DISSERTATION

OCCURRENCE AND BIODEGRADATION OF ANTIBIOTIC COMPOUNDS IN THE AQUATIC ENVIRONMENT

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JONGMUN CHA ENTITLED "OCCURRENCE AND BIODEGRADATION OF ANTIBIOTIC COMPOUNDS IN THE AQUATIC ENVIRONMENT" BE ACCEPTED AS FULFILLING, IN PART, THE REQUIREMENTS FOR DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

OCCURRENCE AND BIODEGRADATION OF ANTIBIOTIC COMPOUNDS IN THE AQUATIC ENVIRONMENT

The occurrence and fate of antibiotics in the aquatic environment are an emerging area of interest due to the potential impact of these compounds on the environment. This study applied rapid, sensitive and reliable analytical methods for the determination of β-lactam and polyether ionophore antibiotics in surface water, wastewater, sediment, lagoon water, and animal manure. The method incorporated solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry (LC-MS-MS) with selected reaction monitoring. Specifically, the method was applied to evaluate the occurrence of these compounds in a small watershed in northern Colorado. The study also investigates the potential for on-farm removal of these compounds by measuring the biodegradation kinetics of oxytetracycline, sulfamethoxazole, tylosin, and monensin in bioreactors set-up to simulate a dairy wastewater lagoon.

Several product ions for LC-MS-MS detection of β-lactam and ionophore compounds have been identified and documented with their proposed fragmentation pathways. This study describes statistical analysis for determination of the method detection limit, accuracy and precision of the analytical method. Consequently, the average recovery of β-lactam and ionophore compounds from all the sample matrices was above 70 % (excluding amoxicillin and ampicillin). Few of the β-lactams under investigation could be detected due to the poor stability of the β-lactam ring. These results indicate that the release of β-lactams, which are commonly used for human applications, from agricultural operations may not be an environmental concern since they rapidly hydrolyze or biodegrade. The concentration of ionophores ranged from 0.03 to 0.05 μg/L for surface water samples, and from 0.5 to 3.1 μg/kg for sediment samples. Monensin (94-1077 μg/L) was also measured in runoff ponds at beef feedlots.

The research demonstrated biodegradation of oxytetracycline, sulfamethoxazole, tylosin, and monensin in dairy lagoon water at both 20 °C and 4 °C. However, the study found that within 175 days at 20 °C and 242 days at 4 °C, all antibiotics did not completely degrade under aerobic or anaerobic treatment. These results showed that degradation of antibiotics was faster under aerobic conditions, compared to anaerobic conditions and lower temperature (4 °C) reduced the biodegradation rate of antibiotics.

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Chapter 1

Introduction

According to the Union of Concerned Scientists (1), 15 thousand tons of antibiotics were used in the U.S.A. in 1998. Specifically, 9 % were used for human, while the remaining 91 % were used for the sum of animal (3 %) and livestock (88 %). In case of Switzerland, the total used amount was 90 tons in 1997. 38 % and 62 % were used for human and veterinary, respectively (Table 1.1). Antibiotics such as tetracyclines (TCs), sulfonamides (SAs), macrolides (MLs), polyether ionophores (PEs), and β-lactams (BLs) are a crucial group of pharmaceuticals in today's human and veterinary medicine. Antibiotics as a veterinary medicine have been widely used as feed additives to promote growth in confined animal feeding operations (CAFOs) as well as for prevention and

Table 1.1. Human and veterniary antibiotic use

	The United States (1998) Total tons	Switzerland (1997) Total tons
Total antibiotic use	15,026	90
Human uses	1,361 (9%)	34 (38%)
Livestock uses		
Medicinal use	907 (6%)	
Feed efficiency and growth promotion	12,509 (82%)	
Companion animal uses	453 (3%)	
Veterinary uses	13, 869 (91%)	56 (62%)

Based on estimates by Union of Concerned Scientists treatment of disease (2-5). Intake pathways into the aquatic environment result from their applications in both human and veterinary medicine. After application, the antibiotics used for animals and their metabolites are emitted into the soil with their manure (6-7). They also can be part of the runoff from soils that then passes into surface water. Ground water can be exposed to antibiotic residues leaching from farmland fertilized with manure or through sewage disposal by spray and broad irrigation in agricultural areas. Outdated and unused antibiotics are sometimes disposed of down household drains and can end up in the sediments where they are either degraded or slowly leach back into the surrounding water (Fig. 1.1).

The significant processes impacting the fate and transport of veterinary antibiotics from farm to nearby bodies of water are followed as:

Point source and local emissions

Downstream of intense urbanization and livestock production as well as surface/groundwater proximal to CAFOs as point sources (including animal feed operations (AFOs) as nonpoint source) and waste lagoons would be susceptible to contamination, thus causing the increase of antibiotic concentration. Local emissions of antibiotics from fields fertilized with manure might be a source of antibiotics in groundwater due to runoff from soil and might result in high concentrations even in groundwater (7, 8).

Runoff and rainstorm

The antibiotics that pass through the animal and their metabolites are carried by runoff from animal feedlots and end up in streams or groundwater. Antibiotics disposed along with feces in

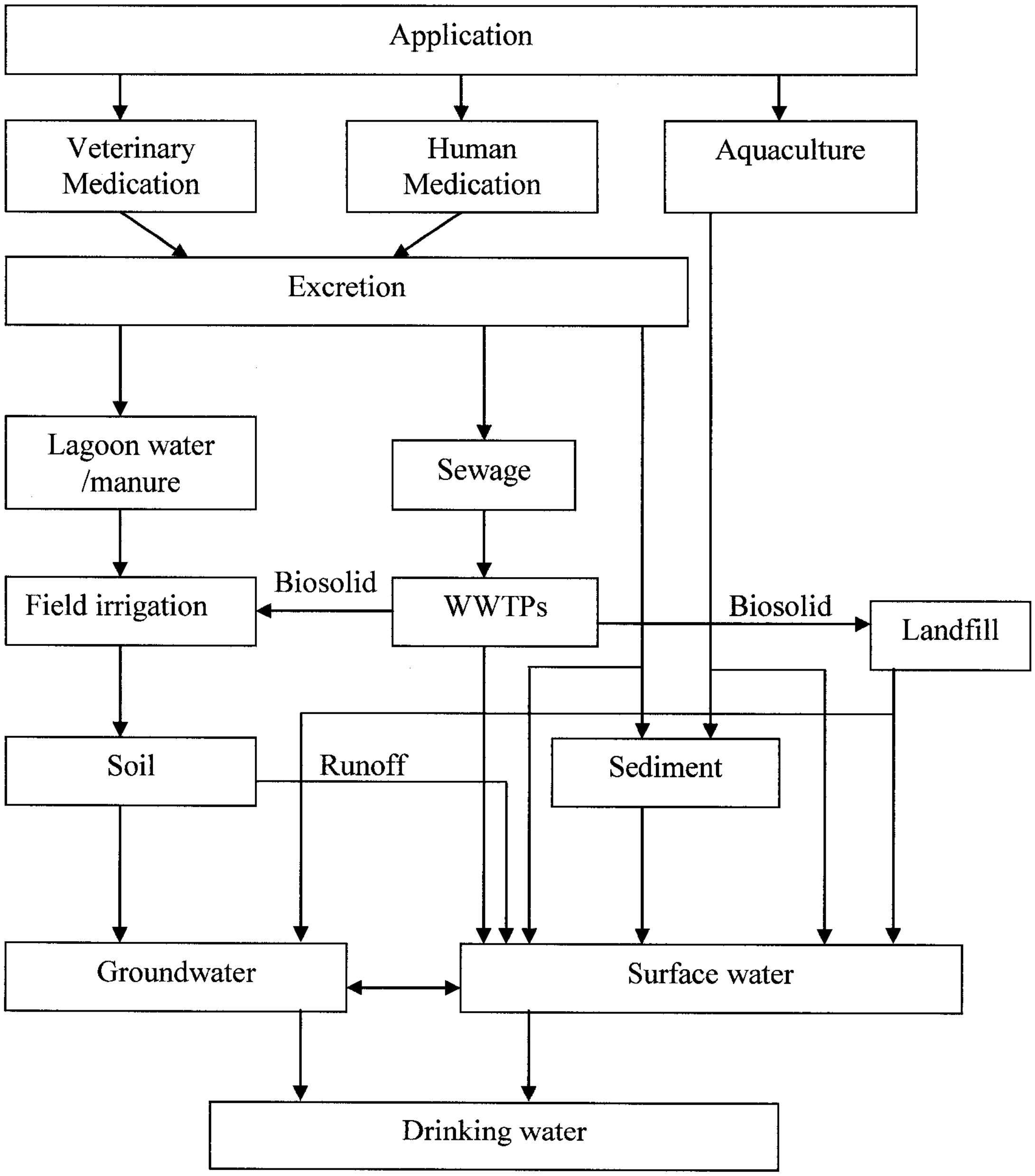

Fig. 1.1. Possible pathways of antibiotics into the aquatic environment.

waste lagoons sometimes overflow in streams during rainstorms.

1.1. The factors that affect antibiotic concentration in the environment

Antibiotics reaching the environment may undergo different reactions resulting in partial or complete elimination or attenuation of the parent compound. Therefore, it is necessary to consider the elimination process like biodegradation, photodegradation and hydrolysis in order to assess the factors that can affect antibiotic concentrations. Based on the degradation behavior, antibiotics can be divided into the following groups:

1) Antibiotics and metabolites that are mineralized by microorganisms and therefore completely eliminated
2) Antibiotics that are partly degraded
3) Antibiotics that persist in the environment.

1.1.1. Biodegradation

Some antibiotics like penicillins seem to be degradable in communal sewage treatment plants. Due to the chemically unstable β-lactam ring, they are readily susceptible to hydrolysis and will be easily eliminated (10). Others like sulfanoamides are poorly degradable or not degradable at all (11). Sulfanoamides like sulfadimethoxine are sufficiently stable in manure to maintain significant residual activity until field application. Microbial degradation in surface water is slower than that in the sewage treatment system due to lower density of bacteria. Antibiotics occurring in soil and sediment proved to be quite persistent in field studies and antibiotics applied to fish farming had long half-lives in soil and sediment (1).

1.1.2. Photodegradation and hydrolysis

If antibiotics are sensitive to light, photodecomposition may be of major significance in the elimination process. Photodecomposition takes place mainly in surface water, since soil and sediment prevent antibiotics from undergoing photochemical degradation due to the lack of light in these matrices (1, 7). Another kind of abiotic elimination of antibiotics is hydrolysis. It has to be noted that the results of bio- or photodegradability studies are conditioned on temperatures, composition of matrix etc. The half-lives of antibiotics vary due to differences in temperature and light intensity etc. Antibiotics that are readily biodegradable in one environment may be long-lived in another because of environmental factors.

1.1.3. Mobility of antibiotics due to complex formation and adsorption

Because sulfonamides are water-soluble and demonstrate little chelating ability and have a low binding constant for soil, they have the potential to enter groundwater and surface water with higher concentration. However, tetracyclines have been shown to be strong chelaters, sorb to soil strongly, and form complexes with ions present in several matrices resulting in the loss in antibiotic activity and detectability (12). Therefore it was hypothesized that although tetracyclines might be detected in surface water, they may not occur in groundwater.

1.2. Surface water antibiotic concentrations

Several recent studies have focused on antibiotic presence in surface water. Hirsch et al. (6) found Eryhtromycin-H_2O with a maximum concentration of 1.7 μg/L based on sampling of several surface water samples in Germany. They also reported that the maximum values of roxithromycin, clarithromycin, sulfamethoxazole, chlorampheicol, and trimethorin were below 0.5 μg/L. Christian et al. (13) reported that amoxicillin, ampicillin, mezlocillin, flucloxacillin, and piperacillin in surface water could be found at concentrations up to 48 ng/L. Hao et al. (14) detected monensin (0.02-0.22 μg/L) in surface water. U.S. Geological Survey reported that 95 organic wastewater contaminants (OWCs) including antibiotics were found in 80% of the 139 streams sampled (15). Kolpine et al. (15) frequently found erythromycin and lincomycin in surface water. Lindsey et al. (16) measured tetracyclines and sulfonamides in surface water. The detected concentrations of tetracyclines and sulfonamides ranged from 0.11 to 1.34 μg/L and from 0.06 to 15 μg/L, respectively. Yang and Carlson (17, 18) monitored tetracyclines, sulfonamides and macrolides along the Cache la Poudre (Poudre) River in northern Colorado, U.S.A. They determined five sampling sites to find the influence of human and/or agricultural activity on antibiotic concentration; pristine, urban, and agriculture area (Fig. 1.2). No tetracyclines, sulfonamides, and macrolides were found in the pristine river section (Site 1) since there are no urban or agricultural influences. Democlocycline and tetracycline were measured at the fringes of the urban area (Site 2), and the increases in these two tetracyclines occurred downstream of a wastewater treatment near the urban area (Site 3; Fort Collins Drake Water Reclamation Facility).

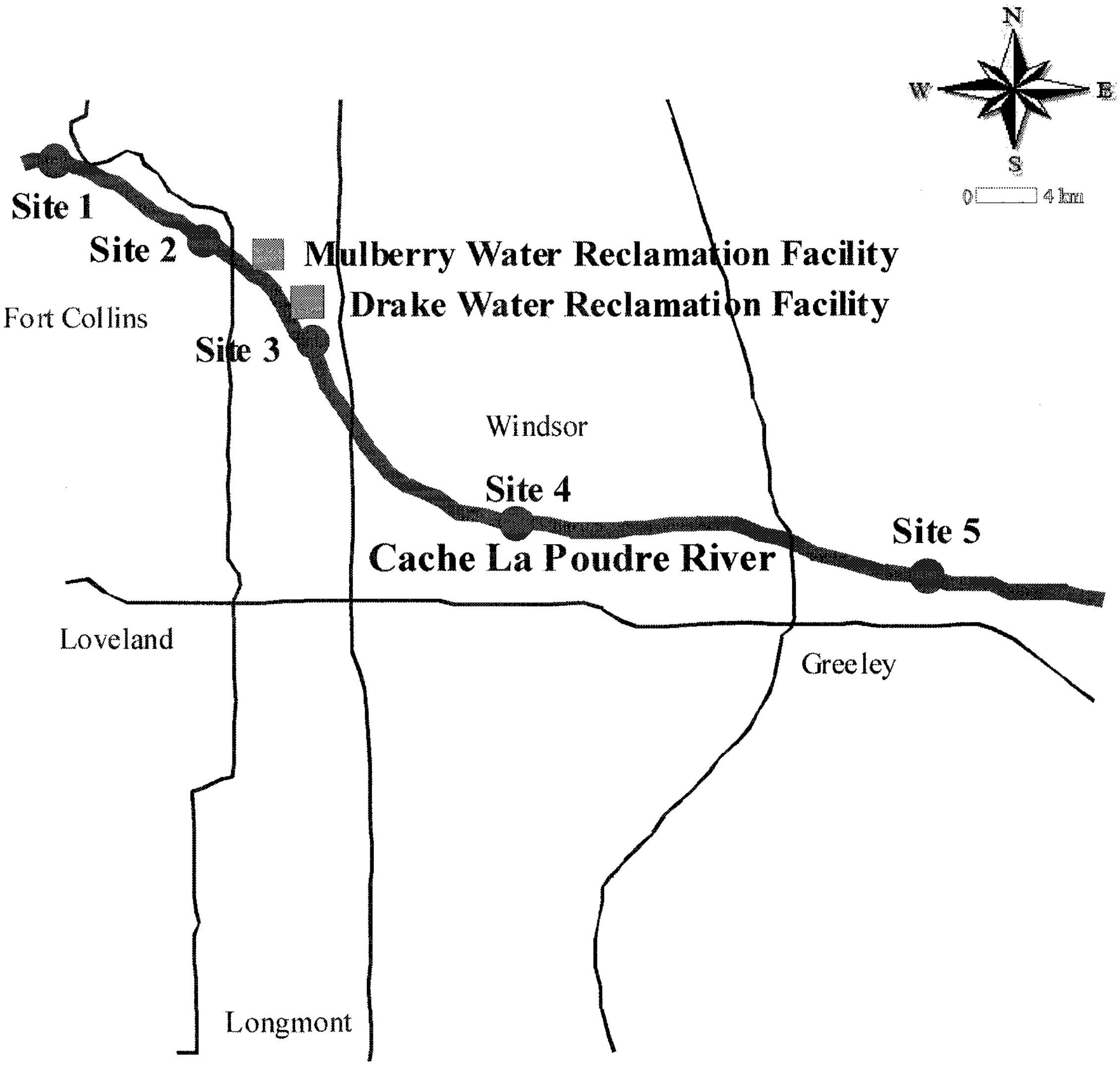

Fig. 1.2. Sampling sites along the Cache la Poudre River in northern Colorado.

Site 3 was the only location where two sulfonamides and three macrolides were detected. Oxytetracycline was first detected at Site 4, which is influenced by the surrounding agricultural activities. Two more tetracyclines (chlortetracycline and doxycycline) were found at site 5 with the greatest influence of agriculture. Total tetracyclines, sulfonamides and macrolides concentrations in surface water were found at 0.06-0.33 μg/L, 0.13-0.16 μg/L, and 0.04-0.17 μg/L, respectively (Table 1.2).

Table 1.2. Occurrence of antibiotics in the Poudre River

Antibiotic (μg/L)	Poudre River Site 1	Poudre River Site 2	Poudre River Site 3	Poudre River Site 4	Poudre River Site 5
Oxytetracycline	< 0.05	< 0.05	< 0.05	0.07	0.15
Chlortetracycline	< 0.05	< 0.05	< 0.05	< 0.05	0.19
Democlocycline	< 0.05	0.12	0.33	0.14	0.31
Tetracycline	< 0.05	0.06	0.11	0.11	0.16
Doxycycline	< 0.05	< 0.05	< 0.05	< 0.05	0.10
Sulfachloropyridazin	< 0.05	< 0.05	0.16	< 0.05	< 0.05
Sulfamethoxazole	< 0.05	< 0.05	0.13	< 0.05	< 0.05
Erythromycin-H_2O	< 0.07	< 0.07	0.17	< 0.07	< 0.07
Roxithromycin	< 0.03	< 0.03	0.04	0.06	< 0.03
Tylosin	< 0.05	< 0.05	0.13	< 0.05	< 0.05

1.3. Investigated antibiotics

One of the objectives of this study was to investigate the occurrence of two classes of seldom-studied compounds, β-lactam antibiotics (BLs) and the ionophores (PEs). In particular, the β-lactam antibiotics (BLs) are comprised of a great variety of semisynthetic penicillins (e.g. amoxicillin (AMOX), ampicillin (AMP), penicillin G (PEN G), oxacillin (OXA), cloxacillin (CLOX)) and cephalosporins (e.g. cephapirin (CEP)). All BLs have as their basic structure a β-lactam ring responsible for the antibacterial activity and variable side chains that account for the major differences in

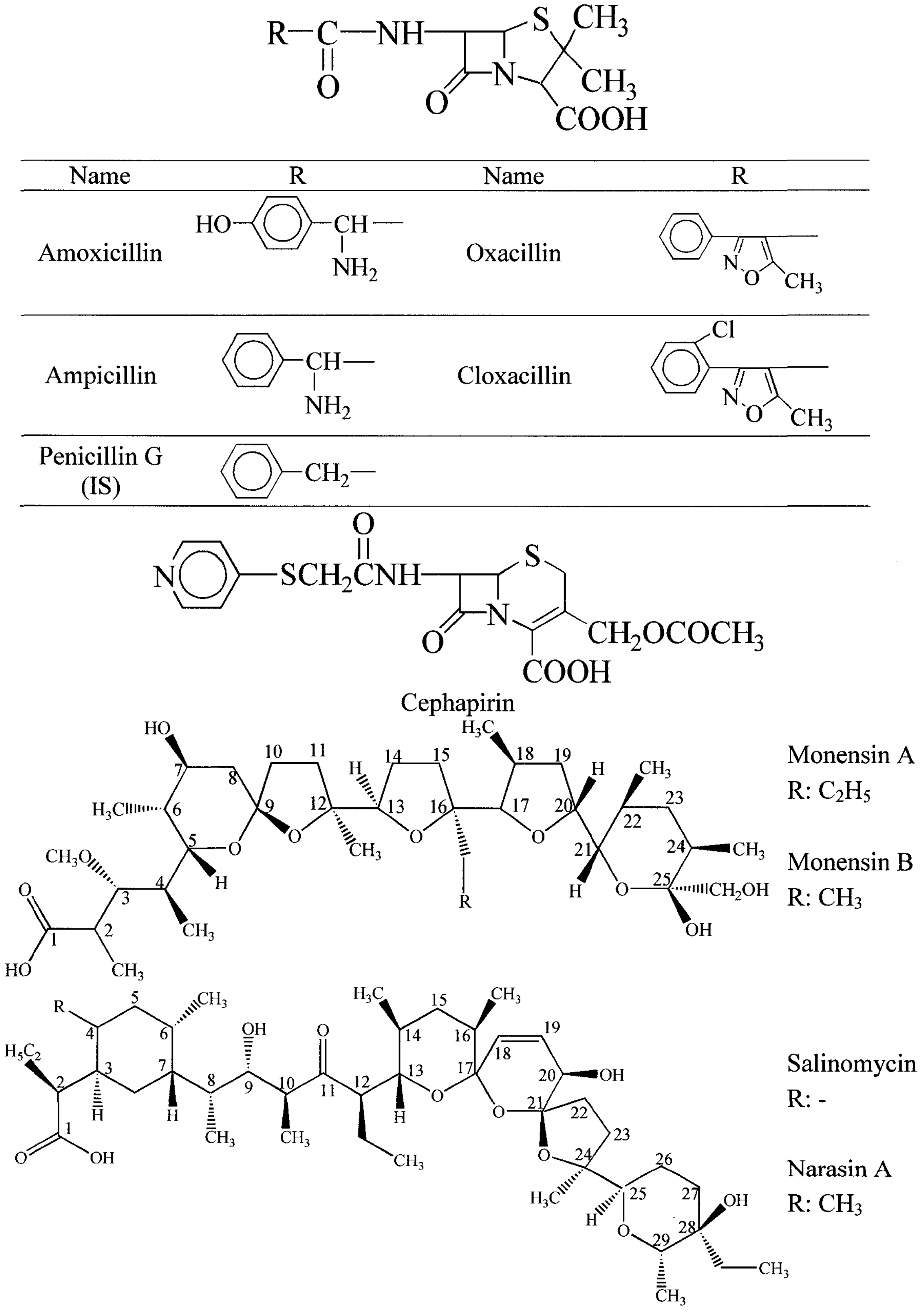

Fig. 1.3. Chemical structures of penicillins, cephapirin, and polyether ionophores.

their chemical and pharmacological properties (Fig. 1.3). The BLs are widely used for their antimicrobial activity against both gram-positive and gram-negative organisms (4). The ionophore antibiotics (e.g. monensin (MON), salinomycin (SAL), narasin (NAR), etc.) produced by various strains of *Streptomyces* have microbiological activities against gram-positive bacteria, fungi and protozoa. These antibiotics are used in veterinary applications as feed additives (coccidiostats) for poultry and livestock and as growth promoters for ruminants (5, 19). These ionophores (PEs) readily form electrically neutral pseudo-macrocyclic complexes with polar mono and divalent cations, i.e. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+} in solution and are lipid soluble (19-21).

Table 1.3. The investigated antibiotics approved for use in human and veterinary medicine in the United States.

Antiobiotics	Veterniary medicine	Human medicine
BLs		
AMOX	Yes	Yes
AMP	Yes	Yes
OXA	No	Yes
CLOX	Yes	Yes
PEN G	Yes	Yes
CEP	Yes	Yes
PEs		
MON	Yes	No
SAL	Yes	No
NAR	Yes	No
TCs		
OTC	Yes	Yes
SAs		
SMX	No	Yes
MLs		
TLS	Yes	No

Antibiotics used biodegradation are reported in boldface

Four antibiotics such as oxytetracycline (OTC), sulfamethoxazole (SMX), tylosin (TLS), and monensin (MON) were selected to determine the biodegradation kinetics in anaerobically and aerobically operated bioreactors that are fed with an actual animal waste that is amended with additional pharmaceutical compounds that may not be present (Table 1.3) (22-23).

1.4. Description of the investigated river area and wastewater treatment plant

The Cache la Poudre (Poudre) River in northern Colorado, U.S.A., was selected as a small mixed-use watershed (Fig. 1.2). The Poudre River originates near the continental divide in the Rocky Mountain National Park, and flows east through steep mountainous terrain for approximately 43 miles before entering the Front Range city of Fort Collins. After traveling through Fort Collins, the river moves through approximately 45 miles of mostly agricultural landscape before joining the South Platte River in Greeley, Colorado. Specifically, Drake Water Reclamation Facility (DWRF) and Mulberry Water Reclamation Facility (MWRF) are the wastewater treatment plants (WWTPs) that are explored in this document. The two facilities serve a population of approximately 128,000 in Fort Collins. The DWRF treats from 45,000 to 50,000 m^3/d of domestic sewage (95%) and industrial wastewater (5%) by a combination of pretreatment; primary, intermediate, and secondary clarification; and chlorine disinfection. The MWRF treats from 9,200 to 9,600 m^3/d of domestic sewage (75%) and industrial wastewater (25%) by pretreatment; primary, intermediate, and secondary clarification; and ultraviolet (UV) light disinfection. The five sampling sites along the Poudre River are Sites 1 and 2 that are 22 and 7 miles upstream of the DWRF, respectively. Sites 3, 4 and 5 are 0.5, 15 and 40 miles downstream of the plant, respectively.

1.5. Hypotheses and objectives of this study

1.5.1. Hypotheses

There are three hypotheses that have been developed to be tested with this study.

1) BLs are widely used human medicine to treat disease and infection. The widespread use of these compounds for both veterinary and human applications indicates that they will be present in the waste streams from animal production facilities and domestic wastewater plants. Literature suggests that these compounds are not stable and therefore we expect to find minimal amounts in the natural environment.

2) PEs are used exclusively in the animal production industry for growth promotion. These compounds will be detected on farm at the source at high concentrations in both the lagoon water and manure. We will also detect these compounds with high frequency at high concentrations in nearby streams in both the water and the sediment indicating that antibiotics are transported from farm to the watershed.

3) Veterinary antibiotics including tetracyclines, sulfonamides, macrolides, and polyether ionophores can be biodegraded during storage in a waste lagoon. Cold temperatures will slow down the degradation significantly and aerobic degradation will be much more effective than anaerobic biodegradation. Typical

operation of lagoons currently is not effective at providing conditions for greater than 90% removal of antibiotics before the waste is land applied.

1.5.2. Objectives of this study

The goal of this study is to develop a rapid, sensitive and reliable analytical method for the determination of β-lactam and ionophore antibiotics in surface water, WWTP influent and effluent wastewater, sediment, lagoon water and animal manure by using the solid-phase extraction (SPE) and liquid chromatography-ion trap tandem mass spectrometry (LC-MS-MS) with positive ion electrospray ionization, ESI (+) and selected reaction monitoring (SRM), and to investigate the occurrence and fate of β-lactam and ionophore antibiotics in a small watershed, several lagoon water and animal manure samples in northern Colorado. The objective of this study was also to demonstrate the biodegradation of oxytetracycline, sulfamethoxazole, tylosin, and monensin in dairy lagoon water and determine the biodegradation kinetics of these compounds.

1.6. Published and expected papers

1) Published papers

1. S. Yang, J.M. Cha, K.H. Carlson, Trace analysis and occurrence of anhydro-erythromycin and tylosin in influent and effluent wastewater by liquid chromatography combined with electrospray tandem mass spectrometry, Anal. Bioanal. Chem. 385 (3): 623-636 2006

2. J.M. Cha, S. Yang, K.H. Carlson, Trace determination of β-lactam antibiotics in surface water and urban wastewater using liquid chromatography combined with electrospray tandem mass spectrometry, J. Chromatogr. A 1115 (1-2): 46-57 2006

3. S. Yang, J.M. Cha, K.H. Carlson, Simultaneous extraction and analysis of 11 tetracycline and sulfonamide antibiotics in influent and effluent domestic wastewater by solid-phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry, J. Chromatogr. A 1097 (1-2): 40-53 2005

4. J.M. Cha, S. Yang, K.H. Carlson, Rapid analysis of trace levels of antibiotic polyether ionophores in surface water by solid-phase extraction and liquid chromatography with ion trap tandem mass spectrometric detection, J. Chromatogr. A 1065 (2): 187-198 2005

5. S. Yang, J.M. Cha, K.H. Carlson, Quantitative determination of trace concentrations of tetracycline and sulfonamide antibiotics in surface water using solid-phase extraction and liquid chromatography/ion trap tandem mass spectrometry, Rapid Commun. Mass Spectrom. 18 (18): 2131-2145 2004

2) Expected papers

Chapter 4

Occurrence and fate of β-lactam and polyether ionophore antibiotics in surface water, urban wastewater and sediment will be submitted to Environ. Sci. Technol.

Chapter 5

Occurrence and fate of β-lactam and polyether ionophore antibiotics in lagoon water and animal manure will be submitted to Water Research.

Chapter 6

Biodegradation of antibiotics in anaerobic and aerobic lagoon waters will be submitted to Environ. Sci. Technol.

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Chapter 2

Rapid analysis of trace levels of antibiotic polyether ionophores in surface water by solid-phase extraction and liquid chromatography with ion trap tandem mass spectrometric detection

This chapter was published in J. Chromatogr. A 2005, 1065, 187-198.

Abstract

The occurrence of antibiotics in surface and ground water is an emerging area interest due to the potential impacts of these compounds on the environment. This paper details a rapid, sensitive and reliable analytical method for the determination of monensin A and B, salinomycin and narasin A in surface water using solid-phase extraction (SPE) and liquid chromatography-ion trap tandem mass spectrometry (LC-MS-MS) with selected reaction monitoring (SRM). Several product ions as sodiated sodium salts for MS-MS detection have been identified and documented with their proposed fragmentation pathways. Statistical analysis for determination of the method detection limit (MDL), accuracy and precision of the method is described. The average recovery of ionophore antibiotics in pristine and wastewater-influenced water was 96.0 ± 8.3 % and 93.8 ± 9.1 %, respectively. No matrix effect was seen with the surface water. MDL was between 0.03 and 0.05 μg/L for these antibiotic compounds in the surface water. The accuracy and day-to-day variation of method fell within acceptable ranges. The method is applied to evaluate to the occurrence of these compounds in a small watershed

in Northern Colorado. The method verified the presence of trace levels of these antibiotics in urban and agricultural land use dominated sections of the river.

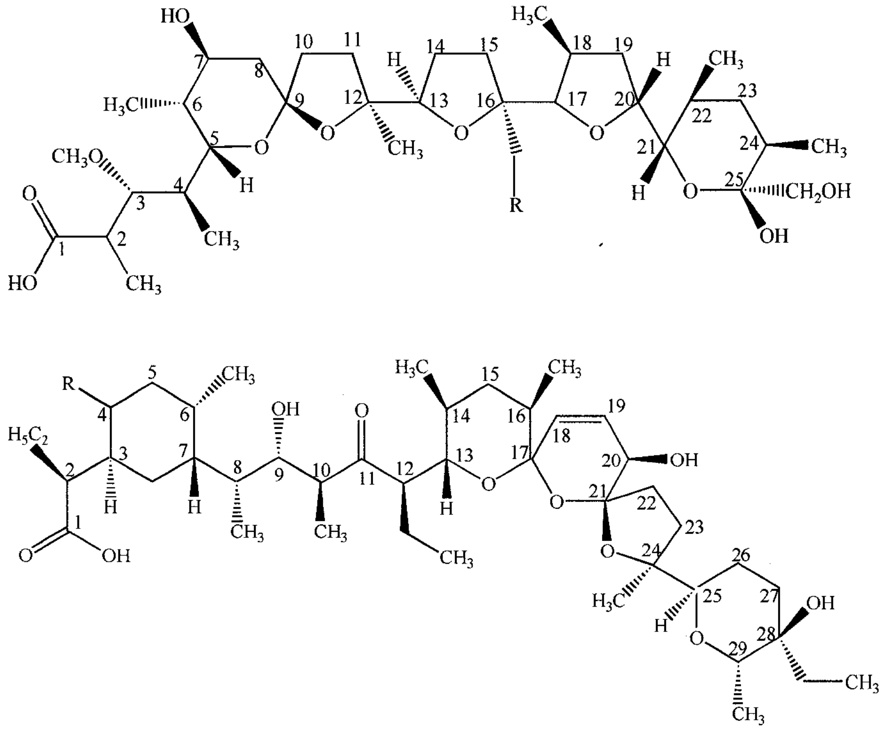
Key words: antibiotics; polyether ionophores; river; agricultural

2.1. Introduction

The ionophore antibiotics (e.g. monensin (MON), salinomycin (SAL), narasin (NAR), etc.) produced by various strains of *Streptomyces* have microbiological activities against Gram-positive bacteria, fungi and protozoa. These antibiotics are used in veterinary applications as feed additives (coccidiostats) for poultry and livestock and as growth promoters for ruminants [1, 2].

Their basic structure consists of multiple cyclic ethers, a free carboxylic acid group at one end of the molecule and a terminal alcohol group at the other, such that they are described as polyether antibiotics (Fig. 2.1). These ionophores readily form electrically neutral pseudo-macrocyclic complexes with polar mono and divalent cations, i.e. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+} in solution and are lipid soluble [2-4]. The transport of cations across the cell membrane by the ionophores is the main feature of their pharmacological activity.

When applied in veterinary medicine, a fraction of these drugs are metabolized to inactive compounds, but a significant amount is excreted as active metabolites unchanged via urine or feces. A variety of residual antibiotics have been found in wastewater treatment plant (WWTP) effluents with concentrations as high as 6 μg/L [5]. U.S. Geological Survey reported that 95 organic wastewater contaminants (OWCs) including antibiotics were found in 80% of the 139 streams sampled [6]. Antibiotic concentrations as high as 1.9 μg/L were found and only 10 of 24 compounds measured were not detected in any of the streams [6]. Donoho [7] revealed the presence of MON in cattle feces and urine, and Catherman et al. [8] found NAR (1.0 – 725.0 μg/kg) in poultry feces and manure.



Monensin A
R: C_2H_5

Monensin B
R: CH_3

Salinomycin
R: -

Narasin A
R: CH_3

Fig. 2.1. Chemical structures of carboxylic polyether ionophores.

The presence of antibiotics in the aquatic environment has created two concerns. The immediate concern is the potential toxicity of these compounds to aquatic organisms and humans through drinking water. In addition, there is growing concern that release of antibiotics to the environment contributes to the emergence of strains of disease-causing bacteria that are resistant to even high doses of these drugs [9].

The origin of antibiotic contamination in surface and ground waters is considered to be point and non-point source discharges of municipal and agricultural wastewater [10]. Since few studies have been conducted on the occurrence, fate and transport of antibiotics in the environment [11] there are several questions that need to be answered on local and watershed levels. Thus, there is a need for sensitive and reliable analytical methods to measure concentrations of polyether antibiotics (PEs) in both natural and wastewater environments.

Numerous methods for analytical determination of one or more of these ionophores in biological matrices (e.g. feeds, eggs, liver, human plasma, poultry tissue) have been reported in the literature [2, 3, 12, 13]. Because ionophore antibiotics do not exhibit any significant UV absorbance [12], derivatization to form a UV-absorbing compound is generally required for their analysis by high- performance liquid chromatography (HPLC). Liquid chromatography-mass spectrometry (LC-MS) [3, 13-15] or LC-MS-MS [2, 16] has been used in the analysis of ionophore antibiotics because of its high sensitivity and ability to provide compound confirmation. Researchers have varied methodological approaches for a variety of biological matrices and site-specific equipment. The majority of LC-MS or LC-MS-MS methods for PEs in biological matrices are for single or triple quadrupole mass spectrometers [2, 3, 13-15] and ion trap tandem mass spectrometers [3]. However, Kiehl et al. [3] did not fully report electrospray ionization (ESI) data and procedures for the investigated MON using an ion trap tandem mass spectrometer. In water matrices, no study of the analytical method for the determination of MON A and B, SAL and NAR A has been conducted with single, triple quadrupole or ion trap mass spectrometers.

