09	0.05	0.00	0.00
	Column 3	0.00	0.07	0.16	0.04	0.00	0.06	0.00
	Column 4	0.00	0.04	0.07	0.00	0.03	0.07	0.01
		CTC (μg/L)						
0.2 μm – 1.2 μm	Column 1	0.00	0.06	0.72	0.09	0.25	0.18	0.09
	Column 2	0.00	0.00	0.23	0.00	0.20	0.04	0.00
	Column 3	0.00	0.00	0.04	0.00	0.06	0.02	0.00
	Column 4	0.00	0.00	0.04	0.02	0.00	0.00	0.02
1.2 μm – 2.7 μm	Column 1	0.00	0.01	0.07	0.02	0.03	0.00	0.00
	Column 2	0.00	0.00	0.01	0.00	0.00	0.00	0.00
	Column 3	0.00	0.00	0.02	0.00	0.00	0.00	0.00
	Column 4	0.00	0.02	0.00	0.00	0.00	0.00	0.02

Table A4-10. Measured concentration of STZ and SMT in colloid phase of column leached effluent

Particle Size		STZ (μg/L)						
	Dates	**0**	**6**	**12**	**18**	**24**	**30**	**36**
0.2 μm – 1.2 μm	Column1	0.00	0.25	33.33	5.57	3.33	1.12	0.69
	Column2	0.00	0.18	11.64	3.59	3.17	0.36	0.24
	Column3	0.00	12.44	28.80	4.64	3.58	1.10	0.42
	Column4	0.00	10.80	20.28	3.57	1.05	0.49	0.47
1.2 μm – 2.7 μm	Column1	0.00	0.24	24.24	4.31	1.93	0.82	0.46
	Column2	0.00	0.18	16.66	3.21	3.08	0.33	0.31
	Column3	0.00	14.78	49.87	7.85	5.61	3.39	0.78
	Column4	0.00	5.87	19.65	4.72	2.44	1.40	0.31
		SMT (μg/L)						
0.2 μm – 1.2 μm	Column1	0.00	0.13	35.37	5.84	3.32	1.59	0.58
	Column2	0.00	0.13	24.53	8.88	5.06	0.82	0.34
	Column3	0.00	25.54	44.72	6.08	3.19	0.98	0.38
	Column4	0.00	29.00	25.80	4.15	0.82	0.55	0.51
1.2 μm – 2.7 μm	Column1	0.00	0.13	37.10	5.17	2.87	0.73	0.46
	Column2	0.00	0.13	35.36	8.67	8.67	0.75	0.48
	Column3	0.00	26.17	73.39	11.87	5.38	3.07	0.64
	Column4	0.00	19.41	25.02	7.64	2.56	1.93	0.43

Table A4-11. Measured concentration of ETM and TYL in colloid phase of column leached effluent

Particle Size		ETM (μg/L)						
	Dates	**0**	**6**	**12**	**18**	**24**	**30**	**36**
0.2 μm – 1.2 μm	Column1	0.00	2.57	7.35	1.46	0.37	0.21	0.11
	Column2	0.00	3.33	8.65	1.92	0.39	0.18	0.29
	Column3	0.00	2.07	2.86	1.04	0.58	0.47	0.30
	Column4	0.00	1.48	2.56	0.68	0.35	0.34	0.22
1.2 μm – 2.7 μm	Column1	0.00	2.65	6.93	1.48	0.53	0.19	0.12
	Column2	0.00	2.98	10.36	1.94	0.35	0.16	0.13
	Column3	0.00	1.28	3.14	1.11	0.49	0.38	0.33
	Column4	0.00	1.30	1.77	0.77	0.59	0.35	0.37
		TYL (μg/L)						
0.2 μm – 1.2 μm	Column1	0.00	0.98	4.40	1.06	0.35	0.15	0.08
	Column2	0.00	1.24	7.55	1.53	0.29	0.15	0.06
	Column3	0.00	0.74	1.21	0.63	0.68	0.45	0.14
	Column4	0.00	0.20	1.09	0.39	0.25	0.15	0.08
1.2 μm – 2.7 μm	Column1	0.00	0.74	4.39	1.04	0.27	0.13	0.07
	Column2	0.00	1.56	8.91	1.33	0.25	0.14	0.08
	Column3	0.00	0.01	1.70	0.88	0.50	0.35	0.20
	Column4	0.00	0.11	0.62	0.42	0.37	0.17	0.09

