

LINCOMYCIN AND SPECTINOMYCIN:
PERSISTENCE IN LIQUID SWINE MANURE AND THEIR
TRANSPORT FROM MANURE-AMENDED SOIL

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ABSTRACT

Antimicrobials administered to livestock can be excreted up to 80% in the feces and urine. Liquid swine manure from confined animal feeding operations is generally retained in lagoon storage until it is applied as a nutrient source to cropland. Thus, the applied manure becomes a possible source of antimicrobials to aquatic ecosystems. Veterinary antimicrobials have been detected in surface and ground waters in Canada, the United States and Europe, however, their environmental fate is not well known. Lincomycin and spectinomycin are two antimicrobials administered as a mixture to swine in the prairie region of Canada for the prevention of post-weaning diarrhea. In order to assess the potential for contamination of prairie wetlands, concentrations of both antimicrobials were monitored in the liquid manure from the nursery area of a commercial-scale barn during a 5-week study, and their persistence during simulated manure storage investigated. The potential for transport of lincomycin and spectinomycin to surface waters via surface runoff and to leach to groundwater was also assessed. This was achieved by monitoring manure-amended soil, simulated rainfall runoff, snow melt runoff and groundwater over a two-year period at two study sites in Saskatchewan, Canada following fall application of liquid swine manure from two commercial barns to crop and pasture land. Liquid chromatography coupled with tandem mass spectrometry was used to quantitate these antimicrobials in all matrix extracts.

In the nursery area of a commercial-scale barn, concentrations of lincomycin and spectinomycin in the cumulating liquid manure at the end of the study were equivalent to 32 and 3.0%, respectively, of doses administered in the feed. In a laboratory study, using fortified liquid manure, concentrations of both antimicrobials showed a rapid initial decrease during simulated lagoon storage, followed by a slower dissipation over a period of 5 months. The average time required for 50% dissipation of lincomycin was greater than one year (365 d) and was approximately 90 d for spectinomycin.

Lincomycin concentrations in soil (46.3 to 117 $\mu\text{g kg}^{-1}$) collected immediately after fall manure application, decreased to non-detectable levels by mid-summer the following year. Lincomycin was present in simulated rainfall runoff (0.1 to 2.7 $\mu\text{g L}^{-1}$) immediately after manure application

with similar concentrations present in snow melt runoff the following spring. Concentrations in groundwater were generally $<0.005 \mu\text{g L}^{-1}$. Spectinomycin was not detected in the manure applied at the study sites nor in soil, runoff water or groundwater samples. This study confirms that some antimicrobials, including lincomycin, may be present in lagoon manure. Thus, the management practice of utilizing livestock manure from confined animal feeding operations as a plant nutrient source on cropland may result in antimicrobial transport to surface and ground waters.

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LIST OF ABBREVIATIONS

~ = approximately
≈ = approximately equal to
< = less than
> = greater than
μg = microgram
μg kg⁻¹ = microgram per kilogram
μg L⁻¹ = microgram per liter
μg m⁻² = microgram per square meter
μL = microliter
μL min⁻¹ = microliter per minute
μm = micrometer
AB = Alberta
ACS = American Chemical Society
APCI(+) = atmospheric pressure chemical ionisation, positive ion mode
APCI = atmospheric pressure chemical ionisation
ASE = accelerated solvent extraction
cm = centimeter
°C = degree centigrade
C = carbon
C₈ = silica based chromatography column coated with a C₈ polymer
C₁₈ = silica based chromatography column coated with a C₁₈ polymer
CA = California
CAS = chemical abstracts service
d = day
DI = deionized
DT50 = the time required for 50% dissipation.
EC₁₀ = a ten percent reduction in endpoint
EC₅₀ = median effective concentration
ESI(+) = electrospray ionization, positive ion mode
ESI = electrospray ionization
EU = European Union
F = degrees of freedom
FEP = fluorinated ethylene-propylene
g = gravity
g = gram
g ha⁻¹ = grams per hectare
g kg⁻¹ = grams per kilogram
GI = gastrointestinal
h = hour
ha = hectare
H = hydrogen
HFBA = heptafluorobutyric acid
HILIC = hydrophilic interaction liquid chromatography
HLB = hydrophilic lipophilic balance

H₂O = water
 HPLC = high performance liquid chromatography
 id = internal diameter
 IL = Illinois
 kg = kilogram
 kg cm⁻³ = kilogram per cubic centimeter
 kg month⁻¹ = kilogram per month
 km = kilometer
 K_{ow} = octonal/water co-efficient
 L = liter
 LC = liquid chromatography
 LC-MS = liquid chromatography coupled with mass spectrometry
 LC-MS/MS = liquid chromatography coupled with tandem mass spectrometry
 LD₅₀ = median lethal dose
 L d⁻¹ = liters per day
 L h⁻¹ = liters per hour
 L ha⁻¹ = liters per hectare
 L min⁻¹ = liters per minute
 LOQ = limit of quantification
 L-S 20 = lincomycin spectinomycin premix formulation
 m = meter
 mA = milliamp
 mbar = millibar
 mg = milligram
 mg kg⁻¹ = milligram per kilogram
 mg kg⁻¹ d⁻¹ = milligram per kilogram per day
 mg L⁻¹ = milligram per liter
 min = minute
 mL = milliliter
 mm = millimeter
 mM = millimolar
 m/z = mass to charge ratio
 M = molar
 MΩ = mega ohm
 (M+H)⁺ = parent ion plus hydrogen
 (M+H+H₂O)⁺ = parent ion plus hydrogen plus water
 (M+H+CH₃OH)⁺ = parent ion plus hydrogen plus methanol
 MA = Massachusetts
 ML = Mega liters
 MO = Missouri
 MRM = multiple reaction monitoring
 MS = mass spectrometry
 MS/MS = tandem mass spectrometry
 n = number of samples
 nd = not detected
 ng = nanogram

ns = no sample available
N = nitrogen
O = oxygen
pH = $-\log [H^+]$
psi = pounds per square inch
P = significance
PEC = predicted environmental concentration
PLE = pressurized liquid extraction
PNEC = predicted no effect concentration
PVC = polyvinyl chloride
QC = Quebec
 R^2 = regression coefficient
s = second
SD = standard deviation
SE = standard error
SK = Saskatchewan
SPE = solid phase extraction
TOC = total organic carbon
US = United States
USA = United States of America
USP = United States Pharmacopeia
v/v = volume for volume
V = volts
WCX = weak cation exchange
WS = watershed
x = times

1.0 INTRODUCTION AND BACKGROUND

1.1 Antimicrobial use in the livestock industry

In Canada, pork sales exceeded 1 billion dollars in 1999 and, with more than 20 million swine slaughtered every year, pork production is Canada's second largest agricultural export (Reid and Friendship, 2002). With the rapid growth of intensive livestock operations, resulting in animal crowding and the use of common waste pits, there is a need to control disease outbreaks. Generally, this is accomplished through the use of antimicrobials.

There are three situations where antimicrobials are used in the livestock industry: 1) in feed, for use either therapeutically or subtherapeutically, 2) as over the counter drugs for injection or addition to water, 3) veterinarian prescribed drugs, administered for short periods of time to treat or prevent disease and are not given in feed (Prescott, 1997). It has been estimated that the swine industry administers approximately 60% of all the antimicrobials used in animal production (Dunlop et al., 1998). Dewey et al. (1999) report that, in the United States, an average of 51% of feeds administered to swine contained antimicrobials for disease prevention, compared to 4% of feeds which contained antimicrobials for treatment of specific outbreaks over a short period of time.

1.1.1 Disease prevention

Antimicrobial use in livestock and poultry operations significantly reduces illness, suffering and deaths due to infection (Cromwell, 2002). In the swine industry, about 10 to 15% of pigs die before they are weaned, mainly due to gastrointestinal infections, and the resulting loss in revenue is approximately 200 to 300 million dollars per year in Canada (Reid and Friendship, 2002).

Swine dysentery (post-weaning diarrhea) affects young swine during the post-weaning period (Barragry, 1994). When piglets are weaned they are submitted to a variety of stressors including: loss of mother, being placed with pigs from other litters and changes in environment and diet (Tsiloyiannis et al., 2001). These factors combined with overcrowding, poor hygiene

and common waste collection can lead to the spread of this highly infectious gastrointestinal disease (Barragry, 1994). Many different organisms, present in combination, are responsible for this disease, including *Teponema hydysenteriae* and various *Escherichia coli* strains (Barragry, 1994; Tsiloyiannis et al., 2001). This disease causes a severe loss of productivity in the swine industry due to reduced growth rates, and mortality (Barragry, 1994). The expense of preventative treatment can be easily justified based on the potential economic losses due to an outbreak (Barragry, 1994). Lincomycin and spectinomycin can be administered together as a premix in the feed to prevent and control post-weaning diarrhea.

1.1.2 Growth promotion

Antimicrobials administered to weanling pigs have been reported to increase their average daily weight gain by 10 to 30% and increase their feed efficiency by 6 to 15% (Cromwell, 2002; Prescott, 1997). In two specific studies performed to gauge antimicrobial use among swine producers in Ontario (Dunlop et al., 1998) and the United States (Dewey et al., 1999), both determined that approximately 95% of feeds administered to younger pigs (between weaning and 45 kg live weight) contained antimicrobials and that older animals were less likely to be administered antimicrobials in their feed to prevent disease. This is because young pigs are more susceptible to disease and it is this early growth period that is most positively affected by antimicrobial therapy (Dewey et al., 1999; Dunlop et al., 1998). It is very difficult to accurately determine the actual amount of antimicrobials being used, as sales figures from pharmaceutical companies do not necessarily mean use (Isaacson and Torrence, 2002). Also, different countries have different regulations regarding the use of antimicrobials. In the United States (US), many veterinary antimicrobials are available without a prescription, whereas in Denmark all antimicrobials are administered via veterinarian's prescription and accurate records of use are kept and reported (Isaacson and Torrence, 2002).

1.1.3 Banning of antimicrobials

Antimicrobials used as growth promoters in feed were banned in Sweden in 1986 and phased out in Denmark as of January 2000. In 1999, a partial ban on antimicrobials used as growth promoters was implemented in all European Union (EU) countries. When these bans first came

into effect, it was important to change husbandry practices to compensate for the lack of antimicrobials as growth promoters.

Immediately after the ban, Sweden saw an increase in antimicrobials used therapeutically to treat post-weaning diarrhea (Wierup, 2001). It was seen that swine production was significantly impacted by factors such as hygiene and segregation, depth and size of waste pits and feed composition (Wierup, 2001). In both Sweden and Denmark, removing antimicrobials as growth promoters in nursery pigs increased mortality, increased feed consumption, and decreased daily weight gain, therefore requiring up to 5 to 6 more days to reach target weights (Stein, 2002; Wierup, 2001). The estimated lost revenues from these infections were 0.50 to 1.00 US dollars per pig in Denmark (Stein, 2002). In Sweden, zinc oxide was added to nursery pig feed as an alternative to antimicrobials starting in 1993 (Wierup, 2001). Zinc oxide was found to have a similar preventative effect as antimicrobials on post-weaning diarrhea (Holmgren, 1994, cited in Wierup, 2001). Over time with better management practices, therapeutic antimicrobial use in nursery pigs declined and by 1999 only 5% were administered antimicrobial supplemented feed (Wierup, 2001).

Overall, the removal of antimicrobial growth promoters in grower-finisher pigs did not significantly affect the animals or the amount of therapeutic antimicrobials being administered to these animals in Sweden (Stein, 2002; Wierup, 2001). However, the literature is not in agreement on the benefits of a complete ban on antimicrobial growth promoters. Mudd et al. (1998) argue that there has been a misrepresentation of the facts. They state that, due to the ban, livestock and poultry producers have compensated by using more potent antimicrobials. Therefore though they are using less kilograms of active ingredient, when the potency of the drugs being used is considered, they are actually using the equivalent or more than before the ban.

Casewell et al. (2003) reviewed how the EU bans on growth promoters affected overall animal health. They concluded that these bans have decreased overall antimicrobial use, however the bans also brought to light previously unknown preventative benefits to overall animal health.

Use of therapeutic antimicrobials has increased and, despite changes in management practices, animal welfare has suffered.

1.1.4 Regulation of antimicrobials

The veterinary pharmaceutical industry is very highly regulated with the hope of minimizing misuse. In Canada, this is overseen by the Food and Drugs Act, administered by Health Canada as well as the Veterinary Drugs Directorate within Health Canada (Government of Canada, 2004). New drugs must be rigorously tested before being approved; proper labelling of all drugs and residue testing of animal products is required (Government of Canada, 2004). These precautions are taken in order to promote proper use, dosing, storage, and withdrawal times before slaughter. Any other, non specified use of an antimicrobial must be under the supervision of a veterinarian. Slaughtered animals are routinely monitored for residue levels in tissues. The most common cause of antimicrobial residues in animal products in the United States is not following recommended withdrawal times as well as not keeping accurate records of treatment (Dewey et al., 1999). Overall, antimicrobial residue violations have generally decreased in the US since 1990 (Dewey et al., 1999). These precautions help to protect our food supply and minimize misuse. There are penalties for not adhering to these rules including fines and imprisonment.

Use of pharmaceuticals in livestock raises both human health and environmental concerns (Khachatourians, 1998). The Canadian public is becoming increasingly concerned with the potential for long-term low level exposure to contaminants through food and water. These contaminants include pesticides, industrial wastes, endocrine disruptors, and pharmaceuticals such as antimicrobials. Little is known about the fate and persistence of chemicals, such as antimicrobials, in the environment (Daughton and Ternes, 1999). The misuse and overuse of antimicrobials has been blamed for the development of antimicrobial resistant bacteria (Franklin, 1999); however an absolute agreement in the literature has not been reached.

It has been reported that, in general, over 70% of bacteria are resistant to at least one antimicrobial (Hirsch et al., 1999). However, animal production is not solely responsible. Cromwell (2002) compared resistance data from a swine farm administering antimicrobials

(tetracyclines) in the feed, with swine at the University of Kentucky which have not been administered any antimicrobials since 1972. It was found that resistance to tetracyclines had developed in the non-medicated group, and that stressors such as housing conditions, moving and age also affected resistance patterns. Antimicrobial resistant bacteria have the potential to become a major public health problem. Programs that monitor antimicrobial use, their efficacy, their presence in the environment, and the development of resistance are one way of assessing their potential human health risk. France, Spain, and Denmark have set up special agencies that specifically monitor the presence of antimicrobial resistant bacteria (Bager, 2000; Bager et al., 2000; Martel et al., 2000; Moreno et al., 2000; Wray and Gnanou, 2000).

1.2 Potential sources of antimicrobials to the environment

Antimicrobials, along with other pharmaceuticals can enter the environment via many different pathways (Figure 1.1). In human medicine, measurable quantities of pharmaceuticals and personal care products and their potentially biologically active metabolites have been detected in ground water, wastewater effluents and waterways downstream of municipal sewage treatment plants (Lindberg et al., 2005). Wastewater treatment does not necessarily remove or breakdown all chemical residues so there is the potential for these chemicals to be present in municipal drinking water (Heberer, 2002).

Aquaculture is also a source of environmental contamination. Antimicrobials are added to fish feed to promote growth or prevent infection (Jorgensen and Halling-Sorensen, 2000). There is great variation within the industry regarding the size and type of water body, location, temperature of water, and the needs of a particular fish species (Isaacson and Torrence, 2002). Sediments under fish farms are known to contain high levels of antimicrobials that have been excreted, or were administered with the feed and not consumed by the animals (Halling-Sorensen et al., 1998).

In livestock and poultry production, antimicrobials can potentially contaminate the environment through a variety of pathways (Figure 1.1). Agricultural soils may be contaminated when manure, containing antimicrobials, is applied to crop and pasture land as a nutrient source. Subsequently, uptake of antimicrobials by plants may take place. Runoff and leaching from

feedlots and manure-treated crop land and pasture land have the potential to contaminate surface and ground water, respectively. Environmental concerns about manure applied to crop land were initially focused on environmental levels of the nutrients phosphorus and nitrogen, as well as metals and pathogens. Now this focus also includes pharmaceuticals and endocrine disrupting chemicals which may be present in livestock manure. Antimicrobials administered to livestock have been shown to be excreted up to 80% in the feces and urine (Calamari et al., 2003; Hornish et al., 1987; Jjemba, 2002). With an increase in intensive animal production operations, there is an increase in the quantities of manure which will be applied to relatively small areas of land. If the antimicrobials are administered to grazing livestock, excretion will be concentrated directly on the pasture land (Jorgensen and Halling-Sorensen, 2000). Some antimicrobials are chemically stable enough to remain active in manure for long periods of time, others are not. Migliore et al. (1995) report that sulfonamides remain active in manure applied to soil long enough to potentially have a negative effect on crop production. However, Loke et al. (2000) found that Tylosin A has a half-life of less than 2 d in the manure under anaerobic conditions.

Other minor sources to consider are the improper disposal of medications and medicated feed. These sources are very difficult to track and the amounts are largely unknown (Jorgensen and Halling-Sorensen, 2000). Crop production also plays a very small role with antimicrobials being used rarely to combat major disease outbreaks (Isaacson and Torrence, 2002).

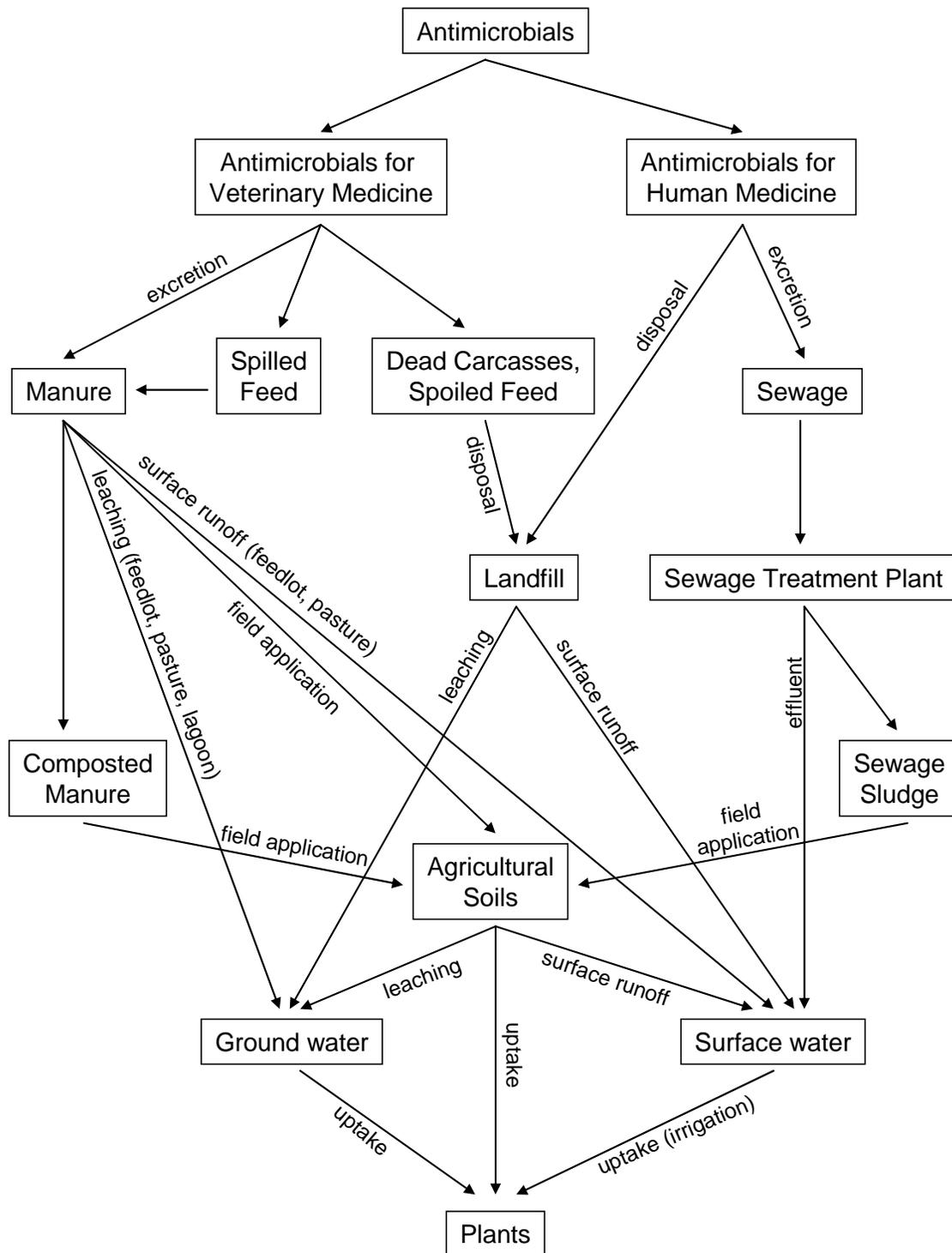


Figure 1.1. Possible transport routes of antimicrobials and other pharmaceuticals to the environment (modified from Jjemba, 2002).

1.3 Chemistry and analysis of antimicrobials in the environment

The physical and chemical characteristics (lipid solubility, water solubility, polarity, binding and adsorption capabilities, photostability, and biodegradability) of a pharmaceutical are very important when considering its fate and transport in the environment. Drugs or metabolites that are water soluble are more likely to end up in surface water and ground water than those that are lipid soluble. Lipid soluble compounds are generally persistent and tend to accumulate in lipid compartments of the environment. Some drugs accumulate in soils and sediments due to sorption.

Excreted antimicrobials and, in many cases, their conjugates retain antimicrobial activity. Conjugates have the potential, given the correct conditions, to reform their parent compound. Residues of antimicrobials have been detected in soil, manure, surface and ground waters in the United States and Europe. Liquid chromatography coupled with mass spectrometry, or tandem-mass spectrometry is the most sensitive and simplest way to analyse for antimicrobials in a variety of matrices. There are a number of published methods available for extracting and analysing antimicrobials in surface and ground waters, sewage treatment plant effluent, soil, manure, honey, animal feed, animal tissues, plasma and milk. Methods published in the literature are presented and discussed in their respective chapter introductions.