Analysis of the ionophore antibiotics presents substantial problems. The polyether compound exhibits lower limited solubility in water due to the formation of lipid-soluble cyclic complexes with alkali metal cations. The compounds with hemiactetal or ketal structures tend to be acid labile. While their lipophilic property facilitates their extraction from aqueous matrices, the equal lipophilicity of their salts is sensitive to the type of acid-base extraction. The appearance of multiple charge adducts for a single analyte of polyether ionophores, which readily form cyclic complexes with polar cations is an issue that some analytical techniques face.

This paper details a rapid, sensitive and reliable analytical method for the determination of MON A and B, SAL and NAR A in surface water using solid-phase extraction (SPE) and ion trap LC-MS-MS with ESI (+) and SRM. This study discusses product ions by fragmentation of ionophore-sodium ion complexes in an ion trap mass spectrometer. Several product ions as sodiated sodium salts for MS-MS detection have been identified and documented. Statistical analysis for determination of the method detection limit

(MDL), accuracy and precision of the method is described. The method is applied to evaluate the occurrence of these compounds as the veterinary medicine in a small watershed in northern Colorado that is influenced by WWTP effluents and agricultural landscapes.

2.2. Experiment section

2.2.1. Materials and reagents

Monensin sodium salt (purity, 90 - 95 %), salinomycin (purity, 96 %), narasin (purity, 97 %) and Na_2EDTA (purity, 99 %) were obtained from Sigma-Aldrich (St. Louis, MO). Simatone, the internal standard (1000 mg/L in methanol) was purchased from Absolute Standards Inc. (Hamden, CT). Stock solutions of the standards were prepared by dissolving each compound in methanol at a concentration of 100 mg/L and stored at -20 °C in the dark. Fresh stock solution was prepared monthly. Working solutions (10, 1 and 0.1 mg/L) were prepared fresh weekly by diluting the stock solution with deionized water and stored at 4 °C in the dark. Internal standard working solutions (1.0 mg/L) were prepared by diluting the standard solution (1000 mg/L in methanol) with deionized water, stored at 4 °C, and replaced with a fresh solution each week.

2.2.2. Description of the investigated area

A watershed-scale field study was conducted on the Cache la Poudre (Poudre) River in northern Colorado, USA (Fig. 2.2). The Poudre River originates near the continental divide in Rocky Mountain National Park flowing through steep mountainous terrain for approximately 43 miles before entering the Front Range city of Fort Collins. After traveling through Fort Collins, the river moves through approximately 45 miles of mostly agricultural landscape before joining the South Platte River in Greeley, Colorado.

2.2.3. Sample collection and preparation

Approximately 45 samples were collected from five locations on the Cache la Poudre (Poudre) River in northern Colorado, USA (Fig. 2.2) over a period of six months from January 1, 2004 to June 30, 2004. The samples were collected in triplicate in the center of the stream as a depth composite using a water grab sampler.

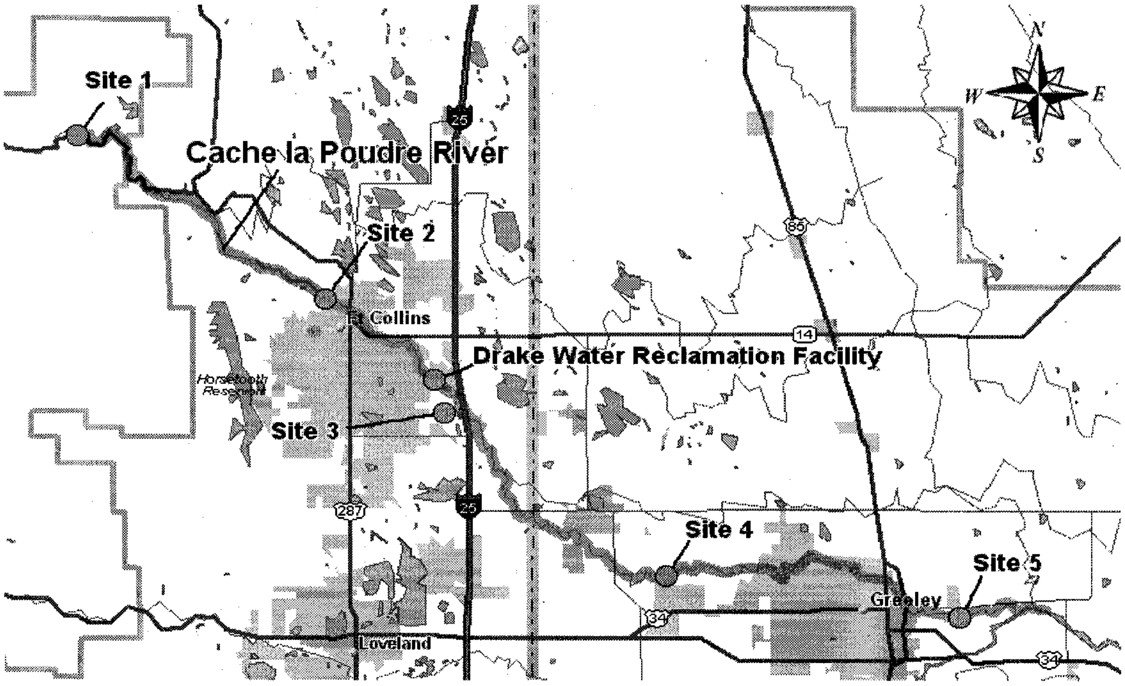


Fig. 2.2. Sampling sites along the Cache la Poudre River in northern Colorado.

All water samples were filtered through 0.4-micron glass fiber filters (Millipore, MA) and stored at 4°C in refrigerators until they were extracted, typically within one week. SPE and measurement were performed on the same day since the solubility of the extracted PEs during freezing and thawing was variable.

2.2.4. Preparation of ionophore-sodium salt

To convert the investigated ionophores into a single sodium adduct species, appropriate amounts (0.005 – 1 % (w/v)) of sodium chloride as a surplus of sodium were dissolved in water samples and then the samples were left to stand for 30 min prior to SPE and LC-MS-MS analyses. The added amount of sodium chloride depends on the sum of cation concentrations (e.g. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+}) in water samples.

2.2.5. Solid-phase extraction

Because the ionophores form complexes with alkali metal cations, all glassware used was heated for 1h at 450 °C, cooled, rinsed with 5 mL of 5.0 % Na_2EDTA and then 10 mL of HPLC grade water three times, and air-dried prior to SPE. SPE experiments were conducted using 60 mg/3 mL Oasis HLB cartridges (Waters, Millford, MA). Cartridges were preconditioned with 3 mL of MeOH, 3 mL of 0.5M HCl and 3 mL of deionized water at 8 in Hg on a vacuum manifold (PrepSep 12 port, Fisher scientific, PA). 120 mL of aqueous samples added at 0.005 – 1.0 % (w/v) of sodium chloride were prepared for extraction. For controls and calibration curves, appropriate amounts of working solution containing each of the analytes was added. Because the investigated ionophores are acid and/or base labile, extraction using the HLB cartridges was performed with the neutral sample pH adjusted by 0.01M NaOH to 7.5 immediately prior to extraction. To test the behavior of PEs spiked into a natural water matrix, a water sample was collected from the Poudre River Site 1. This water was used as a matrix because it has been shown in other studies by the authors [17, 18] that this water was devoid of pharmaceutical compounds since it is essentially snow runoff. Before it was used as a matrix, the water was again analyzed using the developed method in this study and no PEs was detected.

Water samples were passed through the cartridges at 5 mL/min and then, rinsed with 3 mL of deionized water. The analytes were eluted with 5 mL of MeOH into a test tube containing 12 μL of the internal standard, 1.0 mg/L simatone. Simatone was chosen as an internal standard because it eluted within the same chromatographic time frame as the analytes, responded well in ESI (+) mode, and had no noticeable matrix effects. The extracts were concentrated under a flow of N_2 gas to about 50 μL using a nitrogen evaporation system (N-Evap, Organermation Associates Inc., MA). To this, 70 μL of mobile phase A was added. The resulting solutions were transferred to 0.5 mL amber autosampler vials to prevent photodegradation of PEs.

2.2.6. Liquid chromatography

The LC system was a HP 1100 LC (Agilent, Palo Alto, CA) with a variable wavelength UV detector. The UV monitoring wavelength was 520 nm for PEs. The mass spectrometry was a Finnigan LCQ Duo ion trap (ThermoQuest, CA) equipped with a heated capillary interface, and an electrospray ionization source. ThermoQuest Xcalibur software was employed to control the mass spectrometric conditions. PEs were separated using a short Xterra MS C_{18} column (2.1 x 50 mm) with a 2.5 µm pore size (Waters, Millford, MA) in combination with a guard column of the same type (2.1 x 4 mm) from Phenomenex Inc. (Torrance, CA). An injection volume 40 µL and a ternary gradient with a flow rate of 0.30 mL/min were used. The LC column temperature was kept at 25°C. Mobile phase A was water with 0.1 % formic acid, mobile phase B was methanol and mobile phase C was acetonitrile. Separations of PEs were achieved with the following mobile phase gradient program: at 0 min A/B/C = 18:74:8, 9 min A/B/C = 13:1:86, 10 min A/B/C = 18:74:8. The investigated PEs eluted in only 5.84 min. A 10-minute post time allowed re-equilibration of the column.

2.2.7. Ion trap tandem mass spectrometry

Full scan mode was used to acquire mass spectra, precursor ions as protonated sodium salts and product ions as sodiated sodium salts from standard PE solutions. Mass spectral data shown in this study were acquired on a LCQ Duo ion trap tandem mass spectrometer equipped with an ESI source operated in positive ion mode.

Infusion into the ion trap tandem mass spectrometer was performed as follows: the flow of standard compounds (7 mg/L) coming from an integrated syringe pump at a flow rate of 5 µL/min was mixed with mobile phases A/B/C at a 15:32:53 ratio through a T-piece for tuning the mass spectrometer and optimizing the ESI source. The ESI source and MS-MS parameters were automatically optimized and saved in a tune file. Spray needle voltage was set at 4.5 kV for PEs, automatic gain control (AGC) was on, maximum isolation time was 300 ms, and three microscans per scan were acquired. Voltages on capillary and tube lens were 38 and 25 V. These were set by automatic optimization using the LCQ autotune program on the mass spectrometer instrument. Nitrogen was used as a sheath and auxiliary gas. Helium was used as the collision gas in the ion trap. The optimized tune conditions were as follows: sheath and auxiliary gas flow rate each was set at 50 units (a scale of arbitrary units in the 0 to 100 range defined in the LCQ system) and capillary temperature was 175 °C. MS-MS parameters for PEs including their proposed structures, and collision energy and isolation width (*m/z*) are summarized in Table 2.1.

2.2.8. Quantitation

The product ion, $[M+Na-H_2O]^+$ as a sodiated sodium salt producing the highest intensity was used for SRM and quantitation to increase analytical sensitivity and selectivity in LC-MS-MS mode. For the internal standard, the protonated molecular ion, $[M+H]^+$ was chosen for the SIM.

For SRM the product ion of the highest intensity for the investigated MON A and B, SAL and NAR A was reported in boldface in Table 2.1. Quantitation was based on a detector response defined as the ratio of the base peak ion (the specific product ion of interest) to the base peak ion of the internal standard. Calibration curves constructed for PEs spiked into water samples before extraction ranged from 0.05 to 5 μg/L in deionized water and surface water (Poudre River Sites 1 and 3).

Table 2.1

MS-MS parameters for analysis of ionophore antibiotics

Formula of MON A	Product ions, *m/z*	Formula of MON B	Product ions, *m/z*
Nominal molecular mass (Da)	670.5	Nominal molecular mass (Da)	656.4
Isolation width (*m/z*)	3.3	Isolation width (*m/z*)	3.2
Normalized collision energy (%)	33	Normalized collision energy (%)	30
Precursor ion		Precursor ion	
Protonated sodium salt,		Protonated sodium salt,	
$[M+Na]^+$, $C_{36}H_{62}O_{11}Na^+$	693.5	$[M+Na]^+$, $C_{35}H_{60}O_{11}Na^+$	679.4
Product ions		Product ions	
Sodiated sodium salts,		Sodiated sodium salts,	
$[M+Na-H_2O]^+$, $C_{36}H_{60}O_{10}Na^+$	**675.5**[a]	$[M+Na-H_2O]^+$, $C_{35}H_{58}O_{10}Na^+$	**661.5**[b]
$[M+Na-2H_2O]^+$, $C_{36}H_{58}O_9Na^+$	657.5	$[M+Na-2H_2O]^+$, $C_{35}H_{56}O_9Na^+$	643.4
$C_{34}H_{58}O_8Na^+$	617.5	$C_{24}H_{42}O_7Na^+$	465.3
$C_{34}H_{56}O_7Na^+$	599.4	$C_{24}H_{40}O_6Na^+$	447.2
$C_{25}H_{44}O_7Na^+$	479.3		
$C_{25}H_{42}O_6Na^+$	461.3		

Formula of SAL	Product ions, *m/z*	Formula of NAR A	Product ions, *m/z*
Nominal molecular mass (Da)	750.5	Nominal molecular mass (Da)	764.5
Isolation width (*m/z*)	3.0	Isolation width (*m/z*)	3.2
Normalized collision energy (%)	35	Normalized collision energy (%)	35
Precursor ion		Precursor ion	
Protonated sodium salt,		Protonated sodium salt,	
$[M+Na]^+$, $C_{42}H_{70}O_{11}Na^+$	773.5	$[M+Na]^+$, $C_{43}H_{72}O_{11}Na^+$	787.5
Product ions		Product ions	
Sodiated sodium salts,		Sodiated sodium salts,	
$[M+Na-H_2O]^+$, $C_{42}H_{68}O_{10}Na^+$	**755.5**[c]	$[M+Na-H_2O]^+$, $C_{43}H_{70}O_{10}Na^+$	**769.5**[d]
$[M+Na-2H_2O]^+$, $C_{42}H_{66}O_9Na^+$	737.4	$C_{30}H_{50}O_7Na^+$	545.3
$C_{29}H_{48}O_7Na^+$	531.4	$C_{29}H_{48}O_7Na^+$	531.4
$C_{29}H_{46}O_6Na^+$	513.3	$C_{29}H_{46}O_6Na^+$	513.3
$C_{23}H_{36}O_6Na^+$	431.3	$C_{23}H_{36}O_6Na^+$	431.3
$C_{23}H_{34}O_5Na^+$	413.2	$C_{23}H_{34}O_5Na^+$	413.2
$C_{19}H_{34}O_5Na^+$	365.1	$C_{20}H_{36}O_5Na^+$	379.1
$C_{13}H_{22}O_4Na^+$	265.1	$C_{14}H_{24}O_4Na^+$	279.1

[a, b,c,d] Product ions (*m/z*) of the highest intensity for SRM and quantitation are reported in boldface.

2.2.9. Statistical analysis

The method detection limit (MDL) was determined using the recommended US EPA method for MDL determination [19] on the basis of the variability of multiple analyses of seven surface water (Poudre River Sites 1 and 3) extracts spiked at a concentration of 0.2 μg/L. To assess the accuracy and day-to-day variation of the LC-MS-MS method, repeatability experiments were carried out with six surface water (Poudre River Sites 1 and 3) extracts spiked with 0.1, 1.0 or 2.0 μg/L of PEs for three days. Each day, six extracts spiked at three different concentrations in the two water matrices were analyzed.

2.3. Results and discussion

2.3.1. Liquid chromatography

The LC method employing a ternary gradient sequence combined with ESI(+)-MS-MS allowed the rapid, sensitive, selective and reliable determination of the investigated PEs in water matrices. The mass peaks corresponding to PEs appeared on the total-ion chromatograms (TICs) monitored at the selected product ion. The data were processed by creating reconstructed total-ion chromatograms (RTICs) for each analyte as shown in Fig. 2.3. These results indicate that efficient separation of PEs was achieved in only 5.84 min by the short C_{18} column using a column temperature (25 °C), a volumetric flow rate of 0.30 mL/min and mobile phases in a ternary solvent system, meaning fast analysis of the investigated ionophore antibiotics.

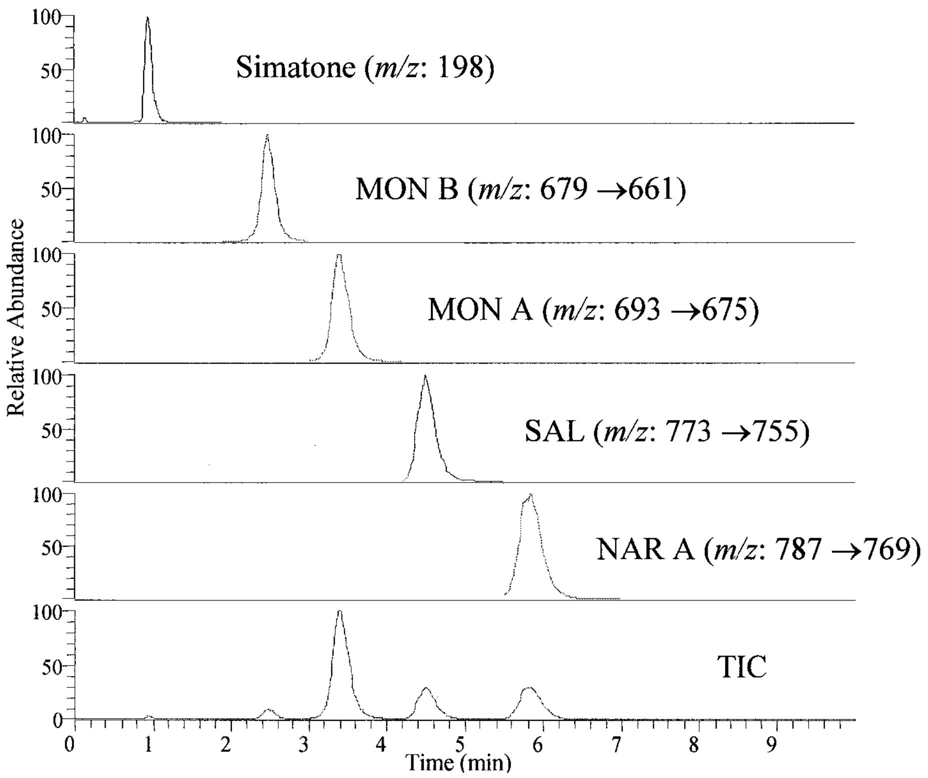


Fig. 2.3. Reconstructed total-ion chromatograms of ionophore antibiotics spiked at a concentration of 2 μg/L before extraction for 120 mL of Poudre River Site 1 as the surface water matrix using LC-MS-MS in SRM. *m/z* indicates precursor ion → product ion used for quantitation.

2.3.2. Determination of ionophore components in standard materials

Interestingly, the ionophore antibiotic standard materials are known to contain their derivatives as impurities. To determine components of derivatives in the ionophore standards (e.g. monensin sodium salt, purity = 90 – 95 %; salinomycin, purity = 96 %; narasin, purity = 97 %) used in this study, 2 mg/L of each standard solution was detected in the LC-MS-MS method with SRM developed in this study, followed by the comparison of mass peak areas. Standard monensin (MON) comprised MON A and B

(Fig. 2.1). MON B (an analogue of MON A that has a methyl instead of an ethyl group) was detected with a 0.91 min shorter retention time as compared to that of MON A (Fig. 2.3). The average composition of MON A and B in the MON standard material used was 87.7 and 12.3 % with a 1.2 % standard deviation, respectively.

Derivatives of standard SAL were not detected in even full scan LC-MS mode. Standard NAR mostly comprised NAR A as the major component (Fig. 2.1). These results for SAL and NAR were consistent with those of Harris et al. [20], reporting the derivatives of less than 5% relative to the main components. Thus, MON A and B, SAL and NAR A were quantitated in LC-MS-MS with SRM in this study.

2.3.3. Electrospray ionization characteristics of ionophore antibiotics

The high affinity of ionophore antibiotics for alkali metal cations as various impurities present in sample matrices and mobile phases has been described in the literature on electrospray ionization [3, 16, 21, 22]. Analysts have tried to force all of the analyte to a particular charge state in a similar manner such as the addition of a particular modifier in the sample matrix and/or mobile phase. Several studies on electrospray ionization showed the predominance of protonated sodium salt, $[M+Na]^+$ and the lack of protonated molecular ion, $[M+H]^+$ [2, 3, 12, 16]. Blanchflower and Kennedy [14, 15] added 0.8 % NaOH solution to egg and poultry tissue extract to quantitate MON, SAL and NAR using the $[M+Na]^+$ ion, followed by the addition of 15 g of sodium sulfate in the 5 g portion of animal tissues by Matabudul et al. [2] and the conversion of ionophores into a single adduct species using dichloromethane (DCM) and alkali chlorides by Volmer and Lock [16]. For ionophores, basic modifiers such as ammonium and sodium acetate were added to the mobile phase in order to ensure sodium adduct formation [3, 13, 15]. Because the modifier and mobile phase are in great excess of the analyte, this can be effective, as the ion formation reaction can be driven far to the right in terms of its equilibrium. These results indicate that it is desirable to quantitate a sodium adduct ion for the ionophores in LC-MS and/or LC-MS-MS. To develop sensitive, specific and reproducible LC-MS and/or LC-MS-MS methods for ionophore antibiotics, it is desirable to convert the ionophores into a single sodium adduct species in the sample matrix and then to enhance the ionization process in the mobile phase containing sodium acetate to ensure a surplus of sodium in order to ensure sodium adduct formation.

To improve sensitivity, selectivity and specificity for the chosen sodium ion of analyte in this study, appropriate amounts (0.005 – 1 % (w/v)) of sodium chloride were dissolved in water samples prior to SPE and LC-MS-MS analyses to convert the investigated ionophores into a single sodium adduct species. The added amount of sodium chloride depends on the sum of cation concentrations (e.g. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+}) in water samples from the investigated Poudre River Sites 1-5. The sum of cation concentrations in the investigated surface water ranged from 20 mg/L (Poudre River Site 1) to 500 mg/L (Poudre River Site 5). Sodium acetate as a basic modifier in the mobile phase may be advantageous to maximize sensitivity and specificity for the sodium adduct ions of PEs in MS-MS analyses but we did not use the basic modifier as a non-volatile compound in the mobile phase due to the problems with clogging of the interface of the mass spectrometer without a Z-spray.

2.3.4. Fragmentation of ionophores in the electrospray ion trap tandem mass spectrometer

This study reports the investigation of protonated sodium adduct ion ($[M+Na]^+$) fragmentation in a positive electrospray ion trap tandem mass spectrometer. Mass spectra and product ions as sodiated sodium salts for PEs were clearly observed in the full MS-MS scan mode of the ion trap tandem mass spectrometer. Fig. 4 shows full scan MS-MS spectra of precursor ion, $[M+Na]^+$ for a standard solution of 7 mg/L MON A and B, SAL and NAR A with ESI (+) source, and their proposed fragmentation pathways. Molecular mass (Da), precursor ions as protonated sodium salts and product ions as sodiated sodium salts at *m/z* for LC-MS-MS including their proposed structures, and collision energy and isolation width (*m/z*) are listed in Table 2.1. Each of the PEs exhibited characteristic fragmentation with the ESI (+) source and the precursor ion observed for all analytes was $[M + Na]^+$.

For MON A and B (Table 2.1 and Fig. 2.4 (A)), fragmentations of MON A and B were the result of openings of the cyclic ether rings as illustrated in Fig. 2.4 (A). Fragmentation of MON A with ion trap MS-MS in this study produced neutral losses of 18 and 36 Da corresponding to the sequent losses of H_2O, $[M+Na-H_2O]^+$ and $[M+Na-2H_2O]^+$ from the precursor ion, $[M+Na]^+$. According to the nomenclature for ionophore antibiotics suggested by Kim et al. [22], fragmentations containing the terminal carboxyl group of the molecule or the terminal hydroxyl group were named as Type A or Type F ions, respectively. It means that these complementary ions correspond to charge retention via the sodium ion on each side of the broken bond for the left-hand and the right-hand of the molecule. MON A exhibited Type-A product ions (e.g. *m/z* 617, 599, 479, 461 due to losses of H_2O from the 617 or 479 ion) and no Type-F product ions were observed in this ion trap MS-MS mode. MON B exhibited the product ions (*m/z* 465 and 447) at 14 Da lower than their counterparts in MON A spectra due to the different alkyl substituents at the C(16) (Fig. 2.4 (A)). However, no *m/z* 603, 585 and 567 in MON B relative to their counterparts (*m/z* 617, 599, 581) in MON A were observed in the ion trap MS-MS of this study.

For SAL and NAR A (Table 2.1, Fig. 2.4 (B)), fragmentation of SAL and NAR A with ion trap MS-MS in this study produced neutral losses of 18 and/or 36 Da corresponding to the subsequent losses of H_2O, $[M+Na-H_2O]^+$ and/or $[M+Na-2H_2O]^+$ from the precursor ion, $[M+Na]^+$ as observed in the fragmentation of MON A and B. Two major fragmentation pathways (Fig. 2.4 (B)) were observed in the ion trap tandem mass spectrometer. The major fragmentation pathways involved ß-cleavage of the oxygen-activated C-C bonds on each side of the C(11) carboxyl function, as illustrated in Fig. 2.4 (B). In the first fragmentation pathway of SAL, ß-cleavage and subsequent hydrogen migration occurred at C(9)-C(10), resulting in two production ions, *m/z* 531 and 265. The 531 ion was then followed by loss of H_2O, resulting in *m/z* 513. The second fragmentation pathway of SAL was due to bond dissociation at C(12)-C(13) to give *m/z*

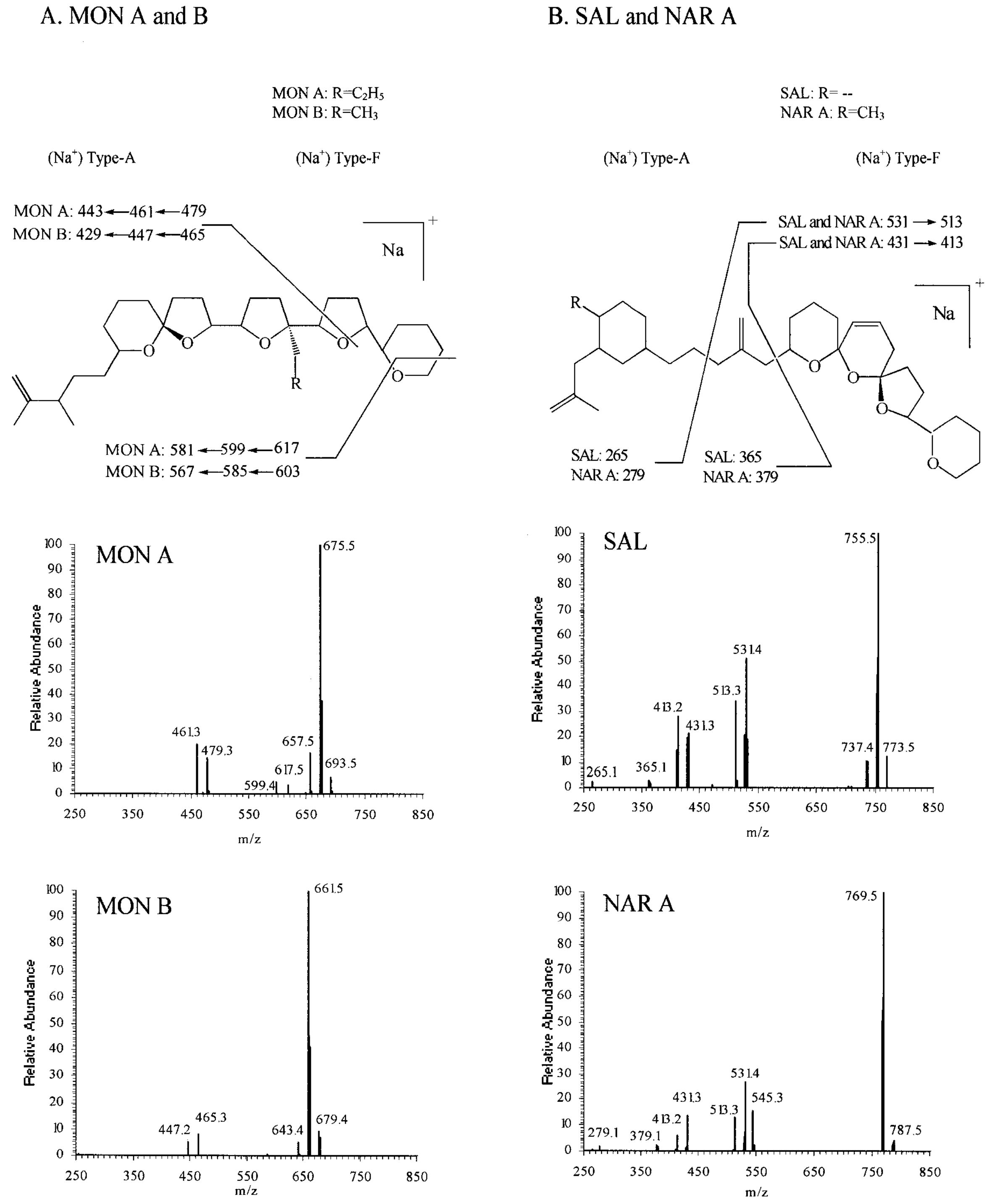


Fig. 2.4. Proposed MS-MS fragmentation pathways of solidated sodium adduct ions and full scan MS-MS spectra of MON A and B, SAL and NAR A.

431 and 365, followed by m/z 413 due to loss of H_2O from the 431 ion. Thus, SAL exhibited both of the Type-A product ions (e.g. *m/z* 265, 365) and Type-F product ions (e.g. *m/z* 531, 513, 431, 413). NAR A exhibited the same fragmentation pathways as compared to those of SAL, indicating that the 531, 513, 431 and 413 ions (Type F product ions) are identical to those in SAL. For Type-A product ions, NAR A exhibited *m/z* 279 and 379 instead of *m/z* 265 and 365 for SAL due to the differences in substitution between the two ionophores at C(4).

Full scan MS-MS spectra for the investigated ionophore antibiotics (Fig. 2.4) clearly exhibited product ions as sodiated sodium salts in the ion trap tandem mass spectrometer. Under the given conditions, no protonated molecular ions, $[M + H]^+$ or multiple charge adducts (e.g. $[M+Cat]^+$, $[M+2Cat-H]^+$, Cat = Na^+, K^+, etc. and $[M+NH_4]^+$) were observed (Fig. 2.4). This indicates that the investigated ionophores were totally converted into a single sodium adduct species by adding sodium chloride to water samples because no multiple charged adducts were observed in this assay. This implies that the compounds show a very high affinity for sodium and the large thermodynamic stability for larger sodium adduct ions versus a smaller proton. It is likely that there is enough 'residual' sodium from other sources (standard monensin sodium salt of the ionophore standards) such that we have no difficulty in generating sodium adduct ions. It is potentially the case that there is already enough sodium present from our current methodology (conversion of ionophores into a sodium adduct ion using an appropriate amount of sodium chloride and addition of sodium hydroxide to adjust sample pH to 7.5) to force the ionization reaction the direction we would like. In addition, formic acid added to the mobile phase in this study had the benefit of improving the spectral quality of mass spectra (Fig. 2.4) because this effect may be due to the protonation of the terminal carboxyl groups at low pH, thereby reducing and/or eliminating the possibility of $[M+2Cat-H]^+$ formation. Also, protonation of the carboxyl function at low pH could lessen the extent of hydrogen bonding between the carboxyl function and the terminal hydroxyl group, thus making cyclic complex formation more difficult. These results indicate that the investigated PEs show considerable sensitivity, selectivity and specificity for the chosen sodium ion ($[M + Na]^+$) and the specific product ion ($[M + Na -H_2O]^+$ of the highest intensity for quantitation of analyte in LC-MS-MS analysis with SRM of this study (Table 2.1, Fig. 2.4).

2.3.5. Recovery comparison

Ionophore antibiotics show multiple charge adduct ions for a single analyte with the various ratio of the multiple charge species in different water matrices, resulting in various recoveries in SPE and then a loss of raw sensitivity and precision in LC-MS-MS analysis. To reduce recovery variability in SPE, the investigated ionophores were converted into a single sodium adduct species by adding sodium chloride to water samples before extraction to promote the formation of a single sodium adduct species.

The recoveries of PEs from the HLB cartridges were measured by extracting analytes from 120 mL of deionized water spiked at 0.07 - 50.0 μg/L and surface water spiked at 0.07 – 5.0 μg/L. Poudre River samples from Sites 1 and 3 were selected as reference matrices for surface water. Recoveries of PEs were determined using the ratio of the concentration of analyte for extract spiked before extraction to the concentration of analyte for extract spiked after extraction. The average recovery in deionized water (Table 2.2) was 99.9 ± 6.8 % at the investigated concentration range, 90.1 ± 8.2 % with a 0.07 μg/L spike, and 93.8 ± 6.9 % with a 1.5 μg/L spike. No concentration dependence was observed. The average recovery of PEs in Poudre River Site 1 was 96.0 ± 8.3 % at the investigated concentration range, 88.3 ± 9.6 % with a 0.07 μg/L spike, and 94.0 ± 9.1 % with a 1.5 μg/L spike, indicating the HLB cartridges also gave reproducible recoveries for PEs and were effective for the isolation of the PEs. Recoveries in Poudre River Site 1 water were similar to those in deionized water indicating that matrix effects in surface water were minimal.

To assess the matrix effects for PEs in more complex surface water, recovery was also determined with water from Poudre River Site 3, immediately downstream of the WWTP. Because MON A and NAR A were detected at the sample site using the developed method, recovery in this matrix over a period of six months was determined using a concentration calculated by subtracting the measured background concentration from the spiked concentration. For Poudre River Site 3 (Table 2.2) the average recovery of PEs was 93.8 ± 9.1 % at the investigated concentration range, 88.2 ± 10.6 % with a 0.07 μg/L spike, and 95.1 ± 10.3 % with a 1.5 μg/L spike, indicating that matrix effects in the more complex surface water matrix were also minimal. In addition, the average recovery of lower concentrations of 0.07 μg/L for the investigated PEs in deionized water and surface water were lower (88.0 – 90.0 %) as compared to more than 92.0 % at higher concentrations but the difference was not statistically significant. Likewise, recoveries of MON A followed the lower and higher concentration trend observed with MON B, SAL and NAR A in deionized water and surface water.

2.3.6. Quantitation

It is desirable that both a compound as an internal standard and target compounds should have structural similarities such that it reflects the properties of the target compounds during the entire analytical procedure. This implies that it would be more ideal to have an internal standard (e.g. isotopically labeled compound, structurally similar compound) for each class of antibiotics. Unfortunately, this equates to the added cost and the difficulties in obtaining the ideal compound. Thus, simatone was chosen as an internal standard for PEs in this study and previous studies [17, 18] because it eluted within the same chromatographic time frame as the analytes, responded well in ESI (+) mode and did not exhibit noticeable matrix effects. As shown in RTICs (Fig. 2.2) of this study, simatone (spike concentration of 0.1 μg/L) also eluted much earlier in the chromatogram and was therefore much less affected by latter eluting interferences. Lindsey et al. [23] and Kolpin et al. [6] reported that internal standard simatone did not exhibit matrix effects in analysis of tetracyclines and sulfonamides in surface water, ground water and U.S. streams.