Table A4-12. Measured concentration of MNS, SLM, and NRS in colloid phase of column leached effluent

Particle Size		MNS (μg/L)						
	Dates	**0**	**6**	**12**	**18**	**24**	**30**	**36**
0.2 μm – 1.2 μm	Column1	0.00	0.13	0.55	0.18	0.04	0.02	0.01
	Column2	0.00	0.12	0.74	0.24	0.03	0.02	0.01
	Column3	0.00	0.19	0.73	0.47	0.24	0.14	0.07
	Column4	0.00	0.32	1.11	0.66	0.23	0.19	0.05
1.2 μm – 2.7 μm	Column1	0.00	0.16	0.54	0.19	0.03	0.02	0.01
	Column2	0.00	0.12	0.63	0.18	0.04	0.02	0.01
	Column3	0.00	0.22	0.86	0.59	0.21	0.13	0.07
	Column4	0.00	0.36	0.86	0.69	0.31	0.18	0.09
		SLM (μg/L)						
0.2 μm – 1.2 μm	Column1	0.00	0.61	0.53	0.05	0.01	0.01	0.01
	Column2	0.00	0.92	0.63	0.05	0.01	0.01	0.01
	Column3	0.00	0.72	0.76	0.49	0.30	0.22	0.14
	Column4	0.00	0.92	0.96	0.53	0.20	0.25	0.04
1.2 μm – 2.7 μm	Column1	0.00	0.70	0.48	0.06	0.01	0.01	0.01
	Column2	0.00	0.90	0.51	0.04	0.01	0.01	0.01
	Column3	0.00	0.87	1.03	0.56	0.27	0.21	0.13
	Column4	0.00	1.16	0.74	0.39	0.37	0.23	0.09
		NRS (μg/L)						
0.2 μm – 1.2 μm	Column1	0.00	0.37	0.90	0.19	0.03	0.02	0.02
	Column2	0.00	0.39	0.99	0.21	0.01	0.02	0.01
	Column3	0.00	0.32	0.97	1.02	0.74	0.56	0.36
	Column4	0.00	0.50	1.49	1.31	0.60	0.74	0.15
1.2 μm – 2.7 μm	Column1	0.00	0.35	0.90	0.22	0.01	0.02	0.02
	Column2	0.00	0.34	0.99	0.20	0.02	0.02	0.01
	Column3	0.00	0.28	1.32	1.15	0.67	0.56	0.33
	Column4	0.00	0.53	1.31	1.05	0.94	0.81	0.31