1.4 Toxicological consequences of antimicrobial residues in the environment

The toxic effects of antimicrobial residues in the environment are largely unknown. There is increasing concern that residues of antimicrobials and their potentially active metabolites have the ability to facilitate antimicrobial resistance, harm non-target species and interfere with sensitive ecosystems. Another area of concern is bioaccumulation and the potential for transport up the food chain, resulting in indirect toxicity. Examples of toxicity testing and risk assessments of antimicrobials are described below.

Isidori et al. (2005) investigated the ecotoxicity of six antimicrobials (erythromycin, oxytetracyclin, ofloxacin, lincomycin, and clarithromycin) on aquatic organisms. They found

that macrolides (lincomycin, erythromycin, and clarithromycin) were the most harmful to the aquatic environment. They determined that the acute toxicity was in the mg L^{-1} range and the chronic toxicity was in the $\mu\text{g L}^{-1}$ range. Algae were determined to be the most sensitive species studied with EC_{50} values ranging between 0.002 and 1.44 mg L^{-1} . Similarly, Halling-Sorensen (2000) reported that the EC_{50} values of different veterinary antimicrobials for the algae species *Selenastrum capricornutum* (green algae) were (mg L^{-1}): streptomycin (0.133), tetracycline (2.2), tylosin (1.38), and tiamulin (0.165). It was also reported that freshwater cyanobacteria were more sensitive to these compounds with corresponding EC_{50} values being 0.007, 0.09, 0.034, 0.003 mg L^{-1} , respectively.

Schallenberg and Armstrong (2004) measured the potential non-target effects of residues of antimicrobials in water in New Zealand. They found that water collected from a lake which received water from an agricultural drain displayed variable, concentration dependant antimicrobial activity on aquatic bacteria. Effects included: reduced numbers of bacteria and decreased respiration. This was a preliminary study and the results were not consistent over time. It was concluded that many factors need to be addressed that could have influenced the results.

Jensen et al. (2003) investigated the threshold levels of the antimicrobials tiamulin, olanquinox and metronidazole to the soil invertebrate species *Flosomia fimetaria* (springtails) and *Enchytraeus crypticus* (enchytraeids). These species were chosen because they normally live in manure. The endpoints used by the authors were a 10% reduction in reproduction or EC_{10} values. The threshold values for the springtails were between 61 and 111 mg kg^{-1} dry soil and for the enchytraeids between 83 and 722 mg kg^{-1} dry soil. They concluded that direct toxic effects to these species at environmentally realistic concentrations were not likely.

Wollenberger et al. (2000) studied the toxicity of some veterinary antimicrobials to *Daphnia magna* using a reproductive endpoint. The acute toxicities, 48 h EC_{50} values, in mg L^{-1} for a selection of antimicrobials tested were found to be: tiamulin (40), sulfadiazine (221), and oxytetracycline (1000). Chronic toxicities (mg L^{-1}) were found at much lower levels: tiamulin (5.4), sulfadiazine (13.7), and oxytetracycline (46.2).

Migliore et al. (1998) studied the phytotoxicity of the antimicrobial sulphadiazine on terrestrial plants. They found that the presence of sulphadiazine in the soil at a concentration of 300 mg L⁻¹ significantly depressed the growth of crop plants and weeds. It was determined that the source of this toxicity was bioaccumulation in the plants.

An Environmental risk assessment of the L-S 20 Premix for treatment in swine is available from the manufacturer (Pharmacia Animal Health Fact Sheet # 194). This was done in accordance to European Union regulations for the registration, or re-registration of veterinary drugs. The test species were *Daphnia magna*, algae, rainbow trout, *Lumbricus terrestris*, earthworms, bacteria, fungi, and plants. They found that all the predicted environmental concentration to predicted no effect concentration (PEC/PNEC) ratios were less than 1, except for one. This was seen to be acceptable because of the conservative nature of these risk assessments, and it was concluded that the L-S 20 premix should not have a significant impact on non-target species.

Spaepen et al. (1997) developed a mathematical model to predict the concentration of pharmaceuticals that would result in soil after manure application. In this model, the following assumptions were made: 1) the active ingredient is fully excreted (metabolites are not taken into account), 2) livestock being treated are housed indoors, 3) all manure is mixed and stored together, and 4) manure is applied to crop land once a year. This can be used as a tool for predicting environmental concentrations and potentially be incorporated into a risk assessment for newly developed pharmaceuticals.

Antimicrobials are an integral part of today's agricultural practice. Consequently, precautions must be taken in order to prevent or minimize environmental contamination, such as: modifying current production and management practices and using alternatives to antimicrobials such as alternative feed additives, probiotics and vaccines (Isaacson and Torrence, 2002; Reid and Friendship, 2002; Tsiloyiannis et al., 2001; Verstegen and Williams, 2002; Wierup, 2000). Antimicrobials being administered to livestock and poultry should be reviewed regularly to determine if they are maintaining efficacy, the benefits still outweigh the costs, and to determine if antimicrobial resistance has developed (Dewey et al., 1999). Environmental risk assessments

of veterinary pharmaceuticals have been required by the EU since 1997 and by the US Food and Drug Administration since 1980 (Boxall et al., 2003). Ideally, an environmental risk assessment would be mandatory in all countries during the initial drug design process, making them safer in the long term.

1.5 Lincomycin and spectinomycin

1.5.1 Lincomycin

Lincomycin (Figure 1.2a) is a member of the lincosamide group of antimicrobials. It can also be described as a macrolide antimicrobial. Its structure is a derivative of an amino acid and a sulfur-containing octose. Lincosamides are generally used as hydrochloride or phosphate salts, to enhance stability and water solubility (Aiello, 1998). The chemical properties of lincomycin and lincomycin hydrochloride are described in Table 1.1.

The mechanism of action of lincosamides is either bacteriostatic or bactericidal depending on their concentration (Aiello, 1998). They are most effective against a variety of anaerobic bacteria and gram-positive bacteria (Aiello, 1998). Most gram-negative bacteria and *Mycoplasma* species are resistant (Aiello, 1998).

Lincomycin is poorly absorbed from the gastrointestinal (GI) tract, especially if administered with food (Aiello, 1998; Hornish et al., 1987). Absorption following oral administration in feed ranges from 20 to 50% in swine (Hornish et al., 1987). When administered intramuscularly, absorption is very high (Aiello, 1998). Levels peak in the plasma in 2 to 4 h, following oral dosing, and in 1 to 2 h following intramuscular injection (Aiello, 1998). Following oral administration, up to 50% of lincomycin is metabolically transformed in the liver (Aiello, 1998). The metabolites normally retain antimicrobial activity (Aiello, 1998). Lincomycin is excreted as the parent compound and various metabolites in the bile and the urine (Aiello, 1998). In swine, following oral administration, 79 to 86% of given dose is excreted in feces, 14 to 21% in urine (Hornish et al., 1987).

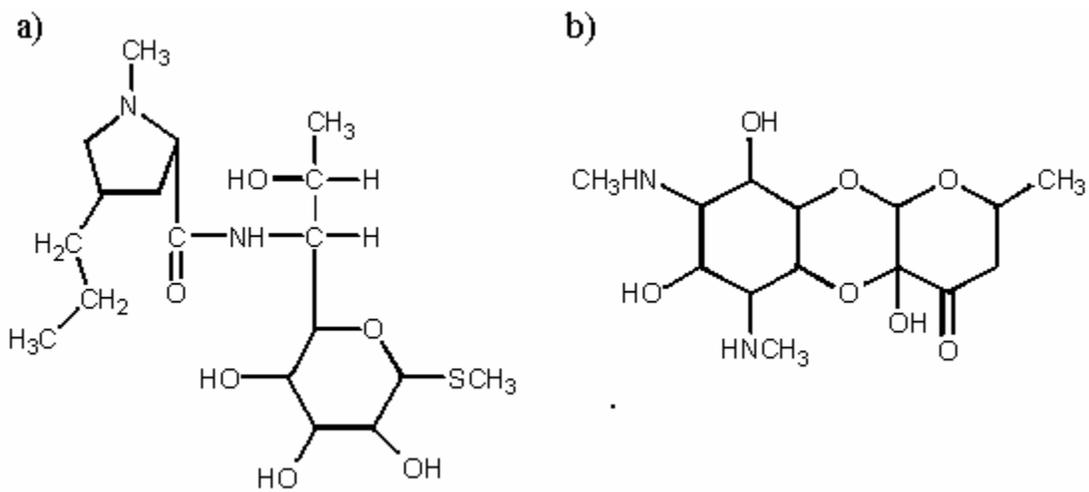


Figure 1.2. Chemical structure of a) lincomycin and b) spectinomycin

Table 1.1. Chemical properties of lincomycin and lincomycin hydrochloride

	Lincomycin	Lincomycin Hydrochloride
Molecular Formula	C ₁₈ H ₃₄ N ₂ O ₆ S	C ₁₈ H ₃₄ N ₂ O ₆ S · HCl · H ₂ O
Molecular weight	406.53	461.02
CAS ¹ Number	154-21-2	859-18-7
Physical Description	not available	White crystalline powder, a very faint odour or odourless, stable in air and light ²
pKa ³ values	7.64	7.64
Solubility	Soluble in methanol, ethanol, ethyl acetate, acetone, chloroform, slightly soluble in water ⁴	Soluble in water, methanol, ethanol, dimethylformamide, slightly soluble in acetone ⁴
K _{ow} ⁵	0.56 ⁶	0.56 ⁶
Half-life of lincosamides (animal unknown)	Generally <3 h ⁷	Generally <3 h ⁷
LD ₅₀ ⁸ in mice, rats (g kg ⁻¹)	1 (Intraperitoneal); 4 (oral) ⁴	1 (Intraperitoneal); 4 (oral) ⁴

¹CAS = Chemical abstracts service; ²The United States Pharmacopeial Convention, 2004a; ³pKa = - log (Ka), where Ka is the ionization constant for the partially ionized acid; ⁴Budavari, 1989; ⁵K_{ow} = octanol/water co-efficient; ⁶Syracuse Research Corporation, 2004; ⁷Aiello, 1998. ⁸LD₅₀ = median lethal dose.

1.5.2 Spectinomycin

Spectinomycin (Figure 1.2b) is described as an “aminoglycoside-like” antimicrobial. It is sometimes referred to as an aminocyclitol antimicrobial. The structure of aminoglycosides consists of a glycoside linkage of aminosugars around the 1,3-diaminocyclohexane unit. Although spectinomycin has the 1,3-diaminocyclohexane ring structure, it does not contain the glycoside linkages with aminosugars. Spectinomycin forms an equilibrium of four diastereoisomers in aqueous solution (Debremaeker et al., 2002). The chemical properties of spectinomycin and spectinomycin hydrochloride are described in Table 1.2.

The antibacterial properties of spectinomycin are expressed by a bacteriostatic mechanism. It is effective against *Mycoplasma* species and a variety of gram-positive and gram-negative bacteria (Aiello, 1998). Spectinomycin is much less toxic than other aminoglycosides, but it has shown increasing bacterial resistance (The United States Pharmacopeial Convention, 2004b).

Spectinomycin is not well absorbed from the GI tract, but it is very well absorbed via intramuscular injection (Aiello, 1998). The distribution of spectinomycin is mainly extracellular, due to its poor ability to penetrate tissues (Aiello, 1998). Spectinomycin undergoes very little metabolic transformation and following intramuscular injection it is excreted up to 80% in the urine over 24 to 48 h (Aiello, 1998). With oral administration, spectinomycin is mostly excreted in the feces, due to its poor GI absorption (The United States Pharmacopeial Convention, 2004b). No information on the excretion of spectinomycin in swine has been reported in the literature. The withdrawal time before slaughter for swine is approximately 3 weeks (Aiello, 1998).

Table 1.2. Chemical Properties of spectinomycin and spectinomycin hydrochloride

	Spectinomycin	Spectinomycin Hydrochloride
Formula	C ₁₄ H ₂₄ N ₂ O ₇	C ₁₄ H ₂₄ N ₂ O ₇ · 2HCl · 5H ₂ O
Molecular weight	332.35	495.35
CAS ¹ Number	1695-77-8	21736-83-4
Physical Description	White crystalline powder ²	White crystalline powder ²
pK _a ³ Values	6.95 and 8.70 ⁴	6.95 and 8.70 ⁴
Solubility	Soluble in water, methanol, ethanol, practically insoluble in acetone, hydrocarbon solvents ⁴	Soluble in water, methanol, propylene glycol, virtually insoluble in benzene, ethanol, chloroform and ether ⁴
K _{ow} ⁵	-0.82 ⁶	-0.82 ⁶
Half-life in pigs	0.98 h ²	0.98 h ²
LD ₅₀ ⁷ in mice (g kg ⁻¹)	> 2 (Intraperitoneal) ⁴	> 2 (Intraperitoneal) ⁴

¹CAS = Chemical abstracts service; ²The United States Pharmacopeial Convention, 2004b; ³pK_a = - log (K_a), where K_a is the ionization constant for the partially ionized acid; ⁴Budavari, 1989; ⁵K_{ow} = octanol/water co-efficient; ⁶Syracuse Research Corporation, 2004; ⁷LD₅₀ = median lethal dose.

1.5.3 L-S 20 Premix

In Saskatchewan, lincomycin and spectinomycin can be administered together as a premix in the feed (Pfizer Animal Health, 2003). It is available in a variety of forms and doses depending on the animal being treated and the illness being prevented or treated (Pfizer Animal Health, 2003). In swine, the L-S 20 Premix is mainly intended for the prevention and control of swine dysentery (post-weaning diarrhea) in growing swine up to 57 kg of body weight (Bayley, 2001). The active ingredients are: 22 g of lincomycin hydrochloride and 22 g of spectinomycin sulfate, for a total of 44 g equivalent of antimicrobial per metric tonne of feed (Bayley, 2001). The combination of the two antimicrobials gives this product broad spectrum antimicrobial activity and it acts quickly and efficiently (Pfizer Animal Health, 2003).

2.0 OBJECTIVES

The overall purpose of my research project was to investigate the environmental sustainability of the use of two antimicrobials, lincomycin and spectinomycin, in swine production with respect to surface and ground water quality. The key objectives were:

- 1) To develop analytical methods, utilizing solid-phase extraction and LC-MS/MS analysis detection, to quantify residues of lincomycin and spectinomycin in liquid swine manure, manure-treated soil, and in surface and ground waters.
- 2) To determine the concentration of lincomycin and spectinomycin in the manure excreted by weanling pigs administered these antimicrobials in their feed in a commercial-scale barn.
- 3) To determine the extent to which both antimicrobials persist in the manure during simulated manure storage and in soil following manure injection into crop land.
- 4) To assess the potential for their transport in surface runoff from manure-treated crop land into surface water bodies using simulated rainfall runoff experiments and by monitoring snow melt runoff.
- 5) To assess the potential for leaching/preferential flow of these two antimicrobials to shallow ground water by monitoring shallow ground water under manure-treated fields.

3.0 DEVELOPMENT OF ANALYTICAL METHODS FOR ANALYSIS OF LINCOMYCIN AND SPECTINOMYCIN IN SURFACE AND GROUND WATERS, MANURE-TREATED SOIL AND LIQUID SWINE MANURE

3.1 Preface

Parts of these methods have been published (reference cited below); a modified version is included in this thesis with the authors' permission.

Peru, K.M., S.L. Kuchta, J.V. Headley, and A.J. Cessna. 2006. Development of a hydrophilic interaction chromatography-mass spectrometry assay for spectinomycin and lincomycin in liquid hog manure supernatant and run-off from cropland. *Journal of Chromatography A* 1107:152-158.

3.2 Introduction

It is estimated that more than 50% of economic losses in weaning pigs are due to *Escherichia coli* infections, which result in either diarrhea or edema disease (Stahl, 2005). Lincomycin and spectinomycin (Figure 1.2a and b) are two commonly administered antimicrobials used to control post-weaning diarrhea. Spectinomycin is a member of the aminocyclitol group of antimicrobials. It is effective against a broad spectrum of gram-positive and gram-negative bacteria by inhibiting protein synthesis through interactions with the 30S ribosomal subunit of bacterial cells (Murray, 1991). Spectinomycin is excreted mostly unchanged in the feces and urine (Aiello, 1998). Lincomycin is a member of the lincomaside group of antimicrobials. It is effective against a variety of anaerobic and gram-positive bacteria, also by inhibiting protein synthesis (Aiello, 1998). Lincomycin undergoes some hepatic metabolism, and is excreted in the bile and urine (Aiello, 1998).

Liquid manure produced in confined animal feeding operations is frequently utilized as a nutrient source on crop land. The applied manure is thus a potential source of antimicrobials to the environment. However, little is established on their environmental fate. In order to study the fate of antimicrobials in the environment, analytical methods are required for the determination of

lincomycin and spectinomycin in liquid swine manure, manure-treated soil, runoff and ground water from manure-treated crop land.

Previously, methods have been reported for the determination of spectinomycin using a variety of microbial assays (El-Sayed et al., 1995; Medina, 2004; Shaikh et al., 1999; Stahl et al., 1991; Tanaka et al., 1996), gas chromatographic assay (Hoebus et al., 1994), thin layer chromatography/densitometry (Krzek et al., 2000), HPLC with electrochemical detection (Debremaeker et al., 2002; Elrod Jr. et al., 1988; Schermerhorn et al., 1995), HPLC with amperometric detection (Phillips and Simmonds, 1994), and HPLC methods requiring either pre- or post-column derivatization (Bergwerff et al., 1998; Burton et al., 1991; Haagsma et al., 1993; Hornish and Wiest, 1998; Myers and Rindler, 1979; Tsuji and Jenkins, 1985). These methods have mainly focused on residues in animal tissues and milk for human consumption. Analysis of spectinomycin by LC-MS/MS has proved challenging largely because of the high polarity and basic properties of the analyte. Chromatographic retention is difficult to achieve and thus poor separation from co-eluting matrix components leads to enhancement and/or suppression of ionization in the source of the mass spectrometer (Peru et al., 2004). Two methods have been reported for the determination of spectinomycin in tissues using ion-pairing reagents with detection by LC-MS (McLaughlin and Henion, 1992) and LC-MS/MS (McLaughlin et al., 1994). More recently, the utility of heptafluorobutyric acid (HFBA) ion-pairing to aid in LC retention and solid-phase extraction was reported (Carson and Heller, 1998). However, it has been observed that the HFBA concentrations required for sufficient retention of spectinomycin and separation leads to poor sensitivity and detection limits when using atmospheric pressure ionization techniques (Peru et al., 2004). A LC-MS/MS method utilising a pH gradient was employed to better retain and resolve spectinomycin from interfering co-eluting compounds (Peru et al., 2004). This method provided improved detection limits compared to the HFBA method, eliminated or significantly reduced the ionization interference in the MS source and gave reproducible results for samples of simulated rainfall run-off. However, in the current study, the method did not provide the required retention and reproducibility for analysis of the more complex liquid swine manure matrix.

Several methods have also been reported for the determination of lincomycin in animal tissues using gas chromatography with derivatization (Farrington et al., 1987; Luo et al., 1996b), HPLC with electrochemical detection in milk and animal tissues (Moats, 1991), ion-pairing HPLC with electrochemical detection in salmon tissues (Luo et al., 1996a), and HPLC with pulsed electrochemical detection in animal feed (LaCourse and Dasenbrock, 1999). Recently, LC-MS/MS with electrospray ionization (ESI) has been used to determine lincomycin in surface waters (Calamari et al., 2003; Kolpin et al., 2002), swine tissues (Sin et al., 2004), and milk (Crellin et al., 2003). One method utilized both a radioimmunoassay and LC-MS with ESI to determine lincomycin residues in samples collected from swine lagoons (Campagnolo et al., 2002). The use of LC-MS/MS with atmospheric pressure chemical ionization (APCI) has also been described for the determination of lincomycin in honey (Thompson et al., 2003). However, there were no examples of reported methods for the application of APCI in combination with hydrophilic interaction liquid chromatography separations for the analysis of antimicrobials.

Hydrophilic interaction liquid chromatography (HILIC) was explored in this work to improve LC retention of the two antimicrobials investigated, particularly spectinomycin. HILIC has been previously described in detail (Alpert, 1990). Briefly, HILIC, based on silica column packing, is an alternative to normal-phase chromatography but utilizes traditional reverse-phase mobile phases (Peru et al., 2005). Hydrophilic interaction liquid chromatography has been used to separate peptides (Yoshida, 2004), histones (Lindner et al., 1996; Lindner et al., 1997) and, more recently, some polar pharmaceuticals (Olsen, 2001; Strege, 1998; Strege et al., 2000) including the antimicrobials neomycin (Oertel et al., 2004), avoparcin (Curren and King, 2002) and three tetracyclines (tetracycline, chlortetracycline and oxytetracycline) (Valette et al., 2004).

The LC/APCI-MS/MS method developed in this work employs HILIC to provide sufficient resolution to reduce interference from the complex liquid swine manure matrix while providing baseline resolution between lincomycin and spectinomycin. Pressurized liquid extraction (PLE; Dionex trade name - ASE for accelerated solvent extraction) was used to extract lincomycin and spectinomycin from manure-treated soils and the solids component of liquid swine manure. Solid-phase extraction (SPE) was used to extract runoff water, ground water, liquid swine manure supernatant, and aqueous PLE extracts of manure-treated soil and the solids component

of liquid swine manure. A weak cation exchange (WCX) solid-phase extraction cartridge was used for spectinomycin and a hydrophilic-lipophilic balance (HLB) cartridge for lincomycin.

3.3 Experimental

3.3.1 Chemicals and reagents

Spectinomycin dihydrochloride ($\geq 98\%$) and lincomycin hydrochloride ($\geq 90\%$) were obtained from Sigma-Aldrich, (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Edmonton, AB, Canada). Deionized water (18 M Ω) containing less than 4 $\mu\text{g L}^{-1}$ total organic carbon was obtained using a Millipore Milli-Q Gradient A10 (with TOC detector) purification system (Millipore, Billerica, MA, USA). Certified ACS grade formic acid, ammonium hydroxide, trisodium citrate, citric acid and 20-30 mesh Ottawa sand were purchased from Fisher Scientific.