Table 2.2

Recoveries of ionophore antibiotics from 120 mL of water samples

Ionophores	Recovery, X ± S. D. (%)								
	Deionized water (μg/L)			Poudre River Site 1 (μg/L)			Poudre River Site 3 (μg/L)		
	0.07	1.5	0.07-50.0	0.07	1.5	0.07-5.0	0.07	1.5	0.07-5.0
MON A	85.6 ± 10.5	95.0 ± 8.3	96.9 ± 8.6	83.6 ± 12.2	92.4 ± 11.7	94.1 ± 11.5	86.1 ± 13.2	92.6 ± 11.8	93.7 ± 12.3
MON B	91.2 ± 6.4	92.7 ± 5.1	99.0 ± 4.9	89.2 ± 6.8	92.7 ± 5.3	94.8 ± 5.7	88.6 ± 7.4	93.4 ± 8.7	92.3 ± 6.9
SAL	90.5 ± 7.1	95.1 ± 7.4	102.2 ± 6.5	91.7 ± 10.1	96.5 ± 10.4	99.3 ± 8.9	89.5 ± 10.9	101.7 ± 11.2	93.9 ± 9.8
NAR A	93.1 ± 8.6	92.0 ± 6.8	101.6 ± 7.2	88.5 ± 9.4	93.2 ± 8.8	95.6 ± 7.2	88.7 ± 10.8	92.9 ± 9.6	95.4 ± 7.2
Average recovery	90.1 ± 8.2	93.8 ± 6.9	99.9 ± 6.8	88.3 ± 9.6	94.0 ± 9.1	96.0 ± 8.3	88.2 ± 10.6	95.1 ± 10.3	93.8 ± 9.1

To evaluate matrix effects of internal standard, we compared the peak area of internal standard (0.1μg/L) in extracts including internal standard and PEs for 120 mL water samples analyzed over a period of four months. The average peak area and standard deviation of the internal standard in deionized water and surface water (Poudre River Sites 1 – 5) was 64,529,774 ± 10.5 % and 61,957,146 ± 13.3 %, respectively. These values in surface water did not differ statistically from those in deionized water. Standard deviation (10.5 verse 13.3 %) of peak areas for the internal standard in the two water matrices is within the recommended acceptable values difference of 15.0 % [24]. This indicates that the internal standards do not exhibit matrix effects in surface water. Thus, concentrations for PEs were calculated reproducibly by using the standard calibration curves, which were constructed using a detector response defined as the ratio of the base peak ion (the specific product ion of highest intensity) to the base peak ion of the internal standard. PEs measured in this surface water were not corrected for the matrix effect of the internal standard.

Calibration curves constructed for PEs spiked into water samples before extraction ranged from 0.05 to 5 μg/L in deionized water and surface water (Poudre River Sites 1 and 3). The calibration curves were linear with correlation coefficients (R^2) > 0.99 for the MS-MS procedure. Because Poudre River Site 3 already contained MON A and NAR A, a calibration curve for these ionophores in this matrix was constructed by subtracting the level concentration from the spiked concentration. The ionophore concentrations in Poudre River samples from Sites 1 - 5 were determined reproducibly by using the standard calibration curves for Poudre River Site 3 since these would include matrix effects that are present in surface water.

2.3.7. Method detection limit, accuracy and precision

The method detection limit (MDL) was determined using the US EPA recommended method for MDL determination [19]. Calculations of MDL using the US EPA method were based on the variability of multiple analyses of seven surface water (Poudre River Site 1 and 3 each) extracts spiked at a concentration of 0.2 μg/L for PEs. The MDL was determined by multiplying the sample standard deviation calculated from each group of the extracts spiked at the concentration by the Student's *t*-variate for a one-sided *t*-test at the 99 % confidence level with *n*-1 degrees of freedom. The MDL for MON A and B, SAL and NAR A extracted from 120 mL of surface water was 0.03, 0.05, 0.04 and 0.04 μg/L, respectively.

The accuracy and the variability of the method were determined using six Poudre River Site 1 and 3 extracts spiked with 0.1, 1.0 and 2.0 μg/L of PEs over a period of three days. Because Poudre River Site 3 (immediately downstream of the WWTP influent) is a more complex water matrix, samples from this site were used to assess the accuracy and precision of the method. The results are summarized in Tables 2.3. The accuracy range was -7.5 to +9.8 % in the two water matrices. This accuracy range is well within the recommended acceptable values of -30 % to + 20 % [25]. The relative standard deviations (RSDs) calculated from these experiments ranged from 5.0 to 10.6 % and 6.1

to 13.9 % in the Poudre River Sites 1 and 3 samples, respectively. No concentration dependence was observed.

2.3.8. Occurrence, distribution, and fate of ionophore antibiotics

The average concentrations of four PE compounds measured along the Poudre River are shown in Fig. 2.5. As expected, no PE compounds were detected in the pristine river section (Site 1) since there are no urban or agricultural influences. No PE compounds were detected at the fringes of the urban area (Site 2) either indicating minimal urban or agricultural influences.

The first occurrence of MON A (0.03 μg/L) and NAR A (0.04 μg/L) is at Site 3, immediately downstream of the WWTP, around the lower density animal feed operations (AFOs) and concentrated animal feed operations (CAFOs) near to urban area. These trace concentrations are most likely due to the influence of agricultural since all four of these antibiotics have only veterinary applications.

As agricultural land use pre-dominates, i.e. Site 4 (around the greater density AFOs and CAFOs) and Site 5 (around the greatest density AFOs and CAFOs), a general increase in MON A and NAR A concentration is noted relative to Site 3. The results indicate that the two ionophore compounds are present at higher concentrations in the river with when the land use is dominated by agriculture activity. SAL (0.04 μg/L) was first detected along the river with the greatest influence of agriculture. No MON B compound were detected at any of the sites.

Table 2.3

The accuracy and day-to-day variation of the LC-MS-MS method in surface water (the Poudre River Sites 1 and 3) extracts from 120 mL spiked with 0.1, 1.0 or 2.0 µg/L of ionophore antibiotics

			MON A			MON B			SAL			NAR A		
Samples	n	Spike Concentration ug/L	Mean concentration ug/L	Accuracy %	RSD[a] %	Mean concentration ug/L	Accuracy %	RSD[a] %	Mean concentration ug/L	Accuracy %	RSD[a] %	Mean concentration ug/L	Accuracy %	RSD[a] %
Day 1:														
Poudre River, Site 1	6	0.10	0.10	3.6	8.3	0.10	-1.3	7.4	0.11	6.4	6.4	0.11	8.7	8.6
	6	1.00	1.03	3.3	10.1	1.00	-0.1	5.0	1.01	0.6	5.5	1.09	8.6	7.6
	6	2.00	2.04	1.9	7.2	1.98	-0.8	6.5	1.96	-1.8	7.2	2.02	0.9	6.7
Poudre River, Site 3	6	0.10	0.11	9.4	13.9	0.09	-7.5	8.5	0.11	8.6	7.8	0.10	-1.8	9.2
	6	1.00	0.99	-1.4	11.4	1.04	3.8	6.6	1.10	9.6	8.6	1.07	7.3	11.9
	6	2.00	1.96	-1.8	8.0	1.96	-2.2	7.1	2.04	1.8	6.6	1.93	-3.3	7.0
Day 2:														
Poudre River, Site 1	6	0.10	0.10	-2.5	10.6	0.10	-3.7	7.0	0.10	-1.4	7.0	0.11	5.4	8.8
	6	1.00	0.97	-3.5	9.6	1.02	1.8	7.5	1.04	3.7	7.3	0.96	-3.6	9.3
	6	2.00	2.02	0.8	7.3	2.01	0.7	6.2	2.10	4.9	6.6	2.00	0.1	7.1
Poudre River, Site 3	6	0.10	0.10	-2.9	12.3	0.11	5.1	8.9	0.10	-1.6	9.2	0.11	8.8	12.4
	6	1.00	1.05	5.3	8.6	1.10	9.8	8.4	0.96	-3.6	8.6	1.06	6.3	8.3
	6	2.00	2.02	1.2	10.1	1.86	-6.8	6.5	1.99	-0.6	6.0	1.97	-1.5	7.3
Day 3:														
Poudre River, Site 1	6	0.10	0.09	-5.2	10.4	0.10	-1.7	7.3	0.10	-0.2	7.1	0.09	-5.2	9.8
	6	1.00	1.06	5.6	9.1	1.10	9.7	6.8	0.98	-2.4	6.2	0.98	-2.2	6.1
	6	2.00	1.98	-1.1	7.8	1.99	-0.5	6.0	2.09	4.4	6.7	1.94	-3.2	8.5
Poudre River, Site 3	6	0.10	0.10	-0.8	12.4	0.10	-4.3	9.5	0.11	6.4	8.9	0.09	-6.5	11.7
	6	1.00	1.06	6.3	10.1	1.01	1.4	6.1	1.06	6.4	9.1	0.97	-3.1	8.5
	6	2.00	2.02	0.9	9.7	2.06	2.9	7.4	2.03	1.7	5.6	2.08	3.8	6.2

[a] RSD: Relative standard deviation

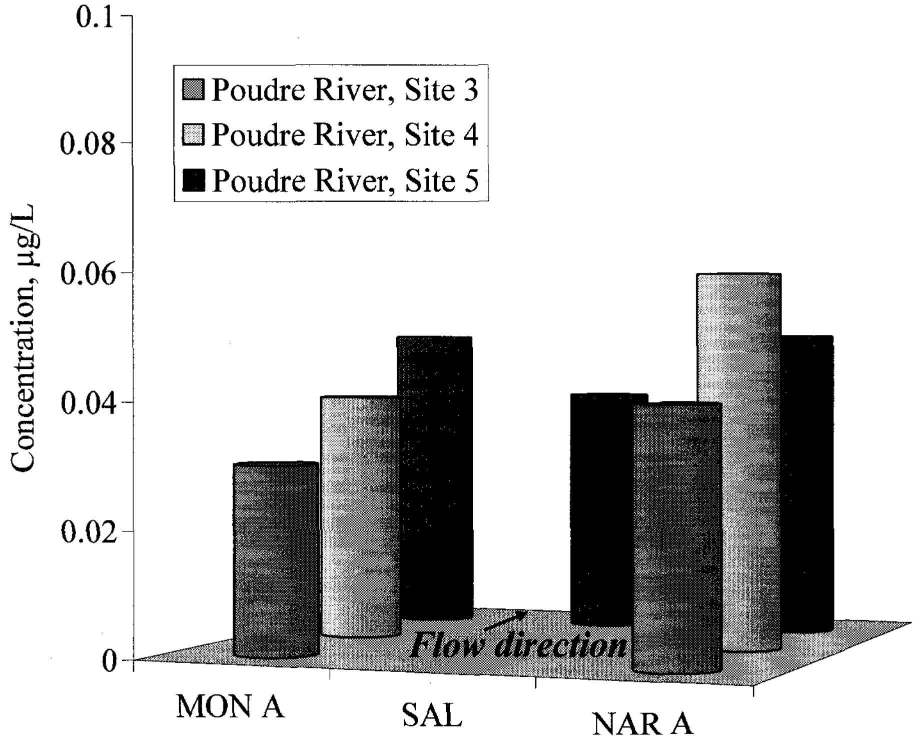


Fig. 2.5. Occurrence of ionophore antibiotics in the Poudre River. Concentrations shown are the triplicate average of three samples at each site over a period of six months. N (Number of samples at each site) = triplicates * three samples * five frequencies = 45.

Four PE compounds found in this study were present at trace levels (0.03 - 0.06 µg/L) in the river water, although the compounds have been used as a growth promoter and/or coccidiostats in cattles, chickens and turkeys. This reflects that the PE compounds exhibit lower limited solubility in water matrices and form complexes with alkali metals cations in the sample matrices. This indicates that these compounds may be present at higher concentrations in sediments, manures and lagoon waters. Thus, it is desirable to evaluate the occurrence and fate of ionophore antibiotics in these matrices as well as surface water. The SPE-LC-MS-MS method in this study was first applied to evaluate the occurrence of four ionophore antibiotics in the Cache la Poudre River influenced by WWTP effluents and the surrounding agricultural activities, and therefore will be applied to develop analytical methods for these compounds in more complex environmental matrices of manure and lagoon water eventually.

2.4. Conclusions

A SPE-LC-MS-MS method with SRM has been shown to be accurate, reliable and robust for the determination of three ionophore veterinary antibiotics. The method was able to quantify two analogues of monensin (A and B) and the results showed a significant fraction of the total (approximately 12%) was monensin B.

Ionophores will readily form different complexes with alkali metal cations in different water matrices, resulting in various recoveries in SPE and then a loss of raw sensitivity and precision in LC-MS-MS analyses. Sensitivity, selectivity and specificity for the chosen protonated sodium ion and the specific product ion of analyte in LC-MS-MS analysis with SRM were improved by adding NaCl before extraction to promote the formation of a single sodium adduct species.

The average recovery of ionophore antibiotics in pristine and wastewater-influenced water was 96.0 ± 8.3 % and 93.8 ± 9.1 %, respectively. No matrix effect was seen with surface water. The MDL was between 0.03 and 0.05 μg/L for MON A and B, NAR A, SAL with an accuracy of better than ± 10 %. The method verified the presence of trace levels (0.03 – 0.06 μg/L) of these antibiotics in urban and agricultural land use dominated sections of the river.

Acknowledgements

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Chapter 3

Trace determination of β-lactam antibiotics in surface water and urban wastewater using liquid chromatography combined with electrospray tandem mass spectrometry

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Abstract

A sensitive and reliable method using liquid chromatography-electrospray tandem mass spectrometry has been developed and validated for the trace determination of β-lactam antibiotics in natural and waste water matrices. Water samples were enriched by solid-phase extraction. The analytes included amoxicillin, ampicillin, oxacillin, cloxacillin and cephapirin. Average recoveries of β-lactams in fortified samples were generally above 75% (except amoxicillin) with the standard deviations lower than 10% in water matrices. Amoxicillin was not quantified due to poor recovery (less than 40%) in the investigated water matrices. Matrix effects were found to be minimal when measuring these compounds in water matrices. The accuracy, within- and between-run precision of the assay fell within acceptable ranges of 15% absolute. The method detection limit was estimated to range between 8-10 ng/L in surface water, 13-18 ng/L in the influent and 8-15 ng/L in the effluent from a wastewater treatment plant. A large number of actual water samples were analyzed using this method in order to evaluate the occurrence of the β-lactams in a river and a wastewater treatment plant in northern Colorado. Most of the samples were negative for all analytes. These compounds were found at 15-17 ng/L in the three influent samples and at 9-11 ng/L in three surface water samples out of a total of 200 samples. This indicates that contamination by β-lactam antibiotics is of minor importance to the small mixed-watershed.

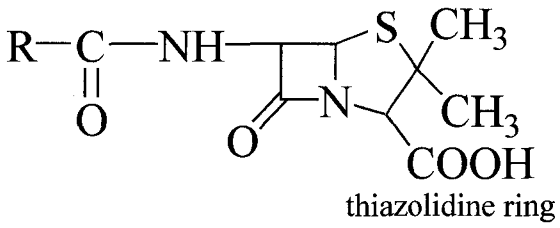
Key words: Antibiotics; β-lactams; River, Wastewater; WWTPs; Agricultural

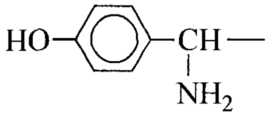
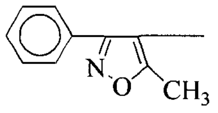
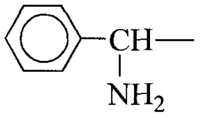
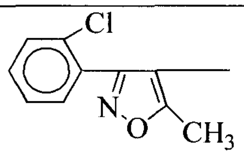
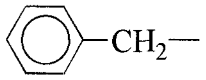
3.1. Introduction

The β-lactam antibiotics are comprised of a great variety of semisynthetic penicillins (e.g. amoxicillin (AMOX), ampicillin (AMP), penicillin G (PEN G), oxacillin (OXA), cloxacillin (CLOX)) and cephalosporins (e.g. cephapirin (CEP)). All β-lactams (BLs) have at their basic structure a β-lactam ring responsible for the antibacterial activity and variable side chains that account for the major differences in their chemical and pharmacological properties. The primary distinguishing structural difference between penicillins and cephalosporins is the ring system fused to the β-lactam ring. Penicillins have a five-membered thiazolidine ring while cephalosporins have a six-membered dihydrothiazine ring (Fig. 3.1). BLs are chemically reactive due to the strained β-lactam ring as well as the lability of their carbonyl carbon towards nucleophilic attack [1].

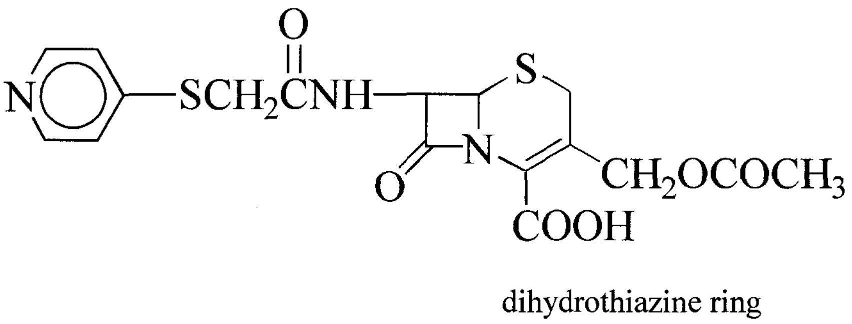
The BLs are widely used for their antimicrobial activity against both gram-positive and gram-negative organisms. These antibiotics are used as human medicine for the

Penicillins



Name	R	Name	R
Amoxicillin (AMOX)	 *pK_{a1} 2.8, pK_{a2} 7.2*	Oxacillin (OXA)	 *pK_a 2.7*
Ampicillin (AMP)	 *pK_{a1} 2.7, pK_{a2} 7.3*	Cloxacillin (CLOX)	 *pK_a 2.7*
Penicillin G (PEN G, IS)	 *pK_a 2.8*		

Cephalosporins



Cephapirin (CEP), *pK_a 2.7*

Fig. 3.1. Chemical structures of β-lactam antibiotics.

treatment of bacterial infections of skin, ear, respiratory tract, and urinary tract [2]. These compounds have been widely used both for prevention and treatment of disease and as feed additives to promote growth in animal feeding operations [3]. There are concerns about β-lactam residues in food because of the potential for allergic reactions in sensitive individuals. Interest in the analysis of antibiotic residues in the environment arises from suspicion that they are responsible for the appearance of bacterial strains that are resistant to important classes of antibiotics.

A high percentage of antibiotics consumed by humans and animals in hospitals or by prescription are excreted unchanged via urine and feces into domestic sewage, and are discharged to wastewater treatment plants (WWTPs). In WWTPs these compounds are only partially removed and there is a potential for residues of antibiotics to be released through the WWTP effluent into the aquatic environment. Thus, the origin of antibiotic contamination in surface and ground waters is considered to be point and non-point source discharges of municipal and agricultural wastewater [4]. Christian et al. [5] reported that AMOX, AMP, mezlocillin, flucloxacillin, and piperacillin in surface water could be found at concentrations up to 48 ng/L. AMOX was found at less than 10 ng/L in 13% of 32 river water samples [5]. Different water compartments were searched for BLs (including WWTP effluents [6], surface water [6-9] and groundwater [9]); however, none of the BLs under investigation could be detected. This is explicable considering the poor stability of the β-lactam ring. It can be opened by β-lactamases, a widespread enzyme in bacteria, or by chemical hydrolysis. Due to this, intact BLs do not occur frequently in the environment [5]. However, we cannot exclude the presence of these

BLs in aquifers, where manure or sludge is applied to soil, or where hospital wastewater is discharged, via a relatively small sewage treatment plant, into the nearest stream. Since there has been a lack of study on the occurrence, fate and transport of β-lactam antibiotics in the environment, there are several questions that need to be answered on local and watershed levels. Therefore, there is a need for sensitive and reliable analytical methods to measure concentrations of BLs in both natural and wastewater environments.

Liquid chromatography-mass spectrometry (LC-MS) [6, 10] or LC-MS-MS [5, 7-9] has been used in the analysis of BLs because of its high sensitivity and ability to provide compound confirmation. A number of researchers have determined BLs in biological samples (e.g. milk, kidney tissue) [11-14]. The methods for analytical determination of BLs in environmental matrices (e.g. natural, ground and waste water) have been reported in the literature [5-9]. Researchers have varied methodological approaches for a variety of matrices and site-specific equipment. The majority of LC-MS or LC-MS-MS methods for BLs are for single or triple quadrupole mass spectrometers [6-9, 11, 12] compared to ion trap tandem mass spectrometers [13, 14]. To our knowledge, only one study has been conducted on an analytical method (LC-MS) for BLs in WWTP influent wastewater [6] and no study for CEP in environmental matrices.

AMOX, AMP, OXA, CLOX and CEP are the most important β-lactam antibiotics approved in the United States for use in human and/or veterinary medicine [13]. Thus, this paper details a sensitive and reliable analytical method for the determination of these compounds in surface water, WWTP influent and effluent wastewater using solid-phase extraction (SPE) and ion trap LC-MS-MS with positive ion electrospray ionization, ESI (+) and selected reaction monitoring (SRM). The paper describes the impact of matrix effects when measuring these compounds at environmentally relevant concentrations. We explore the difficulties of quantification for AMOX due to matrix effects. This study discusses different product ions on the basis of their proposed fragmentation pathways in an ion trap MS-MS. Statistical analysis for determination of the method detection limit (MDL), accuracy, within- and between-run precision of the assay is described. The method is evaluated by studying the occurrence of these compounds in a small watershed in northern Colorado.

3.2. Experiment section

3.2.1. Chemicals and standards

All antibiotics (purity, >90%), formic acid (>95%), trifluoroacetic acid (>98%) and Na_2EDTA (99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethanol and acetonitrile were HPLC grade (Sigma-Aldrich, St. Louis, MO, USA). Individual stock solutions were prepared weekly by dissolving each compound in acetonitrile-ethanol-water (25:25:50) at a concentration of 1000 mg/L and stored at -20 ^{o}C in the dark. Mixed working solutions (10, 1 and 0.1 mg/L) were prepared fresh daily by diluting the individual stock solution with the same solvent and stored at 4 ^{o}C in the dark. PEN G, the internal standard working solution (1 mg/L) was prepared by diluting the standard solution with the solvent, stored at 4 ^{o}C and replaced with a fresh solution every day.

3.2.2. Description of the investigated river area and wastewater treatment plant

The Cache la Poudre (Poudre) River in northern Colorado, USA was selected as a small mixed-use watershed (Fig. 3.2). The Poudre River originates near the continental divide in Rocky Mountain National Park and flows east through steep mountainous terrain for approximately 43 miles before entering the Front Range city of Fort Collins. After traveling through Fort Collins, the river moves through approximately 45 miles of mostly agricultural landscape before joining the South Platte River in Greeley, Colorado. Drake Water Reclamation Facility (DWRF) is the WWTP that was studied in this paper. The plant serves a population of approximately 125,000 in Fort Collins, Colorado. It treats 45,000 – 50,000 m^3/d of

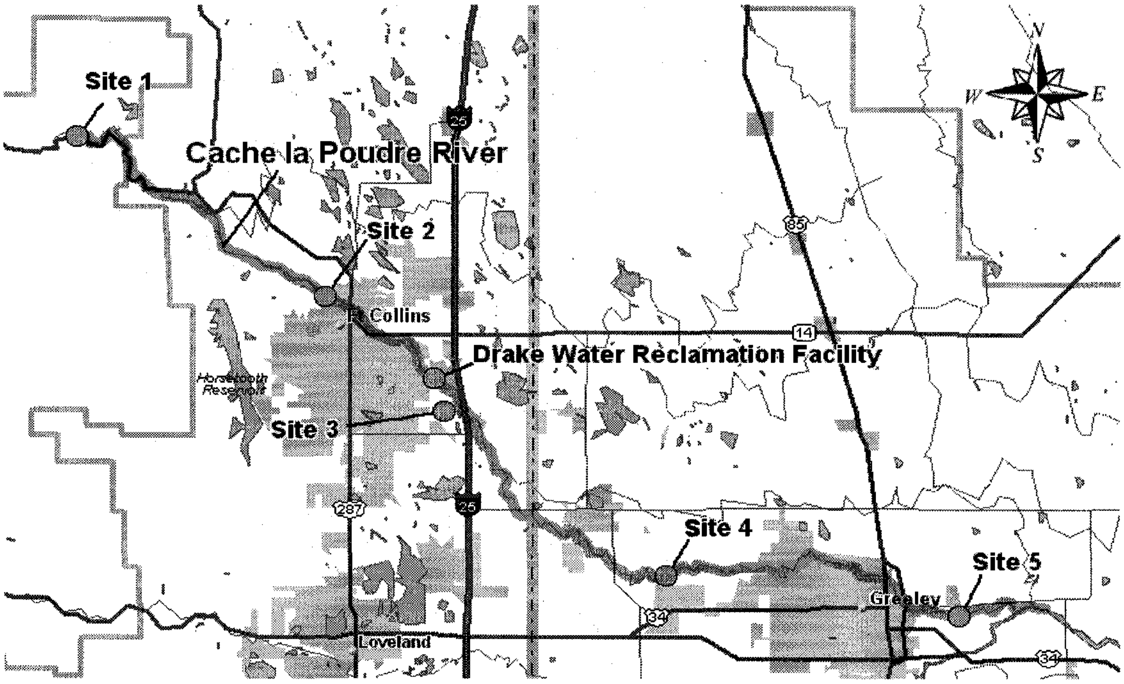


Fig. 3.2. Sampling sites along the Cache la Poudre River in northern Colorado.

domestic (95%) and industrial sewage (5%) by pretreatment, primary clarification, intermediate clarification, secondary clarification and chlorine disinfection. The five sampling points along the Poudre River are Sites 1 and 2 which are 22 and 7 miles upstream of the DWRF, respectively. Sites 3, 4 and 5 are 0.5, 15 and 40 miles downstream of the plant (Fig. 3.2), respectively.

3.2.3. Sample collection and preparation

Approximately 60 samples were collected from five locations on the Poudre River (Fig. 3.2) over a period of twelve months from July 1, 2004 to June 30, 2005. The samples were collected in the center of the stream as a depth composite using a water grab sampler. Twenty-four hour composite samples of raw influent and final effluent were collected from the DWRF over the same period of twelve months mentioned above. A flow proportioned automatic sampler, whereby the 24-h composite samples of the final effluent were collected time-related to the influent, carried out sampling. The DWRF samples were collected in triplicate twice a month.

The influent water samples were centrifuged at 3000 rpm for 40 min at 4 °C in a centrifuge (IEC Centra CL 3R, MA, USA) with a cooling system. All aqueous samples were filtered through 0.4-micron glass fiber filters (Millipore, MA) and stored at 4 °C. SPE and measurement were performed within 12 hours after collection of samples due to the tendency toward biodegradation and/or hydrolysis of the labile β-lactam compounds.

3.2.4. Solid-phase extraction

It was found that various metal ions catalyzed the rate of inactivation or hydrolytic opening of β-lactams [15]. Thus, all glassware used was heated for 1h at 450 °C, cooled, rinsed with 5 mL of 5% Na_2EDTA and then 10 mL of HPLC grade water three times, and air-dried prior to SPE. To prepare more matrix-matched reference matrices, several surface water and DWRF water samples collected were analyzed to verify that they did not contain detectable quantities of the analytes of interest. The wastewater-influenced water (Poudre River Site 3) and DWRF influent and effluent samples with no β-lactam compounds were selected as the reference matrices. For controls and calibration curves, appropriate amounts of working solution containing each of the analytes was added to the reference water and deionized water samples.

Oasis HLB cartridge (Waters, Millford, MA, USA), a copolymer made from a balanced ratio of hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene, was used to clean up and preconcentrate the target BLs in surface water and wastewater. SPE experiments were conducted using 60 mg/3 mL Oasis HLB cartridges. The cartridges were preconditioned with 3 mL methanol, 3 mL 0.5N HCl and 3 mL deionized water at 8 in Hg on a vacuum manifold (PrepSep 12 port, Fisher scientific, PA, USA). Water samples were prepared for extraction by adding 1 mL of Na_2EDTA and 20 µL of PEN G (1.0 mg/L) as the internal standard (IS) to a flask containing 200 mL of water. Sample pH was adjusted with 8% NH_4OH to 7.5 immediately prior to extraction.

Water samples were passed through the cartridges at 5 mL/min. After isolation, cartridges were rinsed with 3 mL of deionized water, and the analytes were eluted with 5 mL of methanol. The extracts were concentrated under a flow of N_2 gas to about 50 μL using a nitrogen evaporation system (N-Evap, Organermation Associates Inc., MA, USA). To this, 70 μL of mobile phase A (0.1% formic acid in water) was added. The resulting solutions were transferred to 0.5 mL amber autosampler vials to prevent photodegradation of the BLs. For recovery studies, appropriate amounts (0.1, 0.5 and 2.0 μg/L) of BLs and a constant amount (0.1 μg/L) of the internal standard were spiked in 200 mL of deionized water or reference water samples before extraction, and in 5 mL extracts after extraction.

3.2.5. Stability of β-lactam antibiotics in acetonitrile-ethanol-water

The stability of stock solutions of individual antibiotics was studied. Stock solutions were stored in a freezer at -20 °C for 10 days in acetonitrile-ethanol-water (25:25:50). Two mixed working solutions were made at 1 mg/L of each substance by dilution with the same solvent from each the fresh stock solution and the 10-day old stock solution. In recovery experiments to determine the extent of degradation observed for the compounds in the solvent during the interval of 10 days, 200 mL deionized water samples were spiked at three levels of 0.1, 0.5 and 2.0 μg/L for analytes, and at a constant level of 0.1 μg/L for the internal standard before extraction using each of the two working solutions. The extracts from the fortified samples were analyzed on the basis of this 10-day interval.

3.2.6. Liquid chromatography and mass spectrometry

The LC system was a HP 1100 LC (Agilent, Palo Alto, CA, USA) which consisted of an autosampler with a 100 µL loop and a quaternary pump. BLs were separated using a short Xterra MS C_{18} column (2.1 x 50 mm) with a 2.5 µm particle size distribution (Waters, Millford, MA, USA) in combination with a guard column (2.1 x 4 mm) from Phenomenex Inc. (Torrance, CA, USA). Mobile phase A (0.1% formic acid in water), phase B (methanol) and phase C (acetonitrile) were used to produce a ternary elution gradient with a flow rate of 0.25 mL/min. The separations of BLs were achieved with the following linear mobile phase gradient program: at 0 min A/B/C = 90:5:5 (v/v/v), 8 min A/B/C = 50:40:10, and 20 min A/B/C = 90:5:5. The investigated BLs eluted within 20 min. A 10-minute post time allowed re-equilibration of the column. Column temperature and injection volume were 45°C and 40 µL.

The mass spectrometer was a Finnigan LCQ Duo ion trap (ThermoQuest, CA, USA) equipped with a heated capillary interface, and a positive electrospray ionization source. ThermoQuest Xcalibur software was employed to control the mass spectrometric conditions. Full scan mode was used to acquire mass spectra, precursor ions and product ions from standard BL solutions. Infusion into the ion trap tandem mass spectrometer was performed as follows: the flow of standard compounds (3 mg/L) coming from an integrated syringe pump at a flow rate of 5 µL/min was mixed with mobile phases A/B/C at a 80:14:6 ratio through a T-piece for tuning the mass spectrometer and optimizing the ESI source. The ESI source and MS-MS parameters were automatically optimized and saved in a tune file. Spray needle voltage was set at 4.5 kV for BLs,

Table 3.1

MS-MS parameters for analysis of β-lactam antibiotics

Beta-lactams	AMOX	AMP	OXA	CLOX	CEP	PEN G^{g}
Nominal molecular mass (Da)	365.4	349.4	401.4	435.9	423.5	334.4
Isolation width (*m/z*)	2.0	2.0	3.0	3.5	3.1	3.0
Normalized collision energy (%)	26	26	35	40	25	25
Precursor ion, $[M+H]^+$, (*m/z*)	366.4	350.4	402.4	436.9	424.5	335.4
Product ions (*m/z*), (Relative abundance, %)						
$[M\text{-}OCOCH_3]^+$					363.9 (74.5)	
$[M\text{-}OCOCH_3\text{-}CO_2]^+$					**319.9**^{e} (100)	
$[M\text{-}OCOCH_3\text{-}CO_2\text{-}CO]^+$					292.0 (71.8)	
$[C_7H_6NOS]^+$					152.0 (15.0)	
$[M+H\text{-}NH_3]^+$	**348.9**^{a} (100)					
$[C_6H_9NO_2S+H]^+$	160.0 (7.0)	**160.0**^{b} (100)	160.0 (73.1)	160.0 (52.8)		**160.0**^{f} (100)
$[C_6H_9NO_2S\text{-}COOH]^+$		114.0 (5.0)				113.9 (5.5)
$[M+H\text{-}C_6H_9NO_2S]^+$	207.0 (5.2)	190.9 (13.1)	**243.0**^{c} (100)	**277.1**^{d} (100)		176.1 (28.7)
$[C_8H_{10}NO_3S\text{-}CO+H]^+$		174.0 (29.5)				
$[C_6H_5CHNH_2]^+$		106.0 (22.5)				
$[M+H\text{-}C_6H_7NO_3]^+$		192.0 (9.3)				
$[C_8H_{11}N_2O_3S+2H]^+$						217.0 (6.0)
$[C_9H_5NO+H]^+$			144.1 (14.1)			
$[C_9H_4ClNO+H]^+$				178.1 (15.0)		

[a, b, c, d, e, f] Product ions (m/z) of the highest intensity for SRM and quantitation are reported in boldface; [g] Internal standard.

automatic gain control (AGC) was on, maximum isolation time was 200 ms, and three microscans per scan were acquired. Voltages on the capillary and tube lens were 29 and 25 V, respectively. These were set by automatic optimization using the LCQ autotune program on the mass spectrometer instrument. Nitrogen was used as a sheath and auxiliary gas. Helium was used as the collision gas in the ion trap. The optimized tune conditions were as follows: sheath and auxiliary gas flow rate each was set at 50 units (a scale of arbitrary units) and capillary temperature was 175 °C. MS-MS parameters for BLs including their proposed structures, collision energy and isolation width (*m/z*) are summarized in Table 3.1.

3.2.7. Method validation study

The product ion producing the highest intensity was used for SRM and quantification to increase analytical sensitivity and selectivity in LC-MS-MS mode. For SRM the product ion of the highest intensity for the investigated AMOX, AMP, OXA, CLOX, CEP, and PEN G (IS) was reported in boldface in Table 3.1. Quantification was based on a detector response defined as the ratio of peak area of the base peak ion (the specific product ion of interest) to peak area of the base peak ion for the IS. Calibration curves were constructed for the β-lactam extracts spiked at concentrations of 0.1, 0.5, 1.0, 1.5 and 2.0 μg/L in deionized water, surface water (Poudre River Site 3), and influent and effluent wastewater before extraction.

The method detection limit (MDL) was determined using the recommended US EPA method for MDL determination [16] on the basis of the variability of multiple analyses of seven surface water (Poudre River Site 3) and wastewater (influent and effluent) extracts spiked at a concentration of 0.3 μg/L. To assess the accuracy, within- and between-run precision of the assay, repeatability experiments were carried out with the Poudre River Site 3, influent and effluent extracts spiked with 0.1, 1.0 or 2.0 μg/L of BLs. These six replicates were all run (n = 6) on three different days.

3.3. Results and discussion

3.3.1. Standard Stability

When determining trace concentrations of β-lactam antibiotics in biological or environmental samples, the behavior of the analytes is influenced by a number of factors such as low pH and organic solvents. Exposure of BLs to organic solvents during stock/working solution preparation, sample preparation or analysis can lead to degradation and/or loss of the compounds. Several studies reported the stability of β-lactam standard in various organic solvents during their storage. Tyczkowska et al. [10] found no degradation of CLOX in acetonitrile-ethanol-water (25:25:50), the least degradation of CLOX in water for one week and complete degradation of CLOX in methanol-water (50:50) in two weeks. However, Fagerquist et al. [17] did not observe any significant decline of AMOX, AMP, OXA and PEN G at 10, 1 and 0.1 mg/L in methanol-water (50:50) for 12 days at -20 °C (except perhaps for a 20% decline for CLOX and 10 % decline for dicloxacillin). Lindberg et al. [18] reported that no significant degradation of 80 mg/L AMOX and AMP in deionized water was apparent at 4 °C after one month of storage. They also presented that AMOX, AMP and cefadroxil stored at 4 °C in particular were degraded more than 30 % under acid conditions (pH 3). Nagata and Saeki observed that stock solution of 100 mg/L AMP in a phosphorous buffer solution (pH 6) stored at 10 °C was stable for 18 days [19].