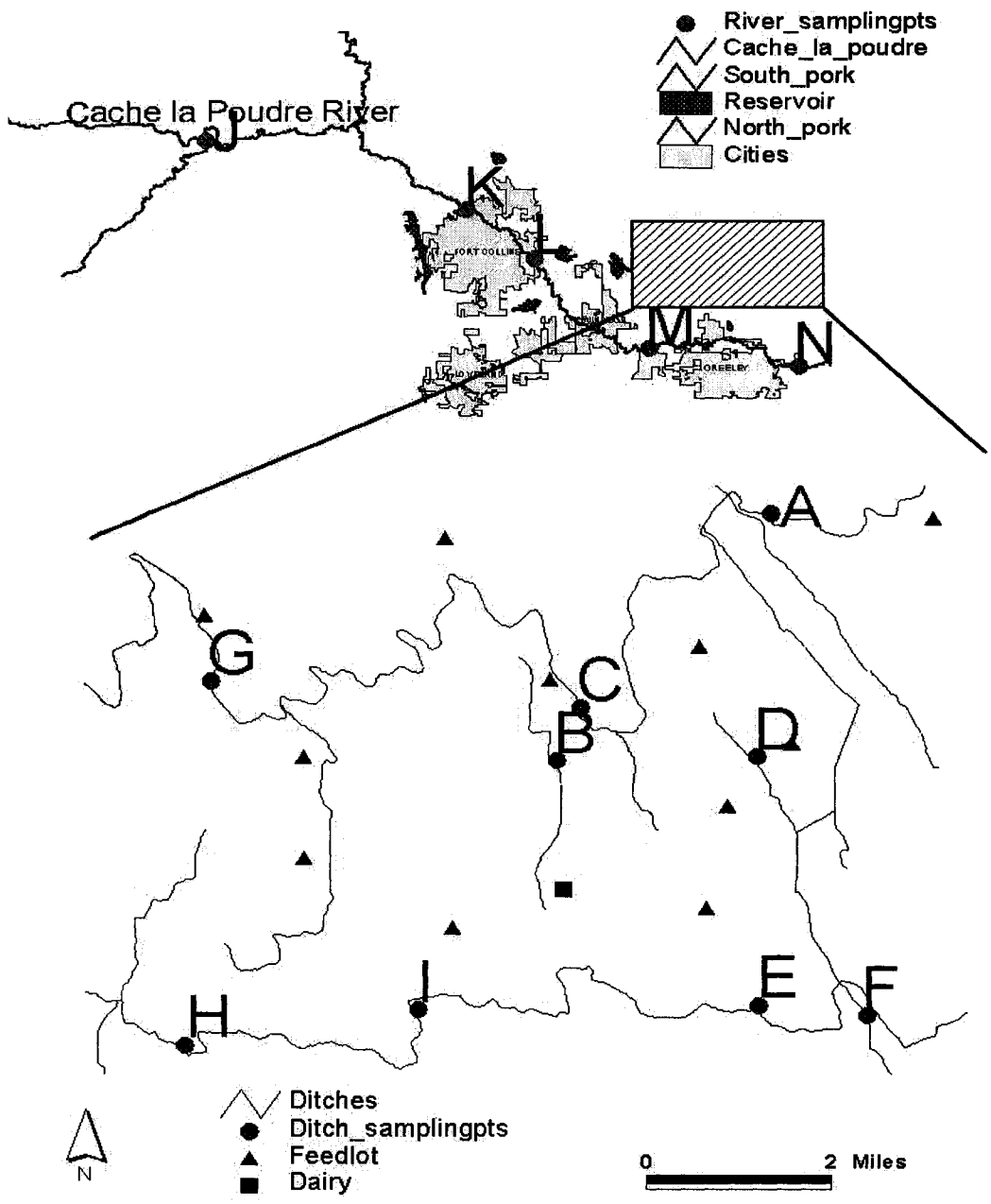

Figure A5-1. Map of studied irrigation ditches and watershed

Table A5-1. Measured concentration of TC, CTC, and OTC in aqueous at irrigation ditches and watershed

			TC (μg/L)			
Sampling locations	August 2004		July 2005		August 2005	
A	0.61	0.02	0.00	0.00	0.11	0.00
B	0.22	0.02	0.00	0.01	0.00	0.03
C	0.05	0.02	0.00	0.00	0.00	0.01
D	0.04	0.01	0.00	0.00	0.00	0.00
E	0.14	0.02	0.00	0.00	0.00	0.00
F	0.20	0.01	0.00	0.01	0.00	0.00
G	0.19	0.02	0.00	0.01	0.00	0.00
H	0.10	0.02	0.00	0.01	0.00	0.00
I	0.07	0.00	0.00	0.00	0.00	0.00
J					0.00	0.00
K	0.03	0.02			0.01	0.00
L	0.06	0.01			0.00	0.00
M	0.03	0.02	0.00	0.00	0.00	0.00
N	0.03	0.01	0.00	0.00	0.01	0.01
			CTC (μg/L)			
Sampling locations	August 2004		July 2005		August 2005	
A	0.89	0.07	0.03	0.13	0.22	0.00
B	0.44	0.04	0.02	0.10	0.06	0.04
C	0.07	0.01	0.06	0.01	0.05	0.00
D	0.07	0.01	0.01	0.01	0.01	0.00
E	0.20	0.13	0.02	0.02	0.02	0.00
F	0.30	0.03	0.02	0.01	0.02	0.00
G	0.37	0.05	0.01	0.02	0.00	0.00
H	0.24	0.02	0.01	0.02	0.02	0.00
I	0.10	0.01	0.02	0.00	0.00	0.00
J					0.00	0.00
K	0.03	0.02			0.00	0.00
L	0.07	0.03			0.04	0.02
M	0.05	0.00	0.01	0.00	0.02	0.05
N	0.04	0.00	0.01	0.00	0.00	0.02
			OTC (μg/L)			
Sampling locations	August 2004		July 2005		August 2005	
A			0.14	0.24	0.01	0.03
B			0.06	0.10	0.07	0.20
C			0.05	0.07	0.00	0.04
D			0.12	0.11	0.06	0.60
E			0.05	0.04	0.04	0.07
F			0.04	0.08	0.01	0.11
G			0.01	0.06	0.00	0.05
H			0.06	0.03	0.00	0.03
I					0.05	0.10
J					0.01	0.01
K			0.06	0.03	0.01	0.04
L			0.08	0.04	0.00	0.01
M			0.06	0.06	0.32	0.18
N			0.07	0.09	0.04	0.05