3.3.2 Preparation of standards and solutions

Stock standard solutions (1000 mg L^{-1}) of lincomycin and spectinomycin were prepared by weighing and dissolving each antimicrobial in 50% aqueous acetonitrile. The stock solutions, stored in the dark at 4°C, were used for a maximum of one month. Nine calibration standards were prepared (0.1 $\mu\text{g L}^{-1}$ – 500 $\mu\text{g L}^{-1}$) in mobile phase (acetonitrile/H₂O, 75:25, v/v containing 0.1% formic acid). Due to differences in detection limits for lincomycin and spectinomycin, a subset of 5 calibration solutions (10 $\mu\text{g L}^{-1}$ – 500 $\mu\text{g L}^{-1}$) was used to generate the calibration curve for spectinomycin while all nine (0.1 $\mu\text{g L}^{-1}$ – 500 $\mu\text{g L}^{-1}$) were used for lincomycin.

3.3.3 Sample preparation and extraction

Deionized (DI) water, runoff and ground water: DI, runoff and ground water samples were extracted using SPE as described previously (Peru et al., 2006). For the extraction of lincomycin, the pH of a 100-mL subsample was adjusted to pH 9 with 1 M ammonium hydroxide solution and passed through an Oasis HLB cartridge (Waters, Milford, MA, USA)

under vacuum. The cartridge was then eluted with acetonitrile (10 mL). For extraction of spectinomycin, a subsample (100-mL) was similarly passed through an Oasis HLB cartridge stacked on top of an Oasis WCX cartridge (Waters, Milford, MA, USA). The HLB cartridge was then discarded, and the WCX cartridge was washed with 25 mM citrate buffer (pH 5) followed by methanol and then eluted with acetonitrile containing 3% formic acid (10 mL).

Liquid swine manure: *Sample preparation:* Liquid swine manure (10 mL) was centrifuged at 3000 x g for 15 min at room temperature and the supernatant decanted. The supernatant was similarly centrifuged twice more to maximize solids removal. The solids components were combined for subsequent extraction.

Liquid component (supernatant): Liquid swine manure supernatant (2 mL) was diluted to 200 mL with distilled water and extracted using the SPE procedures described previously for the surface and ground water samples (Peru et al., 2006).

Solids component: The combined solids components were extracted by pressurized liquid extraction followed by SPE cleanup of the PLE extract (Kuchta et al., 2007).

Air-dried manure solids component (1 g) was mixed with sufficient diatomaceous earth (~ 2 g) and Ottawa sand (~ 4 g) to fill a 33-mL PLE cell equipped with three glass fiber filters placed at the exit end of the cell. The packed cell was then extracted by PLE (ASE 200; Dionex, Sunnyvale, CA) as follows: the PLE cell was heated to 100°C and extracted for 5 min with deionized water (static mode) at a pressure of 1500 psi. The cell was then flushed with 30% volume of deionized water and purged with nitrogen gas for 1.5 min (150 psi). Optimal extraction was achieved by extracting each cell twice. The resulting aqueous extracts (~30 mL each) were combined, diluted to 200 mL with deionized water, and then similarly subjected to SPE clean-up.

Manure-treated soil: Soil samples were air-dried, mixed well, and sieved (2 mm) in preparation for analysis. Sieved soil (2 g) was mixed with sufficient diatomaceous earth and Ottawa sand to fill a 33-mL PLE cell equipped with three glass fiber filters placed at the exit end of the cell.

The PLE and subsequent SPE clean-up parameters for the soil samples were the same as those described above for the solids component of the liquid swine manure (Kuchta et al., 2007).

3.3.4 Fortification studies

Deionized water, surface water and liquid swine manure supernatant: DI water (100 mL), South Saskatchewan River water (100 mL), used as a control matrix, and supernatant from the control sample of liquid swine manure (2 mL diluted to 200 mL) were fortified with lincomycin and spectinomycin at 1, 10 and 100 $\mu\text{g L}^{-1}$, by adding appropriate amounts of both lincomycin and spectinomycin dissolved in methanol (100 μL). The average recoveries of lincomycin and spectinomycin from DI water (n=9) were $103 \pm 10\%$ and $108 \pm 7\%$ for lincomycin and spectinomycin, respectively (Peru et al., 2006). Corresponding average recoveries from fortified river water (n=9) were $89 \pm 8\%$ and $95 \pm 8\%$, respectively. Whereas corresponding values for liquid swine manure supernatant (n=9) were $78 \pm 7\%$ and $84 \pm 2\%$. Recoveries of spectinomycin from liquid manure supernatant were poor at the lowest fortification level (1 $\mu\text{g L}^{-1}$). These recoveries are very similar to those reported earlier by Peru et al. (2006). Additionally, river water was similarly fortified with lincomycin at 0.01 $\mu\text{g L}^{-1}$. Average recoveries were $91 \pm 4\%$ (n=9).

Soil: Air-dried soil (2 g) was fortified by the addition of 2 and 200 ng of lincomycin and spectinomycin, respectively, dissolved in methanol (1 mL), resulting in concentrations of 1 and 100 $\mu\text{g kg}^{-1}$ (n = 9), respectively. The soil was then mixed well and placed in a fume hood until evaporation of the methanol was complete. The fortified soil was extracted the following day. Control soil was similarly treated but with methanol only. The average recoveries of lincomycin and spectinomycin from fortified soil (n = 9) were $74 \pm 8\%$ and $66 \pm 15\%$, respectively.

Solids component of the liquid swine manure: Control liquid swine manure was centrifuged and the solids component isolated and air-dried. Air-dried solids component (1 g) was fortified by the addition of 1, 50 and 100 ng of both lincomycin and spectinomycin dissolved in methanol (1 mL), resulting in concentrations of 1 (n = 5), 50 (n = 4) and 100 (n = 5) $\mu\text{g kg}^{-1}$. The solids component was then mixed well and the fortified manure placed in a fume hood until evaporation of the methanol was complete. The fortified solids were extracted the following

day. Control manure solids were similarly treated but with methanol only. Reliable recoveries were achieved from the solids component of the liquid swine manure for both antimicrobials at the 50 and 100 $\mu\text{g kg}^{-1}$ fortification levels. Average recoveries were $70 \pm 10\%$ and $62 \pm 12\%$ ($n = 9$) for lincomycin and spectinomycin, respectively. Recoveries at the lowest fortification level ($1 \mu\text{g kg}^{-1}$) were poor, being $<10\%$ for both antimicrobials.

Whole manure: Additionally, control liquid swine manure was fortified at 10 and 100 $\mu\text{g L}^{-1}$ and aged for 7 days at 4°C . Samples (10 mL) were centrifuged to separate the solids and liquid component and extracted separately as described above. Total recoveries ($n=12$) were $77.4 \pm 14\%$ and $68.6 \pm 9\%$ for lincomycin and spectinomycin, respectively.

3.3.5 Liquid chromatography coupled with tandem mass spectrometry analysis

Liquid chromatography system

An Alliance 2695 Separations Module (Waters, Milford, MA, USA) consisting of a LC pump, column oven, solvent degasser and auto-sampler was used for all chromatographic separations. HILIC separations were carried out using a silica based Altima HP hydrophilic interaction column (2.1 mm id x 150 mm, 3 μm ; Alltech Associates, Deerfield, IL, USA), maintained at 36°C . Eluents A and B were: 90/10 acetonitrile/water + 0.1% formic acid and 90/10 water/acetonitrile + 0.1% formic acid, respectively. Sample injection volume was 15 μL . Separation was achieved with isocratic conditions ($200 \mu\text{L min}^{-1}$) of 65% eluent A and 35% eluent B. Under these conditions, retention times were 3.6 and 6.3 min for lincomycin and spectinomycin, respectively.

Mass spectrometer

All experiments were conducted on a Micromass Quattro Ultima (Waters, Milford, MA, USA) triple quadrupole mass spectrometer equipped with an APCI interface operated in positive ion mode. Optimized instrumental settings were as follows: corona 0.20 mA, cone 60 V, source temperature 120°C , desolvation temperature 550°C , cone gas flow rate of $153 \text{ L h}^{-1} \text{ N}_2$, desolvation gas flow rate of $199 \text{ L h}^{-1} \text{ N}_2$, nebulizer gas N_2 at maximum flow rate, collision

energy of 30 V and multiplier 700 V. Multiple reaction monitoring (MRM) was used for quantitative analysis with the following settings: 0.5 s dwell time and 0.1 s inter-channel delay while monitoring 2 channels. Argon was used as the collision gas at a pressure sufficient to increase the pirani gauge of the collision cell to a reading of 2.41×10^{-4} mbar.

3.4 Results and discussion

3.4.1 Extraction method

Methods for the extraction of lincomycin are numerous in the literature (Calamari et al., 2003; Farrington et al., 1987; Kolpin et al., 2002; LaCourse and Dasenbrock, 1999; Luo et al., 1996a; Luo et al., 1996b; Moats, 1991; Thompson et al., 2003) in combination with methods for the determination of other antimicrobials. Lincomycin is amenable to SPE using traditional packings (C_{18} , C_8) giving good recoveries from various matrices. In contrast, few SPE methods along with data on the levels of spectinomycin are reported (Bergwerff et al., 1998; Carson and Heller, 1998; Haagsma et al., 1993; Stahl et al., 1991). All require the addition of an ion-pairing reagent prior to extraction to aid in analyte retention (Bergwerff et al., 1998; Carson and Heller, 1998; Haagsma et al., 1993; Stahl et al., 1991). This may be due, in part, to poor retention of the analyte on reverse-phase packings leading to poor SPE recoveries and inadequate chromatographic retention on traditional reverse-phase LC packings. Using stacked SPE cartridges, such that the Oasis HLB cartridge was used for sample cleanup (removal of non-polar compounds) followed by trapping of spectinomycin on the second cartridge containing a weak cation exchange packing (Oasis WCX), enabled the extraction of spectinomycin. The cation exchange capacity of the WCX packing results from carboxylic acid groups that allow protons to be exchanged with basic ionic compounds at pH 5 or lower. Acetonitrile containing 3% formic acid is passed through the cartridges to re-protonate the carboxyl groups and allow elution of the trapped basic analytes. Improved chromatography (with reproducible retention times and fewer matrix effects) was observed using stacked cartridges versus the WCX column alone. This can be attributed to the non-polar fraction being retained on the HLB cartridge, resulting in reduced fouling of the WCX packing and a cleaner final extract.

In this study, lincomycin was extracted using an Oasis HLB cartridge. To suppress ionization and achieve acceptable recovery, samples were adjusted to pH 9 using ammonium hydroxide solution. This required step, unfortunately, circumvented the use of the WCX cartridge for simultaneous extraction of spectinomycin. This is due to the fact that the pH adjustment would have been detrimental to the WCX extraction process, which requires spectinomycin to be in its ionized form for retention.

3.4.2 Mass spectrometry

Prior to developing an alternative to ion-pairing for sufficient LC separation, MS parameters were evaluated to determine the most sensitive ionization technique available in our laboratory. This evaluation was performed by infusing a 1 mg L⁻¹ solution of spectinomycin and lincomycin in 50:50 methanol/water and, for comparison, 50:50 acetonitrile/water. Both ESI and APCI in positive ion mode produced an intense (M+H)⁺ for lincomycin (m/z 407) regardless of solvent (Figure 3.1). The optimal MRM transition for quantitation of lincomycin was m/z 407>126.

However, unlike previously reported ion trap data (Carson and Heller, 1998), both ESI and APCI produced a very weak (M+H)⁺ at m/z 333 for spectinomycin (Figure 3.2a,b). Adjusting instrumental and eluent properties did not improve the (M+H)⁺ abundance for spectinomycin. Because the (M+H)⁺ was so weak, adduct ions were considered for quantification. The mass spectrum for spectinomycin generated using ESI(+) resulted in fragmentation and adduct ion formation (Figure 3.2a). Solvated adduct ions were observed at m/z 351, 365, and 396 (Figure 3.2a). Whereas, the mass spectrum generated using APCI(+) resulted in reduced fragmentation and intense adduct ion [(M+H+H₂O)⁺, m/z 351 and (M+H+CH₃OH)⁺, at m/z 365] formation (Figure 3.2b). Though the intense adduct ion at m/z 365 was produced with both ESI(+) and APCI(+), the transition m/z 365 > 333 showed low reproducibility, thus the optimal MRM transition for quantation of spectinomycin was m/z 351 > 333 and APCI(+) was better suited for this analysis. Although monitoring the loss of H₂O for spectinomycin is not a highly specific transition, the compromise was made to optimize detection limits.

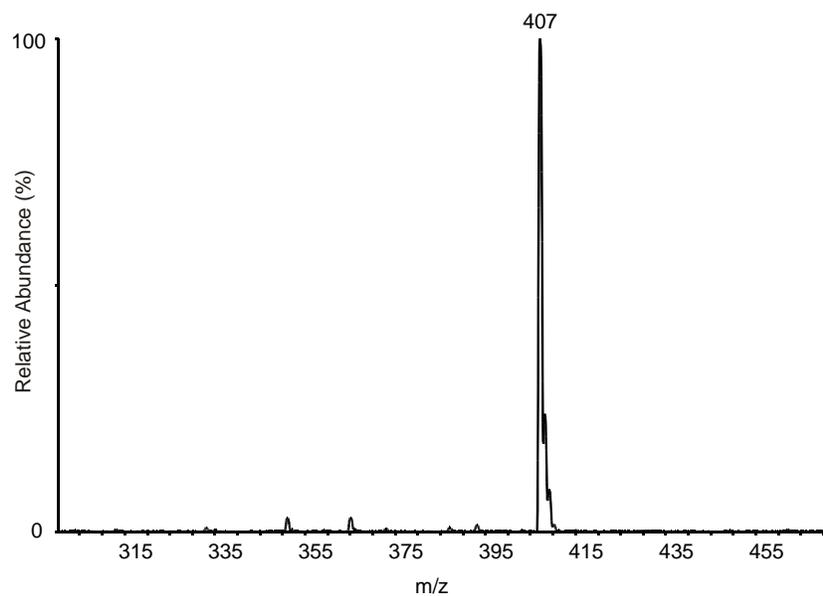


Figure 3.1. Mass spectrum of lincomycin using atmospheric pressure chemical ionization.

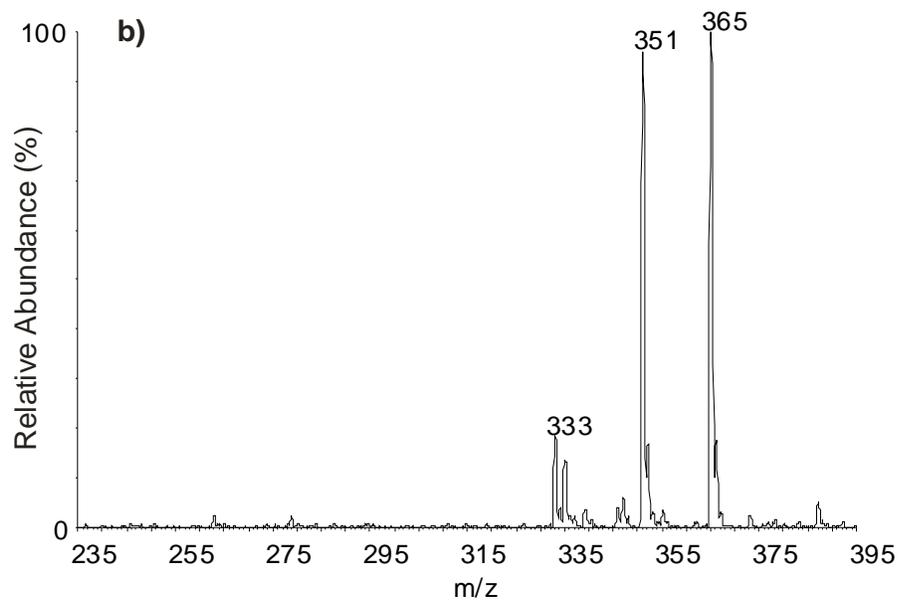
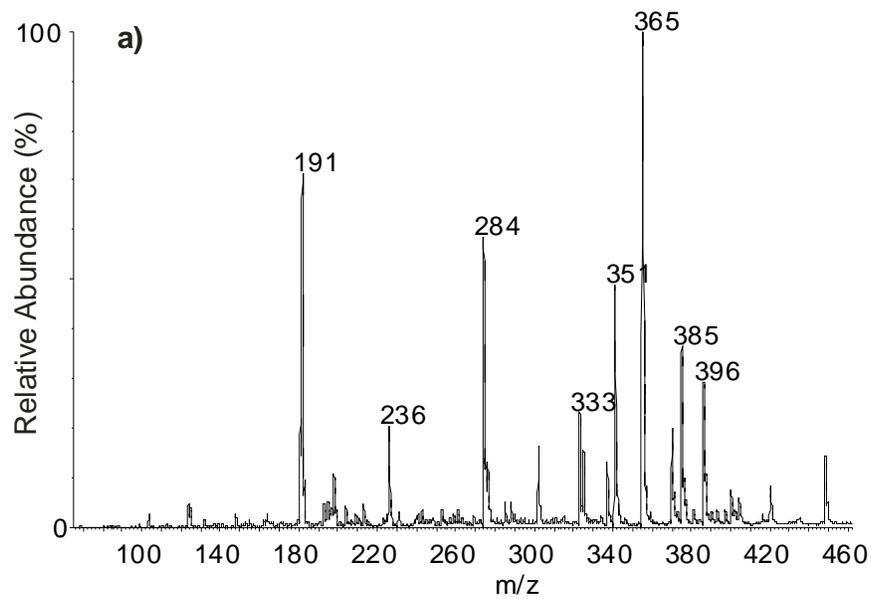


Figure 3.2. Mass spectrum of spectinomycin using (a) electrospray ionization and (b) atmospheric pressure chemical ionization

3.4.3 Chromatography

Initial results using C₈ or C₁₈ HPLC columns with a variety of eluent conditions generated little to no retention of spectinomycin (Figure 3.3a) leading to matrix interference. In addition, peak tailing was observed, most likely due to interactions between the residual silanols on the column surface and positively charged basic compounds. Up to a 200% recovery for spectinomycin was observed due to ionization enhancement of matrix effects when elution of the analyte was at or near the void volume. The use of HILIC increased analyte retention and decreased peak tailing (Figure 3.3b) while reducing or eliminating ionization enhancement and/or matrix interference. Even under isocratic conditions, HILIC provided excellent retention and separation from matrix components while achieving baseline resolution of lincomycin and spectinomycin (Figure 3.3b). Furthermore, the addition of formic acid to the eluents to promote source ionization increased overall sensitivity, provided better peak shape and improved the reproducibility of the analyte retention times. The retention order of the two antimicrobials is reversed from that on reverse-phase columns because more polar compounds are better retained using HILIC. The chromatograms in Figure 3.4 illustrate typical results from each MRM channel of both the WCX and HLB extracts (fortified liquid swine manure supernatant).

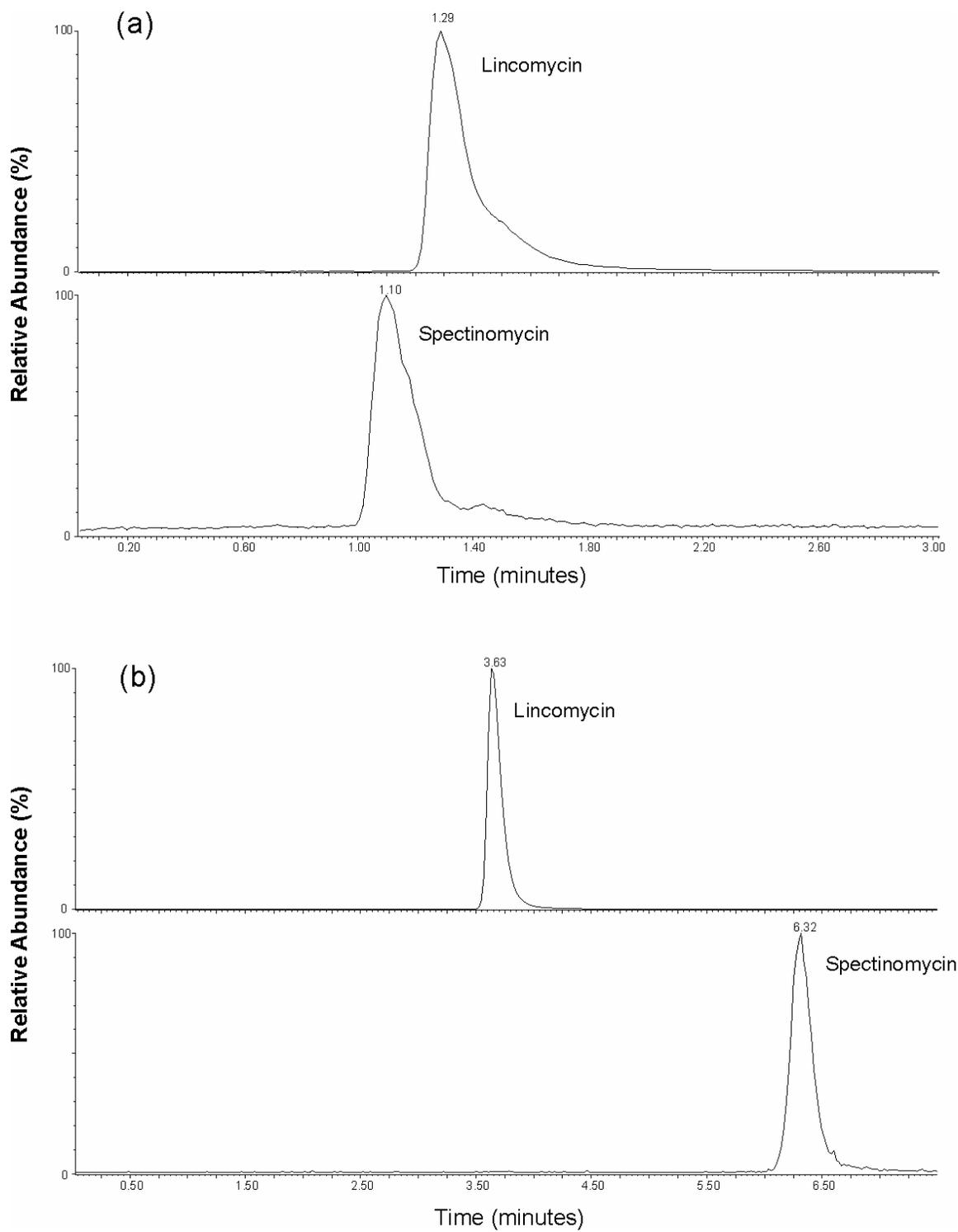


Figure 3.3. Liquid chromatography chromatograms illustrating retention of spectinomycin and lincomycin using (a) C₁₈ analytical column and (b) hydrophilic interaction liquid chromatography analytical column.