Table 3.2

Stability of stock solutions at -20 °C after 10 days of storage

β-lactams	Recovery [a], X ± S.D. (%) (n = 2)			
	0.1 μg/L	0.5 μg/L	2.0 μg/L	Average
AMOX	93.8 ± 4.7	96.2 ± 5.6	101.5 ± 2.9	98.2 ± 4.4
AMP	100.3 ± 6.5	98.5 ± 3.1	105.6 ± 4.4	101.5 ± 4.7
OXA	97.2 ± 4.3	95.4 ± 0.8	103.8 ± 1.4	98.8 ± 2.2
CLOX	101.5 ± 2.8	99.8 ± 4.6	97.7 ± 1.1	99.7 ± 2.8
CEP	96.4 ± 2.6	96.7 ± 2.3	98.9 ± 3.8	97.3 ± 2.9
PEN G (IS)	99.6 ± 1.8			

[a] Recovery = peak area of analyte after 10 days of storage / peak area of analyte based on fresh stock solution × 100.

In this study, stock solutions of each analyte in acetonitrile-ethanol-water (25:25:50, pH 6.5) were stored in the dark at -20 °C for 10 days. To verify that β-lactam compounds would not degrade under the storage conditions, the stability of the target compounds in the extracts from 200 mL deionized water samples was studied. Working solutions were made from each the fresh stock solution and the 10-day old stock solution. Water samples were prepared for extraction by spiking appropriate amounts of BLs from each working solution. Table 3.2 shows average recovery of BLs determined using the ratio of the peak area of analyte for extract originating from the 10-day old stock solution to the peak area of analyte for extract originating from the fresh stock solution. No significant degradation of any of the antibiotic substances or internal standard was observed during this period. Therefore, the stock solutions were prepared weekly and a working solution was prepared daily using acetonitrile-ethanol-water in this study. These results indicate that no significant loss of the compounds was apparent with our current methodology (SPE and analysis procedures).

3.3.2. Liquid chromatography and mass spectrometry

To improve mass peak separation quality and maximize the method sensitivity, the mobile phase composition was investigated on the basis of different organic modifiers (methanol, acetonitrile with or without formic acid or trifluoroacetic acid). Several isocratic, binary and ternary gradient elution conditions were evaluated with the Xterra MS C_{18} column (2.1 x 50 mm) using different mobile phase compositions of water, methanol and/or acetonitrile at different volumetric flow rates. The measured quantification data sets (e.g. peak height, signal-to-noise (S/N) ratio) and resolution have been compared to select the separation procedures with the greatest sensitivity. The investigated isocratic elution gradients did not provide adequate separation of analytes with an analysis time of 20 min in the column. The optimal LC condition was found to be a ternary gradient of 0.1% formic acid in water, methanol and acetonitrile as described in the 'Experimental Section'. The ternary elution gradient provided the most reproducible and sensitive analytical results (e.g. the highest S/N ratio), indicating that the LC conditions of the method produced good peak shapes and sufficient separation. Reconstructed total-ion chromatograms (RTICs) for each analyte are shown in Fig. 3.3.

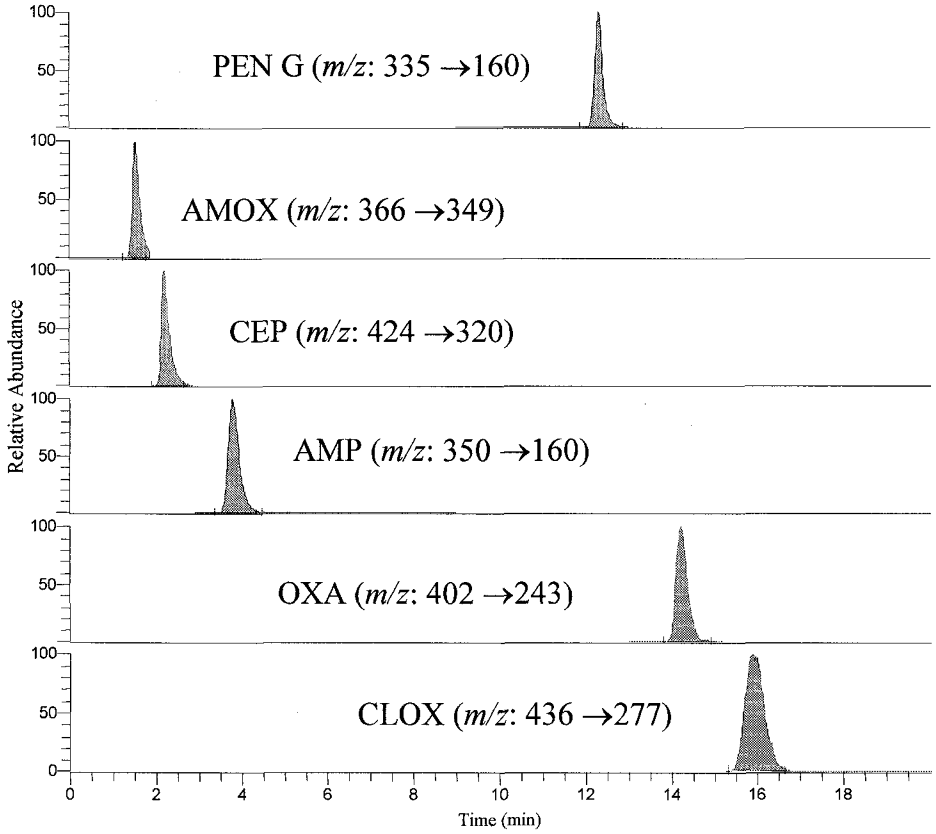


Fig. 3.3. Reconstructed total-ion chromatograms of β-lactam antibiotics spiked at a concentration of 1 μg/L for analytes and 0.1 μg/L for PEN G (internal standard) before extraction for 200 mL of Poudre River Site 3 as the surface water matrix using LC-MS-MS in SRM; *m/z* indicates precursor ion → product ion used for quantification.

Mass spectra and product ions for BLs were clearly observed in the full scan MS-MS mode (from *m/z* = 100 to 500) of the ion trap tandem mass spectrometer. Fig. 3.4 shows MS-MS spectra of precursor ion, $[M+H]^+$ for a standard solution (3 mg/L) of analytes with ESI (+) source, and their proposed fragmentation pathways. Molecular mass (Da), precursor ions, product ions, proposed product ion structures, collision energy and

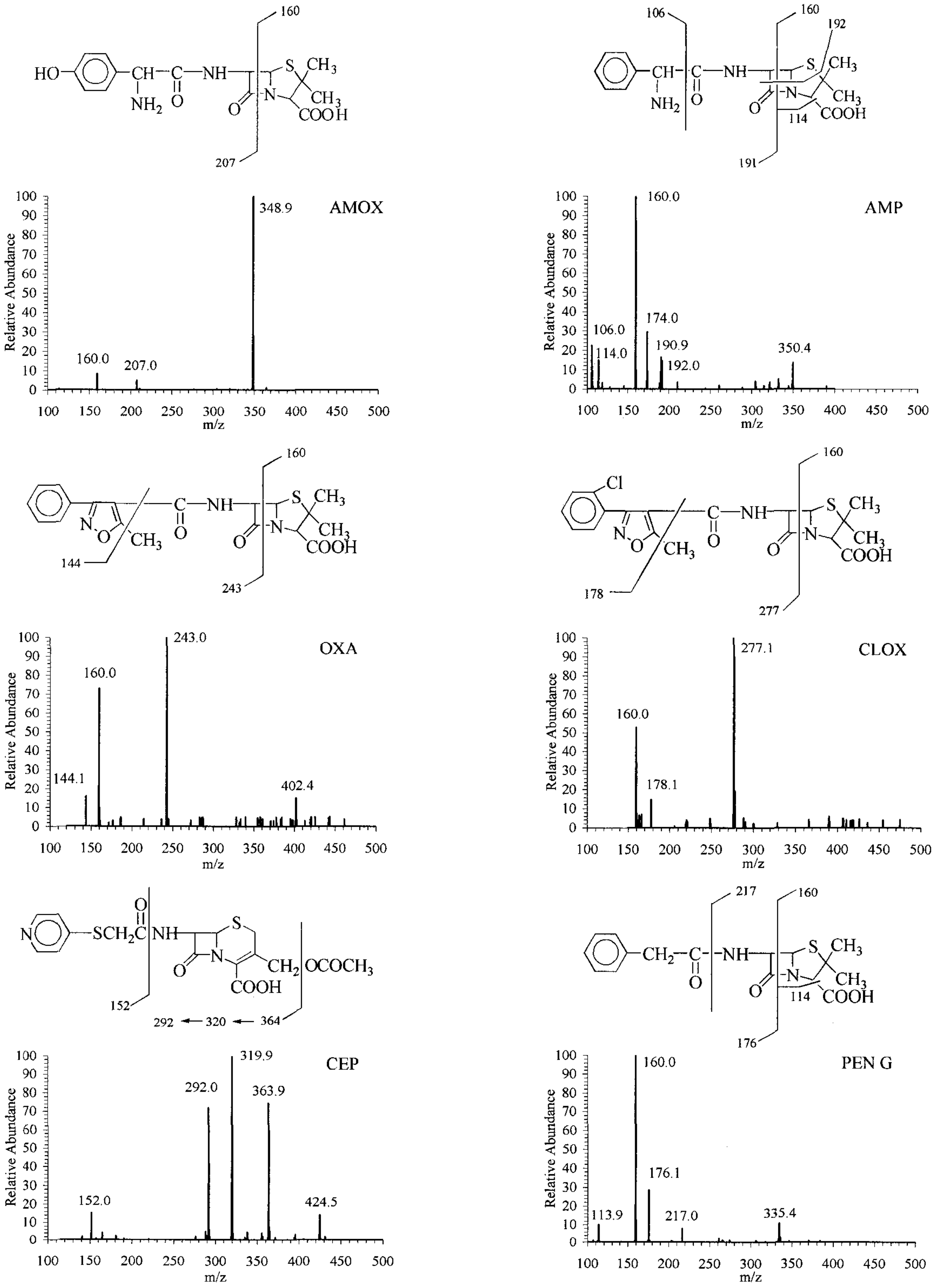


Fig. 3.4. Proposed MS-MS fragmentation pathways and full scan MS-MS spectra of β-lactams.

isolation width (*m/z*) are listed in Table 3.1. When analyzing compounds (e.g. BLs) containing an acidic group in the molecular structure by LC-MS and/or LC-MS-MS in the ESI (+) mode, formic acid as an acidic agent has often been added to the LC mobile phase both to improve peak shape and to force production of $[M+H]^+$ ions. Formic acid added to the mobile phase A in this study had the benefit of improving the spectral quality of mass spectra (Fig. 3.4). Under acidic conditions, the spectra of BLs were dominated by an abundant $[M+H]^+$ ion. These results indicate that BLs can be protonated with high efficiency by ESI (+) source when the solution contains small amounts of formic acid. Thus, each of the BLs exhibits characteristic fragmentation, and the precursor ion for all analytes was $[M + H]^+$.

Fragmentation of penicillins (AMOX, AMP, OXA, CLOX, and PEN G) was the result of the opening and cleavage of the β-lactam ring as illustrated in Fig. 3.4. Therefore, fragmentation of penicillins produced the class-specific product ion, $[C_6H_9NO_2S+H]^+$ at *m/z* 160 representative of penicillin compounds (Table 3.1, Fig. 3.4). AMOX, AMP, OXA, CLOX and PEN G exhibited the product ion (*m/z* 207, 191, 243, 277 or 176) of $[M+H-C_6H_9NO_2S]^+$, corresponding to the loss of $C_6H_9NO_2S$ from the precursor ion. Fragmentation of AMP and PEN G produced the product ion ($[C_6H_9NO_2S-COOH]^+$) at *m/z* 114 due to the loss of COOH (45 Da) from the product ion at *m/z* 160. The 192 ion ($[M+H-C_6H_7NO_3]^+$) for AMP resulted from the cleavage of the other β-lactam ring (Fig. 3.4). AMOX also exhibited the 349 ion due to the neutral loss of NH_3 (17 Da) from the precursor ion. CLOX is similar in structure to OXA, with the aromatic ring modified by an additional chloride group. Their fragmentation showed the product ion at *m/z* 144 or

178 ion, corresponding to $[C_9H_5NO+H]^+$ or $[C_9H_4ClNO+H]^+$. Fragmentation of CEP first produced the 364 ion due to the loss of $OCOCH_3$ (59 Da). CEP then exhibited the product ion at *m/z* 320 due to the loss of CO_2 (44 Da) from the 364 ion, followed by the 292 ion due to the loss of CO (28 Da) from the 320 ion.

3.3.3. Recovery

When analyzing BLs, silanization of all glassware is recommended in order to avoid the formation of epimers that are catalyzed by the presence of metal ions and by basic media in which the thiazolidine ring can be cleaved quite rapidly [20]. This effect was eliminated by silanization with 5% Na_2EDTA and HPLC grade water. BLs in aqueous solution are present as neutral, anionic or zwitterionic due to the presence of carboxylic and amino groups in the molecule (Fig. 3.1). Thus, their extraction behavior will be pH dependent. Because BLs are readily degraded under strong acidic and basic conditions as a result of the hydrolysis of the nucleophilic β-lactam ring, extraction using the HLB cartridges was performed with a neutral sample pH adjusted to 7.5 immediately prior to extraction.

The recoveries of BLs from the HLB cartridges were measured by extracting analytes from 200 mL of deionized water, surface water and wastewater (influent and effluent) spiked at 0.1-2.0 μg/L. Recoveries of BLs for SPE were determined using the ratio of the concentration of analyte for extract spiked before extraction to the concentration of analyte for extract spiked after extraction. Recoveries of BLs are the average of duplicates of 0.1, 0.5, and 2.0 μg/L of BLs spiked in water samples before and after extraction (Table 3.3). To assess the matrix effects for BLs in more complex surface water matrix, the wastewater-influenced water (Poudre River Site 3) was selected as the reference matrix for surface water.

Table 3.3

Recoveries of β-lactam antibiotics from 200 mL of water samples

β-lactams	Recovery, X ± S.D. (%) (n = 2)					
	Deionized water (μg/L)			Poudre River Site 3 (μg/L)		
	0.1	0.5	2.0	0.1	0.5	2.0
AMP	80.0 ± 5.1	82.2 ± 5.4	87.6 ± 6.0	84.7 ± 6.6	85.8 ± 6.4	77.1 ± 6.2
OXA	91.5 ± 6.3	84.6 ± 5.9	92.2 ± 7.1	91.8 ± 9.1	90.0 ± 9.3	89.4 ± 9.6
CLOX	93.1 ± 6.4	88.7 ± 7.3	94.4 ± 7.5	94.3 ± 8.8	83.9 ± 8.2	93.3 ± 8.6
CEP	94.9 ± 5.2	82.0 ± 5.0	96.8 ± 5.6	92.6 ± 6.3	91.3 ± 7.1	92.0 ± 6.5
PEN G (IS)	92.2 ± 8.6			93.8 ± 7.4		
Average recovery	90.3 ± 6.3	84.4 ± 5.9	92.8 ± 6.6	91.4 ± 7.6	87.8 ± 7.8	87.9 ± 7.7
β-lactams	Recovery, X ± S.D. (%) (n = 2)					
	Influent (μg/L)			Effluent (μg/L)		
	0.1	0.5	2.0	0.1	0.5	2.0
AMP	78.3 ± 8.2	76.5 ± 6.7	79.0 ± 6.4	82.2 ± 6.1	76.9 ± 6.3	86.7 ± 6.5
OXA	89.7 ± 9.5	94.3 ± 8.3	90.3 ± 9.8	86.6 ± 9.1	88.2 ± 9.7	95.3 ± 9.2
CLOX	87.6 ± 8.7	92.1 ± 9.5	95.9 ± 8.9	90.4 ± 9.0	94.5 ± 8.6	93.1 ± 7.4
CEP	91.8 ± 9.9	88.9 ± 8.4	89.4 ± 9.5	93.8 ± 6.3	90.7 ± 7.2	92.5 ± 6.6
PEN G (IS)	94.4 ± 8.3			95.7 ±7.8		
Average recovery	88.4 ± 8.9	87.9 ± 8.2	88.6 ± 8.6	89.7 ± 7.7	87.6 ± 7.9	91.9 ± 7.4

The average recovery of BLs (except for AMP and AMOX) including the internal standard (PEN G) from all the sample matrices was generally above 80% (Table 3.3). No concentration dependence was observed. Recoveries of BLs in the surface water and influent and effluent wastewater were similar to those in deionized water, indicating that matrix effects were minimal (except AMOX). Recoveries of BLs in the influent were similar to those in the effluent, indicating that BLs did not exhibit matrix effects in the more complex influent water matrix. The results for matrix effects in this study are consistent with findings in water matrix studies by other research groups [6, 9, 21]. These results indicate that the HLB cartridges also gave reproducible recoveries for the BLs and were effective for the isolation of the BLs. The high extraction efficiency can be due to the strong hydrophobic interactions between the analytes and the retention sorbent, resulting in the excellent performance of HLB cartridges for the extraction of BLs. For amphoteric penicillins (AMP and AMOX), recoveries were generally above 70% for AMP in all water matrices, and between 10% (influent) and 70% (deionized water) for AMOX, resulting in lower recoveries compared to the other BLs (more than 80%). It is likely that these lower values are due to the different chemical structure of AMP and AMOX with a primary amino group compared to the other investigated BLs (Fig. 3.1). AMOX especially exhibited the lower average recovery of 10, 25 and 33% in the influent, effluent and Poudre River Site 3, respectively. This indicates that matrix effects are important due to the presence of organic matter (OM) and/or natural organic matter (NOM) in the water matrices. The higher recovery of AMOX in the effluent and Poudre River Site 3 relative to the influent can be due to the lower total organic carbon concentration (9-10 mg/L in the effluent and 5-7 mg/L in the Poudre River Site 3) compared to 80-100 mg/L in the influent. These results indicate that AMOX associates with OM and/or NOM which may bind to the HLB cartridges irreversibly and can not be easily eluted separately. The lower recovery values for AMOX in this study agree with those in surface water and/or wastewater reported in the literature [9, 21-23]. Thus, AMOX was not quantified in this study. In addition, this study on the BLs using LC-

ESI-MS-MS in SRM mode confirmed suppression of the analyte signal in the influent, effluent and surface water (Poudre River Site 3) as compared with that in deionized water. The extent of suppression of analyte signal in a more complex influent was greater than that in effluent or surface water. Thus, we continue to investigate ionization surpression or enhancement of BLs in water matrices depending on ionization mode (e.g. ESI, APCI).

3.3.4. Quantification

The internal standard, PEN G was selected on the basis of its similarity to the analytes in terms of structure and retention time. It eluted within the same chromatographic time frame as the analytes (Fig. 3.3), responded well in ESI (+) mode and did not exhibit noticeable matrix effects. No PEN G was detected in any of the water samples selected as the reference matrices for quantification. Concentrations of BLs were calculated reproducibly by using the matrix-matched internal standard calibration curves to correct the minimal matrix effects to a certain extent. Calibration curves were constructed for analyte extracts spiked at a range of 0.1 to 2 μg/L in 200 mL of deionized water, Poudre River Site 3, and DWRF influent and effluent. The calibration curves were linear with correlation coefficients (R^2) > 0.99 for the MS-MS procedure. Considering the labile degradation of BLs, AMP, OXA, CLOX and CEP in the investigated water matrices were quantified within twelve hours after sample collection.

3.3.5. Method detection limit

The method detection limit (MDL) was determined using the US EPA recommended method for MDL determination [16]. Calculations of MDL using the US EPA method were based on the variability of multiple analyses of seven surface water and wastewater extracts spiked at a concentration of 0.3 μg/L for BLs. The MDL was determined by multiplying the sample standard deviation calculated from each group of the extracts spiked at the concentration by the Student's *t*-variate for a one-sided *t*-test at the 99% confidence level with *n*-1 degrees of freedom. The MDL (Table 3.4) for β-lactam compounds was between 8-10 ng/L in surface water, 13-18 ng/L in influent and 8-15 ng/L in effluent.

3.3.6. Accuracy and precision

The accuracy and the variability of the method were determined using six surface water and wastewater (influent and effluent) extracts spiked with 0.1, 1.0 and 2.0 μg/L of BLs over a period of three days. The results are summarized in Table 3.4. The accuracy range in surface water was -6.2 to +6.8% for four BLs. The accuracy range in the influent and effluent wastewater was -9.7 to +9.8% and -7.5 to +8.7%, respectively. These results are well within the recommended acceptable values of -30% to +20% at each concentration level [24]. The precision as the relative standard deviations (RSDs) calculated from these experiments ranged from 5.0 to 11.3% in surface water. The within-run precision (RSDs) in the influent and effluent was 5.4 to 14.2% and 5.9 to 13.8%, respectively.

Table 3.4

The accuracy, within-run precision and MDL of the assay for β-lactam antibiotics in surface water, influent and effluent wastewater

Samples	n	Spike Conc. µg/L	AMP Mean Conc. µg/L	AMP Accuracy %	AMP RSDs[a] %	OXA Mean Conc. µg/L	OXA Accuracy %	OXA RSDs[a] %	CLOX Mean Conc. µg/L	CLOX Accuracy %	CLOX RSDs[a] %	CEP Mean Conc. µg/L	CEP Accuracy %	CEP RSDs[a] %
Day 1:														
Poudre River 3	6	0.1	0.095	-5.3	6.4	0.098	-1.9	9.8	0.094	-6.2	8.8	0.106	5.5	7.4
	6	1.0	0.988	-1.2	5.0	0.964	-3.6	10.9	0.975	-2.5	8.4	1.016	1.6	5.6
	6	2.0	1.925	-3.7	6.7	2.016	0.8	7.3	1.928	-3.6	6.2	1.963	-1.9	7.3
Influent	6	0.1	0.092	-8.3	7.2	0.093	-6.9	14.2	0.107	6.8	10.3	0.093	-6.6	10.2
	6	1.0	1.047	4.7	6.6	1.032	3.2	11.0	1.082	8.2	11.7	0.969	-3.1	9.8
	6	2.0	2.070	3.5	8.1	2.042	2.1	7.8	1.949	-2.6	6.4	1.977	-1.2	7.0
Effluent	6	0.1	0.106	6.1	6.5	0.109	8.6	12.2	0.097	-2.8	9.8	0.108	7.6	7.9
	6	1.0	1.087	8.7	5.9	0.985	-1.5	11.6	1.072	7.2	11.9	1.055	5.5	9.1
	6	2.0	1.865	-6.8	7.3	1.964	-1.8	8.1	1.934	-3.3	6.7	2.035	1.7	6.0
Day 2:														
Poudre River 3	6	0.1	0.099	-0.7	5.7	0.103	2.6	11.0	0.106	5.7	6.6	0.098	-2.4	8.1
	6	1.0	1.068	6.8	7.0	1.032	3.2	9.1	1.064	6.4	8.3	1.036	3.6	7.4
	6	2.0	1.968	-1.6	6.2	2.037	1.8	7.2	2.019	0.9	7.0	2.097	4.8	6.6
Influent	6	0.1	0.109	9.1	7.5	0.109	9.4	11.8	0.090	-9.7	13.8	0.106	5.6	9.6
	6	1.0	1.096	9.6	8.3	0.955	-4.5	9.5	1.092	9.2	8.2	1.098	9.8	7.8
	6	2.0	1.885	-5.8	6.5	1.954	-2.3	10.4	1.946	-2.7	7.3	2.031	1.5	5.9
Effluent	6	0.1	0.094	-6.5	9.4	0.096	-3.8	12.8	0.093	-7.5	13.5	0.105	5.4	6.7
	6	1.0	1.039	3.9	8.8	1.062	6.2	7.6	0.968	-3.2	9.0	0.963	-3.7	8.4
	6	2.0	1.957	-2.2	6.3	2.037	1.9	10.1	2.075	3.7	7.2	2.033	1.7	12.2
Day 3:														
Poudre River 3	6	0.1	0.096	-3.9	6.5	0.097	-3.2	11.3	0.104	4.4	8.0	0.105	5.1	7.5
	6	1.0	1.048	4.8	7.2	1.055	5.5	9.2	0.963	-3.7	6.4	1.005	0.5	6.1
	6	2.0	2.014	0.7	5.8	1.977	-1.2	7.9	2.031	1.5	8.1	1.963	-1.9	7.2
Influent	6	0.1	0.092	-8.5	8.9	0.095	-4.8	14.0	0.092	-8.5	13.0	0.106	6.4	10.8
	6	1.0	1.052	5.2	10.6	1.082	8.2	10.1	0.958	-4.2	8.5	1.093	9.3	9.0
	6	2.0	1.937	-3.2	7.7	2.026	1.3	9.7	2.090	4.5	6.3	2.043	2.2	5.4
Effluent	6	0.1	0.095	-5.3	8.6	0.096	-3.7	13.8	0.108	7.8	8.3	0.097	-2.6	10.1
	6	1.0	1.015	1.5	6.9	1.051	5.1	10.3	1.062	6.2	7.7	1.063	6.3	9.2
	6	2.0	2.060	3.0	7.4	2.022	1.1	9.7	1.969	-1.6	6.5	1.915	-4.3	6.6
MDL	Poudre River 3		10			8			8			8		
(ng/L)	Influent		17			14			13			18		
	Effluent		13			8			9			15		

[a]RSDs: Relative standard deviations.

The analytical data of 18 replicates of each surface water, influent and effluent sample over three days of validation study allowed the determination of between-run precision.

Table 3.5

The accuracy, between-run of the assay for β-lactam antibiotics in surface water, influent and effluent wastewater

			AMP			OXA			CLOX			CEP		
Samples	n	Theoretical Conc. µg/L	Mean Conc. µg/L	RSDs[a] %	RE[b] %	Mean Conc. µg/L	RSDs[a] %	RE[b] %	Mean Conc. µg/L	RSDs[a] %	RE[b] %	Mean Conc. µg/L	RSDs[a] %	RE[b] %
Poudre River 3	18	0.1	0.097	6.2	3.4	0.099	10.7	0.7	0.101	7.8	-1.4	0.103	7.7	-2.9
	18	1.0	1.035	6.4	-3.5	1.017	9.7	-1.7	1.001	7.7	-0.1	1.019	6.3	-1.9
	18	2.0	1.969	6.3	1.5	2.010	7.5	-0.5	1.993	7.1	0.4	2.007	7.0	-0.4
Influent	18	0.1	0.097	7.9	2.6	0.099	13.3	0.8	0.096	12.4	3.8	0.102	10.2	-1.8
	18	1.0	1.065	8.5	-6.5	1.023	10.2	-2.3	1.044	9.5	-4.4	1.053	8.8	-5.3
	18	2.0	1.964	7.4	1.8	2.008	9.3	-0.4	1.995	6.7	0.3	2.017	6.1	-0.8
Effluent	18	0.1	0.098	8.2	1.9	0.100	12.9	-0.4	0.099	10.5	0.8	0.103	8.2	-3.5
	18	1.0	1.047	7.2	-4.7	1.033	9.8	-3.3	1.034	9.6	-3.4	1.027	8.9	-2.7
	18	2.0	1.961	7.0	2.0	2.008	9.3	-0.4	1.992	6.8	0.4	1.994	8.3	0.3

[a] RSDs: Relative standard deviations; [b] RE (Relative error) = ((Theoretical conc. - Mean conc.) / Theoretical conc.) x 100.

The results are reported in Table 3.5. Relative error (RE) was determined by comparing the theoretical concentration of each sample with the mean concentration determined from three days of analysis. Acceptable values for the between-run precision and RE were defined as ≤15%. The RSDs were 6.1 – 13.3% for the four BLs in water matrices. RE was between 0.1 and 6.5% absolute for β-lactam compounds.

3.3.7. Occurrence, distribution, and fate of BLs

The developed method was successfully applied to evaluate the occurrence and fate of the four β-lactam antibiotics in both wastewater from the DWRF and surface water from the Cache la Poudre River through pristine, urban and agricultural landscapes in northern Colorado. A total of 60 surface water, 72 influent and 72 effluent wastewater samples were analyzed. The average concentrations of those compounds detected above their MDL indicated a relative percent difference of 8% on analysis of duplicate samples.

Most of samples were negative for all analytes. However, in three DWRF influent samples, CLOX was detected at 15 ng/L once and OXA was found at less than 20 ng/L twice. No β-lactam compounds were detected in any of the effluent samples. As expected, no β-lactam antibiotics were found in the pristine river section (Site 1) since there are no urban or agricultural influences. None of the β-lactam compounds were also measured at the fringes of the urban area (Site 2) either indicating minimal urban or agricultural influences. OXA was found at 10 ng/L once at Site 3, immediately downstream of the WWTP near the urban area. Its occurrence is most likely due to the influence of urban activity since OXA has only human applications. β-lactam analytes were not detected in any of the samples at Site 4, which is influenced by the surrounding agricultural activities. At Site 5 with the greatest influence of agriculture, AMP and CEP were first detected once at concentrations of 11 and 9 ng/L, respectively. Fig. 3.5 shows RTICs for antibiotic compounds found in water samples. The low concentration levels (less than 20 ng/L), and low detection frequency of these compounds in most of the samples is counter to other studies by the authors in which tetracyclines, sulfonamides, macrolides and ionophore antibiotics were detected at the higher sub μg/L range in water samples from the same river sites and DWRF of this study [25-28]. These results indicate that β-lactam antibiotics appear to be less persistent in the aquatic environment, since these compounds readily undergo hydrolysis due to the chemically unstable β-lactam ring. Thus, this preliminary occurrence study revealed that intake from their human and veterinary applications to the small mixed-watershed is of minor importance.

3.4. Conclusions

A sensitive, reliable and reproducible LC-MS-MS method in SRM mode coupled with SPE has allowed the detection of five β-lactam compounds in natural and waste water. High extraction recovery combined with high sensitivity of the method generated MDLs below 10 ng/L for these antibiotics in surface water and less than 20 ng/L in wastewater. The within-run and overall precision of the method was found to be less than 15% absolute for these compounds.

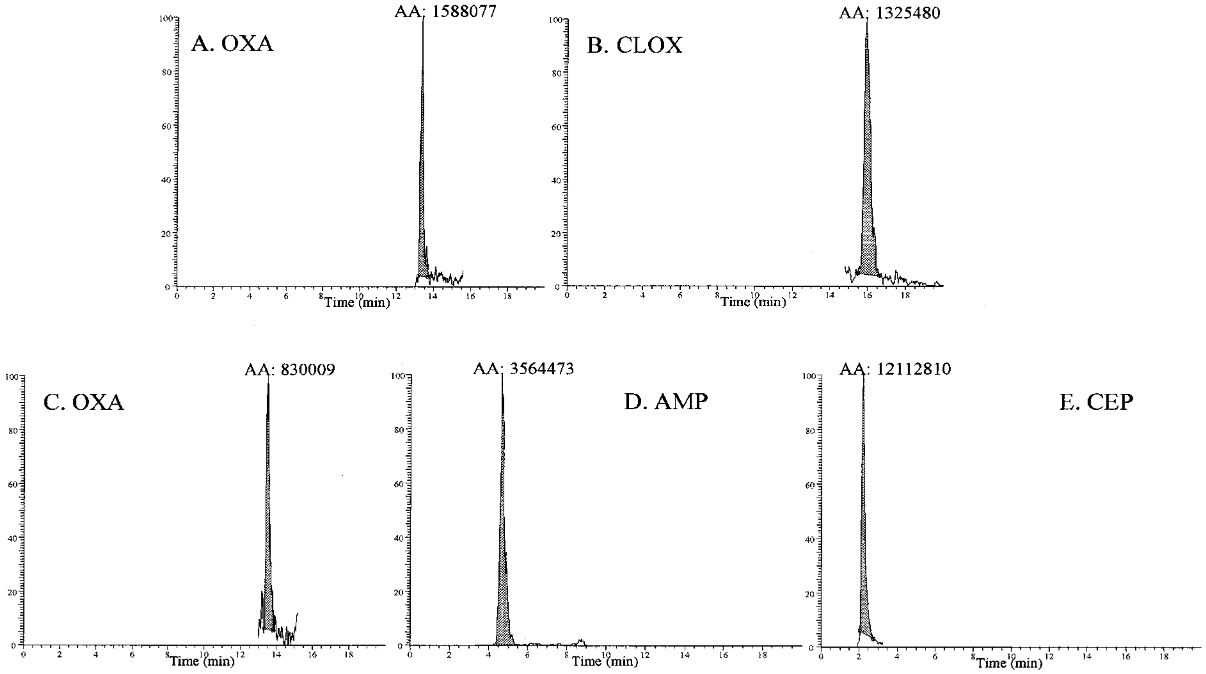


Fig. 3.5. Reconstructed total-ion chromatograms (RTICs) for β-lactam antibiotics found positive in five water samples; A: OXA at 17 ng/L in the influent wastewater; B: CLOX at 15 ng/L in the influent wastewater; C: OXA at 10 ng/L in the Poudre River Site 3; D: AMP at 11 ng/L in the Poudre River Site 3; E: CEP at 9 ng/L in the Poudre River Site 5; AA: peak area of analytes.

The method was applied by analyzing 200 surface water and wastewater samples to evaluate the occurrence of the four β-lactam antibiotics (AMP, OXA, CLOX and CEP) on a river and in a wastewater treatment plant in northern Colorado. No β-lactam compounds were detected in most of samples. The measured concentration levels of the compounds were between 15-17 ng/L in three influent waste water samples and 9-11 ng/L in three surface water samples. The occurrence rate in the samples was about 3% based on 6 positive samples out of a total of 200. To our knowledge, this is the first report on the analytical method for and occurrence of cephapirin in water matrices.

As expected, we verified that β-lactam antibiotics are not frequently found above the low ng/L range in the aquatic environment. Because the presence of these compounds in the aquatic and terrestrial environments can not be ruled out, there is a need to continuously predict the concentrations of these antibiotic compounds and to design strategies to minimize exposure to these compounds in more complex aqueous matrices (e.g. hospital and lagoon wastewater) and solid matrices (e.g. sediment, soil, sewage sludge, bio-solid, animal manure).

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Chapter 4

Occurrence and fate of β-lactam and polyether ionophore antibiotics in surface water, urban wastewater, and sediment

Abstract

The occurrence and fate of antibiotic compounds in the aquatic environment has been recognized as an emerging area of interest in environmental chemistry. An analytical method was developed and validated for determining β-lactam and polyether ionophore antibiotics in surface water, urban wastewater, and sediment based on solid-phase extraction (SPE) and liquid chromatography-ion trap tandem mass spectrometry (LC-MS-MS). The average recovery of β-lactams and polyether ionophores from all the fortified samples was generally better than 80% (except for amoxicillin and ampicillin). A total of 400 actual water and sediment samples were analyzed using SPE-LC-MS-MS to evaluate the occurrence and fate of these antibiotic compounds in a river and a wastewater treatment plant in northern Colorado. Most samples were negative for β-lactam compounds. In those samples with positive results, β-lactam compounds were found at 15-17 ng/L in influent wastewater samples, 9-11 ng/L in surface water samples, and 0.13-0.36 μg/kg in sediment samples. No β-lactam compounds were detected in any effluent wastewater samples. The β-lactams are expected to be easily eliminated in wastewater treatment plants due to the lability of the β-lactam ring towards chemical and microbial degradation. The average concentration of ionophores ranged 0.03-0.05

μg/L in surface water samples and 0.5-3.1 μg/kg in sediment samples. These results confirm that β-lactam and ionophore antibiotics are adsorbed by sediment.

Key words: antibiotics; β-lactams; polyether ionophores; surface water; wastewater; sediment

4.1.Introduction

The β-lactam antibiotics (BLs) consist of a variety of semi-synthetic penicillins – including amoxicillin (AMOX), ampicillin (AMP), penicillin G (PEN G), oxacillin (OXA), and cloxacillin (CLOX) – and cephalosporins such as cephapirin (CEP). All BLs have at their basic structure a β-lactam ring, responsible for their antibacterial activity, and variable side chains that account for the major differences in their chemical and pharmacological properties (Fig. 4.1). The BLs are widely used for their antimicrobial activity against both gram-positive and gram-negative organisms (1). The ionophore antibiotics, including monensin (MON), salinomycin (SAL), and narasin (NAR), are produced by various strains of *Streptomyces* and have microbiological activities against gram-positive bacteria, fungi, and protozoa. In veterinary application, these antibiotics are used as feed additives (coccidiostats) for poultry and livestock and as growth promoters for ruminants (2, 3).