Table A5-2. Measured concentration of STZ and SMT in aqueous at irrigation ditches and watershed

	STZ (μg/L)					
Sampling locations	August 2004		July 2005		August 2005	
A	0.02	0.00	0.01	0.02	0.00	0.00
B	0.00	0.00	0.17	0.14	0.00	0.00
C	0.00	0.00	0.00	0.01	0.00	0.04
D	0.00	0.00	0.01	0.01	0.00	0.01
E	0.01	0.00	0.00	0.01	0.00	0.02
F	0.01	0.00	0.00	0.00	0.00	0.05
G	0.02	0.00	0.00	0.00	0.00	0.00
H	0.00	0.00	0.00	0.00	0.00	0.02
I	0.00	0.00	0.00	0.01	0.00	0.00
J					0.00	0.00
K	0.00	0.00			0.00	0.00
L	0.01	0.01			0.00	0.09
M	0.00	0.00	0.00	0.00	0.00	0.01
N	0.00	0.00	0.00	0.00	0.00	0.00

	SMT (μg/L)					
Sampling locations	August 2004		July 2005		August 2005	
A	0.04	0.44	0.00	0.00	0.00	0.00
B	0.00	0.31	0.00	0.00	0.00	0.00
C	0.05	0.25	0.00	0.57	0.00	0.00
D	0.01	0.11	0.00	0.00	0.00	0.00
E	0.01	0.08	0.00	0.00	0.00	0.00
F	0.01	0.12	0.00	0.00	0.14	0.14
G	0.01	0.00	0.00	0.00	0.18	0.26
H	0.01	0.12	0.00	0.00	0.26	0.31
I	0.01	0.15	0.01	0.25	0.09	0.07
J					0.00	0.00
K	0.00	0.01			0.00	0.00
L	0.02	0.05			0.00	0.00
M	0.01	0.10	0.00	0.00	0.00	0.00
N	0.01	0.04	0.00	0.00	0.00	0.00

Table A5-3. Measured concentration of ETM and TYL in aqueous at irrigation ditches and watershed

	ETM (μg/L)					
Sampling locations	August 2004		July 2005		August 2005	
A	0.87	0.13	0.01	0.02	0.01	2.14
B	0.02	0.02	0.01	0.02	0.02	0.43
C	0.02	0.03	0.01	0.01	0.10	0.01
D	0.02	0.02	0.01	0.01	0.04	0.01
E	0.02	0.02	0.01	0.02	0.01	0.01
F	0.02	0.02	0.01	0.17	0.04	0.07
G	0.03	0.04	0.01	0.01	0.01	0.01
H	0.02	0.02	0.01	0.02	0.02	0.02
I	0.03	0.02	0.02	0.02	0.02	0.26
J					0.07	0.07
K	0.02	0.02			0.42	0.02
L	0.20	0.23			0.09	0.07
M	0.02	0.04	0.02	0.03	0.05	0.02
N	0.02	0.02	0.01	0.04	0.11	0.29

	TYL (μg/L)					
Sampling locations	August 2004		July 2005		August 2005	
A	0.12	0.17	0.00	0.02	0.00	0.06
B	0.02	0.39	0.00	0.01	0.00	0.03
C	0.03	0.08	0.00	0.00	0.03	0.01
D	0.02	0.06	0.00	0.00	0.00	0.07
E	0.03	0.06	0.00	0.00	0.00	0.01
F	0.02	0.20	0.00	0.00	0.00	0.01
G	0.00	0.08	0.00	0.00	0.00	0.00
H	0.01	0.03	0.00	0.00	0.00	0.00
I	0.01	0.01	0.00	0.00	0.00	0.02
J					0.00	0.00
K	0.01	0.09			0.00	0.08
L	0.02	0.05			0.00	0.12
M	0.02	0.10	0.00	0.00	0.00	0.13
N	0.02	0.06	0.00	0.00	0.00	0.12