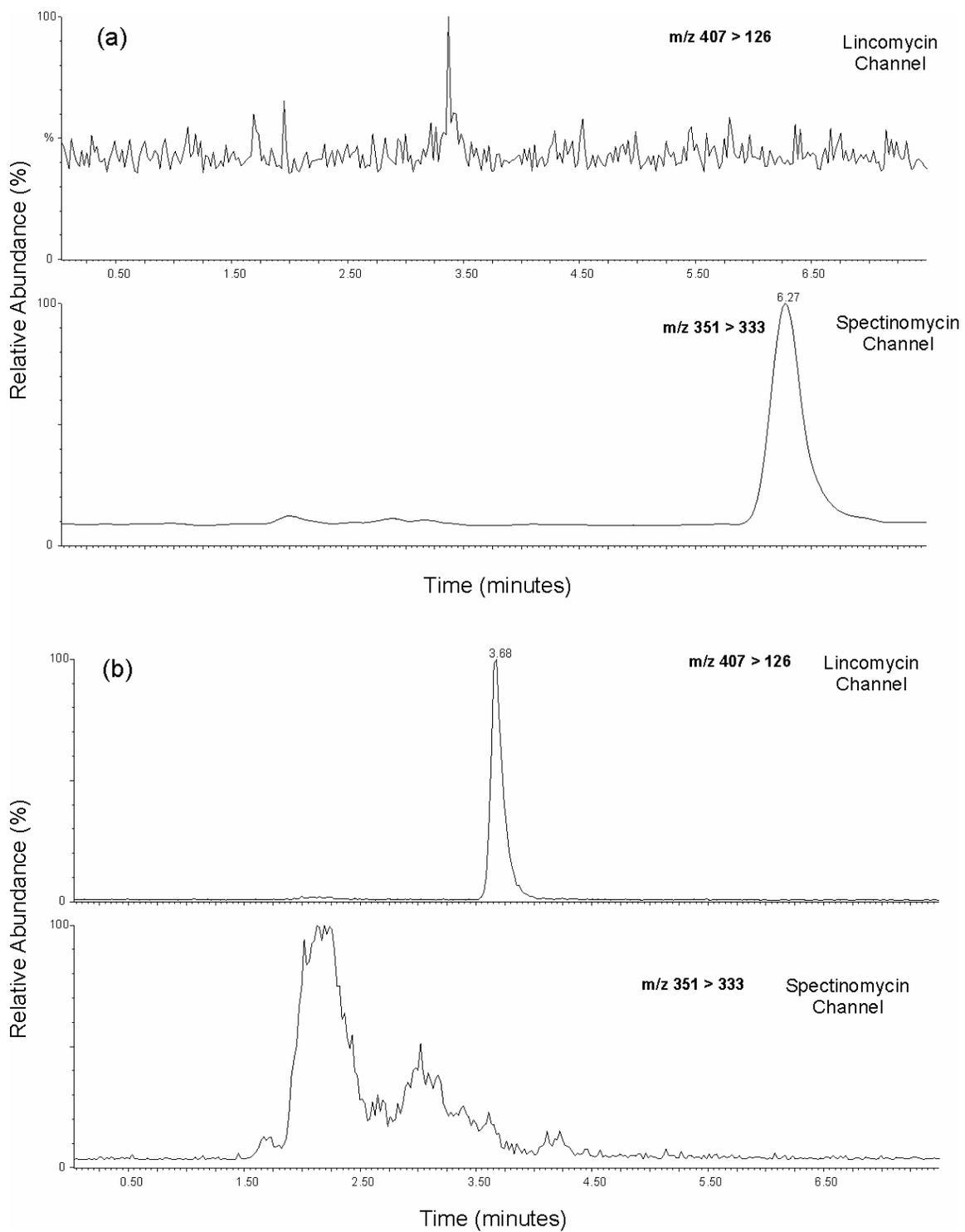


Figure 3.4. Chromatogram of each multiple reaction monitoring channel for extracts on (a) weak cation exchange and (b) hydrophilic lipophilic balance (liquid swine manure supernatant).

3.4.4 Quantification

Calibration curves were generated for both lincomycin (0.1, 0.5, 1, 5, 10, 50, 100, 250, 500 $\mu\text{g L}^{-1}$) and spectinomycin (5, 10, 50, 100, 250, 500 $\mu\text{g L}^{-1}$). Over the tested concentration ranges, linear regression of observed peak areas versus concentration gave excellent linearity with R^2 values of 0.990 or greater (Figure 3.5). In order to minimize enhancement/suppression of analyte ionization within the source of the mass spectrometer due to co-eluting matrix components, hydrophilic interaction liquid chromatography was used to ensure that both compounds eluted well after the void-volume of the column and to simultaneously achieve baseline separation for quantification (Peru et al., 2006).

Limits of quantification (LOQ) were defined as one half the lowest fortification level for each matrix. Limits of quantification for lincomycin and spectinomycin, respectively, were 0.005 and 0.5 $\mu\text{g L}^{-1}$ for runoff and ground water, 0.5 and 50 $\mu\text{g kg}^{-1}$ for soil, 0.5 and 6.0 $\mu\text{g L}^{-1}$ for the liquid component (supernatant) of swine manure and 25 and 50 $\mu\text{g kg}^{-1}$ for the solids component of liquid swine manure (Peru et al., 2006).

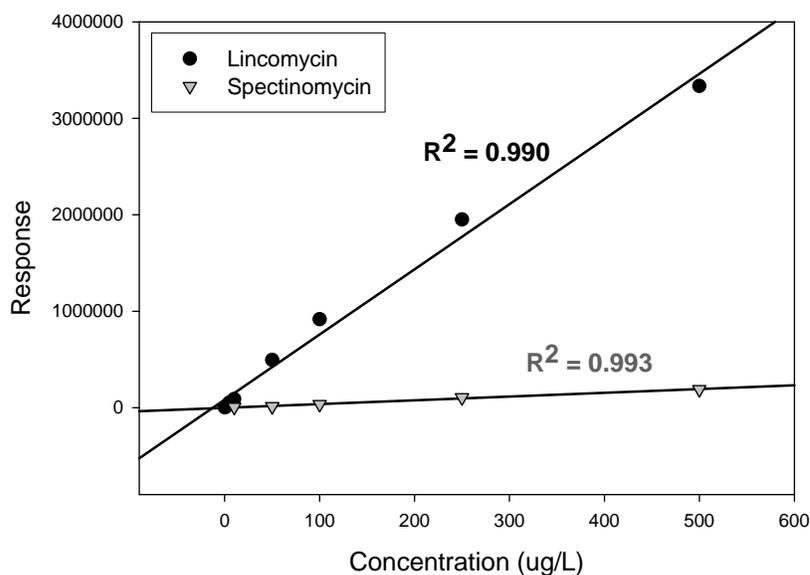


Figure 3.5. Calibration curve for lincomycin and spectinomycin standard solutions (0.1, 0.5, 1, 5, 10, 50, 100, 250, 500 $\mu\text{g L}^{-1}$ for lincomycin and 5, 10, 50, 100, 250, 500 $\mu\text{g L}^{-1}$ for spectinomycin).

3.5 Conclusions

Pressurized liquid extraction was an effective way to extract both lincomycin and spectinomycin from soil and the solids component of liquid swine manure. Weak cation exchange SPE provided adequate recovery of spectinomycin from all matrix extracts. Stacking an Oasis HLB cartridge on top of the WCX cartridge provided the clean-up prior to analysis which was necessary to reduce or eliminate suppression or enhancement of ionization in the source of the mass spectrometer. Hydrophilic interaction chromatography provided better retention on the HILIC column and excellent separation of lincomycin and spectinomycin from interfering matrix components. It also provided a baseline separation without the need for ion-pairing reagents. HILIC facilitated the use of formic acid as a mobile phase additive that was compatible with atmospheric pressure ionization techniques and provided good ionization efficiency. Atmospheric pressure chemical ionization in positive ion mode produced intense ions that were conducive to trace analysis using MS/MS. Instrument sensitivity for lincomycin was greater than for spectinomycin, and thus the LOQ's were lower for lincomycin than spectinomycin.

4.0 LINCOMYCIN AND SPECTINOMYCIN CONCENTRATIONS IN LIQUID SWINE MANURE AND THEIR PERSISTENCE DURING SIMULATED MANURE STORAGE

4.1 Introduction

Increasingly, swine, poultry and cattle are being produced in large confined animal feeding operations. With such intensive animal production, antimicrobials are administered, regardless of the health of the animals, to prevent disease and, when fed at milligrams per kilogram of feed, to promote growth by increasing the rate of weight gain. Since many antimicrobials are poorly absorbed through the gut, they can be excreted up to 80% or more in the feces and urine (Aiello, 1998), and some have been detected in manure (Winkler and Grafe 2001; Campagnolo et al. 2002). Consequently, because of antimicrobials present in the manure, the application of manure to provide plant nutrients to crop and pasture land can be a potential source of antimicrobial contamination of surface and ground water via surface runoff and leaching, respectively.

Water quality has become an important issue with the Canadian public both in terms of safety of drinking water and in terms of protecting and conserving aquatic ecosystems. Recently, antimicrobials used in the livestock industry have been detected in surface waters in Canada (Forrest et al., 2006) the United States (Kolpin et al., 2002; Lindsay et al., 2001) and Europe (Calamari et al., 2003; Hirsch et al., 1999). Contamination of surface and ground waters by veterinary antimicrobials is a cause for concern because it may accelerate the development of antimicrobial-resistant bacteria. Although antimicrobials administered to humans are generally not the same as those given to animals, their structures may be similar enough that veterinary antimicrobials can cause resistance to those used to treat humans. For example, Khachatourians (1998) showed that when streptococci and staphylococci developed resistance to tylosin, a common animal feed additive, they also developed a resistance to erythromycin used in human medicine.

The threat that antimicrobials pose to surface and ground waters, following land application of manure, depends, in part, on the extent to which they are excreted in the feces and urine and on their stability in stored manure. Amounts of antimicrobials excreted in manure and their fate and persistence in stored manure are not well understood, but are becoming areas of increasing

interest. Schlusener et al. (2006) studied the fate of the antimicrobials erythromycin, roxithromycin, salinomycin and tiamulin in liquid swine manure during a 180-d degradation experiment. Calculated half-lives during manure storage were 41, 130 and 6 d for erythromycin, roxithromycin and salinomycin, respectively. In contrast, the concentration of tiamulin remained unchanged during the entire experiment. Arikan et al. (2006) and De Liguoro et al. (2003) monitored oxytetracycline in manure from cattle orally administered the antimicrobial and reported half-lives of 56 and 30 d, respectively. Loke et al. (2000) and Teeter and Meyerhoff (2003) reported half-lives for tylosin of <2 and 7.6 d in liquid swine manure, respectively. Winckler and Grafe (2001) measured the excretion of tetracycline from swine administered the antimicrobial in a low and high dose treatment and reported 42 and 72%, respectively, excreted within 7 d of starting the exposure. In the same study, the half-life of tetracycline (20 mg L⁻¹) in the liquid manure in an outdoor experiment was 105 d whereas in an indoor experiment, in which the liquid manure was maintained at 8°C, the half-life was 56 d. In contrast, Kuhne et al. (2000) reported the time for 50% dissipation of tetracycline in aerated and non-aerated liquid swine manure to be 4.5 and 9 d, respectively. With the exception of these studies, there is very little information on the fate and persistence in liquid swine manure of many commonly used veterinary antimicrobials.

With intensive swine operations, liquid swine manure is generally stored in lagoons until it is utilized as a nutrient source on crop or pasture land. In Saskatchewan, the lagoons are generally emptied once or twice a year, usually in the spring and/or fall. Thus, the results from the studies discussed above indicate that some antimicrobials, such as erythromycin, roxithromycin, oxytetracycline, and tetracycline, have the potential to persist in liquid swine manure until land application in the prairie region of Canada.

Lincomycin and spectinomycin (Figure 1.2a and 1.2b) are antimicrobials that are commonly administered as a mixture in feed to help prevent and control post-weaning diarrhea in weanling pigs. These antimicrobials have been reported to be excreted in both the urine and feces of swine following administration in their feed (Hornish et al., 1987). To our knowledge at the time of this study, there was no information available in the literature on concentrations of lincomycin and spectinomycin in liquid manure from swine administered these antimicrobials or their

persistence in the stored manure: information necessary for assessment of possible contamination of prairie wetlands following land application of manure. To fill these data gaps, studies were carried out to determine (i) the concentrations of lincomycin and spectinomycin in the manure excreted by weanling pigs administered these antimicrobials in their feed in a commercial-scale barn, and (ii) their persistence in liquid swine manure during simulated lagoon storage over a period of several months.

4.2 Materials and methods

4.2.1 Commercial-scale barn and associated lagoon

The study to determine the concentrations of lincomycin and spectinomycin in manure from weanling pigs was carried out at the Prairie Swine Centre Inc. which is located 5 km northeast of Elstow, Saskatchewan. This facility housed a total of approximately 6000 swine at any one time, of which approximately one third were in the nursery area of the barn. Barn personnel provided information regarding the diet administered, feed consumed and water utilized, and the amount of manure produced by the weanling pigs during the study period.

Liquid manure from the barn was retained in an earthen lagoon with a storage capacity of twenty million litres. The lagoon was emptied each fall at which time the volume of liquid manure would be approximately fifteen million litres. The surface of the liquid manure was enclosed throughout the year with a plastic cover to minimize odour.

4.2.2 Antimicrobials administered

Lincomycin and spectinomycin were administered as a mixture to weanling pigs to prevent post-weaning diarrhea until body weight increased from 6 to 35 kg. During this period, the pigs were housed in the nursery area of the barn and administered the L-S 20 Premix (Pfizer Animal Health) in their feed. This premix contains 22 g of lincomycin hydrochloride (the hydrochloric acid salt of lincomycin) and 22 g of spectinomycin sulfate (the sulfuric acid salt of spectinomycin) per kilogram of premix (Bayley 2001). These amounts are equivalent to 19.4 g

lincomycin and 14.6 g spectinomycin per kilogram, respectively and, consequently, the ratio of lincomycin to spectinomycin in the L-S 20 Premix was 1.33:1.

4.2.3 Liquid manure

It was estimated that the total volume of liquid manure produced in the barn was approximately 42,000 L d⁻¹, of which approximately 10% originated from the nursery area of the barn. The total volume of liquid manure included water used for washing pens as well as wastewater from showers, washrooms, laundry facilities, the barn manager's house and the feed mill. However, no human sewage entered the earthen storage lagoon.

4.2.4 Concentrations of lincomycin and spectinomycin in nursery manure.

Nursery: The nursery area of the barn consisted of 8 rooms; each with the potential to house approximately 270 pigs. Each room was divided into 16 pens with slatted floors, 8 on either side of the room. There was a manure pit on each side of the room under the slatted floor such that manure from 8 pens was collected in each pit (Figure 4.1). For this study, the liquid manure from one room of 270 nursery pigs was monitored during a 5-week period.

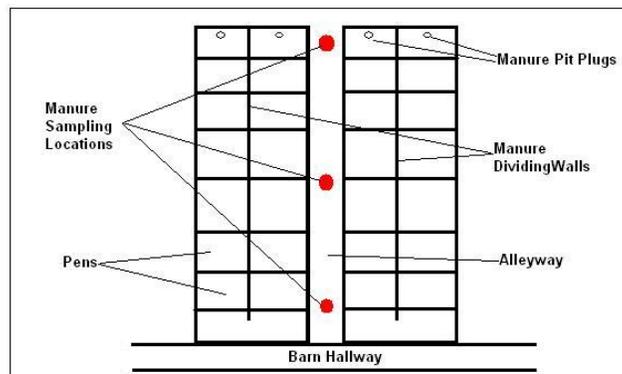


Figure 4.1. Layout of nursery study room and manure sampling locations in the study room

Calibration of the manure pits: To prepare the nursery study room for this study, the floor slats were removed and the room was completely cleaned, and disinfected by barn personnel. The volumes of the manure pits were calibrated using water. A known volume of water was pumped into each pit and the water depth was measured at six locations. This procedure was repeated with increasing volumes of water until the depth of water in the pits reached 0.45 m. Calibration curves were plotted using the depth measurements and water volumes and these curves were used to estimate the volume of manure produced by the weanling pigs in each pit during the study. Before the study began, water was added to each manure pit (1,900 and 1,750 L for pits 1 and 2, respectively) in the study room to ensure the excreted waste was in the form of a slurry.

Administration of Lincomycin and Spectinomycin: The nursery diet consisted of three phases with respect to administration of lincomycin and spectinomycin. The level of antimicrobials in the feed and the amount of medicated feed consumed by the weanling pigs during the first phase was considered confidential information by barn personnel and was not made available. During the second phase, the feed contained 0.1% L-S 20 Premix. During phase three, no premix was added to the feed.

The weanling pigs were administered phase 1 and began phase 2 of their diet in a different nursery room during a 5-d period before being moved to the study room. This ensured that the pigs were excreting both antimicrobials before the study began. After being moved into the study room, they continued to be fed phase 2 of their diet. A total of 5,540 kg of medicated feed was consumed during phase 2. Once the phase 2 diet was consumed, the phase 3 diet was automatically fed out to the pigs; thus, there was no well-defined date at which the diet shifted from phase 2 to phase 3. An estimated 2,500 kg of the phase 3 diet had been fed to the pigs when the last liquid manure sample was collected to terminate the study.

Liquid manure sampling: Manure samples were collected after one week in the study room and then weekly for an additional 4 weeks. Manure, in each pit was sampled by the following procedure: floor slats at three locations in the hallway were removed (Figure 4.1). A round bottomless plastic garbage container (120 L) was lowered into the first pit and the manure contained within was thoroughly mixed using a power drill attached to a 1.5-m long pipe and

equipped with a paint mixer. At each of the three sampling locations in the first pit, a mixed liquid manure subsample (~5 L) was removed with a long-handled ladle and the three subsamples were combined in a 19-L plastic pail. This composite sample (~15 L) was mixed again with the power drill and a 1-L subsample for antimicrobial analysis was transferred to a sample bottle (1-L Nalgene Teflon FEP). This procedure was repeated for the second manure pit. Thus, at each sampling time, two composite manure samples, one from each pit, were collected for residue analysis. The manure samples were maintained at -40°C until analysis.

4.2.5 Antimicrobial persistence during simulated manure storage

Studying lincomycin and spectinomycin persistence in stored manure by directly sampling liquid manure from the storage lagoon was not attempted. With this approach, dissipation of antimicrobial concentrations in the liquid manure would have been compromised by the continual input of manure containing the antimicrobials from the barn. Instead, two 15-L samples of control manure from the grower-finisher area of the barn (where the pigs were not administered antimicrobials) were fortified with lincomycin and spectinomycin and the dissipation of the antimicrobials monitored in the laboratory.

The control manure samples (15 L) were transferred to 20-L stainless steel storage containers equipped with clip-down covers (Cole-Parmer, Anjou, QC). Each sample was then fortified by adding 0.68 and 8.9 mg of the hydrochloric acid salts of lincomycin (HCl, H₂O) and spectinomycin (2HCl, 5H₂O), respectively, dissolved in 500 mL of deionized water. These amounts were equivalent to 0.60 and 6.0 mg of lincomycin and spectinomycin, respectively, and resulted in corresponding concentrations of 38.7 and 387 $\mu\text{g L}^{-1}$ in the fortified manure (~ 15.5 L). After fortification, the containers were placed in a fume hood at room temperature for six months to approximate the time that liquid swine manure is generally stored prior to land application. To simulate covered lagoon storage, the clip-down covers were placed on the storage containers throughout the experiment, except for when the manure was sampled. Prior to each sampling, the fortified manure was thoroughly stirred, and 100-mL sub-samples were then collected to permit duplicate analyses, if required. Sub-samples were collected on days 0, 1, 3, 6, 10, 15 and then bi-weekly for the duration of the study. The samples were maintained at -40°C until extraction.

4.2.6 Analytical method

Liquid swine manure samples were extracted and analyzed using methodology described in Chapter 3.0.

4.3 Results and discussion

4.3.1 Concentrations of lincomycin and spectinomycin in nursery manure

The total amount of feed consumed by the 270 weanling pigs during diet phases 2 to 3 of the study was 8,040 kg, of which 5,540 kg were fed during phase 2 when the pigs were administered the LS-20 Premix. The total amounts of lincomycin and spectinomycin administered in phase 2 were 107.6 and 80.7 g, respectively. Because the amounts of the two antimicrobials administered during phase 1 were kept confidential, these values are somewhat of an underestimation of the total amounts administered.

The total volume of liquid manure in both manure pits increased linearly with time ($r^2 = 0.985$). The 270 pigs in the study nursery room produced approximately 13,760 L (8,090 L in pit 1 and 5,670 L in pit 2) of liquid manure during the study period (Table 4.1). The average solids content of the liquid manure produced was 2.4% (Table 4.2). If one was to assume that 100% of the antimicrobials was excreted and there was no subsequent degradation in the stored manure, then, based on the amount of antimicrobials administered to the pigs and the total volume of the manure produced, maximum concentrations in the liquid manure at the end of the study would have been $7,820 \mu\text{g L}^{-1}$ of lincomycin and $5,865 \mu\text{g L}^{-1}$ of spectinomycin. Because the amounts of the antimicrobials administered in phase 1 were not known, these maximum possible antimicrobial concentrations in the liquid swine manure are somewhat underestimated.

If the same assumption is made, then the ratio of lincomycin to spectinomycin expected in the manure would have been that of the relative amounts of the antimicrobials in the L-S 20 Premix; that is, 1.33:1. However, the average ratio (\pm SE) of the concentrations of lincomycin to spectinomycin over the course of the study was $14.9 \pm 1.5:1$ (from concentration data in Table 4.1). The approximate order of magnitude increase in this ratio indicates either that a lower

proportion of the administered dose of spectinomycin was excreted by the weanling pigs and/or that spectinomycin was less stable in the nursery manure. Antimicrobial concentrations at the end of the study (sampling week 5) were 2,524 $\mu\text{g L}^{-1}$ and 173 $\mu\text{g L}^{-1}$ for lincomycin and spectinomycin, respectively (Table 4.1) and represented 32 and 3%, respectively, of the maximum possible end-of-study concentrations.