Surface and groundwater contamination by such antibiotics is generally considered to be due to point and non-point sources of municipal wastewater and agricultural runoff (4).

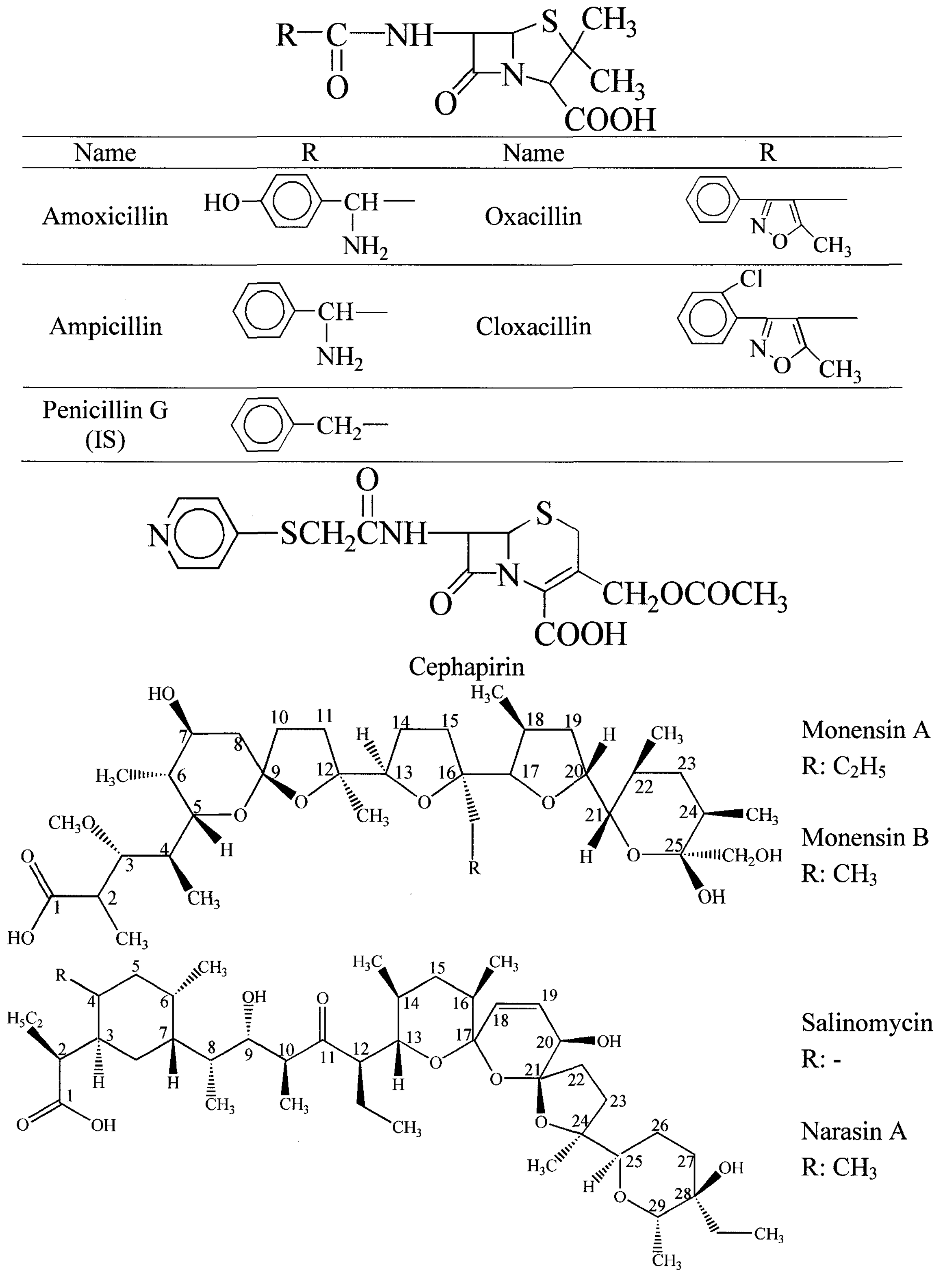


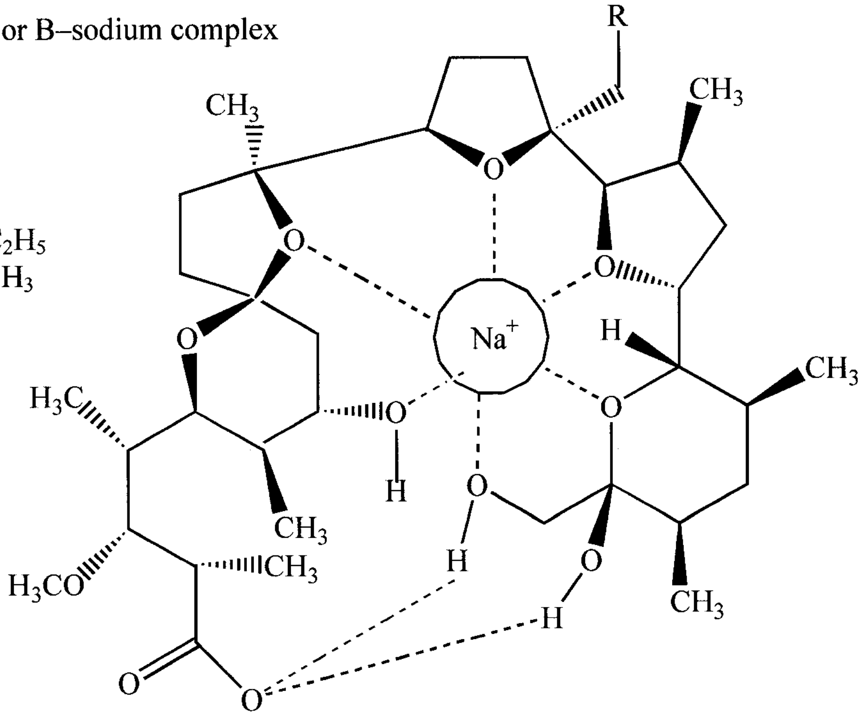
Fig. 4.1. Chemical structures of penicillins, cephapirin, and polyether ionophores.

A significant percentage of these antibiotics consumed by humans is excreted, unchanged, and enters wastewater treatment plants (WWTP) in domestic sewage (5, 6). Because these compounds are not removed completely from domestic wastewater during treatment, there is potential for antibiotic residues to be released in WWTP discharge to the environment. Similarly, a high proportion of antibiotics consumed by animals is excreted in urine and feces and enters the environment via agricultural runoff.

The occurrence and fate of these antibiotics in the aquatic environment are an emerging area of interest due to their potential impacts on the environment (7, 8). Christian et al. (9) found AMOX, AMP, mezlocillin, flucloxacillin, and piperacillin in surface water at concentrations up to 48 ng/L; in 28 of 32 river water samples, the investigators found a maximum concentration of AMOX no greater than 10 ng/L. Campagnolo et al. (10) measured PEN G levels from 2.1 to 3.5 μg/L in lagoon water, and Zhou et al. (11) found 3.2 mg/L AMP in pharmaceutical wastewater. Brown et al. (12) and Kümmerer (13) reported that PEN G and BLs levels in hospital wastewater measured from 850 to 5,200 ng/L and from 20 to 80 μg/L, respectively. AMOX was detected in concentrations from 15 to 120 ng/L in effluents collected from several sewage treatment plants (14). Donoho (15) documented the presence of MON in cattle feces and urine, and Catherman et al. (16) measured NAR concentrations of 1.0 to 725.0 μg/kg in poultry feces and manure. In a previous study, the current investigators reported MON, SAL, and NAR in surface water from 0.03 to 0.06 μg/L (17).

A. Monensin A or B–sodium complex

MON A: R=C_2H_5
MON B: R=CH_3



B. Salinomycin or narasin A–sodium complex

SAL: R= --
NAR A: R=CH_3

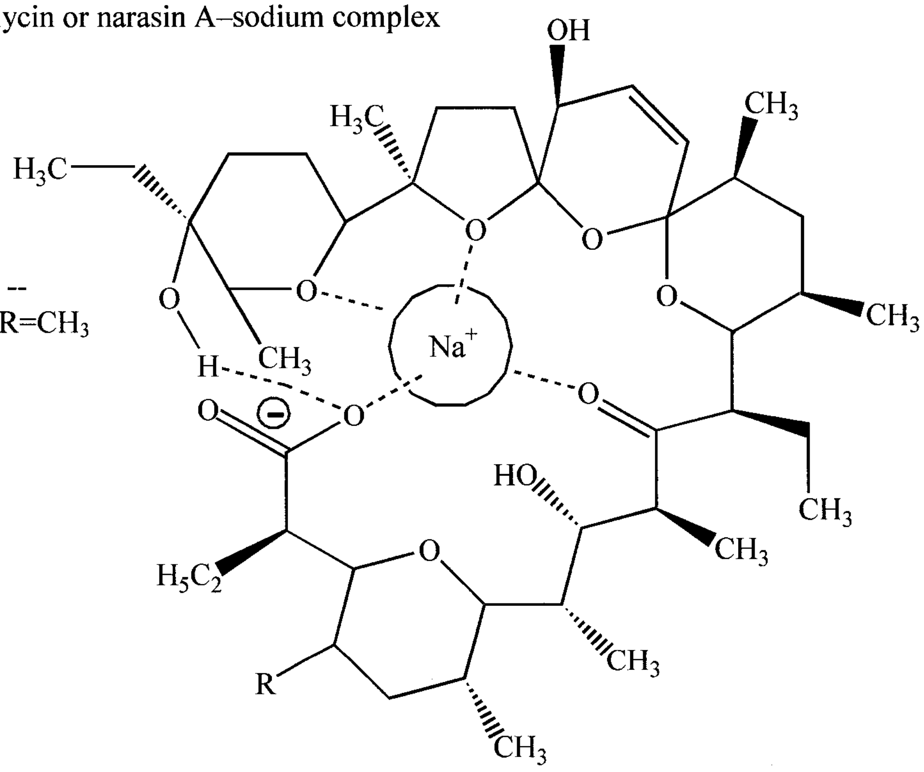


Fig. 4.2. Schematic representations of the cyclic structures of ionophores-sodium complexes.

However, several researchers have reported not detecting the subject BLs in different water compartments, including WWTP effluents (18), surface water (5, 18-20) and groundwater (20). Intact BLs do not occur frequently in the environment, because the β-lactam ring is subject to opening by chemical hydrolysis or by β-lactamases, a widespread enzyme in bacteria (9). Chemical detection of the ionophore antibiotics is complicated by their limited solubility in water, owing to the formation of lipid-soluble, pseudo-macrocyclic complexes with alkali metal cations in solution such as Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+} (Fig. 4.2) (3,21,22). The formation of neutral complexes from multiple-charged adducts of a single polyether ionophore presents measurement limitations for some analytical techniques. Further, the compounds with hemi-actetal or ketal structures tend to be acid labile (Fig. 4.1). While their lipophilic property facilitates their extraction from aqueous matrices, the same lipophilic character of their salts limits the type of acid-base extraction that can be used with those compounds.

Due, in part, to the difficulty of analytical determination of these antibiotics, there has been a lack of study on the occurrence, fate, and transport of β-lactam and ionophore antibiotics in the environment, at the local and watershed level. More specifically, little data are available on the occurrence of pharmaceuticals such as these antibiotics in natural sediments and the role of sediments in removing antibiotics from the environment (23). The current study was designed to address the need for sensitive and reliable analytical methods to measure concentrations of BLs and PEs in the aquatic environments, and this report details a method for the determination of AMOX, AMP, OXA, CLOX, CEP, MON, SAL, and NAR in surface water, WWTP influent and effluent, and sediments, using solid-phase extraction (SPE) and liquid chromatography-ion trap tandem mass spectrometry (LC-MS-MS) with positive ion electrospray ionization, ESI (+) and selected reaction monitoring (SRM). This report also discusses the occurrence and fate of BLs and PEs in a small watershed in northern Colorado, U.S.A.

4.2. Experimental

4.2.1. Materials for Chemical Analysis

All antibiotics (purity, >90%), formic acid (>95%) and Na_2EDTA (99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethanol, and acetonitrile were HPLC grade (Sigma-Aldrich, St. Louis, MO, USA). Individual stock solutions of BLs were prepared weekly by dissolving each compound in acetonitrile-ethanol-water (25:25:50) at a concentration of 1,000 mg/L, and stock solutions of the polyether standards were prepared monthly by dissolving each compound in methanol at a concentration of 100 mg/L. All stock solutions were stored at -20 °C in the dark. Mixed working solutions (10, 1 and 0.1 mg/L) were prepared daily by diluting the individual stock solution with the same solvent and were stored at 4 °C in the dark. PEN G and simatone, the internal standard working solution (1 mg/L), were prepared by diluting the standard solution with the solvent and stored at 4 °C and replaced with a freshly prepared solution every day.

4.2.2. Description of the investigated river area and wastewater treatment plant

The Cache la Poudre (Poudre) River in northern Colorado was selected as a small mixed watershed (Fig. 4.3). The Poudre River originates near the continental divide in Rocky Mountain National Park and flows east through steep, mountainous terrain for approximately 69.2 km, courses through the Front Range city of Fort Collins, then flows through approximately 72.4 km of mostly agricultural land before merging with the South Platte River in Greeley, Colorado. The Drake Water Reclamation Facility (DWRF) and the Mulberry Water Reclamation Facility (MWRF) are the WWTPs noted

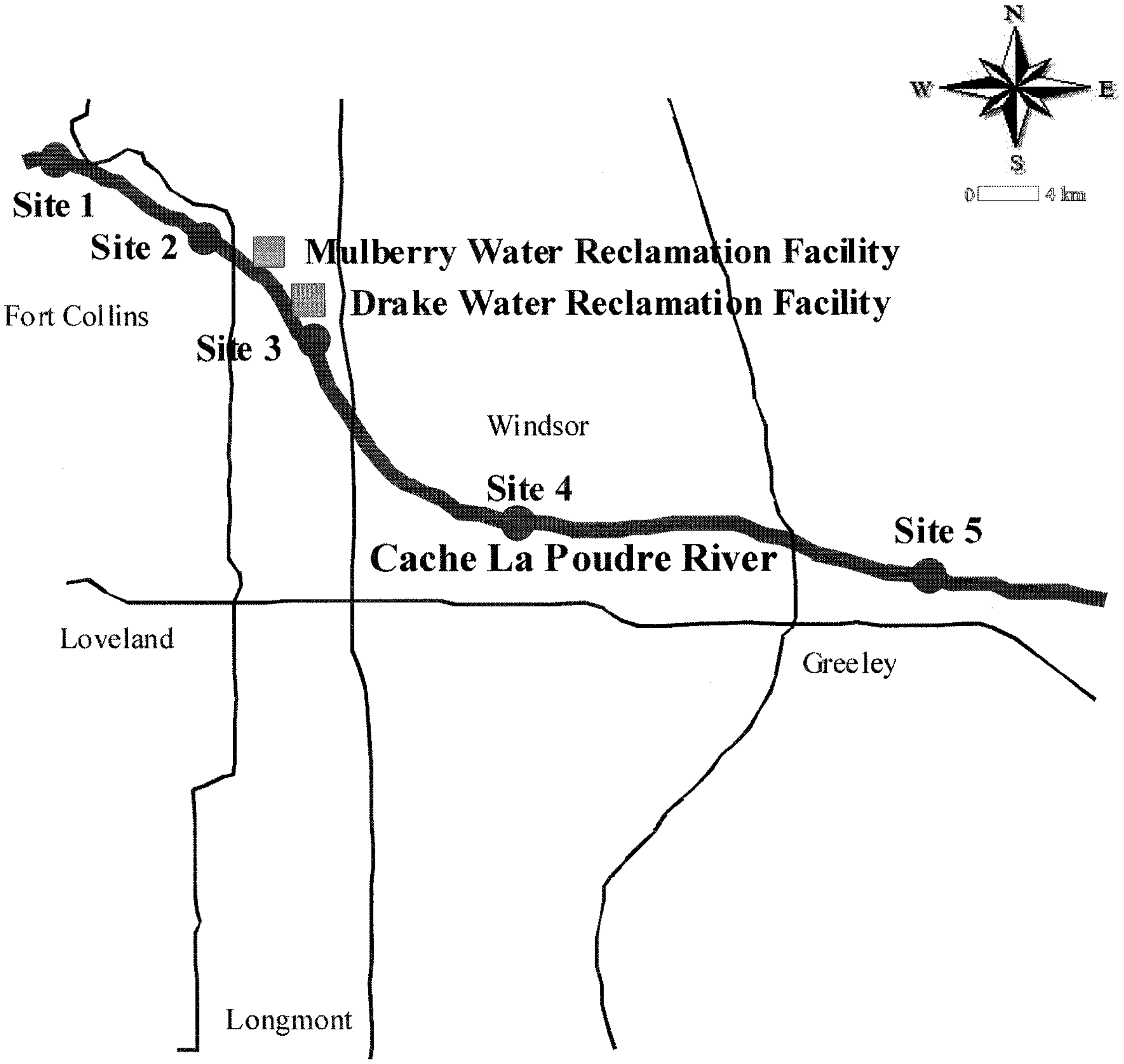


Fig. 4.3. Sampling sites along the Cache la Poudre River in northern Colorado.

in this study (Fig. 4.3). The two facilities serve a population of approximately 128,000 in Fort Collins. The DWRF treats from 45,000 to 50,000 m^3/d of domestic sewage (95%) and industrial wastewater (5%) by a combination of pretreatment; primary, intermediate, and secondary clarification; and chlorine disinfection. The MWRF treats from 9,200 to 9,600 m^3/d of domestic sewage (75%) and industrial wastewater (25%) by pretreatment; primary, intermediate, and secondary clarification; and ultraviolet (UV) light disinfection.

4.2.3. Sample collection and preparation

Approximately 200 surface water and sediment samples were collected from five locations on the Poudre River over a period of twenty months from July 2004 to February 2006. Sites 1 and 2 were 35.4 and 11.3 km upstream of the DWRF,

Table 4.1. Characterization of the raw influent and treated effluent of the DWRF and MWRF

		Raw influent		Final effluent	
Parameter		DWRF	MWRF	DWRF	MWRF
pH		6.7	6.8	7.2	7.2
Conductivity	(mS/cm)	707	771	616	678
NH_4-N	(mg/L)	16.8	15.9	0.9	0.3
TOC	(mg/L)	90.5	62.7	8.7	4.9
BOD	(mg/L)	200.1	164.3	7.4	7.0
COD	(mg/L)	336	311	51	56
SS	(mg/L)	215.6	132.5	8.1	7.2

DWRF: Drake Water Reclamation Facility
MWRF: Mulberry Water Reclamation Facility

respectively (Fig. 4.3). The samples were collected in the center of the stream as a depth composite using a water grab sampler and soil auger. A total of 200, 24-hour composite samples of raw influent and final effluent were collected from the DWRF and MWRF over the same period of twenty months. A flow-proportioned automatic sampler captured the 24-hour effluent composite sample based on influent flow. Table 4.1 shows typical characteristics for the influent and effluent constituents of DWRF and

Table 4.2. Characterization of Poudre River sediment samples

Parameter		Poudre River Site 1	Poudre River Site 2	Poudre River Site 3	Poudre River Site 4	Poudre River Site 5
pH		5.90	5.75	7.10	7.33	8.01
Conductivity	(mS/cm)	0.5	1.2	0.7	2.1	0.6
Dry matter	(%)	87.5	82.4	84.0	68.8	90.1
CEC	(meq/100g)	2.9	4.8	5.8	16.7	4.9
NH_4-N	(mg/kg)	3.8	8.9	4.2	9.8	5.3
Total N	(%)	0.018	0.047	0.023	0.154	0.010
Ca	(mg/kg)	322	501	515	1207	707
Mg	(mg/kg)	47.5	121	48.9	426	86.5
Na	(mg/kg)	92.2	98.4	98.1	175	108
K	(mg/kg)	7.8	8.5	5.3	55.9	3.4
Total Cd	(mg/kg)	0.84	0.93	0.62	1.41	0.93
Total Co	(mg/kg)	1.85	3.62	2.2	4.85	1.84
Total Cu	(mg/kg)	6.6	8.5	7.8	12.3	7.1
Total Fe	(%)	1.081	1.118	0.689	1.418	1.158
Total Zn	(mg/kg)	12.0	2.8	2.2	5.0	2.0
Organic matter	(%)	0.50	0.60	0.90	2.10	1.30
Sand	(%)	94.71	93.63	85.62	64.40	91.31
Silt	(%)	2.86	3.40	5.64	23.19	4.66
Clay	(%)	2.43	2.97	8.73	12.41	4.03
Soil type		sand	sand	loamy sand	sandy loam	sand

MWRF. Characterization of the sediments is shown in Table 4.2.

The influent water samples were centrifuged at 3,000 rpm for 40 min at 4 °C in a centrifuge (IEC Centra CL 3R, MA, USA) with integral cooling system. All aqueous samples were filtered through 0.4-micron glass fiber filters (Millipore, MA, USA) and stored at 4 °C. SPE and measurement were completed within 12 hours after collection of samples due to the tendency toward biodegradation and/or hydrolysis of the labile β-lactam compounds.

4.2.4. Preparation of ionophore-sodium salt

To convert the investigated ionophores into a single sodium adduct species, appropriate amounts (0.005% ~ 2% (w/v)) of sodium chloride as a surplus of sodium were dissolved in water and sediment samples, then the samples were left to stand for 30 min prior to SPE and LC-MS-MS analysis. The added amount of sodium chloride was based on the sum of cation concentrations (e.g. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+}) in the water and sediment samples.

4.2.5. Solid-phase extraction

To prepare additional matrix-matched reference matrices, several collected samples of surface water, DWRF water, and sediment were analyzed to verify that they did not contain detectable quantities of the analytes of interest. The wastewater-influenced water and sediment (Poudre River Site 3) and DWRF influent and effluent samples with no β-lactam and ionophore compounds were selected as the reference matrices. For controls and calibration curves, appropriate amounts of working solution containing each of the analytes were added to the reference water and the sediment samples.

The SPE analyses were conducted using 60 mg/3 mL Oasis HLB cartridges (Waters, Millford, MA, USA). The cartridges were preconditioned on 3 mL-aliquots of methanol, 0.5N HCl, and deionized water at 8-in Hg on a vacuum manifold (PrepSep 12 port, Fisher scientific, PA, USA). Water samples were prepared for extraction of BLs by adding 1 mL of Na_2EDTA and 20 μL of PEN G (1.0 mg/L) as the internal standard (IS) to a flask containing 200 mL of water. Sample pH was adjusted with 8% NH_4OH to pH 7.5 immediately prior to extraction. 120 mL of aqueous samples added at 0.005% ~ 1% (w/v) of sodium chloride and 12 μL of the internal standard, 1.0 mg/L simatone, were prepared for extraction of PEs. Because the investigated ionophores are acid and/or base labile, the extraction using the HLB cartridges was performed with the neutral sample pH adjusted by 0.01M NaOH to pH 7.5 immediately prior to extraction. After 10 g of sediment samples were extracted with 120 mL of 0.001M citric acid and 0.001% formic acid for BLs and PEs, the samples were centrifuged at 3,000 rpm for 10 min at 4 ^{o}C in a centrifuge (IEC Centra CL 3R, MA, USA) with integral cooling system. All samples were filtered through 0.4-micron glass fiber filters (Millipore, MA, USA) and adjusted by 8% NH_4OH and 0.01M NaOH to pH 7.5 immediately prior to extraction, respectively.

Next, aqueous samples were passed through the cartridges at 5 mL/min. After isolation, cartridges were rinsed with 3 mL of deionized water, and the analytes were eluted with 5 mL of methanol. The extracts were concentrated under a flow of N_2 gas to about 50 µL using a nitrogen evaporation system (N-Evap, Organermation Associates Inc., MA, USA). To this, 70 µL of mobile phase A (0.1% formic acid in water) was added. The resulting solutions were transferred to 0.5 mL amber autosampler vials in order to prevent photodegradation of the BLs and PEs.

4.2.6. Liquid chromatography and mass spectrometry

Liquid chromatography (LC) was completed with an HP 1100 LC (Agilent, Palo Alto, CA), which consisted of an autosampler with a 100 µL loop and quaternary pump. BLs and PEs were separated using a short Xterra MS C_{18} column (2.1 x 50 mm) with a 2.5 µm particle size distribution (Waters, Millford, MA, USA) in combination with a guard column (2.1 x 4 mm) from Phenomenex Inc. (Torrance, CA, USA). Mobile phase A (0.1% formic acid in water), phase B (methanol) and phase C (acetonitrile) were used to produce a ternary elution gradient with a flow rate of 0.25 mL/min for BLs and 0.20 mL/min for PEs. The separations of BLs were achieved with the following linear mobile phase gradient program: at 0 min A/B/C = 90:5:5 (v/v/v); 8 min A/B/C = 50:40:10; and 20 min A/B/C = 90:5:5. Separations of PEs were achieved with the following mobile phases: at 0 min A/B/C = 18:74:8; 9 min A/B/C = 13:1:86; and 10 min A/B/C = 18:74:8. A 10-minute post-separation time allowed re-equilibration of the column. Column temperature of BLs and PEs was 45°C and 25°C, respectively.

The mass spectrometer used was a Finnigan LCQ Duo ion trap (ThermoQuest, CA, USA) equipped with a heated capillary interface and a positive electrospray ionization source. ThermoQuest Xcalibur software was used to control the mass spectrometric conditions. Full scan mode was applied to acquire mass spectra, precursor ions, and product ions from standard BL and PE solutions. Mass spectral data shown in this study were obtained from a LCQ Duo ion trap tandem mass spectrometer equipped with an ESI source operated in positive ion mode. Infusion into the ion trap tandem mass spectrometer was performed as follows: the flow of standard compounds for 3 mg/L of BLs and 7 mg/L of PEs, delivered from an integrated syringe pump at a flow rate of 5 μL/min, was mixed with mobile phases A/B/C at a 80:14:6 and 15:32:53 ratio through a T-piece for tuning the mass spectrometer and optimizing the ESI source, respectively. The ESI source and MS-MS parameters were automatically optimized and saved in a tune file. Spray needle voltage was set at 4.5 kV for both compounds, automatic gain control (AGC) was on, maximum isolation time was 200 ms for BLs and 300 ms for PEs, and three microscans per scan were captured. Voltages on the capillary and tube lens were 29 and 25 V for BLs, and 38 and 25 V for PEs, respectively, set by automatic optimization using the LCQ autotune program on the mass spectrometer instrument. Nitrogen was used as a sheath and auxiliary gas. Helium was used as the collision gas in the ion trap. The optimized tune conditions were as follows: sheath and auxiliary gas flow rate each was set at 50 units (a scale of arbitrary units), and capillary temperature was 175 °C. MS-MS parameters for BLs and PEs, including precursor ion, product ion, and collision energy, are summarized in Table 4.3.

Table 4.3. Optimal MS/MS parameters for analysis of selected antibiotics

Analyte	Precursor ion, $[M+H]^+$ (*m/z*)	Product ions (*m/z*)	Normalized collision energy (%)
AMOX	366.4	**348.9**, 160.0, 207.0	26
AMP	350.4	**160.0**, 174.0, 190.9	26
OXA	402.4	**243.0**, 160.0, 144.1	35
CLOX	436.9	**277.1**, 160.0, 178.1	40
CEP	424.5	**319.9**, 363.9, 292.0	25
PEN G (IS)	335.4	**160.0**, 176.1, 217.0	25
Analyte	Precursor ion, $[M+Na]^+$ (*m/z*)	Product ions (*m/z*)	Normalized collision energy (%)
MON A	693.5	**675.5**, 461.3, 657.5	33
MON B	679.4	**661.5**, 465.3, 643.4	30
SAL	773.5	**755.5**, 531.4, 513.3	35
NAR A	787.5	**769.5**, 531.4, 545.3	35

Product ions (*m/z*) of the highest intensity for SRM and quantification are reported in boldface

4.2.7. Method validation study

The product ion producing the highest intensity was used for SRM and quantification to increase analytical sensitivity and selectivity in LC-MS-MS mode. For SRM, the product ion of the highest intensity for the investigated AMOX, AMP, OXA, CLOX, CEP, PEN G (IS), MON A and B, SAL, and NAR A is identified in Table 4.3. In particular, quantification was based on a detector response defined as the ratio of peak area of the base peak ion (the specific product ion of interest) to peak area of the base peak ion for the IS. Calibration curves were constructed with β-lactam extracts spiked at concentrations of 0.1, 0.5, 1.0, 1.5, and 2.0 μg/L in surface water (Poudre River Site 3) and influent and effluent wastewater before extraction. Calibration was made with

PEs spiked into water samples before extraction at 0.05, 1, 2, and 5 μg/L in surface water (Poudre River Site 3). Calibration with β-lactam and ionophore extracts spiked into sediment samples before extraction was made from 1 to 20 μg/kg in sediment (Poudre River Site 3).

Specifically, the method detection limit (MDL) was determined using the methodology recommended by the U.S. Environmental Protection Agency (USEPA) (24), based on the variability of multiple analyses of seven surface water (Poudre River Site 3) and wastewater (influent and effluent) extracts spiked at a concentration of 0.3 μg/L and 0.2 μg/L for BLs and PEs, respectively, as well as seven sediment (Poudre River Site 3) extracts spiked at 3 μg/kg for each antibiotic.

4.3. Results and discussion

4.3.1. Liquid chromatography and mass spectrometry

The LC method employing a ternary gradient sequence combined with ESI(+)-MS-MS allowed the rapid, sensitive, selective, and reliable determination of the investigated BLs and PEs in water and sediment matrices. Specifically, the mass peaks corresponding to BLs and PEs appeared on the total-ion chromatograms (TICs) monitored at the selected product ion. The data were processed by creating the reconstructed total-ion chromatograms (RTICs) for each analyte, as shown in Fig. 4.4.

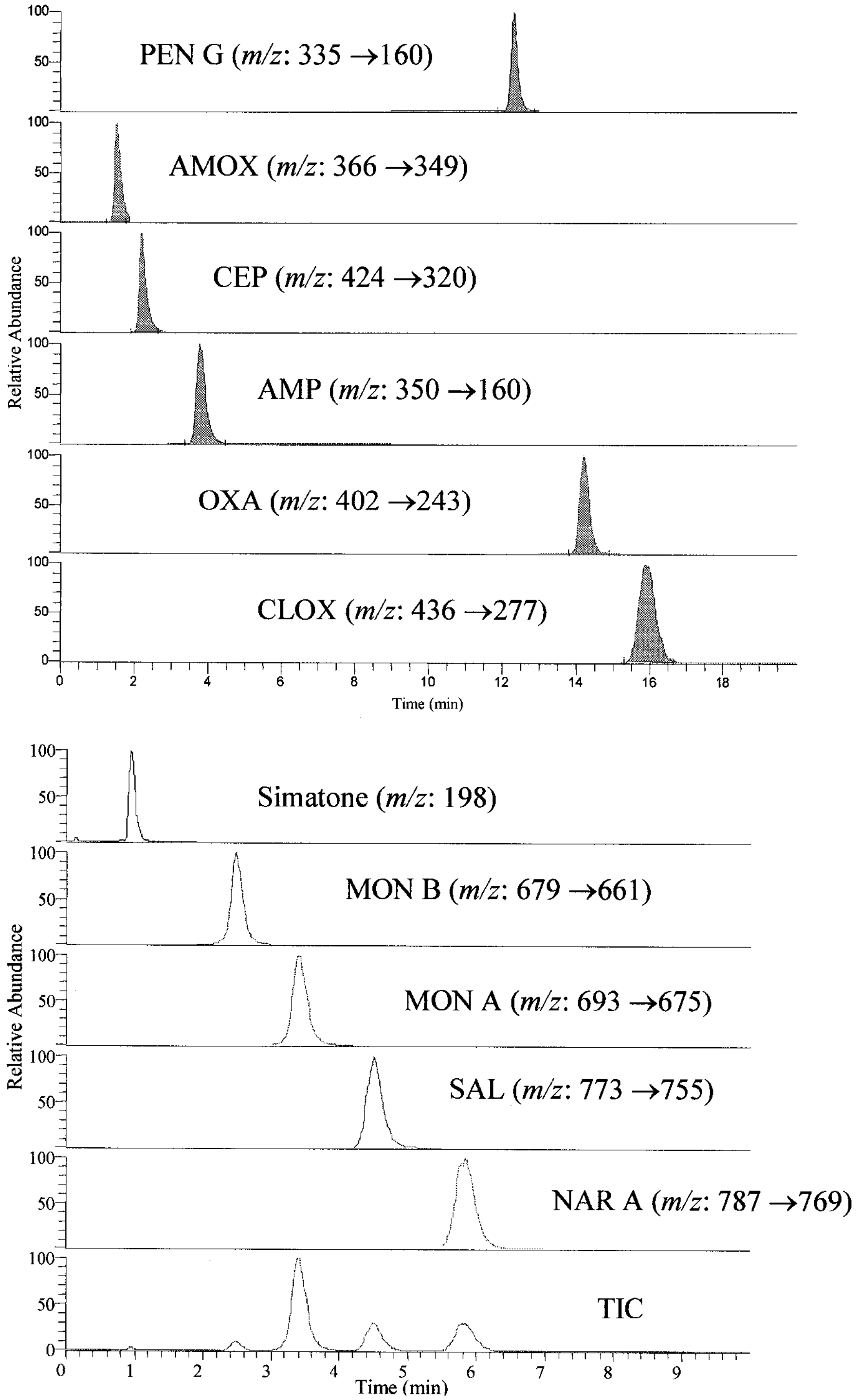


Fig. 4.4. Reconstructed total-ion chromatograms of β-lactam and ionophore antibiotics spiked at a concentration of 20 μg/kg before extraction for 10g of Poudre River Site 3 sediment matrix using LC-MS-MS in SRM. *m/z* indicates precursor ion → product ion used for quantification.

Fragmentation of penicillins (AMOX, AMP, OXA, CLOX, and PEN G) was the result of opening and cleavage of the β-lactam ring and produced the class-specific product ion, $[C_6H_9NO_2S+H]^+$ at *m/z* 160 representative of penicillin compounds (Table 4.3). AMOX, AMP, OXA, CLOX and PEN G exhibited the product ion (*m/z* 207, 191, 243, 277 or 176) of $[M+H-C_6H_9NO_2S]^+$, corresponding to the loss of $C_6H_9NO_2S$ from the precursor ion. AMOX also exhibited the 349 ion due to the neutral loss of NH_3 (17 Da) from the precursor ion. Fragmentation of CEP first produced the 364 ion due to the loss of $OCOCH_3$ (59 Da). CEP then exhibited the product ion at *m/z* 320 due to the loss of CO_2 (44 Da) from the 364 ion, followed by the 292 ion due to the loss of CO (28 Da) from the 320 ion. For MON A and B (Table 4.3), the fragmentation of MON A and B was the result of opening of the cyclic ether rings. The fragmentation of MON A with ion trap MS-MS produced neutral losses of 18 and 36 Da corresponding to the subsequent losses of H_2O, $[M+Na-H_2O]^+$ and $[M+Na-2H_2O]^+$ from the precursor ion, $[M+Na]^+$. SAL and NAR A also exhibited neutral losses of 18 and/or 36 Da corresponding to the subsequent losses of H_2O, $[M+Na-H_2O]^+$ and/or $[M+Na-2H_2O]^+$ from the precursor ion, $[M+Na]^+$, as observed in the fragmentation of MON A and B.