Table A5-4. Measured concentration of MNS, SLM, and NRS in aqueous at irrigation ditches and watershed

			MNS (μg/L)			
Sampling locations	August 2004		July 2005		August 2005	
A	0.012	0.011	0.001	0.008	0.002	0.004
B	0.010	0.008	0.001	0.004	0.001	0.005
C	0.006	0.005	0.000	0.002	0.001	0.002
D	0.009	0.007	0.001	0.002	0.003	0.004
E	0.006	0.004	0.001	0.012	0.001	0.003
F	0.016	0.014	0.000	0.001	0.003	0.004
G	0.006	0.004	0.000	0.000	0.000	0.000
H	0.007	0.005	0.002	0.003	0.000	0.002
I	0.010	0.008	0.001	0.003	0.001	
J					0.000	
K	0.000	0.000			0.000	
L	0.000	0.000			0.001	
M	0.004	0.002	0.001	0.003	0.002	
N	0.004	0.002	0.001	0.005	0.003	
			SLM (μg/L)			
Sampling locations	August 2004		July 2005		August 2005	
A	0.008	0.003	0.004	0.011	0.002	0.002
B	0.006	0.002	0.003	0.004	0.000	0.002
C	0.007	0.002	0.002	0.001	0.001	0.001
D	0.006	0.001	0.004	0.004	0.001	0.001
E	0.006	0.001	0.002	0.006	0.001	0.000
F	0.006	0.001	0.001	0.000	0.001	0.001
G	0.006	0.001	0.006	0.006	0.000	0.000
H	0.006	0.001	0.006	0.002	0.001	0.000
I	0.006	0.001	0.006	0.002	0.000	
J					0.000	
K	0.000	0.001			0.000	
L	0.000	0.001			0.000	
M	0.007	0.002	0.006	0.003	0.001	
N	0.006	0.001	0.003	0.002	0.000	
			NRS (μg/L)			
Sampling locations	August 2004		July 2005		August 2005	
A	0.016	0.003	0.006	0.025	0.003	0.004
B	0.015	0.002	0.002	0.010	0.001	0.002
C	0.015	0.002	0.000	0.002	0.000	0.001
D	0.014	0.001	0.008	0.015	0.000	0.002
E	0.015	0.002	0.000	0.009	0.000	0.001
F	0.014	0.001	0.001	0.001	0.001	0.001
G	0.014	0.001	0.001	0.001	0.000	0.000
H	0.014	0.001	0.000	0.002	0.000	0.001
I	0.014	0.000	0.002	0.004	0.001	
J					0.000	
K	0.000	0.001			0.000	
L	0.000	0.000			0.000	
M	0.014	0.001	0.000	0.001	0.000	
N	0.014	0.000	0.000	0.001	0.000	

Table A5-5. Measured concentration of TC, CTC, OTC, STZ, and SMT in sediment at irrigation ditches and watershed

Sampling locations	August 2004		July 2005		August 2005	
			TC (μg/kg)			
B	2.78	2.98	20.87	22.36	75.17	87.38
D	1.82	1.95	2.08	2.23	1.82	31.00
F			10.04	10.76	24.10	25.89
M	11.27	12.08	21.51	23.05	51.19	51.19
N	14.91	15.98	26.37	28.26	21.34	60.48
			CTC (μg/kg)			
B	42.12	38.69	30.40	27.92	28.04	20.21
D	5.87	5.39	10.03	9.21	5.87	7.11
F			3.08	2.83	9.78	8.54
M	11.41		7.59	1.33	8.52	18.20
N	3.87	3.55	1.60	1.47	10.89	25.97
			OTC (μg/kg)			
B					161.55	148.85
D						40.80
F					46.38	80.55
M					48.41	134.37
N					42.37	179.58
			STZ (μg/kg)			
B			1.46		2.45	3.97
D						1.72
F					1.12	
M	3.25		15.52	2.51	1.80	4.67
N			1.35			2.69
			SMT (μg/kg)			
B						
D			4.94	1.87		
F						
M	14.61	5.52	24.97	9.45		
N			11.94	4.52		