Table 4.1. Total mass (g) and concentration ($\mu\text{g L}^{-1}$) of lincomycin and spectinomycin detected in liquid manure from weanling pigs collected from the study room in the nursery area of the barn.

End of sampling week	Total mass		Total accumulated volume of manure [†] (L) in pits 1 plus 2	Concentration in total accumulated manure	
	Lincomycin (g)	Spectinomycin (g)		Lincomycin ($\mu\text{g L}^{-1}$)	Spectinomycin ($\mu\text{g L}^{-1}$)
1	13.89	0.97	1,420	9,784	686
2	24.47	1.83	3,590	6,818	510
3	31.24	2.63	6,170	5,064	428
4	47.9	2.35	10,230	4,683	230
5	34.73	2.37	13,760	2,524	173

[†] These values do not include the initial volume of water (3,650 L) added to pit 1 plus pit 2.

Table 4.2. Percent of lincomycin and spectinomycin associated with varying solids content of liquid swine manure.

Manure sample	Mean solids content (% \pm SE)	Mean percent associated with solids component (% \pm SE)	
		Lincomycin	Spectinomycin
<i>Nursery manure</i> (n = 10)	2.4 \pm 0.3	0.75 \pm 0.1	0
<i>Grower-finisher manure</i>			
Fortification/recovery (n = 10)	11.3 \pm 0.3	17.4 \pm 1.1	16.2 \pm 0.9
Simulated storage (container 1; n = 12)	25.0 \pm 0.5	44.1 \pm 5.9	18.6 \pm 0.7
Simulated storage (container 2; n = 12)	13.2 \pm 0.4	21.3 \pm 3.5	19.0 \pm 1.9
<i>Lagoon manure</i>			
Elstow barn 2003 (n = 4)	10.0 \pm 0.4	4.0 \pm 1.5	0
Elstow barn 2004 (n = 4)	18.6 \pm 1.1	14.7 \pm 6.8	0
Riverhurst barn 2004 (n = 2)	25.0 \pm 1.0	17.6 \pm 8.0	0

Concentrations of lincomycin in the cumulating liquid manure were generally about an order of magnitude greater than those of spectinomycin over the 5-week period (Table 4.1, Figure 4.2). After the first week, concentrations of both antimicrobials in the cumulating manure decreased with time (Table 4.1, Figure 4.2). Since the amounts of the two antimicrobials in the feed remained constant in diet phase 2, the decrease most likely occurred because of increased water consumption by the pigs as their body weight increased and/or degradation in the cumulating manure. Although concentrations of lincomycin were approximately an order of magnitude greater than those of spectinomycin, the relative amounts of lincomycin and spectinomycin in nursery manure may not represent relative concentrations in lagoon manure that is eventually applied to crop or pasture land since they would be dependent on relative stability of the antimicrobials during lagoon storage.

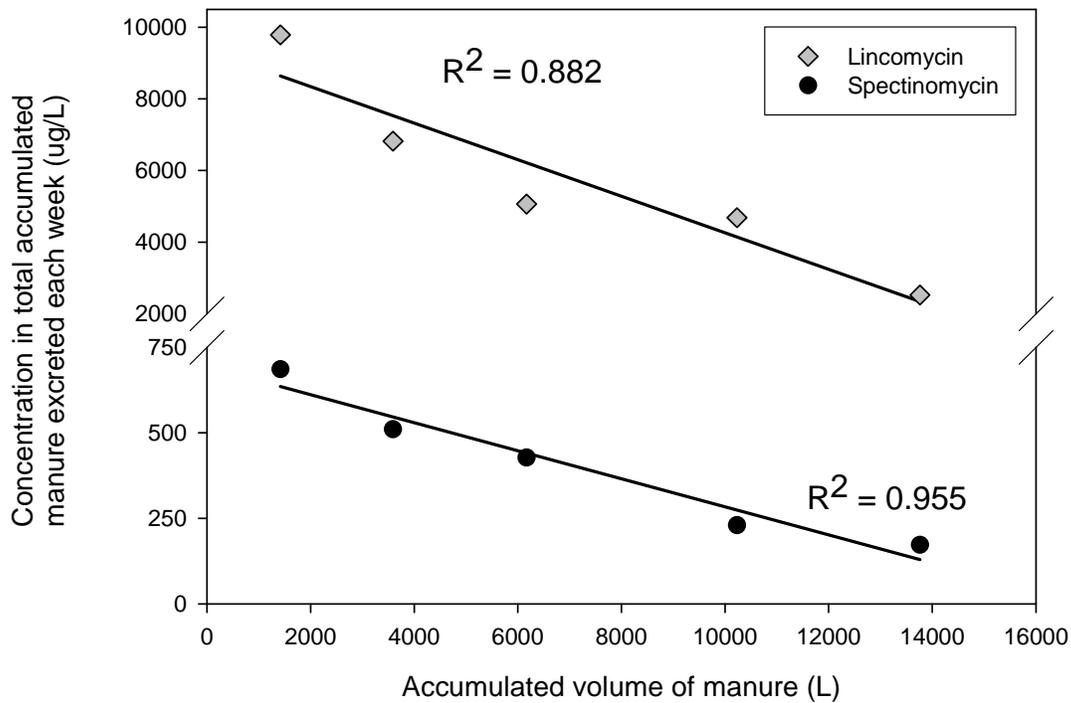


Figure 4.2. Concentrations ($\mu\text{g L}^{-1}$) of lincomycin and spectinomycin in the accumulated volume of manure (L) excreted by weanling pigs.

The mass of both antimicrobials in the cumulating manure increased with time (Figure 4.3) and, as expected from the relative concentrations in the manure (Figure 4.2), the mass of lincomycin increased more rapidly. The increase in mass of lincomycin was best described by a quadratic equation ($f = 0.40 + 1.92x + 1.67x^2$; $F_{2,4} = 184.21$, $P = 0.005$, $R^2 = 0.994$) (Figure 4.3) whereas, the mass of spectinomycin demonstrated a first-order increase over time ($R^2 = 0.950$).

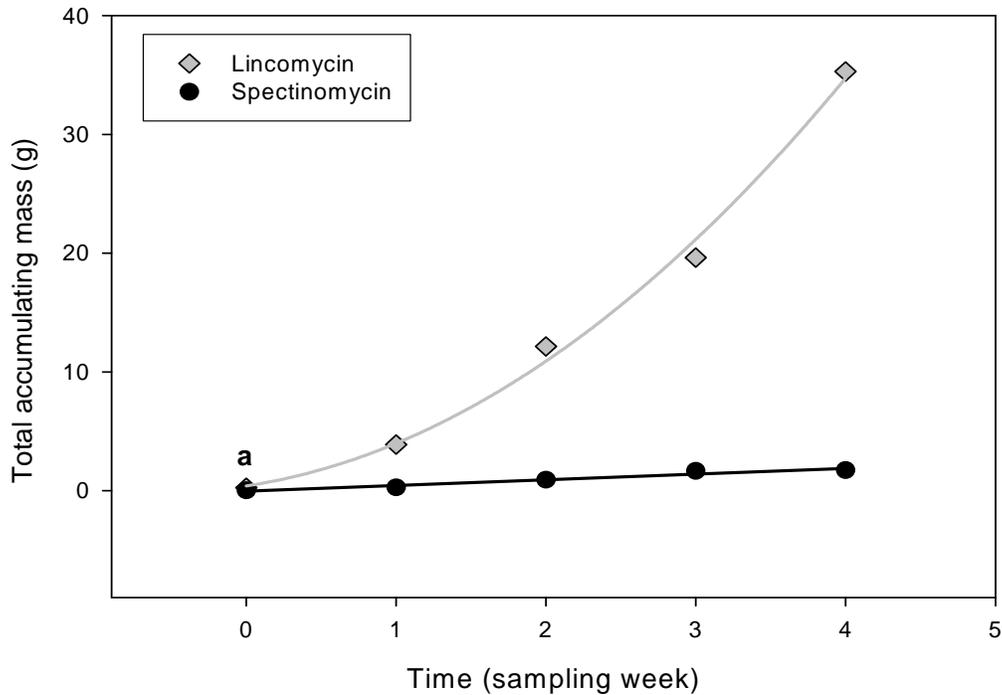


Figure 4.3. Accumulating mass (g) of lincomycin and spectinomycin detected in excreted manure from weanling pigs over time (sampling week). The first points (a) are an estimation because the weanling pigs had been administered feed containing both antimicrobials for 5 days prior to the start of the study (week 0).

Liquid swine manure samples collected from the nursery study room manure pits contained, on average, 2.4% solids (Table 4.2). Extraction of both the solids and liquid components of the manure showed that, in all samples, lincomycin was mainly present in the liquid component, with a maximum of 1.3% detected in the solids component. In contrast, spectinomycin was not detected in the solids component in any of the nursery manure samples.

4.3.2 Persistence during simulated liquid swine manure storage

Because the lagoon associated with the Elstow barn was enclosed with a plastic cover, the liquid manure in the lagoon was most likely anaerobic. Consequently, during the simulated storage study, the persistence of lincomycin and spectinomycin in fortified control (grower-finisher) manure was monitored in the laboratory in covered containers. The average temperature (approximately 20°C) of the manure in the laboratory study would have been higher than that in earthen lagoon storage from spring to early fall, and considerably higher than that for winter lagoon storage from early fall to spring. Thus, the laboratory conditions most likely represent a scenario for maximum antimicrobial degradation.

The control manure used in the antimicrobial persistence study contained higher solids content than the manure from the pits in the nursery area of the barn. The solids content of the liquid manure in containers 1 and 2 were 25% and 13%, respectively (Table 4.2). No lincomycin or spectinomycin was detected in the control manure before fortification.

Spectinomycin: The mean concentration of spectinomycin (containers 1 and 2) in the liquid manure decreased rapidly over the first 6 days (Figure 4.4). The initial concentration 4 h after fortification (262 µg L⁻¹) was substantially lower than the target fortification level of 387 µg L⁻¹. By day 6, the mean concentration of spectinomycin had decreased to 81.8 µg L⁻¹ or 21% of the target concentration. This was unexpected because a similar rapid decrease in spectinomycin concentration was not observed during fortification/recovery experiments in which fortified whole manure was aged 7 d prior to extraction (recovery = 68.6 ± 9%).

The initial rapid decrease in spectinomycin concentration in the simulated storage study (Figure 4.4) could be attributed to several factors including biotic and abiotic degradation and irreversible sorption (that is, formation of non-extractable residues) to manure solids. Since both the recovery and simulated storage studies utilized grower-finisher manure, the more rapid decrease in spectinomycin concentration in the simulated storage study may be explained by higher temperature conditions (~ 20°C) than that (4°C) used in the recovery experiments. A biphasic decrease in concentration (rapid decrease followed by a slow decrease) such as that observed for spectinomycin has also been observed for tylosin in liquid swine manure under

anaerobic conditions (Kolz et al., 2005). These workers attributed the initial rapid decrease in tylosin mainly to irreversible sorption to manure solids which may also explain the initial rapid decrease (~ 80%) in spectinomycin concentrations in the current study. However, some of the initial rapid decrease in spectinomycin concentration may have also resulted from greater degradation of spectinomycin at the beginning of the study when the fortified manure was most likely aerobic. Kuhne et al. (2000) reported more rapid dissipation of tetracycline in aerated compared to non-aerated liquid swine manure.

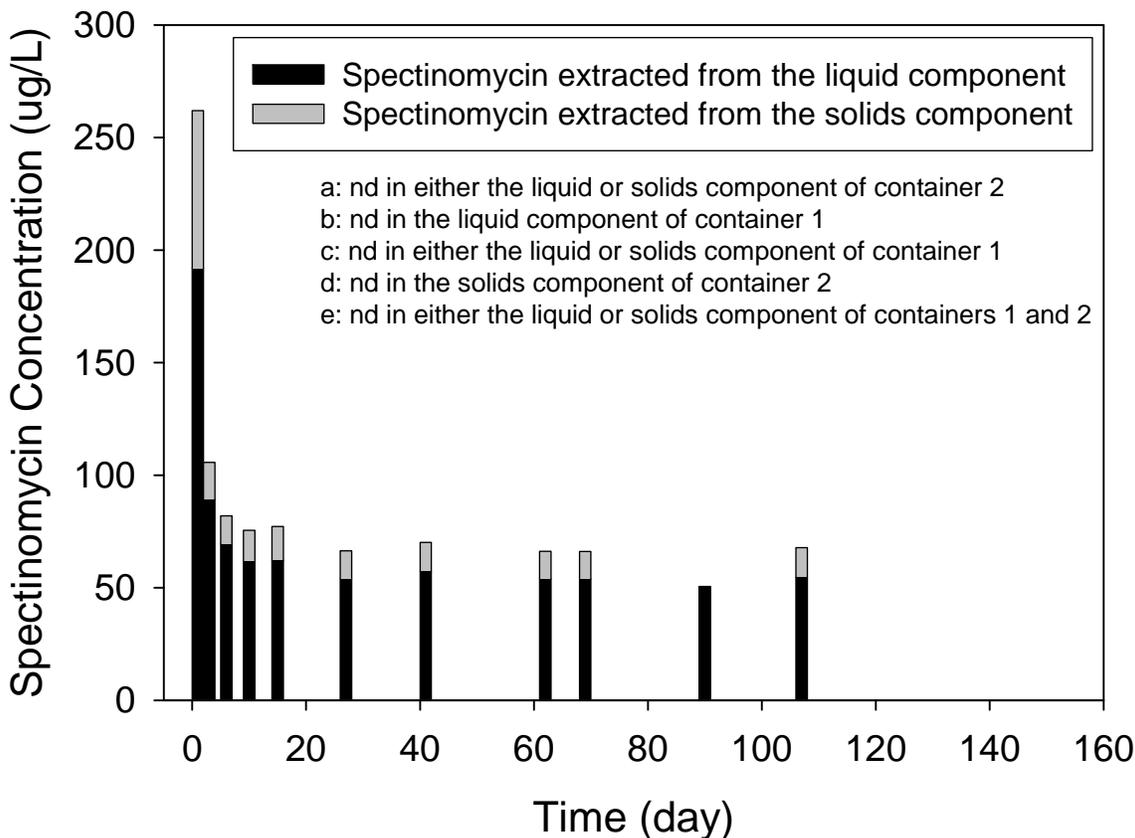


Figure 4.4. Concentrations of spectinomycin detected in the solids and liquid components (mean of two containers) during simulated storage of liquid swine manure versus time.

From days 6 to 107, the mean concentration of spectinomycin decreased at a much lower rate such that concentrations were essentially constant ($65.2 \pm 12.8 \mu\text{g L}^{-1}$; Figure 4.4). Assuming first-order kinetics, there was a weak correlation between spectinomycin concentration (natural

log values) versus time ($R^2 = 0.34$). The average time required for 50% dissipation of spectinomycin during simulated manure storage was approximately 90 d. This value is within the range of previously reported half-lives in liquid swine manure for erythromycin and roxithromycin (Schlusener et al., 2006) and tetracycline (Winckler and Grafe, 2001).

There was an unexplained inconsistency in the analysis of spectinomycin during the simulated manure storage study which was not evident during method development and the recovery of the antimicrobial from fortified manure. For four sampling dates, the antimicrobial was not consistently detected in the solids and liquid components of manure from either container. On days 15 (container 2), 90 (container 1) and 154 (containers 1 and 2), spectinomycin was not detected in either the solids or liquid components but was detected in both components before and after (days 15 and 90) these days (Figure 4.4). On day 41 (container 1), spectinomycin was not detected in the liquid component and on day 90 (container 2) was not detected in the solids component. The analysis of these particular sub-samples was repeated and the same results were obtained. It is not clear why spectinomycin was not consistently detected in the liquid and solids components of the manure; however, the pattern of spectinomycin concentrations in the liquid and solids components (Figure 4.4) suggest that spectinomycin was most likely present in both components on all sampling dates.

Lincomycin: Mean lincomycin concentrations demonstrated a biphasic decrease similar to that for spectinomycin, except that the initial decrease was less rapid (Figure 4.5). At 4 h after fortification (day 1), the mean concentration was $53.4 \mu\text{g L}^{-1}$. This concentration was somewhat higher than the target concentration of $38.7 \mu\text{g L}^{-1}$ and may reflect incomplete mixing of the fortified manure. The mean concentration on day 3 ($41.1 \mu\text{g L}^{-1}$) was close to the target concentration. From day 6 to day 154, the concentration of lincomycin decreased so slowly that it remained almost constant (mean concentration was $33.4 \pm 2.7 \mu\text{g L}^{-1}$).

The less rapid decrease of lincomycin compared to spectinomycin may reflect the smaller molecular size of spectinomycin. Chander et al. (2005) have shown that tetracycline, which is a smaller molecule than tylosin, sorbed in greater amounts to soil, most likely because the smaller tetracycline molecule was able to better penetrate into the inter-clay spaces. The greater

absorption of smaller antimicrobial molecules may also explain why the initial mean concentration of spectinomycin was considerably less than the target concentration only 4 h after fortification.

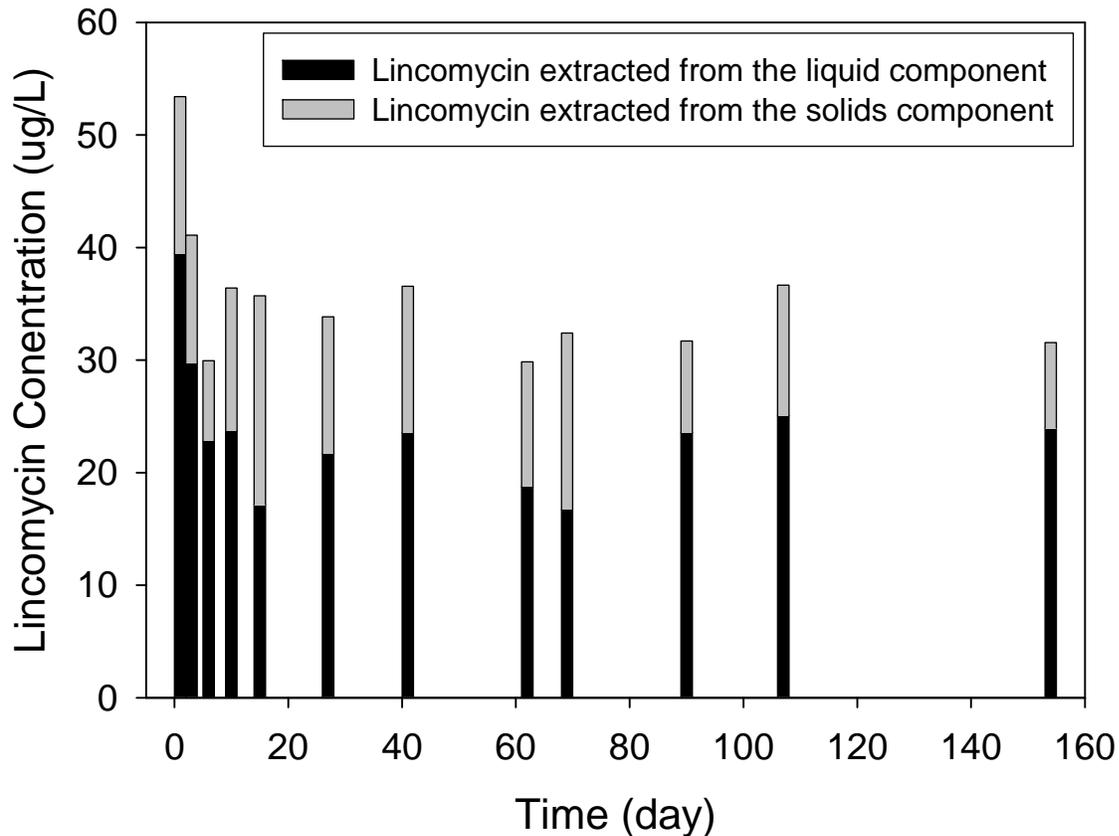


Figure 4.5. Concentrations of lincomycin detected in the solids and liquid components (mean of two containers) during simulated storage of liquid swine manure versus time.

Following the rapid initial decrease, there was relatively little dissipation of lincomycin during simulated storage under anaerobic conditions, even at room temperature (Figure 4.5). A weaker correlation between lincomycin concentration with time ($R^2 = 0.21$) was observed compared to that for spectinomycin and the time required for 50% dissipation of lincomycin was of the order of a year. This timeframe is longer than previously reported half-lives of other antimicrobials in liquid swine manure (Kuhne et al., 2000; Schlusener et al., 2006; Teeter and Meyerhoff, 2003; Winckler and Grafe, 2001) and indicates that lincomycin would persist in the manure during six

months of lagoon storage and be present in manure applied to crop or pasture land as a plant nutrient source. The presence of lincomycin in lagoon manure has been reported by Campagnolo et al. (2002) and Kuchta et al. (2006).

4.3.3 Antimicrobials associated with the solids component of the liquid manure

Spectinomycin: The proportion of spectinomycin associated with manure solids was much higher for the control manure used for simulated storage than for nursery manure (Table 4.2). No spectinomycin was detected in the nursery manure solids and this may be a function of the 11-fold smaller concentration of spectinomycin in the manure compared to that for lincomycin. There was no direct correlation between the proportion of spectinomycin associated with manure solids and the solids content of the manure. For example, an average of $18.6 \pm 2.3\%$ and $19.0 \pm 6.6\%$ of spectinomycin was extracted from the solids component of simulated storage manure even though the solids content in container 1 (25%) was double that in container 2 (13%).

Lincomycin: The proportion of lincomycin associated with the solids component of the liquid manure was much higher for the simulated storage manure than for the nursery manure (Table 4.2) and this most likely reflects the higher solids content of the simulated storage manure (Table 4.2, Figure 4.6). There was a moderately strong direct correlation ($R^2 = 0.507$) when the proportion of lincomycin associated with manure solids was plotted against the solids content of several liquid manures (Figure 4.6) including nursery, grower-finisher and lagoon manures. The weakness in the correlation may, in part, reflect the difficulty in obtaining homogeneous manure samples for analysis (that is, samples with a representative solids content), especially as the solids content of the manure increased.