4.3.2. Recovery

Recoveries of BLs and PEs from the HLB cartridges were measured by extracting analytes from 200 mL and 120 mL of surface water and wastewater (influent and effluent), respectively, spiked at levels from 0.1 to 2.0 μg/L and 0.07 to 5.0 μg/L. The recoveries of BLs and PEs were specifically determined by using the ratio of the concentration of analyte for extract spiked before extraction to the concentration of analyte for extract spiked after extraction. Recoveries are the average of duplicates of 0.1, 0.5, and 2.0 μg/L of BLs and 0.07, 1.5, and 5.0 μg/L of PEs spiked in water samples before and after extraction (Table 4.4). In addition, the recoveries of BLs and PEs were also measured by extracting analytes from 10 g of sediment spiked at levels from 1 to 20 μg/kg. Recoveries are the average of duplicates of 1, 10, and 20 μg/kg of BLs and PEs

Table 4.4. Percent recoveries (± S.D.) (n=6) and Method Detection Limits (MDL) (n=7) for antibiotics spiked into surface water, sediment and wastewater influent and effluent samples

Antibiotic	Surface water		Sediment	
	% recovery (± S.D.)	MDL (μg/L)	% recovery (± S.D.)	MDL (μg/kg)
AMP	82.5 ± 6.4	0.010	85.5 ± 4.2	0.12
OXA	90.4 ± 9.3	0.008	94.6 ± 9.8	0.09
CLOX	90.5 ± 8.5	0.008	95.8 ± 9.4	0.08
CEP	92.0 ± 6.6	0.008	82.7 ± 7.9	0.13
MON A	93.7 ± 12.3	0.03	91.8 ± 6.2	0.2
MON B	92.3 ± 6.9	0.05	88.5 ± 9.3	0.5
SAL	93.9 ± 9.8	0.04	94.1 ± 8.5	0.5
NAR A	95.4 ± 7.2	0.04	96.7 ± 7.4	0.4
Antibiotic	Influent		Effluent	
	% recovery (± S.D.)	MDL (μg/L)	% recovery (± S.D.)	MDL (μg/L)
AMP	77.9 ± 7.1	0.017	81.9 ± 6.3	0.013
OXA	91.4 ± 9.2	0.014	90.0 ± 9.3	0.008
CLOX	91.9 ± 9.0	0.013	92.7 ± 8.3	0.009
CEP	90.0 ± 9.3	0.018	92.3 ± 6.7	0.015

spiked in sediment samples before and after extraction (Table 4.4). For surface water and sediment, Poudre River Site 3 (immediately downstream of the WWTP) was selected to assess the matrix effects for BLs and PEs in a more-complex matrix.

The average recovery of BLs (except for AMOX and AMP) from all the sample matrices was generally better than 80% (Table 4.4). No concentration dependence was observed. Recoveries of BLs in the surface water and influent and effluent wastewater were similar (except AMOX), indicating that matrix effects were minimal. Recoveries of BLs in the influent were similar to those in the effluent, indicating that BLs did not exhibit matrix effects in the more-complex influent water matrix. The results for the matrix effects in this study are consistent with the findings in water matrices studies by other research groups (18, 20, 25). In case of amphoteric penicillins (AMOX and AMP), recoveries were generally better than 70% for AMP in all water matrices and between 10% (influent) and 33% (surface water) for AMOX – lower than recoveries of the other BLs (> than 80%). It is more likely that these lower values are due to the different chemical structure of AMOX and AMP – with a primary amino group – compared to the other investigated BLs (Fig. 4.1). AMOX, particularly, exhibited lower average recoveries of 10%, 25%, 33% and 37% in the influent, effluent, surface water, and sediment, respectively, indicating a significant matrix effect due to the presence of organic matter (OM) and/or natural organic matter (NOM) in the water and sediment matrices. The lower recovery values for AMOX in this study are similar to those in surface water and/or wastewater reported in the literature (20, 25-27). Accordingly, AMOX was not quantified in this study. On the other hand, the average recoveries of

PEs in surface water and sediment (Table 4.4) were 93.8 ± 6.8% and 92.8 ± 7.9%, respectively, over the targeted concentration range, indicating the HLB cartridges also gave reproducible recoveries for PEs and were effective for the isolation of the PEs.

4.3.3. Quantification

Concentrations of BLs and PEs were calculated reproducibly by using the matrix-matched internal standard calibration curves to correct the matrix effects. Specifically, the calibration curves were constructed for BLs extracts spiked at levels from 0.1 to 2.0 μg/L in 200 mL of Poudre River Site 3 and DWRF influent and effluent. The calibration also was made for PEs spiked into water samples before extraction at levels from 0.05 to 5.0 μg/L in surface water (Poudre River Site 3). The calibration was made for β-lactam and ionophore extracts spiked into sediment samples before extraction at levels from 1 to 20 μg/kg in sediment (Poudre River Site 3). The calibration curves were linear, with correlation coefficients (R^2) > 0.99 for the MS-MS procedure. Due to the labile degradation of BLs, AMP, OXA, CLOX and CEP, the investigated water and sediment matrices were quantified within 12 hours after sample collection. Because Poudre River Site 3 already contained MON A and NAR A, a calibration curve for these ionophores in that matrix was constructed by subtracting the level concentration from the spiked concentration. The ionophore concentrations in Poudre River samples from Sites 1 ~ 5 were determined reproducibly by using the standard calibration curves for Poudre River Site 3, representing the matrix effects present in surface water and sediment.

4.3.4. Method detection limit

The MDL was determined by using the US EPA-recommended method for MDL determination (24). The calculations of MDL using the US EPA method were based on the variability of multiple analyses of seven surface water and wastewater extracts spiked at a concentration of 0.3 µg/L and 0.2 µg/L for BLs and PEs, respectively, and seven sediment extracts spiked at 3 µg/kg for each antibiotic. The MDL was specifically determined by multiplying the sample standard deviation calculated from each group of extracts spiked by the Student's *t*-variate for a one-sided *t*-test at the 99% confidence level with *n*-1 degrees of freedom. As a result, the MDL (Table 4.4) for β-lactam compounds ranged from 0.008 to 0.010 µg/L in surface water, 0.013 to 0.018 µg/L in influent and 0.008 to 0.015 µg/L in effluent, and 0.08 to 0.13 µg/kg in sediment. The MDL for MON A and B, SAL, and NAR A extracted from 120 mL of surface water was 0.03, 0.05, 0.04, and 0.04 µg/L, respectively, and the MDL for MON A and B, SAL, and NAR A extracted from 10 g of sediment was 0.2, 0.5, 0.5, and 0.4 µg/kg, respectively.

4.3.5. Occurrence, distribution, and fate of BLs and PEs

Surface and waste water

The developed LC-MS-MS method was successfully used to evaluate the occurrence and fate of the four β-lactam and four ionophore antibiotics in both surface water and sediment samples from the Cache la Poudre River collected in pristine, urban, and agricultural areas in northern Colorado. A total of 300 water and wastewater samples were analyzed.

The frequency of detection of β-lactams was less than 2% on analysis of duplicate samples. However, CLOX and OXA were measured at levels less than 20 ng/L in three DWRF influent samples. No β-lactam compounds were detected in any of the DWRF effluent, MWRF influent or MWRF effluent samples. The β-lactams are expected to be easily eliminated in WWTPs due to the lability of β-lactam ring towards chemical and microbial degradation. At Site 3 (downstream of the DWRF), OXA was found at 10 ng/L, possibly due to that site's urban location, since OXA has only human application. AMP and CEP were detected at Site 5, with the greatest agricultural influence of the study sampling sites.

Table 4.5. Occurrence of ionophore antibiotics in surface water samples

	Average concentration, μg/L			
Sample location	MON B	MON A	SAL	NAR A
Poudre River Site 1	ND	ND	ND	ND
Poudre River Site 2	ND	ND	ND	ND
Poudre River Site 3	ND	0.03	ND	0.04
Poudre River Site 4	ND	0.04	ND	0.05
Poudre River Site 5	ND	0.04	0.04	0.05

ND: less than the MDL of the ionophore antibiotic

The average concentration of four ionophore antibiotics in surface water is shown in Table 4.5. As expected, no ionophore compounds were detected at the pristine river section (Site 1) or at the fringes of the urban area (Site 2). MON A and NAR A were found at 0.03 μg/L and 0.04 μg/L, respectively, at Site 3, which is immediately downstream of the DWRF and near urban area, lower-density animal feed operations (AFOs) and concentrated animal feed operations (CAFOs). Since ionophore antibiotics are only used for veterinary application, the presence of these compounds can be considered to agricultural influence. Samples from Site 4 (near the greater-density of AFOs and CAFOs) and Site 5 (near the greatest-density of AFOs and CAFOs), show a very small, likely insignificant change in MON A and NAR A concentration, compared to samples from Site 3. SAL (0.04 μg/L) was first detected along the course of the river at Site 5, where agricultural influence is greatest. No MON B was detected at any of the sites. MON A was found at trace levels (from 0.03 to 0.04 μg/L) in the river water, though the ionophore antibiotics have been used as a growth promoter and/or coccidiostats in cattle, chickens and turkeys. This reflects the fact that the ionophores exhibit lower solubility and form complexes with alkali metal cations in water matrices. Hao et al. (28) detected MON A at levels from 0.02 to 0.22 μg/L in surface water– a finding that is similar to those reported by the current investigators.

Sediment

Properties of antibiotics such as the octanol/water partition coefficient (K_{ow}) and distribution coefficient (K_d) might help predict whether antibiotics are likely to accumulate in the sediment or soil. Properties of the antibiotics (29-34) are shown in Table 4.6. Antibiotics with high log K_{ow} values may demonstrate affinity to sediment or soil, while high K_d values indicate the tendency of antibiotics to be adsorbed onto sediment or soil materials through the phenomenon of adsorption distribution or solid/liquid partition. Both factors contribute to the increase in their concentrations in the sediment or soil matrix (31).

Table 4.6. Properties of the study compounds

Compound	log K_{ow}	K_d	Photolysis half-life (days)	Soil degradation half-life (days)	Reference
AMP	1.35 - 1.45				27
OXA	2.38				28
CLOX	2.48				29
CEP	-1.15				28, 29
MON	2.75 - 4.24	9.3	43.9	7.5	30, 31, 32
SAL	8.53			1.5	28, 29
NAR	4.85 - 6.2	2	1.5	8.8	28, 29

A total of 100 sediment samples were analyzed for BLs and PEs, and most samples were negative for β-lactam antibiotics. None of the β-lactam compounds were measured in samples from Sites 1 and 2. OXA and CLOX each were detected just once in all the samples from Site 3, at 0.36 μg/kg and 0.21 μg/kg, respectively. AMP was detected at 0.13 μg/kg in one sample from Site 4, which is influenced by surrounding agricultural activitiy. CEP was only detected in one sample (0.16 μg/kg) from Site 5. These results confirm that β-lactam antibiotics, readily hydrolyzed, are not frequently found in the sediment (~ 4 %) and are adsorbed by sediment only in small quantities (6, 35).

Table 4.7. Occurrence of ionophore antibiotics in sediment samples

	Average concentration, µg/kg			
Sample location	MON B	MON A	SAL	NAR A
Poudre River Site 1	ND	ND	ND	ND
Poudre River Site 2	ND	ND	ND	ND
Poudre River Site 3	ND	0.9	ND	0.5
Poudre River Site 4	ND	3.1	ND	1.2
Poudre River Site 5	ND	2.0	1.4	0.7

ND: less than the MDL of the ionophore antibiotic

No ionophore antibiotics were found in sediment from the pristine river section nor at the fringes of the urban area. The first detection of MON A (0.9 µg/kg) and NAR A (0.5 µg/kg) along the course of the river was at Site 3 (Table 4.7). Concentrations of MON A and NAR A were greater at Sites 4 and 5, where agricultural land use predominates. The highest sediment concentrations of MON A and NAR A were measured at 3.1 µg/kg and 1.2 µg/kg, respectively, at Site 4 which had the highest total cation concentrations, clay content and organic matter (Table 4.2). SAL (1.4 µg/kg) was only detected at Site 5. These results support that ionophore antibiotic adsorption by sediment is influenced by cation concentration, the presence of clay minerals and soil organic matter (30-31). Based on measured concentrations in sediment, MON A (with a higher K_d) might be more readily adsorbed by sediment than NAR A, with a lower K_d. Its lower half-life in soil (Table 4.6) might explain why SAL was only detected at Site 5.

4.4. Conclusions

The occurrence and fate of antibiotics in the aquatic environment are an important and emerging environmental issue. This study focused on the detection of four β-lactam and four ionophore compounds in water and sediment samples from a river impacted by WWTP effluents and surrounding agricultural activity. It is the first reported study using the sensitive and reproducible SPE-LC-MC-MS analytical method described.

Average recoveries of BLs and PEs from all samples were generally better than 75%, except for AMOX. In most samples, no β-lactam compounds were detected; in those samples with positive results, the concentration levels of the β-lactams ranged from 15 to17 ng/L in influent wastewater samples, from 9 to 11 ng/L in surface water samples, and from 0.13 to 0.36 μg/kg in sediment samples. These results indicate that β-lactam antibiotics appear to be less persistent in the aquatic environment, since these compounds readily undergo hydrolysis due to the chemically unstable β-lactam ring. Thus, this occurrence study revealed that intake from their human and veterinary applications to the small mixed-watershed is of minor importance. The average concentration of ionophores ranged from 0.03 to 0.05 μg/L in surface water samples and from 0.5 to 3.1 μg/kg in sediment samples, predicting that elevated concentrations of these compounds might be found, particularly, in animal manures and lagoon waters (36-40). As ionophore antibiotics are only used in veterinary applications as feed additives for poultry and livestock and as growth promoters for ruminants, the occurrence of ionophore compounds in the aquatic environment can be considered an agricultural influence. The analytical method applied in this study could be used to develop detection methods for these compounds in more complex environmental matrices such as animal manure, lagoon water, and compost.

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Chapter 5

Occurrence and fate of β-lactam and polyether ionophore antibiotics in lagoon water and animal manure

Abstract

The occurrence and fate of antibiotics in agricultural wastes is an emerging area of interest due to the potential impact of these compounds on the environment. A sensitive and reliable analytical method using liquid chromatography-electrospray tandem mass spectrometry (LC-MS-MS) has been developed and validated for the determination of three β-lactam and three polyether ionophore antibiotics in lagoon water and animal manure matrices. The average recovery of β-lactam and ionophore compounds from all the sample matrices was generally above 70 % (excluding amoxicillin and ampicillin). The method was applied to evaluate the occurrence and fate of these compounds from participating farms in northern Colorado. Seven of the 19 lagoon water samples and two of the six animal manures showed detectable antibiotics using LC-MS-MS. The three targeted β-lactams (cephapirin, penicillin G, cloxacillin) were found at 0.97- 43.31 μg/L in the lagoon water samples. Of the three targeted polyether ionophores, only monensin (94 to 1077 μg/L) was detected in the beef runoff pond water samples. Only cloxacillin was measured in the dairy animal manure samples at levels from 8.09 to 45.20 μg/kg. No cephapirin, penicillin G, cloxacillin, salinomycin, or narasin A were detected in any solid animal manure sample. These results indicate that elevated concentrations of β-

lactam and ionophore compounds might be found in lagoon or runoff pond waters and solid animal manures compared to surface waters as these compounds are used in veterinary applications.

Key words: antibiotics; β-lactams; polyether ionophores; lagoon water; animal manure

5.1. Introduction

The β-lactam antibiotics are widely used for their antimicrobial activity against both gram-positive and gram-negative organisms (1-2). These antibiotics are used in human medicine for the treatment of bacterial infections of skin, ear, respiratory tract, and urinary tract (3). These compounds have been widely used both for prevention and treatment of disease and as feed additives to promote growth in animal feeding operations (2). Therefore, The β-lactam antibiotics are an important and widely used class of drugs for both human and veterinary medicine. There are several β-lactams currently approved by the U.S. Food and Drug Administration (US FDA) for use in lactating dairy cattle, swine and poultry (1-3). The β-lactam antibiotics (BLs) are comprised of a great variety of semisynthetic penicillins (e.g., amoxicillin (AMOX), ampicillin (AMP), penicillin G (PEN G), cloxacillin (CLOX), oxacillin (OXA)) and cephalosporins (e.g. cephapirin (CEP), ceftiofur). The polyether ionophore antibiotics (PEs), (e.g., monensin (MON), salinomycin (SAL), and narasin (NAR)), are used in veterinary applications as feed additives (coccidiostats) for poultry and livestock and as growth promoters for ruminants (4-5) (Fig. 5.1).

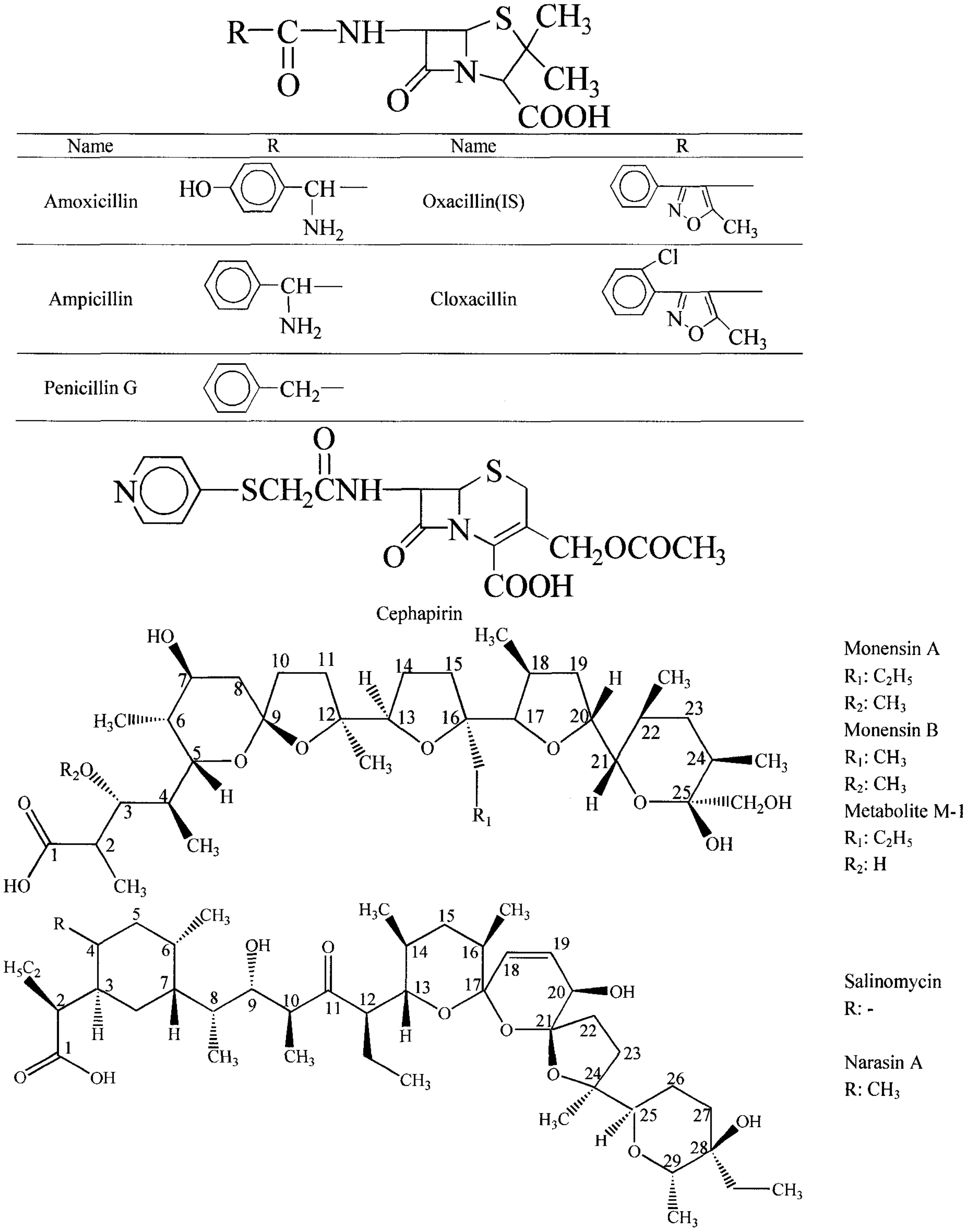


Fig. 5.1. Chemical structures of penicillins, cephapirin, and polyether ionophores.

Only a fraction of any of these antibiotics consumed by animals is metabolized to inactive compounds; instead, a significant quantity of administered antibiotic is excreted as active metabolites, unchanged, via urine or feces (6-7). Thus, the origin of antibiotic contamination in surface and ground waters is considered to be point and non-point source discharges of municipal and agricultural wastewater (8).

Christian et al. (9) reported that AMOX, AMP, mezlocillin, flucloxacillin, and piperacillin in surface water could be found at concentrations up to 48 ng/L. However, in 4 of 32 river water samples, AMOX concentrations did not exceed 10 ng/L(9). Cha et al. (10) found levels of BLs from 9 to 11 ng/L in three surface water samples. Campagnolo et al. (11) reported levels of PEN G from 2.1 to 3.5 μg/L in lagoon water samples. Different water compartments were searched for β-lactams, including surface water (6, 10, 12-14) and groundwater (14), and few of the targeted BLs have been detected. Intact BLs do not occur frequently in the environment (9), due to the poor stability of the β-lactam ring. Some researchers measured MON from 1 to 5 mg/kg (15) and at 4.4 mg/kg (16) in cattle feces. Catherman et al. (17) found NAR from 1.0 to 725.0 μg/kg in poultry feces and manure. Schlüsener et al. (18) reported SAL in manure at 11 μg/kg. Hao et al. (19) detected MON from 0.02 to 0.22 μg/L in surface water. In a previous study, the current authors reported measuring MON A, SAL, and NAR A in surface water from 0.03 to 0.06 μg/L (20).

A few studies have focused on the occurrence, fate, and transport of BL and PE antibiotics in lagoon water and animal manure, and there are several questions to be answered regarding the behavior of these compounds in lagoon water and animal manure. To address these concerns, there is a need for sensitive and reliable analytical methods to measure concentrations of BLs and PEs in these matrices. This study details a sensitive and reliable analytical method for the determination of AMOX, AMP, PEN G, CLOX, CEP, MON, SAL, and NAR in lagoon water and animal manure using solid-phase extraction (SPE) and ion trap liquid chromatography/tandem mass spectrometry (LC-MS-MS) with positive ion electrospray ionization, ESI(+) and selected reaction monitoring (SRM). This paper also addresses the occurrence and fate of BLs and PEs in several lagoon waters and animal manures in northern Colorado.

5.2. Experiment section

5.2.1. Materials for chemical analysis

All antibiotics (purity, >90%), formic acid (>95%) and Na_2EDTA (99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA. Methanol, ethanol, and acetonitrile were HPLC-grade (Sigma-Aldrich, St. Louis, MO, USA). Individual stock solutions of BLs were prepared weekly by dissolving each compound in acetonitrile-ethanol-water (25:25:50) at a concentration of 1,000 mg/L, and stock solutions of the PE standards were prepared monthly by dissolving each compound in methanol at a concentration of 100 mg/L. All stock solutions were stored at -20 °C in the dark. Mixed working solutions (10, 1, and 0.1 mg/L) were prepared daily by diluting the stock solutions with the same solvent and stored at 4 °C in the dark. OXA and simatone, the internal standard working solution (1 mg/L), were prepared by diluting the standard solution with the solvent and were stored at 4 °C and replaced with a fresh solution daily.

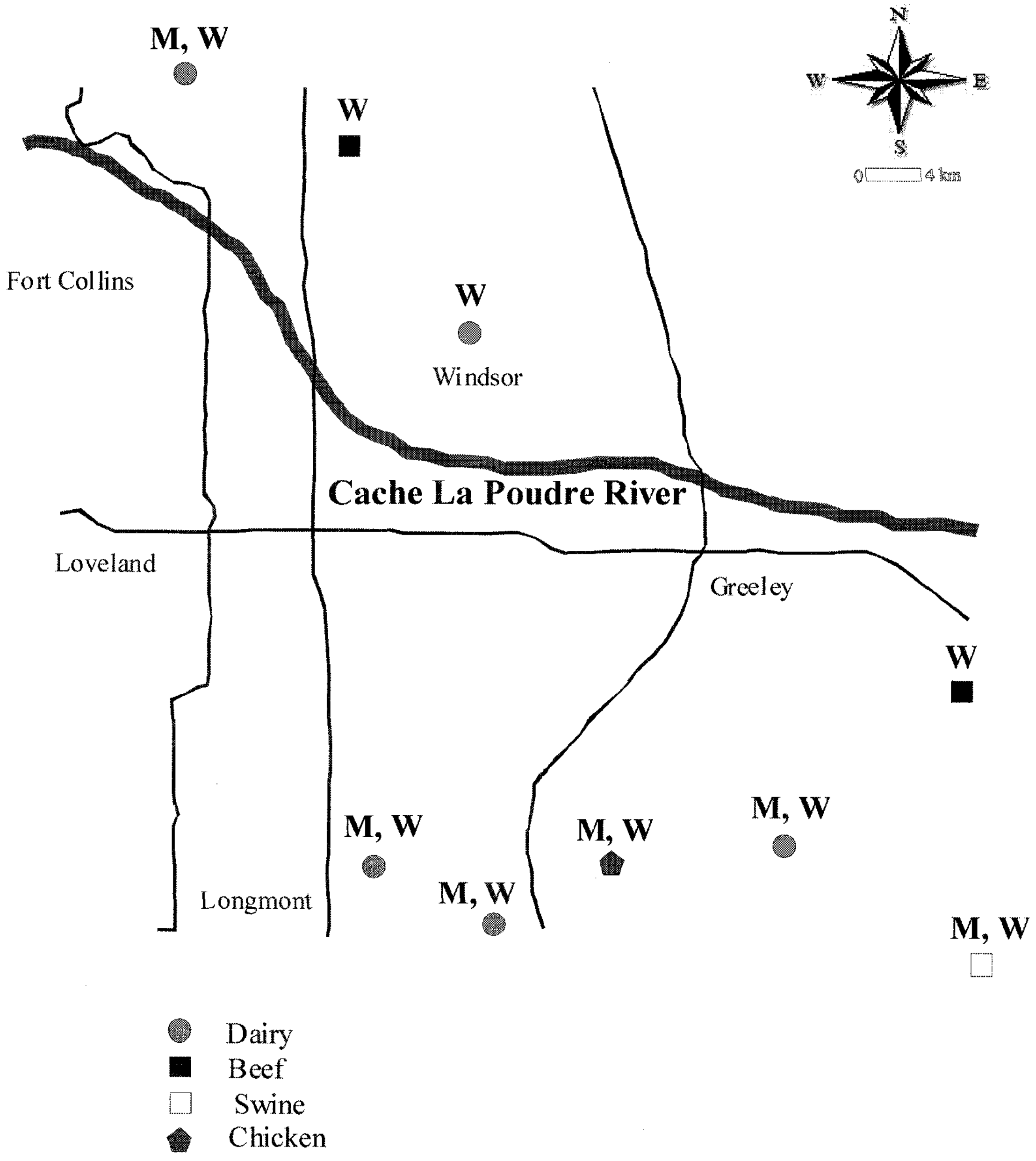


Fig. 5.2. Sample sites of lagoon or runoff pond waters (W) and animal manures (M) in northern Colorado.

5.2.2. Sample collection and preparation

A total of 25 manure waste lagoon or runoff pond water samples (19) and fresh animal manure samples (six) were collected during October and November, 2006, from nine farms in northern Colorado (Fig. 5.2). The lagoon waters from the upper one meter of middle lagoon. The lagoon waters were prepared in a centrifuge with a cooling system (IEC Centra CL 3R, MA, USA) at 3,000 rpm for 40 min at 4 °C. The lagoon water samples were filtered through 0.4-micron glass fiber filters (Millipore, MA, USA) and stored at 4 °C. SPE and quantitative measurement were performed within 12 hours after collection of samples due to the tendency toward biodegradation and/or hydrolysis of the labile β-lactam compounds.

To convert the investigated ionophores to a single sodium adduct species, the appropriate amounts (2% ~ 5% (w/v)) of sodium chloride as a surplus of sodium were dissolved in all samples, which were then left to stand for 30 mins prior to the application of SPE and LC-MS-MS analysis. The quantity of sodium chloride added to each sample was based on the sum of cation concentrations (e.g. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+}) in the sample.

5.2.3. Solid-phase extraction

To prepare matrix-matched reference samples, several additional lagoon water and animal manure samples were analyzed to verify that they did not contain detectable quantities of the analytes of interest. For controls and as calibration standards, the reference lagoon water and animal manure samples were supplemented with appropriate amounts of working solution containing each of the analytes.

SPE was performed using 60 mg/3mL Oasis HLB cartridges (Waters, Millford, MA, USA). These cartridges were preconditioned with 3 mL of methanol, 3 mL of 0.5N HCl, and 3 mL of deionized water at 8 in Hg on a vacuum manifold (PrepSep 12 port, Fisher scientific, PA, USA). All samples were filtered through 0.4-micron glass fiber filters and pH-adjusted immediately prior to extraction. For extraction of BLs, 1 mL of Na_2EDTA and 20 μL of OXA (1.0 mg/L) as the internal standard (IS) were added to a flask containing 20 mL of lagoon water and 80 mL of 0.001M citric acid. Sample pH was adjusted with 8% NH_4OH to pH 7.5. For extraction of PEs, 12 μL of internal standard, 1.0 mg/L simatone, was added to 20 mL of lagoon water samples, and 80 mL of 0.001% formic acid was added at 2% ~ 4% (w/v) of sodium chloride. Because the investigated ionophores are acid and/or base labile, the extraction using the HLB cartridges was performed with the neutral sample pH adjusted by 0.01M NaOH to pH 7.5. BLs and PEs were extracted from 5-g animal manure samples with 120 mL of 0.001M citric acid and 0.001% formic acid, and prepared in the same centrifuge as described above, at 3,000 rpm for 10 min at 4 ^{o}C.

Aqueous samples were passed through the cartridges at 5 mL/min. After isolation, cartridges were rinsed with 5 mL of deionized water, and the analytes were eluted with 5 mL of methanol. The extracts were concentrated under a flow of N_2 gas to about 100 μL using a nitrogen evaporation system (N-Evap, Organermation Associates Inc., MA, USA). To this, 140 μL of mobile phase A (0.1% formic acid in water) was added. The resulting solutions were transferred to 0.5-mL amber autosampler vials to prevent the photodegradation of the BLs and PEs.

5.2.4. Liquid chromatography and mass spectrometry

The liquid chromatography (LC) system used was an HP 1100 LC (Agilent, Palo Alto, CA) that incorporated an autosampler with a 100- μL loop and quaternary pump. BLs and PEs were separated using a short Xterra MS C_{18} column (2.1 x 50 mm) with a 2.5-μm particle size distribution (Waters, Millford, MA, USA) in combination with a guard column (2.1 x 4 mm) from Phenomenex Inc. (Torrance, CA, USA). Mobile phase A (0.1% formic acid in water), phase B (methanol), and phase C (acetonitrile) were used to produce a ternary elution gradient with a flow rate of 0.25 mL/min for BLs and 0.20 mL/min for PEs. The separations of BLs were achieved with the following linear mobile phase gradient program: at 0 min A/B/C = 90:5:5 (v/v/v), 8 min A/B/C = 50:40:10, and 20 min A/B/C = 90:5:5. PE separations were achieved with the following program: at 0 min A/B/C = 18:74:8, 14 min A/B/C = 13:1:86, 15 min A/B/C = 18:74:8. A 10-minute post time allowed re-equilibration of the column. Column temperature for separation of BLs and PEs was 45°C and 25°C, respectively.

The mass spectrometer used was a Finnigan LCQ Duo ion trap (ThermoQuest, CA, USA) equipped with a heated capillary interface and a positive electrospray ionization source. ThermoQuest Xcalibur software was applied to control the mass spectrometric conditions. Full scan mode was used to acquire mass spectra, precursor ions, and product ions from standard BL and PE solutions. Mass spectral data presented in this report were acquired on a LCQ Duo ion trap tandem mass spectrometer equipped with an ESI source operated in positive ion mode. Infusion into the ion trap tandem mass spectrometer was completed with a flow of standard compounds for 3 mg/L of BLs and 7 mg/L of PEs at 5 μL/min from an integrated syringe pump, mixing mobile phases A/B/C at a 80:14:6 and 15:32:53 ratio, respectively, through a T-piece for tuning the mass spectrometer and optimizing the ESI source. The ESI source and MS-MS parameters were automatically optimized and saved in a tune file. Three microscans per scan were acquired with spray needle voltage set at 4.5 kV for both compounds, automatic gain control (AGC) on, and maximum isolation time at 200 ms for BLs and 300 ms for PEs. Voltages on the capillary and tube lens were 29 and 25 V, respectively, for BLs and 38 and 25 V, respectively, for PEs. These were set by automatic optimization using the LCQ autotune program on the mass spectrometer. Nitrogen was used as a sheath and auxiliary gas. Helium was used as the collision gas in the ion trap. The optimized tune conditions included a sheath and auxiliary gas flow rate each set at 50 units (a scale of arbitrary units) and capillary temperature set at 175 ^{o}C. MS-MS parameters for BLs and PEs, including precursor ion, product ion, and collision energy, are summarized in Table 5.1.

5.2.5. Method validation study

The product ions producing the highest intensity and used for SRM and quantification to increase analytical sensitivity and selectivity in LC-MS-MS mode for the targeted AMOX, AMP, PEN G, CLOX, CEP, OXA (Internal Standard, or IS), MON A and B, SAL, and NAR A are listed in Table 5.1. Quantification was based on a detector response defined as the ratio of peak area of the base peak ion (the specific product ion of interest) to peak area of the base peak ion for the IS. Calibration curves were constructed with lagoon water spiked at BL concentrations of 3, 30, 50, 100, and 150 µg/L before extraction. Calibration curves for PEs spiked into lagoon water before extraction were constructed at 3, 50, 100, 200, 400, and 800 µg/L. Calibration curves constructed for BLs and PEs spiked into animal manure samples before extraction ranged from 10, 50, 100, 150 and 200 µg/kg.

Table 5.1. Optimal MS/MS parameters for analysis of selected antibiotics

Analyte	Precursor ion, $[M+H]^+$ (*m/z*)	Product ions (*m/z*)	Normalized collision energy (%)
AMOX	366.4	**348.9**, 160.0, 207.0	26
AMP	350.4	**160.0**, 174.0, 190.9	26
PEN G	335.4	**160.0**, 176.1, 217.0	25
CLOX	436.9	**277.1**, 160.0, 178.1	40
CEP	424.5	**319.9**, 363.9, 292.0	25
OXA (IS)	402.4	**243.0**, 160.0, 144.1	35
Analyte	Precursor ion, $[M+Na]^+$ (*m/z*)	Product ions (*m/z*)	Normalized collision energy (%)
MON A	693.5	**675.5**, 461.3, 657.5	33
MON B	679.4	**661.5**, 465.3, 643.4	30
SAL	773.5	**755.5**, 531.4, 513.3	35
NAR A	787.5	**769.5**, 531.4, 545.3	35

Product ions (*m/z*) of the highest intensity for SRM and quantification are reported in boldface

The method detection limit (MDL) was determined by using the recommended U.S. Environmental Protection Agency (US EPA) method for MDL determination (21) based on the variability of multiple analyses of seven lagoon water extracts spiked at a concentration of 10 μg/L before extraction for BLs and PEs, respectively, and seven animal manure extracts spiked at 30 μg/kg before extraction for each antibiotic.

5.3. Results and discussion

5.3.1. Liquid chromatography and mass spectrometry

The LC method employing a ternary gradient sequence combined with ESI(+)-MS-MS yielded mass peaks corresponding to BLs and PEs on the total-ion chromatograms (TICs) monitored for the selected product ion. The data were processed by creating the reconstructed total-ion chromatograms (RTICs) for each analyte as shown in Fig. 5.3.