Table A5-6. Measured concentration of ETM, TYL, MNS, SLM, and NRS in sediment at irrigation ditches and watershed

Sampling locations	August 2004		July 2005		August 2005	
			ETM (μg/kg)			
B	21.43	27.54	17.94	22.95	16.23	20.71
D	33.49	43.36	6.52	7.97	10.22	12.82
F	6.94	8.52	12.80	16.20	5.68	6.86
M			37.34	48.42	5.77	6.98
N			71.25	92.92	11.43	14.40
Sampling locations			TYL (μg/kg)			
B	12.73	20.19	15.60	24.98	18.51	29.82
D	10.39	16.29	3.00	3.97	20.32	32.84
F	7.87	12.08	7.06	10.73	1.44	1.38
M	38.09	62.45	10.39	16.28	1.44	1.38
N	34.59	56.62	37.86	62.07	2.44	3.04
Sampling locations			MNS (μg/kg)			
B	4.95	4.17	3.30	5.17	9.30	1.46
D	0.86	0.53	9.90	15.51	3.90	6.00
F	1.32	0.71	1.15	1.80	7.20	1.12
M	19.20	30.08	1.37	2.14	1.39	2.19
N	19.99	31.32	1.81	2.83	3.40	5.40
Sampling locations			SLM (μg/kg)			
B	12.13	13.24	6.50	1.10	4.10	6.90
D	6.85	11.36	8.70	1.47	7.20	1.22
F	11.03	9.00	2.40	4.00	6.70	1.14
M	17.90	30.40	6.50	1.10	6.40	1.08
N	13.35	22.67	8.30	1.41	2.80	4.80
Sampling locations			NRS (μg/kg)			
B	0.00	0.00	4.94	9.83	6.63	13.19
D	0.00	0.00	5.45	10.84	7.06	14.05
F	0.00	0.00	6.31	12.55	6.96	13.86
M	3.17	6.30	6.82	13.57	7.03	14.00
N	0.00	0.00	6.97	13.88	6.94	13.82

Table A6-1. Parameters for constructing calibration curve of studied antibiotics in manure

Antibiotics	Slope	Interception	R^2
CTC	0.004	1.596	0.98
TYL	0.033	0.829	0.97
MNS	0.285	1.855	0.99

Table A6-2. Measured concentration of CTC, TYL, and MNS in composting and stockpiling

Plots	0	3	6	18	27	46	60	140
				CTC (μg/kg)				
C-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C-2	167.05	51.81	18.52	14.50	7.82	3.51	1.59	1.05
C-3	125.85	38.21	25.85	16.36	9.37	5.69	1.68	1.18
C-4	119.81	76.22	29.70	15.89	7.51	3.86	1.63	1.05
S-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S-2	128.67	80.54	43.36	21.58	12.50	4.99	4.49	1.81
S-3	160.36	92.72	60.36	25.85	10.03	4.13	4.07	1.56
S-4	112.70	93.29	44.24	16.08	14.05	3.18	3.09	1.61
				TYL (μg/kg)				
C-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C-2	90.62	20.67	9.93	0.51	0.00	0.00	0.00	0.00
C-3	90.44	32.18	6.48	8.10	0.00	0.00	0.00	0.00
C-4	87.72	42.34	16.52	9.66	0.00	0.00	0.00	0.00
S-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S-2	118.83	50.48	33.48	31.08	7.39	6.64	4.20	1.29
S-3	95.18	60.13	24.80	14.86	5.18	8.16	2.61	1.04
S-4	130.57	56.21	17.92	19.16	17.34	8.79	2.22	1.09
				MNS (μg/kg)				
C-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C-2	129.61	53.21	41.76	28.68	21.37	15.65	8.85	8.10
C-3	124.86	42.75	45.55	19.89	33.16	23.50	8.19	7.69
C-4	116.61	46.85	39.34	30.22	20.85	9.21	5.35	3.78
S-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S-2	124.83	80.50	57.17	39.64	37.16	31.62	9.69	11.97
S-3	114.04	85.57	56.61	39.86	36.83	26.84	7.81	9.07
S-4	127.96	91.94	43.23	49.59	21.85	25.25	15.60	8.03