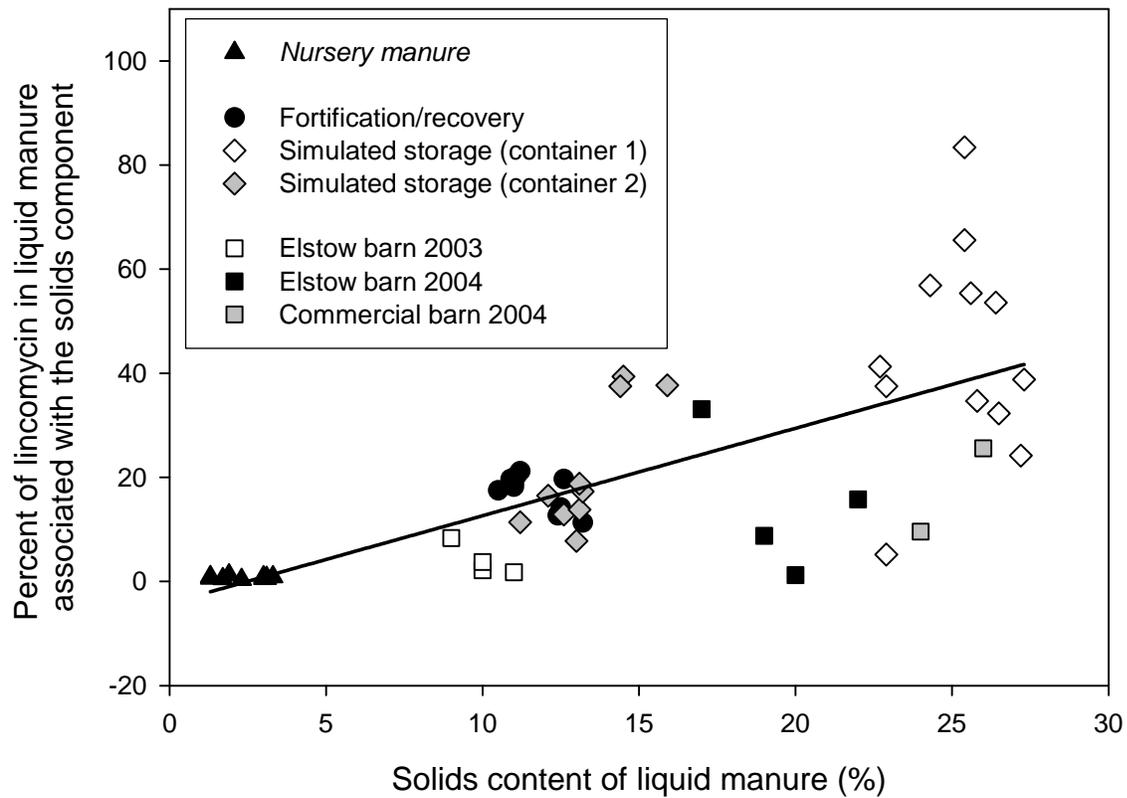


Figure 4.6. Relationship between the solids content of three types of liquid swine manure (nursery manure, grower-finisher manure and lagoon manure) and the amount of lincomycin associated with the solids component of the manures.

5.0 ANTIMICROBIAL DISSIPATION IN MANURE-AMENDED SOIL: TRANSPORT OF LINCOMYCIN IN SURFACE RUNOFF

5.1 Introduction

Swine, poultry and cattle are increasingly being produced in large confined animal feeding operations. With such production, antimicrobials are administered, regardless of the health of the animals, to prevent disease and to promote growth by increasing the rate of weight gain. Since many antimicrobials are poorly absorbed through the gut, they can be excreted up to 80% or more in the feces and urine (Aiello, 1998). For example, Winckler and Grafe (2001) measured the excretion of tetracycline from pigs which were administered the antimicrobial in their feed at 1000 and 2500 mg kg⁻¹ for 5 d. The majority of antimicrobial excretion occurred within 7 d of starting the exposure and was equivalent to 42 and 72%, respectively, of amounts administered. Campagnolo et al. (2002) reported concentrations of different classes of antimicrobials in eight swine lagoons in Iowa and Ohio in the USA. The lagoons contained residues of lincomycin (2.5 – 240.0 µg L⁻¹), tetracycline and oxytetracycline (25.0 – 410.0 µg L⁻¹), chlortetracycline (68.0 – 1000 µg L⁻¹), sulfamethazine (2.5 – 400.0 µg L⁻¹) and erythromycin (2.5 µg L⁻¹). They concluded that total antimicrobial concentrations in storage lagoons could exceed 1 mg L⁻¹. In liquid swine manure from the nursery portion of a commercial barn, lincomycin and spectinomycin were detected in concentrations ranging from 93.3 to 215.5 µg L⁻¹ and 64.1 to 105.4 µg L⁻¹, respectively (Peru et al., 2006). Consequently, because of antimicrobials present in the manure, the application of manure as a nutrient source to crop and pasture land can be a potential source of contamination of surface and ground waters via surface runoff and leaching, respectively.

Recently, antimicrobials used in the livestock industry have been detected in surface waters in Canada (Forrest et al., 2006), the United States (Kolpin et al., 2002; Lindsey et al., 2001) and Europe (Calamari et al., 2003; Hirsch et al., 1999). Contamination of surface and ground waters by veterinary antimicrobials is a concern because it may accelerate the development of antimicrobial-resistant bacteria. Although antimicrobials administered to humans are generally not the same as those given to animals, their structures may be similar enough that veterinary antimicrobials can cause resistance to those used to treat humans. For example, Khachatourians

(1998) showed that when streptococci and staphylococci developed resistance to tylosin, a common animal feed additive, they also developed a resistance to erythromycin used in human medicine.

Two antimicrobials, lincomycin and spectinomycin (Figure 1.2a and 1.2b) are administered in the prairie region of Canada as a mixture to swine for the prevention and control of post-weaning diarrhea. Lincomycin has been detected in surface waters in several countries (Hirsch et al., 1999; Lindsey et al., 2001; Kolpin et al., 2002; Calamari et al., 2003; Forrest et al., 2006). However, although spectinomycin has been detected in milk (Carson and Heller, 1998; Schermerhorn et al., 1995) and animal tissues (Bergwerff et al., 1998; Hornish and Weist, 1998), there have been no reports of its detection in environmental waters.

The possibility that these and other antimicrobials, following land application of manure, will be transported to surface and ground waters depends, in part, on the extent to which they persist in stored manure and in manure-amended soil. Studies have shown that some veterinary antimicrobials persist in manure over time. Schlusener et al. (2006) studied the persistence of erythromycin, roxithromycin, salinomycin and tiamulin in liquid swine manure during a 180-d experiment. Calculated half-lives were 41, 130 and 6 d for erythromycin, roxithromycin and salinomycin, respectively, whereas the concentration of tiamulin remained unchanged during the entire experiment. Arikan et al. (2006) reported a half-life for oxytetracycline of 56 d during anaerobic digestion of manure from beef calves who were administered $22 \text{ mg kg}^{-1} \text{ d}^{-1}$ for 5 d. A somewhat lower half-life of 30 d was reported by De Liguoro et al. (2003) for oxytetracycline in cattle manure following oral administration of $60 \text{ mg kg}^{-1} \text{ d}^{-1}$ for 5 d. In the same study, tylosin concentrations were not sufficient to determine its half-life following administration of $20 \text{ mg kg}^{-1} \text{ d}^{-1}$ for 5 d. Insufficient concentrations may have been due to lower tylosin excretion and/or, based on a predicted half-life of $< 2 \text{ d}$ in the aqueous component of swine manure (Loke et al., 2000), instability in the manure. Winckler and Grafe (2001) reported a half-life for tetracycline in the liquid swine manure of 105 d whereas, in an indoor experiment in which the liquid manure was maintained at 8°C , the half-life was 56 d. In contrast, Kuhne et al. (2000) reported the time for 50% dissipation of tetracycline in aerated and non-aerated liquid swine manure to be 4.5 and 9 d, respectively.

Wang et al. (2006) reported that the degradation of sulfadimethoxine, a sulfonamide antimicrobial, in manure-amended soil was accelerated with increasing manure and moisture content of the soil. Other recent studies have shown that some antimicrobials can persist in manure-amended soil for several months. Aga et al. (2005) reported that the half-life of oxytetracycline in treated soil was approximately 3 wk; however, when the soil samples were analyzed using an enzyme-linked immunosorbent assay, the total tetracycline concentration in the soil remained relatively constant for up to 5 months after manure application. Schlusener et al. (2003) reported that, nine months after application, tiamulin was still present ($0.7 \mu\text{g kg}^{-1}$) in soil that had been fertilised with liquid swine manure containing tiamulin ($43 \mu\text{g kg}^{-1}$) and salinomycin ($11 \mu\text{g kg}^{-1}$). No salinomycin was detected.

The objective of the present study was to assess the susceptibility of lincomycin and spectinomycin to transport in surface run-off and to leach to groundwater from manure-amended soil. This objective was met by determining their dissipation in soil and monitoring their presence in simulated rainfall runoff, spring snow melt runoff, and in groundwater from manure-treated fields.

5.2 Materials and Methods

5.2.1 Study Sites and Manure Applications

Study sites consisted of crop and pasture land adjacent to two commercial swine barns; one near Elstow, SK and the other near Riverhurst, SK (Figure 5.1). In some cases, study sites consisted of multiple watersheds/fields to which liquid manure was applied, and each study site had an untreated watershed/field that served as a control site. With the exception of one liquid manure application made with a center pivot, all manure applications were by injection using the Coulter disc applicator (Bourgault Industries, St. Brieux, SK, Canada). For these treatments, the manure applicator was set to inject the liquid manure into the soil to a 10-cm depth. However, in some places, injection depth was shallower as evidenced by the fact that the surface soil became wet.

The centre pivot application of liquid manure was made from the Riverhurst barn which utilized two lagoons. When the primary lagoon was full, the liquid component of the manure overflowed into the secondary lagoon. Manure from the primary lagoon was applied by injection, whereas the liquid component from the secondary lagoon was applied using a centre pivot.

All arrangements for manure applications and for land to which manure would be applied were made by barn personnel. They also provided information on rates of application of liquid swine manure to crop and pasture land.

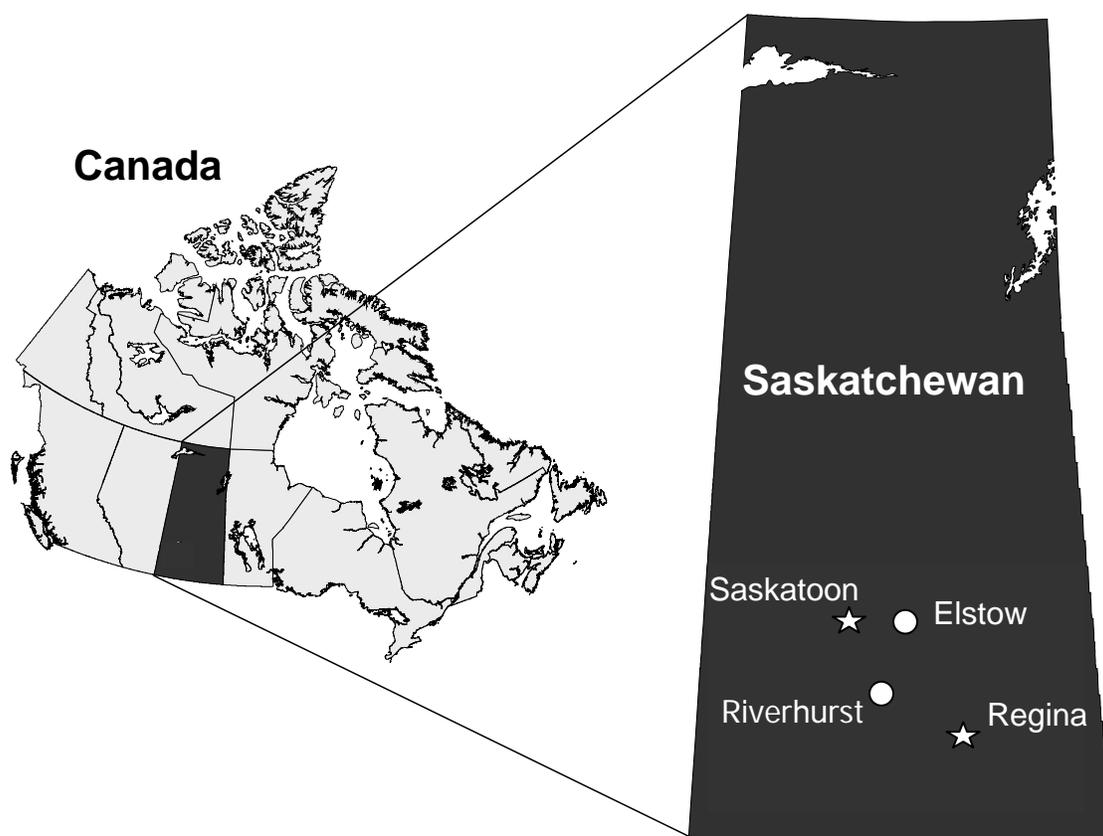


Figure 5.1. Location of Elstow and Riverhurst in Saskatchewan

5.2.2 Elstow Study Sites

All fields are located within 5 km of each other in an area classified as shallow lacustrine plain with gently sloping land (Acton et al., 1978). The soils are dominantly Orthic Brown Chernozemic soils formed in medium to moderately fine textured, moderately calcareous and clayey glacio-lacustrine deposits (Elstow Association) (Acton et al., 1978).

Elstow study site 1 (2003 and 2004): This study site was being used in an ongoing study to determine the impact of the application of liquid swine manure to cropland on surface and ground water quality with respect to nitrogen and phosphorus (Maulé and Elliott, 2006). It consisted of four 64.75-ha fields that were farmed by the same producer. Two of the fields were divided such that one half of each field was treated with liquid swine manure in the fall of 2003 (3 October) at approximately 60,000 L ha⁻¹ and the other half at approximately 95,000 L ha⁻¹. A single watershed was defined in each half of each field and instrumented with a single piezometer. Liquid swine manure was last applied to both fields in fall 2001. A third field, to which liquid swine manure was last applied in fall 2002, acted as a control watershed and was also instrumented with a piezometer. The fourth field, to which liquid swine manure had not been applied in the previous five years, was also used as a control, but no piezometer was installed. All fields were cropland with either canary seed (*Phalaris canariensis* L.) or wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.) stubble. Slopes on all fields ranged from 0.5 to 6.4%.

Elstow study site 2 (2004 and 2005): This site consisted of two 64.75-ha fields, one treated with liquid swine manure and the other used as a control. Both fields were farmed by the same producer and were cropland with canola (*Brassica napus* L.) stubble. The treated field was injected with liquid swine manure in fall 2004 (4 October) at a rate of approximately 78,000 L ha⁻¹. Manure had not been previously applied to these fields in the past five years. There were two ephemeral wetlands on the treated field, and one ephemeral wetland and one depression on the control field. Slopes ranged from 0.5 to 3.7%.

5.2.3 Riverhurst Study Sites

All manure-treated and control fields for study sites 1 to 4 are located within 5 km of each other in an area made up predominately of glacio-lacustrine plains with gently to very gently sloping land (Ellis et al., 1968). The cropped soils of study sites 1, 2 and 3 are dominantly Orthic Brown Chernozemic soils formed in medium to coarse-textured glacio-lacustrine materials (Birsay association) (Ellis et al., 1968). All three sites were farmed by the same producer who was unable to provide an accurate history of manure application. The pasture soils of study site 4, which were farmed by another producer, were dominantly Orthic Brown Chernozemic soils formed in medium to coarse textured glacio-lacustrine materials (Birsay association) with significant inclusions of Orthic Brown Chernozemic soils formed in medium-textured glacial till (Haverhill association) (Ellis et al., 1968).

Riverhurst study site 1 (2004): This field was approximately 32 ha of summer fallow and 48 ha of cereal crop stubble. Manure from the primary lagoon was injected (21 October 2003) into both the summer fallow and stubble portions of this field at a rate of approximately 79,000 L ha⁻¹. There were no wetlands on this site, but there were seven depressions on both the summer fallow and stubble portions of the field. The control field contained three depressions and two ephemeral wetlands.

Riverhurst study site 2 (2004): This site consisted of two 64.75-ha fields with three ephemeral wetlands and eight depressions located in the field of canola stubble and seven depressions in the field of wheat stubble. Liquid swine manure was applied in fall 2003 (25 October) from the secondary lagoon to these fields using the centre pivot at a rate of approximately 3,000 L ha⁻¹. The control field used for study site 1 was also used for this site.

Riverhurst study site 3 (2005): This was the only study site to receive a spring application of liquid swine manure. This site consisted of two 64.75-ha fields of canola stubble with four ephemeral wetlands and seven depressions. Liquid swine manure was injected in the spring of 2004 (mid-May) at a rate of approximately 90,000 L ha⁻¹. The control field contained two depressions.

Riverhurst study site 4 (2005): This site consisted of three 64.75-ha fields of pasture land, two of which were injected with liquid swine manure (15 September 2004) at rates of approximately 88,000 and 110,000 L ha⁻¹, respectively. The third field, which had not been treated with manure in the last five years, served as the control. There was a dugout (small constructed reservoir) situated on each field and, since they derive their water from surface runoff, all were monitored for antimicrobial content. There were four ephemeral wetlands and ten depressions on one of the manure-treated fields (88,000 L ha⁻¹) and twelve depressions on the other. There were two depressions on the control field.

5.2.4 Manure Application

Depending on lagoon size and the barn operation, manure may be applied once or twice a year, usually in the spring and/or the fall. Consultation with barn personnel at both study site locations (Elstow and Riverhurst) indicated that the Elstow lagoon was emptied once in 2003 (fall) and once in 2004 (fall), whereas the Riverhurst lagoons were emptied in both the spring and fall of both years. Information on the rates of manure application was also obtained from barn personnel each year (Table 5.1).

5.2.5 Antimicrobial Use

Barn personnel also provided information regarding average amounts of lincomycin and spectinomycin used per month in their respective barns. Lincomycin and spectinomycin were administered together as a premix (LS-20, Pfizer Animal Health) in the feed at both barns. This premix contained a 1:1 ratio of lincomycin hydrochloride and spectinomycin sulfate (22 g each) per kilogram of premix (Bayley, 2001). Thus, the actual amounts of lincomycin and spectinomycin per kilogram of premix were 20.16 and 16.99 g, respectively. In the Riverhurst barn, lincomycin was also administered in another premix (Linco44, Bio Agri Mix) which contained 44 g of lincomycin hydrochloride per kilogram of premix (Bayley, 2001), equivalent to 40.32 g of lincomycin. There were no major disease outbreaks in either barn for which lincomycin or spectinomycin was administered during the study.

Total amounts of these antimicrobials used since the previous manure application were estimated by multiplying the average monthly amount by the number of months since the previous

application. The Elstow barn used an average of approximately 53 kg of LS-20 premix per month, which was equivalent to 12.8 and 10.8 kg each of the active ingredients lincomycin and spectinomycin, respectively, over a 12-month period. The Riverhurst barn used approximately 14.4 kg of LS-20 premix and approximately 135 kg of Linco44 premix per month. During a six-month period, this use was equivalent to 34.4 kg of lincomycin and 1.46 kg of spectinomycin. However, amounts of antimicrobial used are approximate since it was not possible to determine the exact amount of premix the animals consumed.

Table 5.1. Manure application rates and volumes and lincomycin concentrations in the applied manure.

Study Site	Manure application rate (L ha ⁻¹)	Total volume of manure applied (ML)	Theoretical maximum concentration (µg L ⁻¹)		Mean lincomycin concentration in applied manure (µg L ⁻¹ ± SE)	Mean solids content of applied manure (% ± SE)
			Lincomycin	Spectinomycin		
Elstow 1 (2003)	60,000	3.9	543	458	132 ± 17 (n=4)	10 ± 0.4
	95,000	6.1 5.0 [†]				
Elstow 2 (2004)	78,000	5.0 10.0 [†]	543	458	122 ± 40 (n=4)	19 ± 1
Riverhurst 1 (2004)	79,000	6.3 6.7 [†]	2,640	112	ns [‡]	ns
Riverhurst 2 (2004)	3,000	3.8 9.2 [†]	2,640	112	ns	ns
Riverhurst 3 (2005)	90,000	11.5 1.5 [†]	2,640	112	ns	ns
Riverhurst 4 (2005)	88,000	5.7	2,640	112	32 ± 7 (n=2)	25 ± 1
	110,000	3.6 3.7 [†]				

[†]manure applied to non-study site fields

[‡]ns: no sample was collected by the manure applicator operator

5.2.6 Manure Sampling during Application

Samples of manure were collected by the manure applicator operators during manure application at Elstow study sites 1 and 2 and at Riverhurst study site 4. Samples were collected in Teflon containers (Nalgene, 500 mL) and were stored at -40°C until extraction. The mean percent solids

in these samples were 10 (n = 4) and 19% (n = 4) for Elstow sites 1 and 2, respectively (Table 5.1). Because of the dual lagoon system at the Riverhurst barn where liquid component overflowed into the second lagoon, liquid manure applied to the Riverhurst study site had the highest solids content (25%, n = 2). Corresponding mean pH values (7.8, 7.9 and 8.3, respectively) were inversely correlated ($R^2 = 0.81$) with the solids content of the manure.

5.2.7 Soil Sampling

In the fall of 2003 and 2004, soil samples were collected from all fields at Elstow study sites 1 and 2, respectively, before and within a week of manure application. After snow melt runoff the following spring, soil samples were collected monthly until freeze-up in the fall of 2004 and 2005, respectively. In each watershed/field three soil samples were collected at each sampling time as follows: a metal template (1-m long x 5-cm wide x 5-cm deep) was pounded into the soil such that it encompassed a minimum of three injection furrows. The soil (approximately 2,500 cm³) contained within the template was collected with a narrow trowel. Soil samples were placed in freezer bags and maintained at -40°C until extraction and analysis.

5.2.8 Simulated Rainfall Runoff Experiments

Simulated rainfall runoff experiments were completed within a week of manure application at Elstow study sites 1 and 2 in the falls of 2003 and 2004, respectively. At Elstow study site 1, three rainfall simulation experiments were carried out in each of the defined watersheds. At Elstow study site 2, rainfall simulation experiments were similarly carried out at three locations within the manure-treated field.