Fragmentation of penicillins (AMOX, AMP, PEN G, CLOX, and OXA) resulted from the opening and cleaving of the β-lactam ring, producing the class-specific product ion, $[C_6H_9NO_2S+H]^+$ at *m/z* 160, representative of penicillin compounds (Table 5.1). AMOX, AMP, PEN G, CLOX, and OXA exhibited the product ion $[M+H-C_6H_9NO_2S]^+$ at *m/z* 207, 191, 176, 277 or 243, respectively, corresponding to the loss of $C_6H_9NO_2S$ from the precursor ion. AMOX also exhibited the 349 ion due to the neutral loss of NH_3 (17 Da) from the precursor ion. Fragmentation of CEP first produced the 364 ion due to the loss of $OCOCH_3$ (59 Da). CEP then exhibited the product ion at *m/z* 320 due to the loss of CO_2 (44 Da) from the 364 ion, followed by the 292 ion due to the loss of CO (28

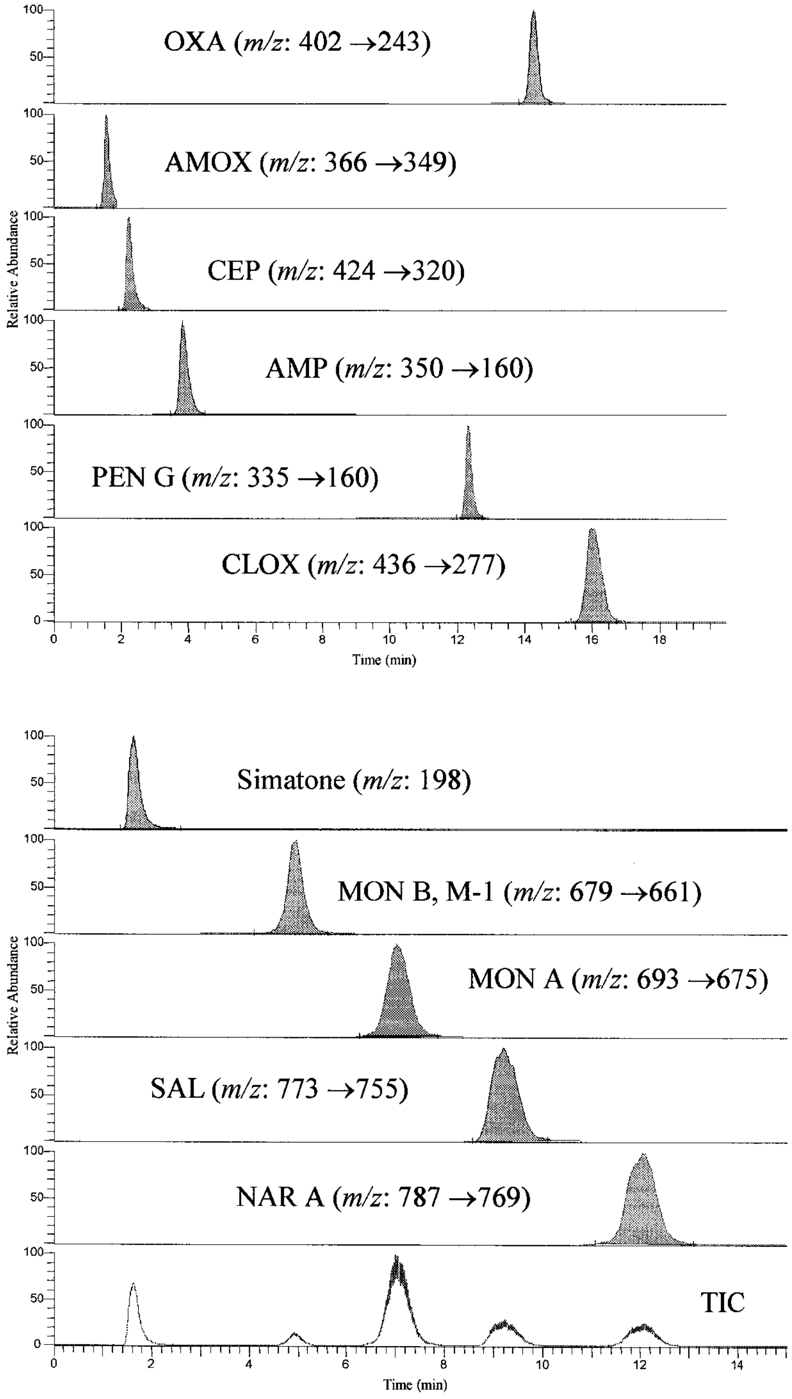


Fig. 5.3. Reconstructed total-ion chromatograms of β-lactam and ionophore antibiotics spiked at a concentration of 10 μg/L before extraction for 20 mL as the lagoon water matrix using LC-MS-MS in SRM. *m/z* indicates precursor ion → product ion used for quantification.

Da) from the 320 ion. Fragmentations of MON A and B resulted from openings of the cyclic ether rings. Fragmentation of MON A with ion trap MS-MS produced neutral losses of 18 and 36 Da corresponding to the sequential losses of H_2O, $[M+Na-H_2O]^+$, and $[M+Na-2H_2O]^+$ from the precursor ion, $[M+Na]^+$. SAL and NAR A also exhibited neutral losses of 18 and/or 36 Da corresponding to the subsequent losses of H_2O, $[M+Na-H_2O]^+$ and/or $[M+Na-2H_2O]^+$ from the precursor ion, $[M+Na]^+$, as observed in the fragmentation of MON A and B. Metabolite M-1 from MON A was isolated from both lagoon water and animal manure samples (22-24). The mass spectrum of metabolite M-1 indicated that M-1 was O-demethylated monensin. The indicated molecular weight was the same as MON B, equivalent to MON A minus CH_2. The fragmentation pattern of M-1 is similar to that of MON A and B. Consequently, MON was quantified as the sum of MON A, B, and metabolite M-1.

5.3.2. Recovery

The recoveries of BLs and PEs from the HLB cartridges were measured by extracting analytes from 20 mL of lagoon water spiked with 3 to 400 μg/L. Recoveries were determined using the ratio of the concentration of analyte for water spiked before extraction to the concentration of analyte in the matrix spiked after extraction. Recovery determinations were calculated as the average of analyses of duplicate lagoon water samples spiked with BLs at 3, 50, and 100 μg/L and with PEs at 3, 100, 200, and 400 μg/L before and after extraction. Similarly, recoveries of BLs and PEs were measured in 5-g samples of animal manure spiked at 10, 100, and 200 μg/kg before and after extraction. All recovery data for BL and PE compounds spiked into lagoon water and animal manure samples is shown in Table 5.2.

Table 5.2. Percent recoveries (± S.D.) (n=6) and Method Detection Limits (MDL) (n=7) for antibiotics spiked into lagoon water and animal manure samples

Antibiotic	Lagoon water		Animal manure	
	% recovery (± S.D.)	MDL (μg/L)	% recovery (± S.D.)	MDL (μg/kg)
PEN G	77.9 ± 8.2	0.15	83.6 ± 2.5	0.34
CLOX	75.8 ± 9.5	0.23	87.1 ± 7.2	0.72
CEP	74.4 ± 6.8	0.76	71.7 ± 4.3	1.86
MON	91.0 ± 5.6	2.13	94.2 ± 3.9	7.38
SAL	84.5 ± 6.4	0.62	73.9 ± 7.5	1.39
NAR A	85.1 ± 3.3	0.47	74.8 ± 8.7	2.94

The average recovery of BLs (except for AMOX and AMP) from all the sample matrices was better than 70%. No concentration dependence was observed. For amphoteric penicillins (AMOX and AMP), recoveries were generally between 8% (lagoon water) and 15% (animal manure). It is likely that these lower values are due to the different chemical structure of AMOX and AMP, which, unlike the other targeted BLs, have a primary amino group (Fig. 5.1). The lower recovery values for AMOX and AMP in the current study agree with those reported by other researchers (14, 25-27). Therefore, AMOX and AMP were not quantified as targeted parameters in this study. The average recovery of PEs in lagoon water and animal manure (Table 5.2) was 86.9 ± 5.1 % and 81.0 ± 6.7 %, respectively, in the investigated concentration range, indicating the HLB cartridges also yielded effective isolation of the PEs. The lower recovery of BLs and PEs from lagoon water and animal manure relative to surface water indicate that matrix effect is important, most likely due to the presence of more organic matter (OM) and/or natural organic matter (NOM) in the lagoon water and animal manure matrices.

5.3.3. Quantification and method detection limit

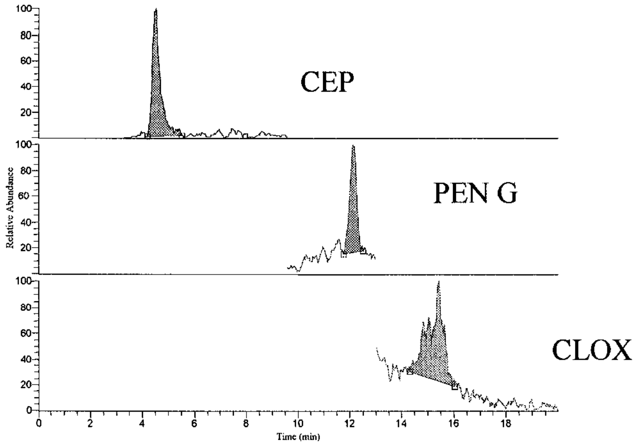
Concentrations of BLs and PEs were calculated reproducibly by using the matrix-matched internal standard calibration curves to correct, to a certain extent, for the minimal matrix effects. Calibration curves were constructed for BLs and PEs extracts spiked into 20 mL of lagoon water before extraction within range of 3 to 150 µg/L and 3 to 800 µg/L, respectively. Calibration curves also were constructed for BL and PE extracts spiked into animal manure samples before extraction at levels from 10 to 200 µg/kg. The resulting calibration curves were linear with correlation coefficients (R^2) > 0.99 for the MS-MS procedure. Because of the labile degradation of BLs, PEN G, CLOX and CEP were quantified in the collected lagoon water and animal manure samples within twelve hours of collection.

Determination of the MDL by the US EPA method (21) was based on the variability of multiple analyses of seven lagoon water extracts spiked at 10 µg/L for BLs and PEs and seven animal manure extracts spiked at 30 µg/kg for each antibiotic. The sample standard deviation calculated from each group of the spiked extracts was multiplied by the Student's *t*-variate for a one-sided *t*-test at the 99% confidence level with *n*-1 degrees of freedom. The MDL (Table 5.2) for β-lactam compounds ranged from 0.15 to 0.76 μg/L in lagoon water and from 0.34 to 1.86 μg/kg in animal manure. The calculated MDLs for MON, SAL and NAR A extracted from 20 mL of lagoon water were 2.13, 0.62, and 0.47 μg/L, respectively; the MDLs for MON, SAL and NAR A extracted from 5 g of animal manure were 7.38, 1.39 and 2.94 μg/kg, respectively.

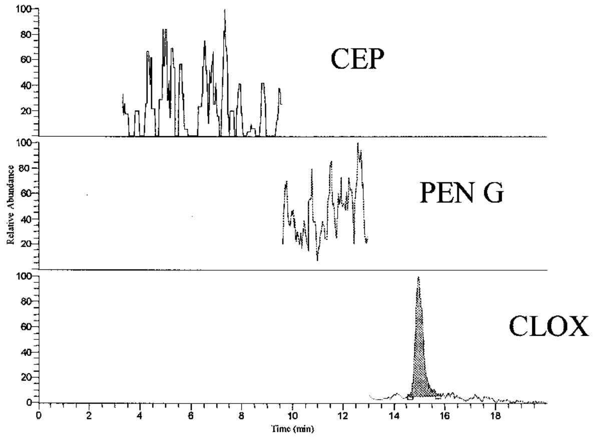
5.3.4. Occurrence and fate of BLs and PEs

The LC-MS-MS method detailed was used to determine the occurrence and fate of three β-lactam and three ionophore compounds in the lagoon or runoff pond water and animal manure samples, representing matrices that might contribute to point and non point agricultural contamination sources. The lagoon or runoff pond waters of raw influent (-1) and lagoon or runoff pond waters (-2, -3) were collected from participating farms in northern Colorado. Fig. 5.4. shows the reconstructed total-ion chromatograms (RTICs) for six BL and PE antibiotics in the lagoon or runoff pond water and animal manure samples, reconstructed on the basis of each class of compound.

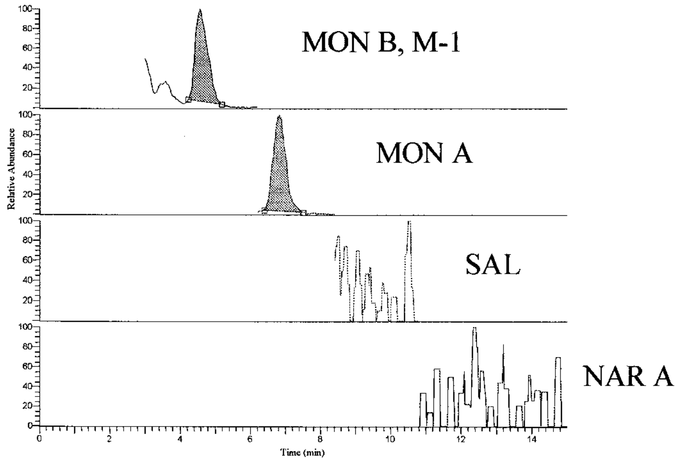
Seven of the 19 lagoon or runoff pond water samples showed detectable antibiotics via the LC-MS-MS method (Table 5.3). The three targeted BLs – CEP, PEN G and CLOX – were found in lagoon water at levels from 0.97 to 43.31 μg/L. CEP (0.97 μg/L) and CLOX (5.05 μg/L) were only found in sample chicken A-1 lagoon water. PEN G (43.31 μg/L) exhibited the highest concentration found in lagoon water. Of the three targeted PEs, only MON was detected in lagoon water samples – specifically, in beef



(a) BLs in the chicken A-1 lagoon water



(b) BLs in the dairy B animal manure



(c) PEs in the beef B-1 runoff pond water

Fig. 5.4. Reconstructed total-ion chromatograms (RTICs) for six BL and PE antibiotics in lagoon or runoff pond water and animal manure samples.

Table 5.3. Occurrence of selected antibiotics in lagoon or runoff pond water (μg/L)

Samples	CEP	PEN G	CLOX	MON	SAL	NAR A
Dairy A-1	ND	1.11	ND	ND	ND	ND
Dairy A-2	ND	ND	ND	ND	ND	ND
Dairy B-1	ND	4.33	ND	ND	ND	ND
Dairy B-2	ND	ND	ND	ND	ND	ND
Dairy B-3	ND	ND	ND	ND	ND	ND
Dairy C-1	ND	ND	ND	ND	ND	ND
Dairy D-1	ND	ND	ND	ND	ND	ND
Dairy E-1	ND	ND	ND	ND	ND	ND
Dairy E-2	ND	ND	ND	ND	ND	ND
Dairy E-3	ND	ND	ND	ND	ND	ND
Chicken A-1	0.97	43.31	5.05	ND	ND	ND
Chicken A-2	ND	ND	ND	ND	ND	ND
Swine A-1	ND	ND	ND	ND	ND	ND
Swine A-2	ND	ND	ND	ND	ND	ND
Swine A-3	ND	ND	ND	ND	ND	ND
Beef A-1	ND	ND	ND	945	ND	ND
Beef A-2	ND	ND	ND	94	ND	ND
Beef B-1	ND	ND	ND	1077	ND	ND
Beef B-2	ND	ND	ND	96	ND	ND

ND: less than the MDL of the selected antibiotic

runoff pond water samples at levels from 94 to 1077 μg/L. No β-lactam and ionophore antibiotic was found in lagoon water from dairy E, as this is an organic farm.

Table 5.4 shows analytical results in samples differentiated as the lagoon or runoff pond waters of raw influent and lagoon or runoff pond waters – those with aerobic treatment, biodegradation and/or hydrolysis treatment. Considering the measured concentrations of BLs and PEs in the lagoon or runoff pond waters, these compounds were removed during aerobic treatment, biodegradation and/or hydrolysis treatment. The removal efficiency for three BL compounds in the lagoon waters was > 86.5 %, except CEP in the chicken A sample. MON was removed with removal efficiencies of 90.1 and 91.1 %, respectively, in beef A and B samples. Degradation of the BL antibiotics is predictable, due to their poor stability.

Table 5.4. Occurrence of lagoon or runoff pond water sample sites

Samples	Analyte	Raw influent (µg/L)	Lagoon or runoff pond water (µg/L)	Removal efficiency (%)
Dairy A	PEN G	1.11	ND[a]	> 86.5[b]
Dairy B	PEN G	4.33	ND	> 96.5
Chicken A	CEP	0.97	ND	> 21.6
	PEN G	43.31	ND	> 99.6
	CLOX	5.05	ND	> 95.4
Beef A	MON	945	94	90.1
Beef B	MON	1077	96	91.1

[a]ND: less than the MDL of the selected antibiotic

[b]Removal efficiency is based on MDL.

Only two of the solid manure samples were found to contain detectable levels of the six targeted antibiotics. Only CLOX (8.09 to 45.20 µg/kg) was found, in dairy manure; no residuals of CEP, PEN G, MON, SAL, or NAR A were detected in any solid manure sample (Table 5.5).

Seven of the lagoon water samples and two of the animal manure samples were found to contain antibiotic compounds via the described LC-MS-MS method. The concentration of MON in Beef lagoon water samples suggests there is a need to measure concentration of MON in beef animal manure samples.

Table 5.5. Occurrence of selected antibiotics in animal manure (μg/kg)

Samples	CEP	PEN G	CLOX	MON	SAL	NAR A
Dairy A	ND	ND	ND	ND	ND	ND
Dairy B	ND	ND	ND	ND	ND	ND
Dairy C	ND	ND	45.20	ND	ND	ND
Dairy D	ND	ND	8.09	ND	ND	ND
Chicken A	ND	ND	ND	ND	ND	ND
Swine A	ND	ND	ND	ND	ND	ND

ND: less than the MDL of the selected antibiotic

The concentrations of antibiotic compounds found in lagoon or runoff pond water and solid manure samples in this study were compared to those reported by other research groups (11, 15-18). PEN G of the BL group was not detected in the swine lagoon water in this study – compared with detection of 2.1 to 3.5 μg/L reported by Campagnolo et al. (11). CEP, PEN G, and CLOX were detected only in chicken lagoon water in the current study and PEN G in two of the five dairy lagoon waters. No BLs were detected in either Swine or Beef lagoon waters. MON, SAL, and NAR A were not detected in solid manure in the current study, but other researchers (15-18) measured MON from 1 to 5 mg/kg, SAL at 11 μg/kg, and NAR from 1.0 to 725.0 μg/kg. No other study has reported concentrations of BLs in animal manure or PEs in lagoon water.

5.4. Conclusions

Animal waste lagoon water and manure are commonly spread on agricultural fields as organic fertilizers. When applied to agricultural land, their components may be transported to surface water or groundwater through runoff or infiltration from fields. Therefore, the occurrence and fate of veterinary antibiotics in lagoon water and animal manure are crucial, emerging environmental issues (28-35). This paper describes a sensitive and reproducible analytical method for quantifying three β-lactam and three polyether ionophore compounds, widely used in veterinary medicine, in lagoon waters and in animal manure samples from farms in northern Colorado using SPE and LC-MS-MS with ESI(+) and SRM.

This analytical methodology allowed successful evaluation of the occurrence and fate of these compounds in the targeted sample matrices. The average recovery of BLs and PEs from all the sample matrices was better than 70 % (excluding chemically unstable AMP and AMOX). Of the targeted six antibiotic compounds, CEP, PEN G and CLOX were found in the lagoon water samples at concentrations ranging from 0.97 to 43.31 μg/L. MON was also measured in runoff pond at levels ranging from 94 to 1077 μg/L. PEN G (43.31 μg/L) and MON (1077 μg/L) reflected the highest concentrations of BLs and PEs, respectively, in the lagoon or runoff pond water samples. Considering the measured concentrations of BLs and PEs in the lagoon or runoff pond waters, these compounds were removed during aerobic treatment, biodegradation and/or hydrolysis treatment. Only CLOX (8.09 to 45.20 μg/kg) was found in solid manure samples.

The successful application of the SPE/LC-MS-MS method detailed in this study to evaluate the occurrence of three β-lactam and three ionophore antibiotics in lagoon water and animal manure warrants further investigation of this methodology in analysis of these compounds in more complex environmental matrices such as sewage sludge (bio-solids) and compost.

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Chapter 6

Biodegradation of antibiotics in aerobic and anaerobic lagoon waters

Abstract

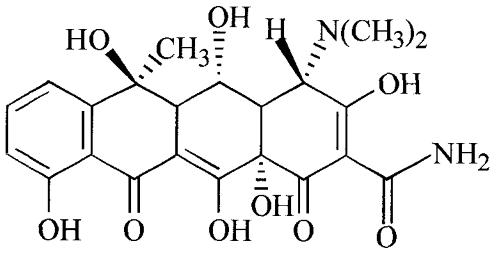
The occurrence and fate of veterinary antibiotics in lagoon water and animal manure are an emerging area of interest due to the potential impact of these compounds on the aquatic environment. The objective of this study was to evaluate the aerobic and anaerobic degradation of oxytetracycline (OTC), sulfamethoxazole (SMX), tylosin (TYL), and monensin (MON) in dairy lagoon water under aerobic and anaerobic conditions. OTC, SMX, TYL and MON were added to samples at 20 mg/L to create Antibiotic (Ab) Spiked matrices, and 50 g/L sodium azide was also added to create Ab Spiked and Killed matrices to eliminate aerobic biological activity. Mercuric chloride (0.1 g/L) also was added to Ab Spiked and Killed samples to remove anaerobic biological activity. Biodegradation of OTC, SMX, TYL and MON occurred at both 20 °C and 4 °C under aerobic and anaerobic conditions. However, all antibiotics did not completely degrade within 175 days at 20 °C under aerobic or anaerobic treatment. Concentrations decreased at 4 °C, but none of these compounds completely biodegraded in either treatment within 242 days at 4 °C. Calculated first-order degradation half-lives of OTC, SMX, TYL and MON at 20 °C were 12, 6.5, 16, and 9.2 days, respectively, under aerobic treatment and 17, 59, 19, and 71 days, respectively, under anaerobic treatment. At 4 °C, half-lives were 80, 204, 46, and 128 days, respectively, under aerobic treatment and 110, >242, 103, and 193 days, respectively, under anaerobic treatment. Therefore, these results show that degradation of antibiotics, in general, is faster under aerobic conditions, compared to anaerobic conditions. It was found that lower temperature (4 °C) reduced the biodegradation rate of antibiotics.

Key words: antibiotics; biodegradation; aerobic; anaerobic; lagoon water

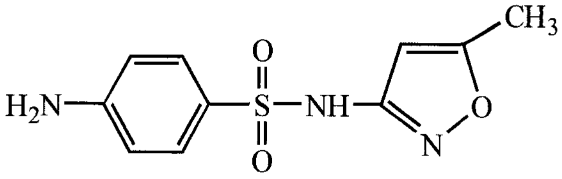
6.1. Introduction

Antibiotics are widely used in human and veterinary health management to treat disease and as growth promoters in confined-animal feeding operations (CAFOs) (1-5). Antibiotic use in veterinary medicine was reported in millions of kilograms in the US in a 1999 survey conducted by the Animal Health Institute (AHI). Tetracyclines, sulfonamides, macrolides, and ionophores make up the majority of these antibiotics, and oxytetracycline (OTC), sulfamethoxazole (SMX), tylosin (TYL), and monensin (MON) are the most widely used veterinary antibiotics within these four classes, respectively (Fig. 6.1) (1).

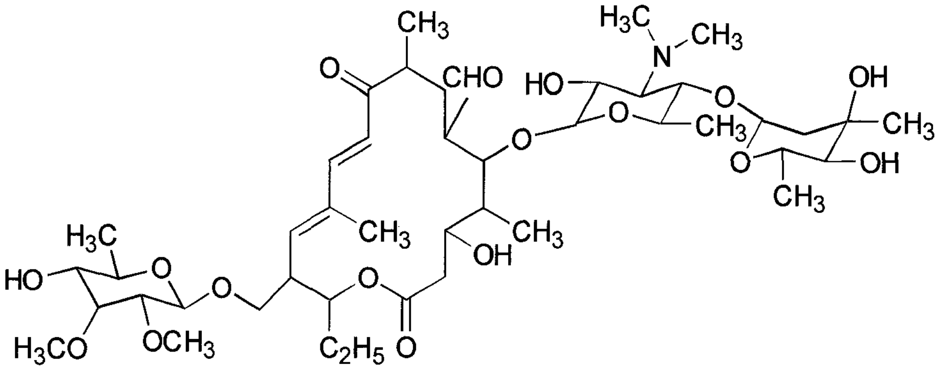
Only a fraction of these drugs consumed by humans and animals is metabolized to inactive compounds; instead, a significant quantity of administered antibiotic is excreted as active metabolites, unchanged, via urine or feces (6-7). Thus, the origin of antibiotic contamination in surface and ground waters may be either point or non-point source discharges of municipal and agricultural wastewater (8). And, agricultural wastewater



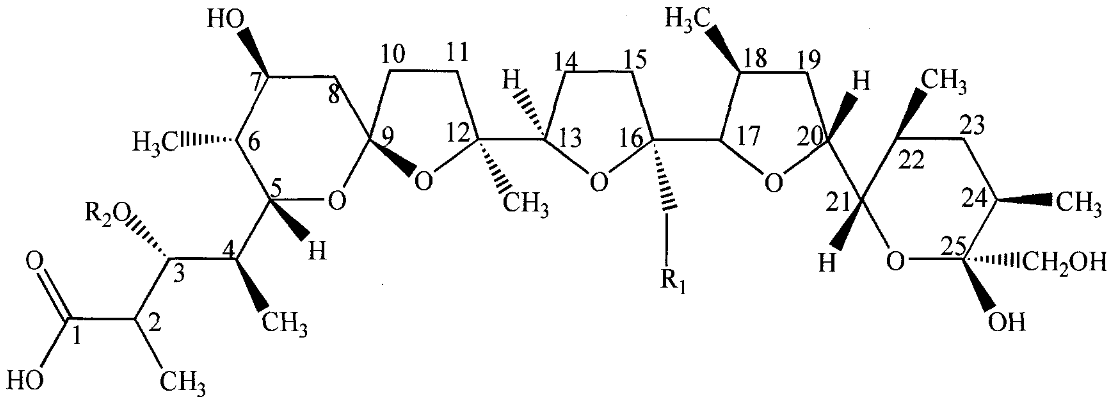
Oxytetracycline



Sulfamethoxazole



Tylosin



Monensin A	Monensin B	Metabolite M-1
R_1: C_2H_5	R_1: CH_3	R_1: C_2H_5
R_2: CH_3	R_2: CH_3	R_2: H

Fig. 6.1. Chemical structures of selected antibiotics.

was often stored in lagoons for six months before it is spread on agricultural fields as organic fertilizer (9).

Various antibiotics have been quantified in lagoon water or animal manure by other researchers. Hamscher et al. (10) reported the presence of tetracycline from 14 to 41 mg/kg, chlortetracycline from 0.9 to 1.0 mg/kg, and sulfamethazine at 7.2 mg/kg in liquid swine manure. Jacobsen and Halling-Sorensen (11) measured tetracyclines from 0.05 to 15.7 μg/g and sulfonamides from 0.015 to 2.1 μg/g. De Liguoro et al. (12) reported detection of OTC and TYL in feces and manure. Campagnolo et al. (13) found tetracycline and OTC at levels from 25 to 410 μg/L in swine lagoon water. Some researchers (14-16) reported finding sulfonamides in lagoon water and manure. Schlüsener et al. (17) measured 11 μg/kg salinomycin in manure. Other researchers measured MON in cattle feces from 1 to 5 mg/kg (18) and at 4.4 mg/kg (19). Catherman et al. (20) measured narasin from 1.0 to 725.0 μg/kg in poultry feces and manure.

Only a few studies examining the biodegradation of antibiotics in water, soils, lagoon water, or manure have been reported. TYL in soil-manure matrices was found to be completely biodegraded within 30 days at temperatures from 20 °C to 30 °C but biodegraded to only 60 % within 30 days at 4 °C (21). Only a small portion of chlortetracycline in soil-manure slurries was found to have degraded within 30 days at 20°C (21). SMX has proven to be very resistant to biodegradation according to recent research (22).

Several studies have reported the half-lives of various antibiotics in marine sediments (23), surface water (24), soil-manure slurries (17, 21, 25), manure (12, 25-27), and soils (25, 28). Degradation half-lives for OTC were reported to be from 30 to 64 days and from 10 to 30 days in marine sediment and manure, respectively (12, 23). The half-life of TYL was reported to be from 4 to 8 days in swine, calf, and chicken manure (12, 27), from 2 to 8 days in soil-manure mixture (29), and from 10 to 40 days in a surface water simulation system (24). Loke et al. (26) reported a half-life of less than two days for TYL in swine slurry under methanogenic conditions. Schlüsener et al. (17) found that the half-life for TYL in soil with manure added was 8.3 days. The half-lives of MON were reported to be from 3.3 to 3.8 days in soil (27) and from 13 to 18 days in soil with manure (25).

The objective of this study was to demonstrate the biodegradation of OTC, SMX, TYL, and MON in dairy lagoon water and determine the biodegradation kinetics of these compounds. Samples of lagoon waters under aerobic and anaerobic conditions were collected and incubated in lab batch reactors, and OTC, SMX, TYL and MON residues were measured using solid-phase extraction (SPE) and ion trap liquid chromatography/tandem mass spectrometry (LC-MS-MS) with positive ion electrospray ionization, ESI(+) and selected reaction monitoring (SRM). Four lagoon water treatments were compared: aerobic conditions at 20 °C and 4 °C and anaerobic conditions at 20 °C and 4 °C.

6.2. Experimental section

6.2.1. Materials for Chemical Analysis

OTC, SMX, TYL, and MON (purity, >90%), citric acid (purity, 99%), formic acid (purity, >95%) and Na_2EDTA (99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethanol, and acetonitrile were HPLC-grade (Sigma-Aldrich, St. Louis, MO, USA). Individual stock solutions of antibiotic standards were prepared monthly by dissolving each compound in methanol at a concentration of 100 mg/L and were stored at -20 ^{o}C in the dark. Mixed working solutions (50, 10, and 1 mg/L) were prepared fresh daily by diluting the individual stock solution with the same solvent and were stored at 4 ^{o}C in the dark. Simatone, the internal standard (IS) working solution (1 mg/L), was prepared by diluting the standard solution with the solvent and was stored at 4 ^{o}C and replaced with a fresh solution every day.

6.2.2. Sample collection and characterization

Samples of lagoon waters from an aerobic lagoon (~1 mg/L dissolved oxygen) and an anaerobic lagoon (0 mg/L dissolved oxygen) were collected from a dairy farm in northern Colorado in October 2005, for a simulated summer-temperature (20 °C) study and in March 2006, for a simulated winter-temperature (4 °C) study. Characterization tests of initial samples determined the pH, dissolved oxygen (DO), temperature, chemical oxygen demand (COD), and total organic carbon (TOC). The background concentrations of antibiotics were analyzed by SPE and LC-MS-MS.

6.2.3. Aerobic and anaerobic studies

Aerobic degradation studies were conducted by transferring 8 L aerobic lagoon water to 9-L bottle reactors. All samples were spiked with 20 mg/L OTC, SMX, TYL and MON to create antibiotic (Ab) Spiked matrices, and 50 g/L sodium azide (NaN_3) was added to remove aerobic biological activity in samples representing Ab Spiked and Killed matrices. Aerobic lagoon waters without antibiotic spikes were included for Background conditions (unamended). Fish tank aeration pumps (Profile Aquarium Air Pump 4000, Taiwan) were used to aerate and mix the aerobic reactors.

Anaerobic degradation studies were structured similarly, except that 0.1 g/L mercuric chloride ($HgCl_2$) was also added to the Ab Spiked and Killed matrices to eliminate anaerobic biological activity. After adding these compounds, the bottles were capped and vortexed for one minute, and the headspace of each anaerobic reactor was immediately purged and filled with nitrogen gas.

Aerobic and anaerobic degradation experiments were repeated at 4 °C to observe reduced-temperature effects on biodegradation kinetic rates. The reactors were setup for each Ab Spiked and each Ab Spiked and Killed condition under aerobic and anaerobic treatments.

6.2.4. Preparation of monensin-sodium salt

To convert the investigated MON into a single sodium adduct species, appropriate amounts (2% ~ 4% (w/v)) of sodium chloride as a surplus of sodium were dissolved in the lagoon water samples, which were then left to stand for 30 min prior to SPE and LC-MS-MS analyses. The amount of sodium chloride added to each sample was based on the sum of cation concentrations (e.g. Na^+, K^+, Li^+, Cs^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cu^{2+}) in the samples.

6.2.5. Solid-phase extraction

To prepare matrix-matched reference samples for use as controls and calibration standards, several additional lagoon water samples were analyzed to verify that they did not contain detectable quantities of the analytes of interest. Samples were spiked with appropriate amounts of working solution containing each of the analytes.

SPE was performed using 60 mg/3 mL Oasis HLB cartridges (Waters, Millford, MA, USA). The cartridges were preconditioned with 3 mL of methanol, 3 mL of 0.5N HCl, and 3 mL of deionized water at 8 in Hg on a vacuum manifold (PrepSep 12 port, Fisher Scientific, PA, USA). For extraction of OTC, SMX and TYL, 1 mL of Na_2EDTA and 12 μL of simatone (1.0 mg/L) as the internal standard were added to a flask containing 20 mL of lagoon water and 80 mL of 0.001M citric acid. Sample pH was adjusted by 40% H_2SO_4 to <3.0 for OTC and SMX, and ~5.0 for TYL immediately prior to extraction, respectively. For extraction of MON, 12 μL of the 1.0-mg/L simatone internal standard was added to 20 mL of lagoon water sample and 80 mL of 0.001% formic acid added at 2% ~ 4% (w/v) of sodium chloride. Because MON is an ionophore and is acid and/or base labile, extraction using the HLB cartridges was performed with a neutral sample pH adjusted by 0.01M NaOH to pH 7.5 immediately prior to extraction (30-33).

Aqueous samples were passed through the cartridges at 5 mL/min. After isolation, cartridges were rinsed with 5 mL of deionized water, and the analytes were eluted with 5 mL of methanol. The extracts were concentrated under a flow of N_2 gas to about 100 μL using a nitrogen evaporation system (N-Evap, Organermation Associates Inc., MA, USA). To this, 140 μL of mobile phase A (0.1% formic acid in water) were added. The resulting solutions were transferred to 0.5 mL amber autosampler vials to prevent photodegradation of the antibiotics.

6.2.6. Liquid chromatography and mass spectrometry

The Liquid Chromatography (LC) system was an HP 1100 LC (Agilent, Palo Alto, CA) that incorporated an autosampler with a 100-μL loop and quaternary pump. The antibiotics were separated using a short Xterra MS C_{18} column (2.1 x 50 mm) with a 2.5-μm particle size distribution (Waters, Millford, MA, USA) in combination with a guard column (2.1 x 4 mm) from Phenomenex Inc. (Torrance, CA, USA). Details of the mobile gradient separation and detection are described in previous reports (30-33).

Table 6.1. Optimal MS/MS parameters for analysis of selected antibiotics

Analyte	Precursor ion, $[M+H]^+$ (m/z)	Product ions (m/z)	Normalized collision energy (%)
OTC	461	**443**, 426, 444	23
SMX	254	**188**, 156, 108	36
TYL	916	**772**	33
Analyte	Precursor ion, $[M+Na]^+$ (m/z)	Product ions (m/z)	Normalized collision energy (%)
MON A	693	**675**, 461, 657	33
MON B	679	**661**, 465, 643	30

Product ions (m/z) of the highest intensity for SRM and quantification are reported in boldface

The mass spectrometer used was a Finnigan LCQ Duo ion trap (ThermoQuest, CA, USA) equipped with a heated capillary interface and a positive electrospray ionization source. ThermoQuest Xcalibur software was applied to control the mass spectrometric conditions. Full scan mode was used to acquire mass spectra, precursor ions, and product ions from the standard solutions. Mass spectral data were acquired on a LCQ Duo ion trap tandem mass spectrometer equipped with an ESI source operated in positive ion mode. Optimal MS-MS parameters for OTC, SMX, TYL and MON, including precursor ion, product ion, and collision energy, are summarized in Table 6.1.