Rainfall simulator: Simulated rainfall was produced from a 1.5-m boom equipped with two nozzles positioned 50 cm apart. The boom was mounted on top of a 1.5-m wide x 1.5-m long x 2-m high frame constructed from plastic pipe. Tarps were attached to each of the four sides of the frame to minimize wind interference of the simulated rainfall. Water, from a municipal well near the Elstow study sites, was pumped at approximately 8 L min⁻¹ to the boom via a hose from a 3400-L tank mounted on the bed of a truck. A sample (1-L) of the rain water used in the experiments was collected for antimicrobial analysis each time the tank was filled.

The tarped frame was placed over a 1-m x 1-m plot of land which encompassed at least three injection furrows on the manure-treated sites. In order to define the 1-m x 1-m plot, three metal sheets (1-m long x 10-cm high) were hammered into the soil at right angles to each other such that approximately 5 cm of each sheet remained above the soil surface. On the fourth side of the plot, a triangular metal tray (flume) was placed approximately 1 cm below ground level in order to collect simulated rainfall runoff from the plot. A hole, dug at the exit of the triangular tray, permitted collection of runoff water from the plot into 1-L amber glass bottles. Rain gauges were placed at the center and back of the 1-m x 1-m plot in order to estimate the amount of simulated rainfall applied to the study plot during each simulation.

Rainfall simulation: Prior to the start of each experiment, tent pegs were used to fasten the lower edges of each tarp to the ground to prevent the simulator from being blown over by the wind. After simulated rainfall was begun and runoff from the plot began to flow over the triangular metal tray, runoff samples (1 L) were collected beginning at 5, 15, and 25 minutes after runoff began to determine how quickly antimicrobial was depleted from the runoff-soil interaction zone. Immediately after collection of the 25-min sample, the pump supplying water to the boom was turned off. However, runoff was still collected so that the total runoff volume could be measured. After each experiment was complete, output from each nozzle was measured by restarting the pump and collecting water for 30 s into a 2-L graduated cylinder. The samples were placed on ice in a cooler and transported to the laboratory where they were maintained at 4°C until extraction.

5.2.9 Snow Melt Runoff Sampling

In the springs of 2004 and 2005, snow melt runoff samples were collected in 1-L amber glass bottles from the control and manure-treated fields at all study sites. During snow melt runoff, samples were collected daily from depressions or ephemeral wetlands where runoff water collected. At Elstow study site 2, runoff was collected as it entered the two ephemeral wetlands. After runoff was complete, samples were collected weekly at all study sites until water in the depressions or wetlands had completely infiltrated. Runoff samples were maintained at 4°C, until extraction.

5.2.10 Groundwater Sampling

At Elstow study site 1, groundwater samples (1-L) were collected from the piezometer (~ 4 m depth) in each defined watershed. In the fall of 2003, groundwater samples were collected both before and after manure application and then, following spring snow melt runoff, monthly until mid-October 2004. Groundwater samples were similarly collected in 2005.

5.2.11 Sample Analysis

All samples (surface and ground waters, soil and liquid swine manure) were extracted and analyzed as described in Chapter 3.0.

5.2.12 Statistical Analysis/Calculations

Statistical analysis: The lincomycin concentrations detected in simulated rainfall runoff, snowmelt runoff, and soil were grouped into box plots. This allowed visual assessment of the median and dispersion of the values. Data were analysed using Minitab 12 (Minitab, State College, PA). The results were evaluated using a one-way Analysis of Variance (ANOVA) with a comparison of means (Tukey's pairwise comparisons). A value of one-half the limit of quantification was used as the concentration of lincomycin for samples in which the antimicrobial was not detected.

Calculations: Lincomycin loading for each manure-treated field was estimated by multiplying the mean concentration ($\mu\text{g L}^{-1}$) detected in the manure by the manure application rate (L ha^{-1}) (Table 1). This value was then divided by 10,000 to give the loading per square meter ($\mu\text{g m}^2$), the plot size used in the simulated rainfall runoff experiments.

The mass of lincomycin transported in runoff during each simulated rainfall runoff experiment was estimated by multiplying the mean antimicrobial concentration (mean of the 5-, 15- and 25-min samples; $\mu\text{g L}^{-1}$) in runoff by the total runoff volume (L). This value (μg) was then divided by the mass of lincomycin applied to a square meter (μg) and multiplied by 100 to determine the percent of the antimicrobial transported in runoff.

5.3 Results and Discussion

5.3.1 Rainfall and Soil Moisture Information

Neither study site was equipped with a micrometeorological station. The Elstow site, at which simulated rainfall runoff, snowmelt runoff, soil persistence and groundwater studies were carried out, was approximately 40 km from the nearest source of weather data (airport in Saskatoon, SK), whereas the analogous distance for the Riverhurst site, where only snow melt studies were carried out, was approximately 56 km from the weather station (Outlook, SK). In 2003, when this study was initiated, total precipitation was less (256 and 301 mm at Elstow and Riverhurst, respectively) than normal (350 and 338 mm) at both study sites and above normal in 2004 (376 and 400 mm) and 2005 (455 and 445 mm) (Environment Canada Climate Normals 2007). The moisture content in soil samples collected for antimicrobial analysis reflects, to a large degree, the annual rainfall. Soil moisture content was lowest in October 2003 ($13.9 \pm 5.1\%$; $n = 15$) and much higher during the spring/summer period of 2004 ($20.9 \pm 4.1\%$; $n = 75$) when rainfall was above normal. Moisture content was even higher in October 2004 ($26.4 \pm 4.8\%$; $n = 12$) and similar during the spring/summer period of 2005 ($23.2 \pm 1.1\%$; $n = 60$) when rainfall remained above normal.

5.3.2 Control Samples

Even though most of the control watersheds/fields had been previously treated with manure, lincomycin and spectinomycin were not detected in any soil, simulated rainfall runoff, or snow melt runoff samples collected from control watersheds/fields at any study site.

5.3.3 Antimicrobial Concentrations in Manure

Based on the amounts of lincomycin (12.8 kg) and spectinomycin (10.8 kg) used and the estimated volume (23.6 ML) of liquid swine manure produced in a 12-month period at the Elstow barn, maximum concentrations of the antimicrobials would have been 543 and 458 $\mu\text{g L}^{-1}$, respectively (Table 5.1). Analogous values for the Riverhurst barn would have been 2,640 and 112 $\mu\text{g L}^{-1}$. In calculating these values, 100% excretion of the antimicrobials was assumed with no metabolism following ingestion, or degradation during lagoon storage.

At the Elstow barn, lincomycin and spectinomycin were administered only to weanling pigs for a 6-week period. During this period, approximately 14,000 L of liquid manure were produced by the weanling pigs and, at the end of the period, the manure contained 2,524 and 173 $\mu\text{g L}^{-1}$ of lincomycin and spectinomycin, respectively (Table 4.1; Chapter 4). Since approximately equal amounts of each antimicrobial were administered to the pigs, the much lower concentration of spectinomycin indicates it was excreted in lower amounts and was less stable in the stored manure (Chapter 4; Kuchta et al., 2004). The extent to which the nursery manure was diluted with manure from untreated pigs would determine antimicrobial concentrations in lagoon manure applied to cropland. Based on the estimated total volume of liquid swine manure produced and the estimated volume of nursery manure produced in the Elstow barn in the 12-month period preceding manure application in fall 2003, antimicrobial concentrations in the nursery manure would have been diluted by a factor of approximately 20; that is, from 2,524 and 173 $\mu\text{g L}^{-1}$ to 126 and 9 $\mu\text{g L}^{-1}$ for lincomycin and spectinomycin, respectively.

In the field studies, lincomycin was detected in all liquid manure samples collected by applicator operators during manure application at both Elstow study sites and at the Riverhurst study site (Table 5.1). Less than 20% of the lincomycin in the manure was associated with the solids component. Concentrations in liquid manure applied to Elstow study sites 1 and 2 ranged from 105 to 179 $\mu\text{g L}^{-1}$ (mean = 132 $\mu\text{g L}^{-1}$) and 45.8 to 231 $\mu\text{g L}^{-1}$ (mean = 122 $\mu\text{g L}^{-1}$), respectively, whereas those for the Riverhurst site were 25.1 to 38.5 $\mu\text{g L}^{-1}$ (mean = 32 $\mu\text{g L}^{-1}$) (Table 5.1). The mean concentrations for the Elstow sites are of the same order of magnitude as the estimated concentration of 126 $\mu\text{g L}^{-1}$ and within the range (2.5 to 240 $\mu\text{g L}^{-1}$) reported by Campagnolo et al. (2002) reported for concentrations of lincomycin in eight swine lagoons in Iowa and Ohio.

In contrast, spectinomycin was not detected in any of the liquid manure samples collected by the manure applicator operators and, consequently, was not expected in any soil, surface runoff or groundwater samples. Based on mean concentrations of lincomycin in lagoon manure applied at the Elstow study sites, expected mean concentrations of spectinomycin would have been approximately 8 to 9 $\mu\text{g L}^{-1}$. Because of its lower stability in stored liquid swine manure (Chapter 4; Kuchta et al., 2004), it is not surprising that spectinomycin was not detected in the manure applied at these sites. At the Riverhurst site, concentrations of spectinomycin would

have been well below the limit of quantification of $6 \mu\text{g L}^{-1}$ since approximately twenty-four times more lincomycin was used compared to spectinomycin (2.86 and $0.12 \text{ kg month}^{-1}$, respectively).

5.3.4 Antimicrobial Content in Soil

Although the manure applicator was set to inject the manure to a 10-cm depth, injection, especially for the higher volumes of manure application, was shallower in some areas of the fields as evidenced by the presence wet surface soil. Consequently, lincomycin was detected in all replicate soil samples collected from Elstow study sites 1 and 2, in fall 2003 and 2004, respectively. Mean concentrations for each watershed/field location ranged from 46.3 to $117 \mu\text{g kg}^{-1}$ in the upper 5 cm of soil. Analysis of variance indicated no significant difference ($P < 0.05$) in lincomycin residues in the upper 5 cm of soil resulted when liquid manure was applied at $60,000$, $78,000$ or $95,000 \text{ L ha}^{-1}$, respectively. As expected, spectinomycin was not detected in any soil samples collected from manure-treated fields at Elstow study sites 1 and 2.

Following snowmelt runoff in spring 2004 and 2005, lincomycin was detected only in about a third of the replicate soil samples at each sampling time, and in lower concentrations. The decreased concentrations observed immediately after snowmelt may have resulted from slow microbial degradation (soil temperatures were $< 0^\circ\text{C}$ from late fall to early spring), loss in snowmelt runoff, and possible leaching of lincomycin during snowmelt. Lincomycin concentrations then decreased as soil temperatures increased and, by 30 July in 2004 and 23 August in 2005, were $< 5 \mu\text{g kg}^{-1}$. This decrease was first-order in nature and the average time for 50% dissipation was 19.4 days in 2004 [$R^2 = 0.81$; plot of lincomycin concentration in soil (natural log values) versus time] and 17.4 days in 2005 ($R^2 = 0.93$). These values are of the same order of magnitude as the half-life in soil reported for oxytetracycline by Aga et al. (2005) but considerably shorter than that reported for tiamulin (Schlusener et al. 2003).

5.3.5 Simulated Rainfall Runoff

Simulated rainfall runoff experiments at both Elstow study sites were completed within a week of manure application each fall. As expected, spectinomycin was not detected in any simulated rainfall runoff samples collected from manure-amended watersheds/fields.

Based on the average concentration of lincomycin in the liquid swine manure and the volume of manure applied per hectare, the application rates of lincomycin were 7.9, 9.5 and 12.5 g ha⁻¹ (equivalent to 792, 952 and 1250 µg m⁻², respectively) for application of liquid manure at 60,000, 78,000 and 95,000 L ha⁻¹, respectively. These application rates of lincomycin are equivalent to those for some pesticides; for example, the sulfonylurea and imidazolinone herbicides.

The volume of simulated rain applied and the proportion that infiltrated versus that moving as runoff were dependent, in large part, on the moisture content of the soil at the start of each experiment. With the very dry soil conditions in fall 2003, the largest average (n = 5) volume (289 ± 40 L) of simulated rain was applied and the greatest proportion (± SE) infiltrated (97.8 ± 1.2%) versus that collected as surface runoff (2.2 ± 1.5%). Under these conditions, only one simulated rainfall runoff experiment could be completed per day. When it became evident that, in some experiments, the time required to collect the 5-min sample would exceed 25 min such that the 15- and 25-min samples could not be collected and a complete data set would not be acquired, it was decided to terminate the fall experiments and carry out a full set of experiments at Elstow study site 1 in spring 2004.

Lincomycin in simulated rainfall runoff: Lincomycin was detected in all simulated rainfall runoff samples from experiments carried out immediately after liquid manure application at Elstow study sites 1 and 2, fall 2003 and 2004, respectively. In order for a chemical to be susceptible to movement in surface runoff, it must be present in the runoff-soil interaction zone or the upper 0.5 to 1 cm of soil (Wauchope 1978; Leonard et al., 1979; Ahuja et al., 1981). Although the manure applicators were set to inject the manure to 10 cm in both years, wet surface soil observed during manure application could account for some liquid manure (and lincomycin) being placed within the soil-runoff interaction zone. In addition, lincomycin from deeper depths may have moved into the runoff-soil interaction zone with the bulk flow of soil water as the soil dried. Such movement has been observed for some herbicides (Taylor and Glotfelty 1988).

In the Elstow study site 2 (fall 2004) simulated rainfall runoff experiments, mean lincomycin concentrations were significantly higher ($P < 0.05$) in the 5-min samples than in the 15- and 25-min samples (Figure 5.2). Although the mean concentration for the 25-min samples was lower, there was no significant difference in lincomycin concentrations for the 15- and 25-min samples. A pattern of decreasing lincomycin concentrations in runoff water with time was expected due to leaching and transport in runoff from the runoff-soil interaction zone. In spring 2004, at Elstow study site 1, when lincomycin concentrations in the simulated runoff water were approximately an order of magnitude lower, there was no significant difference ($P < 0.05$) in concentrations for any of the sampling times

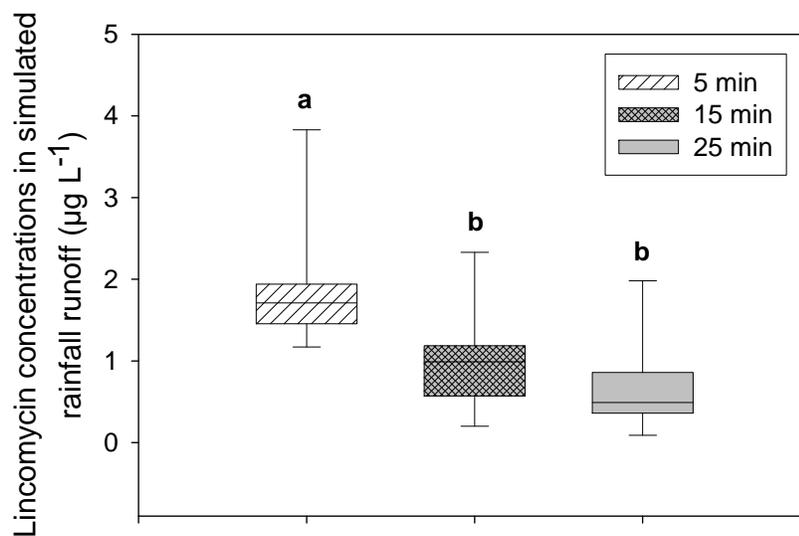


Figure 5.2. Lincomycin concentrations ($\mu\text{g L}^{-1} \pm \text{SE}$; $n=9$) from manure-amended cropland at Elstow study site 2. Runoff samples were collected 5, 15 and 25 min after runoff began. [Means with the same letters (lowercase) are not significantly different. Significance level is $P < 0.05$]

Fall runoff experiments (Elstow study sites 1 and 2): With the wetter soil conditions in fall 2004 when manure was applied at $78,000 \text{ L ha}^{-1}$, the mean lincomycin concentration [$1.18 \pm 0.21 \mu\text{g L}^{-1}$ ($\pm \text{SE}$; $n = 9$)] in simulated rainfall runoff was significantly higher ($P < 0.05$; ANOVA not shown) than that in fall 2003 [$0.11 \pm 0.035 \mu\text{g L}^{-1}$ ($\pm \text{SE}$; $n = 2$)] when manure was applied at $60,000 \text{ L ha}^{-1}$ (Table 5.2). The higher mean concentration in fall 2004 and higher proportion of runoff ($14.5 \pm 2.3\%$ versus $2.2 \pm 1.5\%$) resulted in a significantly higher mean mass of lincomycin transported in the simulated runoff [$54.6 \pm 10.9 \mu\text{g}$ ($\pm \text{SE}$; $n = 9$) versus 0.62 ± 0.10

μg (\pm SE; $n = 2$)]. Consequently, a higher proportion [$5.7 \pm 1.2\%$ (\pm SE; $n = 9$) versus $0.08 \pm 0.01\%$ (\pm SE; $n = 2$)] of the amount of lincomycin applied in the manure was transported in runoff in fall 2004. The much lower proportion transported in fall 2003 was most likely due to the dry soil conditions. Because simulated rainfall preferentially infiltrated into the soil, lincomycin would have concurrently leached below the runoff-soil interaction zone and not been available for transport in the rainfall runoff.

Table 5.2. Lincomycin concentrations ($\mu\text{g L}^{-1}$) detected in simulated rainfall runoff experiments.

Date of experiment	Field cover	Application rate (L ha^{-1})	Number of experiments	Lincomycin concentration	
				Mean ($\mu\text{g L}^{-1} \pm$ SE)	Range of mean values ($\mu\text{g L}^{-1}$)
Manure applied in Fall 2003					
Fall 2003	Cereal stubble	60,000	2	0.11 ± 0.02	-
Spring 2004	Cereal stubble	60,000	6	0.018 ± 0.006	<0.005 - 0.039
Spring 2004	Cereal stubble	95,000	6	0.098 ± 0.069	0.009 - 0.44
Manure applied in Fall 2004					
Fall 2004	Canola stubble	78,000	9	1.18 ± 0.21	0.59 - 2.71

Spring runoff experiments (Elstow study site 1): In spring 2004, lincomycin was detected in only 55% of the simulated rainfall runoff samples. The mean concentration of lincomycin in runoff from watersheds treated with liquid manure at $95,000 \text{ L ha}^{-1}$ was not significantly ($P < 0.05$) greater than that for the $60,000 \text{ L ha}^{-1}$ application rate.

Fall versus spring runoff experiments (Elstow study site 1): Because of the limited number of simulated rainfall runoff experiments accomplished in fall 2003, the only possible comparison of spring and fall experiments involved watersheds treated with liquid manure at $60,000 \text{ L ha}^{-1}$. In spring 2004, the mean lincomycin concentration [$0.018 \pm 0.006 \mu\text{g L}^{-1}$ (\pm SE; $n = 6$)] runoff was significantly ($P < 0.05$; ANOVA not shown) lower than that in fall 2003 [$0.11 \pm 0.035 \mu\text{g L}^{-1}$ (\pm SE; $n = 2$)] (Table 5.2). Lower concentrations in runoff in spring 2004 were expected because the persistence of lincomycin in the upper 5 cm of soil would predict lower concentrations of lincomycin in the soil-runoff interaction zone. The reduced concentrations and frequency of detection of lincomycin in the spring runoff most likely resulted from leaching of lincomycin

below the runoff-soil interaction zone during snowmelt, and possibly some microbial degradation.

5.3.6 Snow Melt Runoff Monitoring

Spectinomycin was not detected in soil from manure-treated watersheds/fields and, consequently, was not detected in corresponding snow melt runoff.

Analysis of soil samples from the Elstow study sites indicated that fall applications of lincomycin would persist until after snow melt the following spring. Thus, any lincomycin present in the runoff-soil interaction zone following the winter months would be available for transport in snow melt runoff. Lincomycin was detected in essentially all snow melt runoff samples collected from depressions and ephemeral wetlands at the Elstow (Table 5.3) and Riverhurst (Table 5.4) study sites.

When liquid manure was applied at two application rates at a single study site, concentrations of lincomycin in snow melt runoff were directly related to the rate at which the manure was applied. For example, at Elstow study site 1, the mean concentration of lincomycin in samples collected from depressions on watersheds treated with liquid manure at 95,000 L ha⁻¹ in fall 2003 was higher (0.31 µg L⁻¹) than that (0.095 µg L⁻¹) from watersheds treated at 60,000 L ha⁻¹ (Table 5.3). Similarly, at Riverhurst study site 4, mean concentrations in runoff from depressions were 0.39 and 0.26 µg L⁻¹ for liquid manure application rates of 110,000 and 88,000 L ha⁻¹, respectively (Table 5.4). (It should be noted that all depressions on the Elstow and Riverhurst study sites were directly treated with liquid manure (either by injection or centre pivot) which may have contributed, in part, to the lincomycin concentrations detected in runoff water from depressions).

Elstow study sites: Snow melt runoff was collected from one depression per watershed in the spring of 2004 and from two ephemeral wetlands on the manure-treated field, and one ephemeral wetland and one depression on the control field in spring 2005. At the latter site, samples were collected as snow melt runoff was flowing into the ephemeral wetlands on the manure-treated field. The presence of lincomycin in these samples [mean concentration (± SE) of 0.82 ± 0.11 µg

L⁻¹; n = 3] directly confirms the transport of lincomycin in snow melt runoff. The lower mean concentration in the wetland water ($0.40 \pm 0.05 \mu\text{g L}^{-1}$; Table 5.3) most likely resulted from dilution of the runoff water by snow melt from snow which had accumulated within the wetland.

Riverhurst study sites: The highest concentrations of lincomycin in snow melt runoff water were from depressions on summer fallow (< 0.005 to $3.2 \mu\text{g L}^{-1}$; Table 5.4) and cereal stubble (0.024 to $4.2 \mu\text{g L}^{-1}$) at Riverhurst study site 1. A shallower injection depth, due to low soil moisture content in fall 2003, such that more lincomycin was placed in the runoff-soil interaction zone may have contributed to these high concentrations. Lower snow accumulation in the winter of 2003-2004 compared to 2004-2005 resulting in less dilution because of a lower volume of runoff may have also contributed to the high concentrations of lincomycin in snow melt runoff water. Lincomycin concentrations in runoff in depressions on cereal stubble were higher than those on summer fallow (Table 5.4) and may reflect even drier soil conditions in stubble because of moisture requirements of the crop.