6.2.7. Method validation study

The product ion producing the highest intensity, identified in Table 6.1, was used for SRM and quantification to increase analytical sensitivity and selectivity in LC-MS-MS mode. Quantification was based on a detector response defined as the ratio of peak area of the base peak ion (the specific product ion of interest) to peak area of the base peak ion for the IS. Calibration curves were constructed with non-extracted lagoon water spiked with target extracts at concentrations of 1, 5, 10, 15, and 20 mg/L.

Determination of the method detection limit (MDL) by the recommended U.S. Environmental Protection Agency (US EPA) method (34) was based on the variability of multiple analyses of seven lagoon water extracts spiked at a concentration of 10 μg/L for each antibiotic compound.

6.3. Results and discussion

6.3.1. Fragmentation of OTC, SMX, TYL and MON in the ion trap tandem mass spectrometer

This study reports the investigation of precursor ion fragmentation in a positive electrospray ion trap tandem mass spectrometer. Precursor ions and product ions for four antibiotics were clearly observed in the full MS-MS scan mode of the ion trap tandem mass spectrometer. Precursor ions and product ions at *m/z* for LC-MS-MS, and collision energy are listed in Table 6.1.

OTC exhibited neutral losses of 17 and 35 Da corresponding to the loss of NH_3, $[M+H-NH_3]$, with the subsequent loss of H_2O (18Da), $[M+H-NH_3-H_2O]^+$. Fragmentation of OTC with ion-trap MS-MS in this study also produced $[M+H-H_2O]^+$ due to the loss of 18 Da without the loss of 17 Da. SMX exhibited the loss of 66 Da corresponding to the loss of H_2SO_2, $[M+H-H_2SO_2]^+$. Fragmentation of TYL produced only a single fragment ion, m/z 772 corresponding to $[M+H-C_7H_{12}O_3]^+$.

Fragmentations of MON A and B resulted from the opening of the cyclic ether rings. Fragmentation of MON A with ion trap MS-MS produced neutral losses of 18 and 36 Da corresponding to the sequent losses of H_2O, $[M+Na-H_2O]^+$ and $[M+Na-2H_2O]^+$ from the precursor ion, $[M+Na]^+$. Metabolite M-1 from MON A was isolated from lagoon water samples, as has been reported by other investigators (35-37). The mass spectrum of metabolite M-1 indicated that M-1 was O-demethylated monensin. The indicated molecular weight was the same as that of MON B, equivalent to MON A minus CH_2. The fragmentation pattern of M-1 was similar to that of MON A and B. Consequently, MON was quantified as the sum of MON A, B, and metabolite M-1.

6.3.2. Recovery and quantification

The recoveries of the four target antibiotics from the HLB cartridges were measured by extracting analytes from 20 mL of lagoon water spiked from 1 to 20 mg/L. Recoveries were determined using the ratio of the concentration of analyte in water spiked before extraction to the concentration of analyte in water spiked after extraction. The average recovery of the four antibiotics in lagoon water (Table 6.2) was 86.0 ± 7.6% over the targeted concentration range, indicating the HLB cartridges yielded effective isolation of the subject compounds.

Concentrations of four antibiotics were calculated reproducibly by using the matrix-matched internal standard calibration curves to correct, to a certain extent, the minimal matrix effects. Calibration curves were constructed with compound extracts spiked from 1 to 20 mg/L in 20 mL of lagoon water. The calibration curves were linear with correlation coefficients (R^2) > 0.99 for the MS-MS procedure.

Table 6.2. Percent recoveries (± S.D.) (n=6) and Method Detection Limits (MDL) (n=7) for antibiotics spiked into lagoon water samples

Antibiotic	Lagoon water	
	% recovery (± S.D.)	MDL (μg/L)
OTC	73.1 ± 8.2	1.79
SMX	87.4 ± 2.7	0.58
TYL	92.8 ± 4.1	0.46
MON	90.5 ± 6.4	2.13

6.3.3. Method detection limit

The MDL was identified by multiplying the sample standard deviation calculated from each group of the spiked extracts by the Student's *t*-variate for a one-sided *t*-test at the 99% confidence level with *n*-1 degrees of freedom. The MDLs (Table 6.2) for the target antibiotics ranged from 0.46 to 2.13 μg/L in the lagoon water samples.

6.3.4. Lagoon water characterization

The characterization of the aerobic and anaerobic lagoon water samples is shown in Table 6.3. The pH of the aerobic and anaerobic lagoon waters was between 7.7 and 8.5.

Aerobic lagoon water had higher total solids, chemical oxygen demand (COD), and nitrogen compared to anaerobic lagoon water, but the total organic carbon (TOC) values were similar. Background concentrations of OTC, SMX and MON were below the MDL, but background concentrations of TYL were 5.72 μg/L in aerobic lagoon water and 5.46 μg/L in anaerobic lagoon water.

Table 6.3. Characterization of initial lagoon water samples (n=2)

Parameter		Aerobic lagoon water	Anaerobic lagoon water
pH		8.5	7.7
Conductivity	(mS/cm)	4.34	4.94
Total solids	(g/L)	3.26	2.42
DO	(mg/L)	0.50	0.13
COD	(mg/L)	975	585
Nitrogen	(mg/L)	100	75
NH_4-N	(mg/L)	82.3	61.4
TOC	(mg/L)	0.33	0.29
Phosphorus	(mg/L)	20.5	17.5
PO_4-P	(mg/L)	0.28	1.25
Ca	(mg/L)	207	162
Mg	(mg/L)	117	85.1
Na	(mg/L)	354	251
K	(mg/L)	257	183
Cu	(mg/L)	0.22	0.58
Fe	(mg/L)	0.77	3.10
Zn	(mg/L)	0.16	0.42
OTC	(μg/L)	ND	ND
SMX	(μg/L)	ND	ND
TYL	(μg/L)	5.72	5.46
MON	(μg/L)	ND	ND

ND: less than the MDL of the selected antibiotic

6.3.5. Biodegradation of antibiotics

The stability of oxytetracycline in the dairy lagoon water matrices incubated at 4 °C and 20 °C is shown in Fig. 6.2. Within 75 days at 20 °C, OTC in the antibiotic (Ab) Spiked matrix had almost degraded under both aerobic and anaerobic treatment, while 22 % and 36 % of added OTC remained in the Ab Spiked and Killed matrix under aerobic and anaerobic treatment,

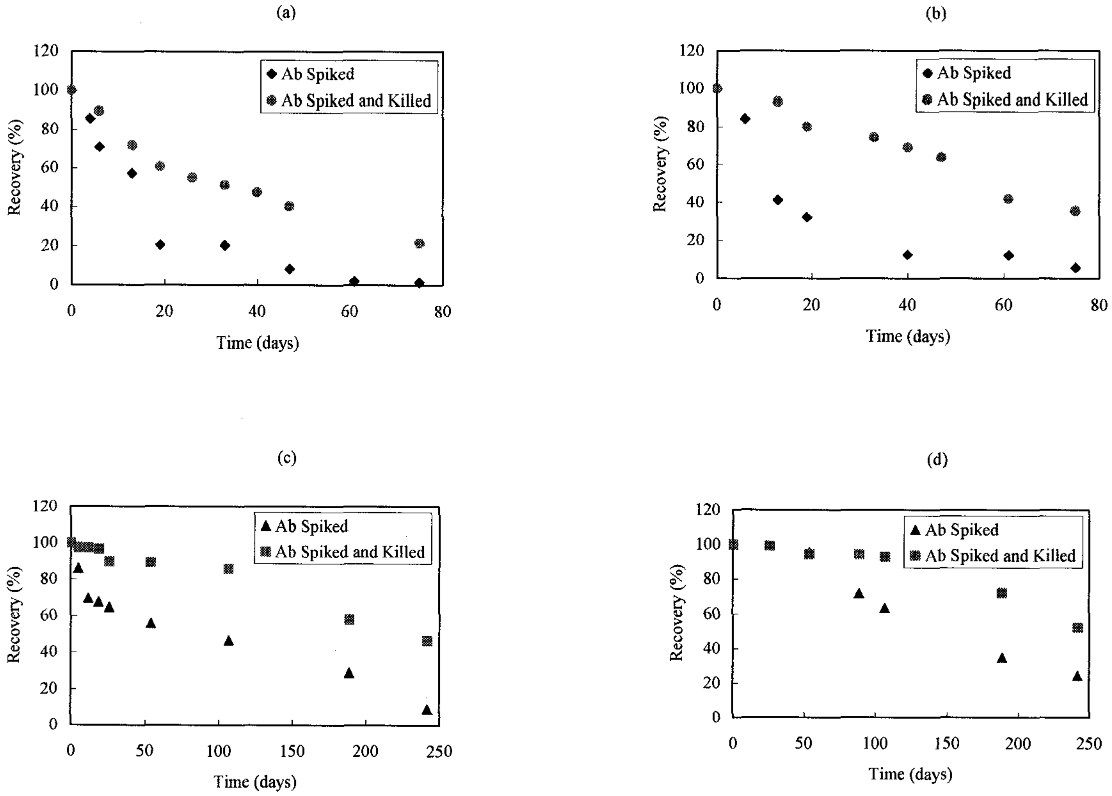


Fig. 6.2. Oxytetracycline (OTC) recovered (as a % of added) during an incubation of four treatments in lagoon water: (a) aerobic at 20 °C, (b) anaerobic at 20 °C, (c) aerobic at 4 °C, and (d) anaerobic at 4 °C.

Note: Ab Spiked = OTC (20 mg/L); Ab Spiked and Killed = OTC (20 mg/L) + NaN_3 (50 g/L) [+$HgCl_2$ (0.1 g/L) for anaerobic treatment samples] respectively. Within 242 days at the reduced temperature (4 °C), OTC in the Ab Spiked matrix almost degraded under aerobic treatment, while 24% of the added OTC remained in the Ab Spiked matrix under anaerobic treatment. There was some reduction in concentration of OTC in the Ab Spiked and Killed matrix at the reduced temperature, but the rate of degradation was much slower than that at 20 °C.

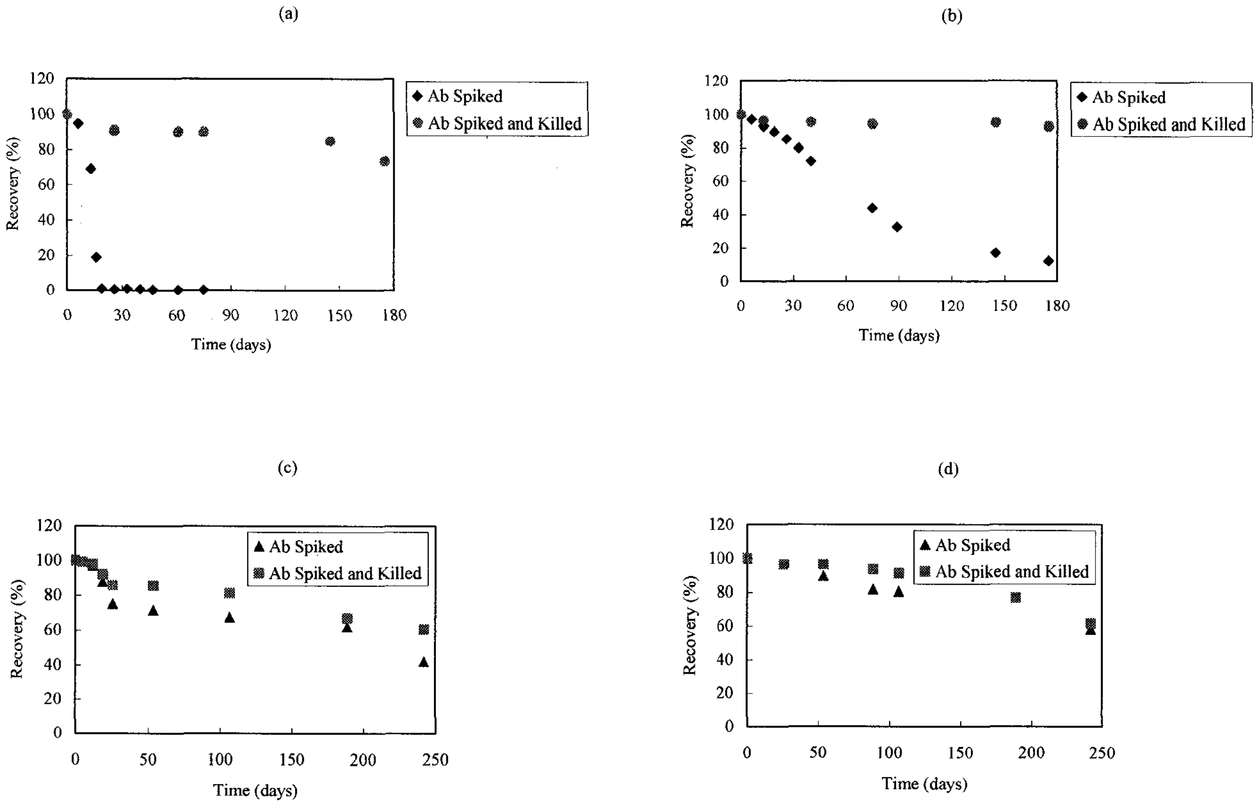


Fig. 6.3. Sulfamethoxazole (SMX) recovered (as a % of added) during an incubation of four treatments in lagoon water: (a) aerobic at 20 °C, (b) anaerobic at 20 °C, (c) aerobic at 4 °C, and (d) anaerobic at 4 °C.

Note: Ab Spiked = SMX (20 mg/L); Ab Spiked and Killed = SMX (20 mg/L) + NaN_3 (50 g/L) [+$HgCl_2$ (0.1 g/L) for anaerobic treatment samples]

The degradation of SMX is shown in Fig. 6.3. In the lagoon waters incubated at 20 °C, SMX concentration in the Ab Spiked matrix diminished rapidly under aerobic treatment and had almost depleted within 19 days. Twelve percent of the original SMX concentration remained in the Ab Spiked matrix within 175 days under anaerobic treatment. SMX in the Ab Spiked and Killed matrix was very resistant to

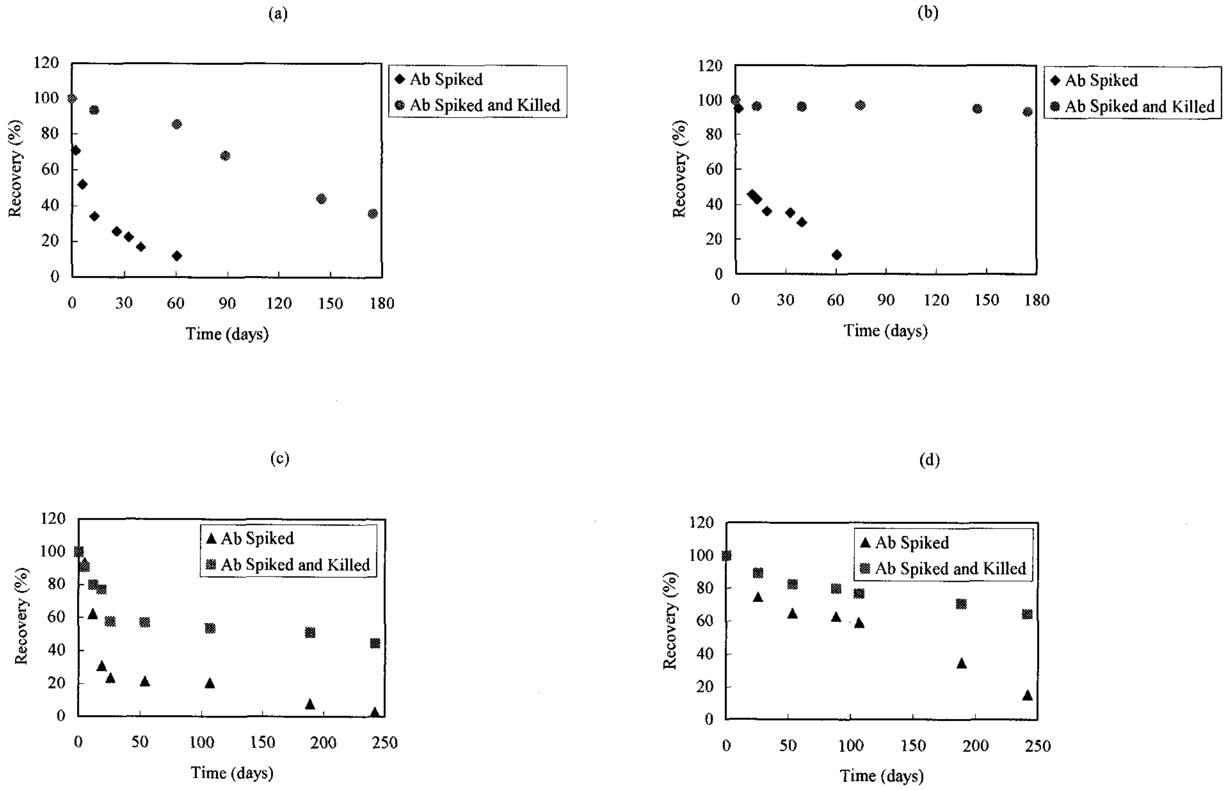


Fig. 6.4. Tylosin (TYL) recovered (as a % of added) during an incubation of four treatments in lagoon water: (a) aerobic at 20 °C, (b) anaerobic at 20 °C, (c) aerobic at 4 °C, and (d) anaerobic at 4 °C.

Note: Ab Spiked = TYL (20 mg/L); Ab Spiked and Killed = TYL (20 mg/L) + NaN_3 (50 g/L) [+$HgCl_2$ (0.1 g/L) for anaerobic treatment samples] biodegradation under both aerobic and anaerobic treatment at 20 °C. Within 242 days at the reduced temperature, SMX in both the Ab Spiked and Ab Spiked and Killed matrices was also very resistant to biodegradation under both aerobic and anaerobic treatment.

Approximately 30 % and 5 % of added TYL in the Ab Spiked matrix had degraded within 2 days under aerobic and anaerobic treatment, respectively, at 20 °C (Fig. 6.4). Within 61 days at 20 °C, 90 % of the original TYL concentration in the Ab Spiked matrix had degraded under both aerobic and anaerobic treatment. Within 175 days at 20 °C, 35 % of TYL remained in the Ab Spiked and Killed matrix under aerobic treatment, while 93 % was recovered from similar samples under anaerobic treatment. TYL in the Ab Spiked matrix had almost degraded under aerobic treatment within 242 days at 4 °C, while 15 % of TYL remained in the Ab Spiked matrix under anaerobic treatment within 242 days at 4 °C. There was some decrease in concentration of TYL in the Ab Spiked and Killed matrix under both aerobic and anaerobic treatment after eight months at 4 °C.

MON in the Ab Spiked matrix had almost degraded under aerobic treatment within 26 days at 20 °C, while 28% of added MON remained in the Ab Spiked matrix under anaerobic treatment within 175 days at 20 °C (Fig. 6.5). Within 175 days at 20 °C, 39 % and 73 % of added MON remained in the Ab Spiked and Killed matrix under aerobic and anaerobic treatment, respectively. Within 242 days at 4 °C, 73% and 68 % of the MON in the Ab Spiked matrix had biodegraded under aerobic and anaerobic treatment,

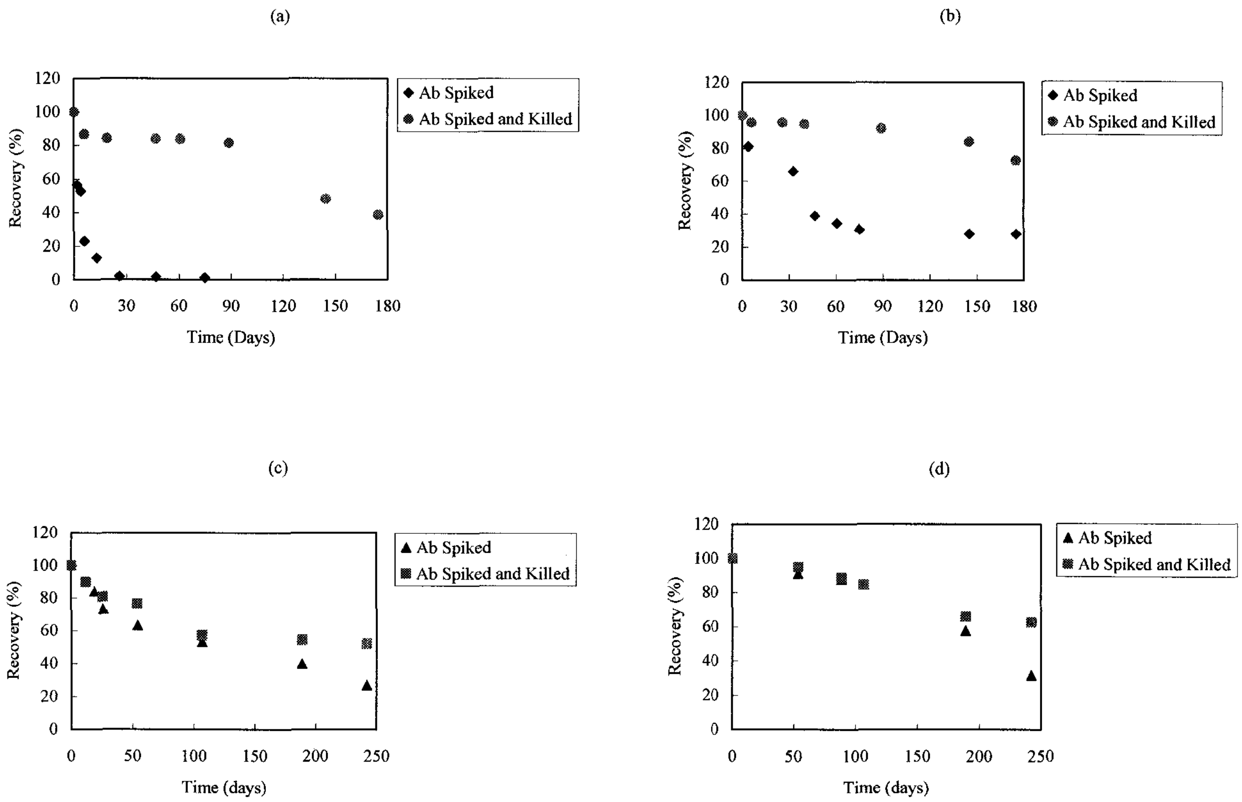


Fig. 6.5. Monensin (MON) recovered (as a % of added) during an incubation of four treatments in lagoon water: (a) aerobic at 20 °C, (b) anaerobic at 20 °C, (c) aerobic at 4 °C, and (d) anaerobic at 4 °C.

Note: Ab Spiked = MON (20 mg/L); Ab Spiked and Killed = MON (20 mg/L) + NaN_3 (50 g/L) [+$HgCl_2$ (0.1 g/L) for anaerobic treatment samples]

respectively, while 48 % and 37 % of MON had biodegraded in the Ab Spiked and Killed matrices under aerobic and anaerobic treatments, respectively.

Some degree of biodegradation of OTC, SMX, TYL, and MON occurred in all Ab Spiked matrices at both 20 °C and 4 °C. within 175 days at 20 °C, OTC, SMX, TYL and

MON in the Ab Spiked matrices had completely degraded under aerobic treatment; similar results were seen under anaerobic treatment, except for SMX and MON. Though some biodegradation of all four antibiotics was observed at 4 °C, no matrix showed 100% biodegradation under either treatment within 242 days.

Table 6.4. Half-lives (in days) for OTC, SMX, TYL, and MON at 20 °C

Treatment	Condition	OTC	SMX	TYL	MON
Aerobic	[a]Ab Spiked	12	6.5	16	9.2
	[b]Ab Spiked and Killec	34	>175	128	144
Anaerobic	Ab Spiked	17	59	19	71
	Ab Spiked and Killed	56	>175	>175	>175

[a]Ab Spiked = OTC+SMX+TYL+MON (each 20 mg/L)
[b]Ab Spiked and Killed = OTC+SMX+TYL+MON+NaN_3 (50 g/L) [+$HgCl_2$ (0.1 g/L) for anaerobic treatment samples]

The half-lives for the four antibiotics were calculated using a first-order degradation model. Biodegradation half-lives in the Ab Spiked matrices at 20 °C followed the order of TYL>OTC>MON>SMX under aerobic treatment, and MON>SMX>TYL>OTC under anaerobic treatment (Table 6.4). Biodegradation half-lives in the Ab Spiked matrices at 4 °C followed the order of SMX>MON>OTC>TYL under both aerobic and anaerobic treatment (Table 6.5). These results show that degradation of antibiotics, in general, is faster under aerobic conditions, compared to anaerobic conditions. The biodegradation rate of all four antibiotics at 4 °C was slower than that at 20 °C.

Table 6.5. Half-lives (in days) for OTC, SMX, TYL, and MON at 4 °C

Treatment	Condition	OTC	SMX	TYL	MON
Aerobic	[a]Ab Spiked	80	204	46	128
	[b]Ab Spiked and Killec	239	>242	173	217
Anaerobic	Ab Spiked	110	>242	103	193
	Ab Spiked and Killed	>242	>242	>242	>242

[a]Ab Spiked = OTC+SMX+TYL+MON (each 20 mg/L)
[b]Ab Spiked and Killed = OTC+SMX+TYL+MON+NaN_3 (50 g/L) [+$HgCl_2$ (0.1 g/L) for anaerobic treatment samples]

6.3.6. Discussion

Drillia et al. (38) showed that in the activated sludge process at 25 °C, SMX in an Ab Spiked sample had almost degraded under aerobic treatment within 5 days. Some researchers reported that SMX is more persistent in the environment (39, 40). Our results showed that SMX in dairy lagoon water had almost biodegraded under aerobic treatment within 19 days at 20 °C.

In this study, 78% of TYL in an Ab Spiked lagoon-water matrix had biodegraded within 33 days at 20 °C under aerobic treatment. Gavalchin et al. (21) reported complete biodegradation in a soil sample amended with manure within 30 days at 20 °C, and Kolz et al. (9) demonstrated that 90% of TYL in swine manure slurries had biodegraded within 0.5 to 1.2 days at 22°C under aerobic treatment. TYL under anaerobic treatment had biodegraded to 26 % of original levels within 26 days at 4 °C in this study. TYL biodegraded to 60 % within 30 days at 4 °C in a soil-manure mixture (21). TYL was found to be adsorbed to lagoon water solids and colloidal materials but is expected to be biodegraded readily due to its structure (41). The half-lives for TYL were seen to be from 16 to 19 days in the lagoon water examined in this study, while other researchers found half-lives of from 2 to 8.3 days in soil with manure (17, 29).

Degradation half-lives for OTC were found to be from 12 to 17 days in the dairy lagoon water examined in this study, while De Liguoro et al. (12) reported half-lives of from 10 to 30 days in manure. In this study, MON showed a biodegradation half-life from 9.2 to 71 days in lagoon water, while the degradation half-lives of MON were reported to be from 13 to 18 days in soil-manure mixtures (25). These results demonstrate that the biodegradation rate of antibiotics may be related to experimental condition and sample matrix.

6.4. Conclusions

The occurrence and fate of veterinary antibiotics in lagoon water and animal manure are considered a critical and emerging environmental issue (42-44). This paper describes the depletion of OTC, SMX, TYL, and MON and the biodegradation kinetics of these compounds in dairy lagoon water samples using SPE and LC-MS-MS with ESI(+) and SRM. Specifically, biodegradation was compared under aerobic and anaerobic treatment at both 20 °C and 4 °C.

OTC, SMX, TYL, and MON showed some biodegradation in all Ab Spiked matrices at both 20 °C and 4 °C. OTC and TYL in the Ab Spiked matrix had completely biodegraded within 175 days at 20 °C under both aerobic and anaerobic treatment, as did SMX and MON in the Ab Spiked matrix under aerobic treatment. There was some decrease in concentration of all four antibiotics at 4 °C, but none showed complete biodegradation in any treatment within 242 days at 4 °C.

OTC, SMX, TYL, and MON in the Ab Spiked at 20 °C followed first-order degradation model under aerobic and anaerobic treatments (Table 6.6). These results show that degradation of antibiotics, in general, is faster and more complete under aerobic conditions, compared to anaerobic conditions (Table 6.6). Biodegradation of the subject antibiotics depends on ambient temperature, with elevated temperature increasing rate of decomposition and lower temperature reducing the biodegradation rate.

Table 6.6. Half-lives (in days) for OTC, SMX, TYL, and MON in the Ab Spiked matrix

Antibiotic	Aerobic		Anaerobic	
	4 °C	20 °C	4 °C	20 °C
OTC	80	12	110	17
SMX	204	6.5	>242	59
TYL	46	16	103	19
MON	128	9.2	193	71

Three factors pertaining to the biodegradation of these antibiotics need to be considered. First, hydraulic retention time (HRT): The HRT of the target compounds in lagoons is generally considered to impact the occurrence and fate of these antibiotics in relation to their efficient removal from lagoons. The results provided in this study recommend that the HRTs are longer than six months. Second, treatment condition: aerobic biodegradation of antibiotics is more effective than anaerobic treatment. Therefore, it is recommended that when possible, lagoons should have some aerobic treatment to remove these antibiotics efficiently. Third, temperature: biodegradation clearly depends on temperature with lower values reducing the removal rate. The lagoon fate and lab biodegradation results collected in this study will be useful for assisting in the future development and implementation of best management practices at concentrated animal feed operations (CAFOs).

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Chapter 7

Conclusions and future research

7.1. Conclusions

The occurrence and fate of antibiotics in the aquatic environment are a critical and emerging environmental issue. Overall, this study focused on the occurrence and fate of selected antibiotics and their biodegradation. Specifically, a SPE-LC-MS-MS method with SRM has been shown to be accurate, reliable and robust for the determination of β-lactam (amoxicillin, ampicillin, penicillin G, oxacillin, cloxacillin, cephapirin) and ionophore (monensin, salinomycin, narasin) antibiotics in aquatic matrices. LC-MS-MS coupled with SPE was successfully used to evaluate the occurrence and fates of these compounds in surface water, wastewater, sediment, lagoon water and animal manure matrices in northern Colorado.

The application of this method resulted in high extraction recovery of β-lactams except amoxicillin and ampicillin and generated method detection limits (MDLs) of 8-10 ng/L in surface water, 8-18 ng/L in wastewater, 0.08-0.13 μg/kg in sediment, 0.15-0.76 μg/L in lagoon water, and 0.34-1.86 μg/kg in animal manure. The average recovery of ionophore antibiotics in pristine water, wastewater-influenced water, lagoon water, and animal manure was 96.0 ± 8.3 %, 93.8 ± 9.1 %, 86.9 ± 5.1 %, and 81.0 ± 6.7 %, respectively. The method detection limit of ionophores ranged from 0.03 to 0.05 μg/L for surface water, from 0.2 to 0.5 μg/kg for sediment, from 0.47 to 2.13 μg/L for lagoon water, and from 1.39 to 7.38 μg/kg for animal manure.

The frequency of detection of β-lactam compounds was about 3 % when considering all sample matrices. In those samples with positive results, the concentration levels of the β-lactams were measured at a range of 15-17 ng/L in influent wastewater samples, 9-11 ng/L in surface water samples, and 0.13-0.36 μg/kg in sediment samples, respectively. The β-lactams are expected to be easily eliminated in wastewater treatment plants due to the lability of the β-lactam ring towards chemical and microbial degradation, and no β-lactam compounds were detected in any effluent wastewater samples. Cephapirin, penicillin G, and cloxacillin were found in lagoon water with a concentration range of 0.97-43.31 μg/L. Only cloxacillin (8.09-45.20 μg/kg) was found in animal manure samples. The average concentration levels of the ionophores detected was 0.03-0.05 μg/L in surface water samples and 0.5-3.1 μg/kg in sediment samples. A wide range of monensin concentrations was measured in the beef runoff pond waters (94-1077 μg/L). These results confirmed that elevated concentrations of these compounds might be found, particularly, in animal manures and lagoon waters. As ionophore antibiotics are only used in veterinary applications as feed additives for poultry and livestock and as growth promoters for ruminants, the occurrence of ionophore compounds in the aquatic environment can be considered an agricultural influence. Therefore, the origin of antibiotic contamination in surface water may be considered to be point and non-point source discharge of municipal and agricultural wastewater.

Next, this study determined the biodegradation kinetics of oxytetracycline (OTC), sulfamethoxazole (SMX), tylosin (TYL), and monensin (MON) in anaerobically and aerobically operated bioreactors that were fed with dairy lagoon water at 20 °C and 4 °C. OTC, SMX, TYL, and MON biodegraded at both 20 °C and 4 °C. However, within 175 days at 20 °C, all antibiotics did not completely degrade under aerobic or anaerobic treatment. Within 75 days at 20 °C, OTC in the antibiotic (Ab) Spiked matrix had almost degraded under both aerobic and anaerobic treatment. In the lagoon waters incubated at 20 °C, SMX concentration in the Ab Spiked matrix diminished rapidly under aerobic treatment and had almost depleted within 19 days. Approximately 30 % and 5 % of added TYL in the Ab Spiked matrix had degraded within 2 days under aerobic and anaerobic treatment, respectively, at 20 °C. MON in the Ab Spiked matrix had almost degraded under aerobic treatment within 26 days at 20 °C, while 28% of added MON remained in the Ab Spiked matrix under anaerobic treatment within 175 days at 20 °C.

There was some decrease in concentration of four antibiotics at 4 °C, but they did not completely degrade in either treatment within 242 days at 4 °C. Within 242 days at the reduced temperature (4 °C), OTC in the Ab Spiked matrix almost degraded under aerobic treatment, while 24% of the added OTC remained in the Ab Spiked matrix under anaerobic treatment. Within 242 days at the reduced temperature, SMX in both the Ab Spiked and Ab Spiked and abiotic matrices was very resistant to biodegradation under both aerobic and anaerobic treatment. TYL in the Ab Spiked matrix had almost degraded under aerobic treatment within 242 days at 4 °C, while 15 % of TYL remained in the Ab Spiked matrix under anaerobic treatment within 242 days at 4 °C. Within 242 days at 4 °C, 73% and 68 % of the MON in the Ab Spiked matrix had biodegraded under aerobic and anaerobic treatment, respectively.

OTC, SMX, TYL, and MON in the Ab Spiked at 20 °C followed first-order degradation model under aerobic and anaerobic treatments (Table 7.1). These results show that degradation of antibiotics, in general, is faster and more complete under aerobic conditions, compared to anaerobic conditions (Table 7.1). Biodegradation of the subject antibiotics depends on ambient temperature, with elevated temperature increasing rate of decomposition and lower temperature reducing the biodegradation rate. Overall, the results from this study provided support for the hypothesis.

Table 7.1. Half-lives (in days) for OTC, SMX, TYL, and MON in the Ab Spiked matrix

Antibiotic	Aerobic		Anaerobic	
	4 °C	20 °C	4 °C	20 °C
OTC	80	12	110	17
SMX	204	6.5	>242	59
TYL	46	16	103	19
MON	128	9.2	193	71

Three factors pertaining to the biodegradation of these antibiotics need to be considered. First, hydraulic retention time (HRT): The HRT of the target compounds in lagoons is generally considered to impact the occurrence and fate of these antibiotics in relation to their efficient removal from lagoons. The results provided in this study recommend that the HRTs are longer than six months. Second, treatment condition: aerobic biodegradation of antibiotics is more effective than anaerobic treatment. Therefore, it is recommended that when possible, lagoons should have some aerobic treatment to remove these antibiotics efficiently. Third, temperature: biodegradation clearly depends on temperature with lower values reducing the removal rate. The lagoon fate and lab biodegradation results collected in this study will be useful for assisting in the future development and implementation of best management practices at concentrated animal feed operations (CAFOs).

7.2. Future research

Through the study, several recommendations are presented for future study.

1) The continued development of accurate and reliable methods of analysis for the determination of antibiotics in agricultural and aquatic matrices is needed.
2) Additional monitoring studies are needed to assess the occurrence and fate of antibiotics in other surface waters and agricultural wastewaters.
3) There is a need to study the antibiotic uptake from by plants from soil fertilized with animal manure.
4) There is a need to research the effectiveness of different wastewater treatment plants in the removal of antibiotics from wastewater.

5) There is a need to investigate the impact of composting on the degradation of antibiotics in manure and the impacts of different manure management practices (temporal and spatial) on the occurrence and fate of antibiotics.