At Riverhurst study site 2, overflow from the second lagoon was applied directly to the soil surface on 25 October 2003 at a low application rate ($3,000 \text{ L ha}^{-1}$) using a centre pivot. Because of the low application volume (equivalent to 3 mm of liquid manure) and the potential for sorption of lincomycin to soil components and soil organic matter, much of the lincomycin most likely remained within the runoff-soil interaction zone, thus accounting for the high lincomycin concentrations detected in essentially all runoff samples (Table 5.4). Concentrations were lower in the ephemeral wetlands, most likely because of dilution due to greater snow accumulation. Infiltration was rapid on these two fields and, with the exception of two depressions on each field, only one sample was collected from each depression. A maximum of five samples was collected from two of the ephemeral wetlands.

Riverhurst study site 3 was the only site injected with liquid manure ($90,000 \text{ L ha}^{-1}$) in the spring (mid-May). Thus, snow melt runoff was sampled approximately ten months after manure application (in contrast to approximately 5 months for fall applications) and this period included the summer months when microbial degradation (because of higher soil temperatures) and leaching of lincomycin would be greatest. Consequently, the lowest concentrations of

Table 5.3. Lincomycin concentrations ($\mu\text{g L}^{-1}$) detected in snow melt runoff samples collected from depressions and ephemeral wetlands in the spring following manure application at Elstow study sites 1 and 2.

Field cover	Application rate (L ha^{-1})	Number of samples [†]	Number of depressions	Number of ephemeral wetlands	Lincomycin concentration			Frequency of detection (%)
					Mean ($\mu\text{g L}^{-1} \pm \text{SE}$)	Median ($\mu\text{g L}^{-1}$)	Range ($\mu\text{g L}^{-1}$)	
Elstow Study Site 1 - Manure injected in fall 2003								
Cereal stubble	60,000	15	2	0	0.10 ± 0.011	0.089	0.038 - 0.18	100
Cereal stubble	95,000	13	2	0	0.31 ± 0.073	0.31	0.058 - 0.80	100
Elstow Study Site 2 - Manure injected in fall 2004								
Canola stubble	78,000	11	-	2	0.40 ± 0.047	0.37	0.23 - 0.73	100

[†]samples from manure-treated fields only

Table 5.4. Lincomycin concentrations ($\mu\text{g L}^{-1}$) detected in snow melt runoff samples collected from depressions, ephemeral wetlands and dugouts in the spring following manure application at Riverhurst study sites 1 to 4.

Field cover	Application rate (L ha^{-1})	Number of samples [†]	Number of depressions	Number of ephemeral wetlands	Number of dugouts	Lincomycin concentration			Frequency of detection (%)
						Mean ($\mu\text{g L}^{-1} \pm \text{SE}$)	Median ($\mu\text{g L}^{-1}$)	Range ($\mu\text{g L}^{-1}$)	
Riverhurst Study Site 1 – Manure injected in fall 2003									
summer fallow	79,000	20	7	-	-	1.2 ± 0.17	1.0	<0.005 – 3.2	95
cereal stubble	79,000	9	7	-	-	1.7 ± 0.45	1.4	0.024 – 4.2	100
Riverhurst Study Site 2 – Centre pivot manure application in fall 2003									
canola stubble	3,000	10	8	-	-	0.81 ± 0.23	0.50	0.13 – 2.2	100
canola stubble	3,000	13	-	3	-	0.46 ± 0.063	0.39	0.16 – 0.83	100
cereal stubble	3,000	9	7	-	-	0.94 ± 0.13	1.0	<0.005 – 1.4	89
Riverhurst Study Site 3 - Manure injected in spring 2004									
canola stubble	90,000	15	7	-	-	0.020 ± 0.009	0.008	<0.005 - 0.15	100
canola stubble	90,000	20	-	4	-	0.012 ± 0.005	<0.005	<0.005 - 0.11	85
Riverhurst Study Site 4 - Manure injected in fall 2004									
pasture	88,000	25	10	-	-	0.26 ± 0.031	0.21	<0.005 - 0.59	100
pasture	88,000	16	-	4	-	0.16 ± 0.023	0.13	0.071 - 0.39	100
pasture	88,000	5	-	-	1	0.12 ± 0.029	0.09	0.044 - 0.20	100
pasture	110,000	32	12	-	-	0.39 ± 0.035	0.40	0.036 - 0.84	100
pasture	110,000	6	-	-	1	0.21 ± 0.017	0.21	0.16 - 0.28	100

[†]samples from manure-treated fields only

lincomycin in snow melt runoff water were detected at this study site. The mean lincomycin concentration (\pm SE) detected in samples collected from this site (0.012 ± 0.05), which received manure the previous spring, was significantly lower ($P < 0.05$) than the mean lincomycin concentration (\pm SE) detected in samples collected from the two ephemeral wetlands at Elstow study site 2 (0.40 ± 0.047), which had received manure the previous fall (Figure 5.3). Even though concentrations were lower at Riverhurst study site 3, lincomycin was still detected in nearly all samples.

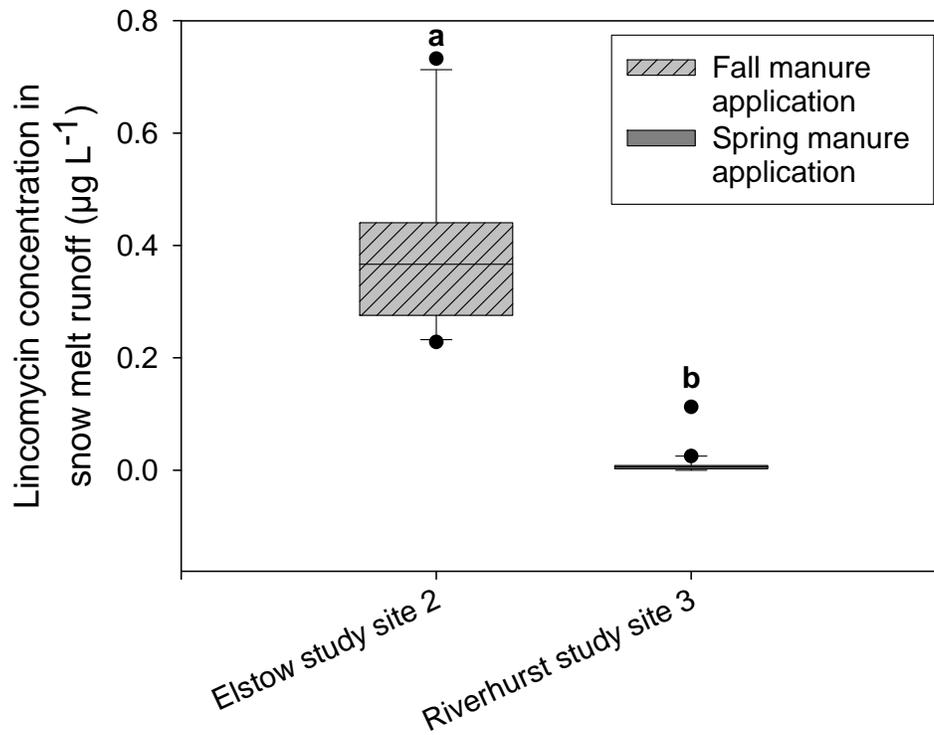


Figure 5.3. Lincomycin concentrations ($\mu\text{g L}^{-1}$) detected in snow melt runoff samples collected in spring 2005 from ephemeral wetlands on Elstow study site 2 following application of liquid swine manure to cropland at $78,000 \text{ L ha}^{-1}$ in fall 2004 and four ephemeral wetlands on Riverhurst study site 3 following application of liquid swine manure to cropland at $79,000 \text{ L ha}^{-1}$ in Spring 2004. [Means with the same letters (lowercase) are not significantly different. Significance level is $P < 0.05$]

Riverhurst study site 4 was the only study site involving pasture land. Lincomycin was detected in all samples and concentrations were in the order depressions > ephemeral wetlands > dugouts (Table 5.4). The lower mean concentration in ephemeral wetlands ($0.16 \mu\text{g L}^{-1}$) compared to depressions ($0.26 \mu\text{g L}^{-1}$) can be attributed to dilution from snow contained within ephemeral wetlands and that, in contrast to depressions, the ephemeral wetlands were not directly injected with liquid manure. The observation that the lowest mean concentration occurred in dugouts can be explained by dilution by water already in the dugouts before snow melt runoff began.

When comparing crop and pasture land (Riverhurst study sites 1 and 4, respectively), concentrations of lincomycin in runoff were much lower on pasture land even though it received higher rates of manure application (Table 5.4). Zhan et al. (2007) reported higher infiltration into grassed soil versus bare soil. In addition, Kumar et al. (2005) reported uptake of chlortetracycline from manure-amended soil by corn (*Zea mays* L.), green onion (*Allium cepa* L.) and cabbage (*Brassica oleracea* L. Capitata group), although none of these crops absorbed tylosin. Thus, the lower lincomycin concentrations in runoff from grassed pasture land may have resulted from increased infiltration of snow melt below the soil-runoff interaction zone and possibly plant uptake. The lower lincomycin concentrations may have also resulted from greater degradation of lincomycin in the runoff-soil interaction zone due to earlier application of the liquid manure to pasture (15 September 2004) compared to the cropland (21 October 2003).

5.3.7 Groundwater Monitoring

Groundwater was monitored for lincomycin and spectinomycin only at Elstow study site 1 from fall 2003 to fall 2005. Samples were collected from single piezometers installed in each of four manure-treated watersheds and one control watershed. In contrast to lincomycin, spectinomycin was not present in detectable concentrations in the soil of these watersheds either in fall 2003 or during the period from spring 2004 to fall 2004 and, subsequently, was not detected in any corresponding groundwater samples collected during these time frames.

Moisture conditions from spring 2003 to fall 2005 played a significant role in the number of groundwater samples collected on each sampling date. Because of the dry conditions in 2003, groundwater was present only in two of the five piezometers: one from the control watershed and

the other from one of the watersheds treated with liquid manure at 60,000 L ha⁻¹. In 2004 when rainfall was above normal, groundwater was present in all five piezometers, but not consistently on a monthly basis. Under wetter conditions in 2005, groundwater was consistently present in all five piezometers at each monthly sampling.

In fall 2003 immediately after manure application, lincomycin was not detected in groundwater from either the single manure-treated watershed or the control watershed. From spring to fall 2004 when rainfall was near normal, lincomycin was present in groundwater from two watersheds: one treated at the higher manure application rate (95,000 L ha⁻¹) and the other at the lower rate (60,000 L ha⁻¹) (Table 5.5). Concentrations ranged from non detectable (nd) to 0.15 µg L⁻¹ and lincomycin was detected more frequently (71 versus 25%) and at higher mean concentrations (0.026 versus < 0.005 µg L⁻¹) in groundwater samples from the watershed which received the higher manure application rate. Lincomycin was not detected in groundwater from the control watershed or in five samples collected from the other two manure-treated watersheds. The only previous detection of lincomycin in groundwater was reported by Campagnolo et al. (2001) who sampled wells adjacent to large-scale confined swine and poultry feeding operations in the USA. These workers detected lincomycin in one well.

In the second year after manure application (spring to fall 2005) when rainfall was well above normal, lincomycin was detected in groundwater samples from all four manure-treated watersheds (Table 5.5). The frequency of detection was higher (78 to 81%) than in the previous year; however, lincomycin concentrations in the groundwater were much lower (nd to < 0.005 µg L⁻¹) and there was no difference in mean concentrations for the low and high manure application rates.

In contrast to the previous year, lincomycin was also present in groundwater samples collected from the control watershed with a frequency of detection of 66% (Table 5.5). Mean, median and maximum concentrations were of the same order of magnitude as those from the manure-treated watersheds. These concentrations most likely originated from the liquid swine manure application made to this field three years previously (fall 2002) and may have been mobilized within the soil by the excessive amount of rain received in 2005. Since the treated watersheds

Table 5.5. Lincomycin concentrations ($\mu\text{g L}^{-1}$) detected in groundwater from Elstow study site 1 over two years

Sampling years	Application rate in fall 2003 (L ha^{-1})	Number of samples [†]	Number of producing piezometers	Lincomycin concentration			Frequency of detection (%)
				Mean ($\mu\text{g L}^{-1}$)	Median ($\mu\text{g L}^{-1}$)	Range ($\mu\text{g L}^{-1}$)	
Elstow Study Site 1							
Fall 2003	60,000	1	1	nd [‡]	nd	nd	0
	95,000	0	-	-	-	-	-
	control	1	1	nd	nd	nd	0
April to October 2004	60,000	11	2	< 0.005	nd	nd to 0.005	25
	95,000	7	2	0.026	0.005	nd to 0.15	71
	control	8	1	nd	nd	nd	0
April to October 2005	60,000	14	2	< 0.005	< 0.005	nd to < 0.005	78
	95,000	16	2	< 0.005	< 0.005	nd to < 0.005	81
	control	9	1	< 0.005	< 0.005	nd to < 0.005	66

[†]Due to dry conditions in the fall of 2003, no groundwater was present in 3 piezometers. Single water samples were collected only from the control watershed and from one manure-treated watershed ($60,000 \text{ L ha}^{-1}$).

[‡]nd: not detected

had also been previously treated with liquid swine manure (fall 2001), it is not clear whether concentrations detected in 2005 originated from previous manure applications or the manure application made in fall 2003. However, the detection of lincomycin in the groundwater confirms that when lincomycin is present in liquid manure applied to cropland as a plant nutrient source, leaching to groundwater can occur under prairie conditions.

5.4 Conclusions

Some antimicrobials, such as lincomycin, will be sufficiently stable during manure storage such that they will be present in the manure when applied to crop or pasture land as a plant nutrient source. Monitoring lincomycin concentrations in manure-amended soil demonstrated that this antimicrobial may persist in the upper 5 cm of soil for several months after fall application. Our knowledge of the implications of such antimicrobials in cropland for human health and aquatic ecosystems is limited. The recent study by Kumar et al. (2005) shows that antimicrobials in the upper layer of soil may be taken up by crops. Ingesting these crops may result in allergic reactions or antimicrobial resistance in humans. Antimicrobials which degrade slowly in soil may be available for transport in surface runoff into receiving water bodies (such as wetlands and farm dugouts) or leaching to groundwater. Monitoring lincomycin in ephemeral wetlands confirmed that this antimicrobial can be quite stable in wetland waters and studies by Isidori et al. (2005) and Halling-Sørensen. (2000) have shown that some antimicrobials at environmentally relevant concentrations can be toxic to some algae. Thus, the consequence of the use of manure on agricultural land may have impacts on human health and on flora and fauna of aquatic ecosystems.

However, producers can make choices to minimize possible human health and environmental effects. Use of antimicrobials, such as spectinomycin, which have reduced stability in stored manure will reduce or prevent transport to surface and groundwaters. The greater snow melt transport of lincomycin following fall application of liquid manure demonstrated that spring application of manure to crop and pasture land will reduce the amount of antimicrobial available for transport in snow melt runoff into surface water bodies. Injection of liquid manure to a deeper depth would most likely minimize antimicrobial transport in surface runoff since less antimicrobial should be placed in the runoff-soil interaction zone. While deeper injection could

potentially increase leaching to groundwater, microbial degradation in soil may take place before significant leaching occurred.

6.0 GENERAL DISCUSSION

Antimicrobials administered to livestock can be excreted up to 80% in the feces and urine. Liquid manure, when applied to crop land as a nutrient source, is thus a possible source of antimicrobials to nearby surface and ground waters through runoff and leaching, respectively. Trace concentrations of veterinary antimicrobials have been detected in surface and ground waters in Canada, the United States and Europe. However, the environmental fate and persistence of these pharmaceuticals is not well known. Lincomycin and spectinomycin are two antimicrobials that are frequently administered as a mixture to swine in the prairie region of Canada for the prevention and control of post-weaning diarrhea.

In order to investigate the occurrence and persistence of these antimicrobials in the environment, analytical methods were developed to extract and analyze lincomycin and spectinomycin in liquid swine manure (solids and liquid components), manure-treated soil, and in surface and ground waters. Solid phase extraction (SPE) was used to extract these antimicrobials from surface and ground waters and from the liquid component of liquid hog manure. Pressurized liquid extraction followed by SPE clean-up was used for manure-treated soil and the solids component of liquid hog manure. LC-MS/MS was used to quantitate these antimicrobials in all matrix extracts. For lincomycin, Oasis HLB SPE provided good recoveries and clean extracts for analysis. Weak cation exchange SPE provided adequate recovery of spectinomycin from all matrices. Stacking an Oasis HLB cartridge on top of the WCX cartridge provided the clean-up prior to analysis which was necessary to reduce or eliminate suppression or enhancement of ionization in the source of the mass spectrometer. Hydrophilic interaction chromatography provided excellent retention and separation of spectinomycin and lincomycin from interfering matrix components and provided baseline separation without the need for ion-pairing reagents. APCI(+) produced intense ions that were conducive to trace analysis using MS/MS. Instrument sensitivity for lincomycin was greater than for spectinomycin, and thus the LOQ's were lower for lincomycin than spectinomycin. Limits of quantification for lincomycin and spectinomycin, respectively, were 0.005 and 0.5 $\mu\text{g L}^{-1}$ for runoff and ground water, 0.5 and 50 $\mu\text{g kg}^{-1}$ for soil, 0.5 and 6.0 $\mu\text{g L}^{-1}$ for the liquid component (supernatant) of swine manure and 25 and 50 $\mu\text{g kg}^{-1}$ for the solids component of liquid swine manure.

The concentrations of lincomycin and spectinomycin excreted in the manure from weanling pigs were investigated to determine the potential for these antimicrobials to be present in lagoon manure which would eventually be applied to agricultural land. Cumulating manure was monitored in the nursery area of a commercial-scale barn in which weanling pigs were administered the antimicrobials in their feed. Concentrations of lincomycin and spectinomycin in the liquid manure at the end of the study were equivalent to 32 and 3%, respectively, of the doses administered. Although lincomycin and spectinomycin were present in liquid manure from within the barn, their presence in lagoon manure at the time of manure application to agricultural land (after approximately 6 to 12 months of lagoon storage) will depend on their stability during manure storage.

The persistence of lincomycin and spectinomycin during simulated manure storage was investigated to determine the potential for environmental contamination. In a laboratory study, using fortified liquid manure, concentrations of both antimicrobials showed a rapid initial decrease during simulated lagoon storage, followed by a slower dissipation over a period of 5 months. The average time required for 50% dissipation of lincomycin was greater than one year and approximately 90 d for spectinomycin. Therefore, with the management practice of storing liquid manure in earthen lagoons and applying the manure to crop or pasture land once or twice a year, both antimicrobials may be present in the lagoon manure when applied as a plant nutrient source.

The persistence of lincomycin and spectinomycin in soil following manure injection into crop land was investigated to determine the potential for contamination of surface water bodies and ground water via runoff and leaching, respectively. Spectinomycin was not detected in lagoon manure applied to crop or pasture land. Consequently, it was not detected in manure-treated soil, in simulated rainfall runoff, in spring snow melt runoff or in ground water. Lincomycin was present in lagoon manure (25.1 - 231 $\mu\text{g L}^{-1}$) applied to crop and pasture land and persisted in the upper 5 cm of soil (46.3 to 117 $\mu\text{g kg}^{-1}$) from fall application until mid-June to mid-July the following year.

In order to assess the potential for lincomycin and spectinomycin to be transported from manure-treated soil to surface waters via surface runoff and to leach to ground waters, simulated rainfall runoff, snow melt runoff and ground water were monitored for lincomycin and spectinomycin. These studies were carried out over a two-year period at two study sites in Saskatchewan, Canada following application of liquid hog manure from two commercial barns to crop and pasture land.

Immediately after fall manure application, lincomycin was detected in simulated rainfall runoff samples at concentrations ranging from 0.1 to 2.7 $\mu\text{g L}^{-1}$. Less than 6% of the lincomycin applied to the field during manure applications was recovered in runoff water. During spring snow melt following fall or spring application of liquid swine manure, lincomycin was detected in snow melt runoff water in essentially all depressions and ephemeral wetlands on manure-treated fields at both study sites (<0.005 to 4.0 $\mu\text{g L}^{-1}$). Lincomycin persisted in the water in depressions and ephemeral wetlands until all of the water had infiltrated/evaporated. Lincomycin was also present in water collected from two dugouts, located on manure-treated land, which had received snow melt runoff water.

Ground water was collected from piezometers (~ 4 -5 m depth) installed on manure-treated and control fields. Lincomycin was detected in ground water with concentrations ranging from <0.005 to 0.150 $\mu\text{g L}^{-1}$, and was detected more frequently in ground water two years after manure application.

This study showed that some antimicrobials used to prevent disease can be excreted in the feces/urine such that concentrations are detectable in the liquid manure. It is also evident that with the management practice of storing liquid manure in earthen lagoons and applying the manure to crop or pasture land once or twice a year, some antimicrobials persist in the lagoon manure until land application. After application, these antimicrobials may persist in the upper layer of soil for several months and a portion of the antimicrobials being available for transport in surface runoff into surface water bodies (such as wetlands and dugouts) or leaching to ground water. Thus, the consequence of current management practices (antimicrobial use and use of

liquid manure as a plant nutrient source) may result in environmental impacts on aquatic ecosystems. Future investigation on this topic could include:

- 1) Further investigation into the rapid decrease of both lincomycin and spectinomycin concentrations during the first 6 days of simulated manure storage. This may be the result of sorption to manure solids. Utilizing different solvents during pressurized liquid extraction could more effectively extract these chemicals from the manure solids.
- 2) Investigating the effectiveness of management practices to minimize the potential for antimicrobial residues in livestock manure being applied to crop and pasture land. These could include the effects of (1) aeration of manure during lagoon storage and (2) composting the manure before applying it to agricultural soils.
- 3) Looking at the effectiveness of application practices to minimize the potential for transport into surface waters. For example, injecting liquid manure to a deeper depth, so that the majority of the antimicrobials are placed below the soil-runoff interaction zone.
- 4) To determine what effect amending soil with manure has on the development of antimicrobial resistance in soil indicator organisms.
- 5) To investigate the biological significance of concentrations of antimicrobials measured in aquatic ecosystems.
- 6) To investigate the potential for antimicrobials present in manure-amended agricultural soils to be taken up by crops and thus, creating an indirect exposure route for both humans and animals through food.

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