Impact of veterinary antibiotics in the environment

Keri Lynn Deppe Henderson

Iowa State University
Impact of veterinary antibiotics in the environment

by

Keri Lynn Deppe Henderson

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Program of Study Committee:
Joel R. Coats, Co-major Professor
Thomas B. Moorman, Co-major Professor
Thomas E. Loynachan
Gary D. Osweiler
Jon J. Tollefson

Iowa State University

Ames, Iowa

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Abstract

The focus of this dissertation is assessment of the potential impact of livestock production practices on terrestrial and aquatic ecosystems by studying the environmental fate and effects of two classes of veterinary antibiotics: macrolides (represented by tylosin) and sulfonamides (represented by sulfamethazine). These antibiotics are widely used in livestock production for disease treatment and prevention, as well as growth promotion. Each has also been widely detected in surface waters in the U.S. The work presented utilized laboratory experiments to study the fate (mobility, dissipation, binding, and degradation) in soil columns and aquatic microcosms, and bioavailability to benthic invertebrates. Additionally, methodologies were developed for using a passive sampling, or biomimetic, device to estimate bioavailability in aquatic ecosystems. Results suggest the potential persistence of some antibiotic residues in sediments, and the level of bioaccumulation in *Lumbriculus variegatus*, an aquatic oligochaete, indicates that uptake by sediment-dwelling invertebrates may be a concern.
Chapter 1. General introduction

Abstract

The focus of this dissertation is assessment of the potential impact of livestock production practices on terrestrial and aquatic ecosystems by studying the environmental fate and effects of two classes of veterinary antibiotics: macrolides (represented by tylosin) and sulfonamides (using sulfamethazine). These antibiotics are widely used in livestock production for disease treatment, prevention, and growth promotion. Each has also been widely detected in surface waters in the U.S. The work presented utilized laboratory experiments to study the fate (mobility, dissipation, binding, and degradation) in soil columns and aquatic microcosms, and bioavailability to benthic invertebrates. Additionally, methodologies were developed for assessing potential impacts of antibiotics on invertebrate gut microflora, and for using a passive sampling, or biomimetic, device to estimate bioavailability in aquatic ecosystems.

Background

Agriculture constitutes a critical portion of the U.S. economy, and the production of swine, cattle, and poultry raised for human consumption represents a significant portion of the U.S. agricultural economy. To maximize production, livestock producers regularly use antibiotics as supplements in animal feed and water to increase rates of weight gain and prevent diseases among their livestock. In swine, for example, it is estimated that antibiotics are used for disease prevention and growth promotion in more than 90% of starter feeds, 75% of grower feeds, 50% of finishing feeds, and 20% of sow
feeds. Equally relevant numbers are seen in beef cattle production (Hayes et al., 1999; USDA APHIS Swine2000; USDA APHIS COFE). It has been well documented that measurable quantities of these antibiotics are excreted, often in original form, in feces and urine of livestock. Livestock waste, containing antibiotics, is often used as fertilizer for farm fields or pastures and may result in nonpoint source pollution of ground or surface waters (Loke et al., 2000). Field application of manure often involves injection or incorporation of the waste, which may bring nutrients and antibiotic residues spatially closer to tile drainage systems. These drainage systems often flow directly into nearby streams, rivers, or other waterways, and may therefore act as a source of waterway contamination.

Although antibiotic residues have been studied extensively in tissues and excrement, relatively little is known about the environmental fate of antibiotics and their metabolites once the excreta reaches soil and water environments (Tolls, 2001; Coats et al., 1976). While sorption and mobility data exist for a few of these chemicals, very few studies have evaluated mobility and degradation in the presence of a manure substrate (Rabølle and Spliid, 2000). Sorption of chemicals onto solid phases, such as soil or sediment, is extremely important because it could affect the fate and impact of these substances in that environment.

Recently, antibiotics, including the veterinary antibiotics tylosin and sulfamethazine, were found in 48% of 139 stream waters tested in 30 states, according to the United States Geological Survey (Kolpin et al., 2002). Recent sediment monitoring studies by Kim and Carlson (2007a and b) detected extractable antibiotic residues up to 0.1 mg/kg in sediment from an impacted river; typical concentrations were 0.001 to 0.03
mg/kg. Antibiotics entering the environment could potentially alter bacterial populations and their activity in soil, sediment, and water, thus affecting biodegradation, nutrient cycling, and water quality. In addition, there is concern that antibiotics in the environment may induce antibiotic resistance, resulting in adverse human health effects. Certainly, there is significant evidence for development of antibiotic resistance within animals and in the excretion of antibiotic-resistant bacteria in manure (Beaucage et al., 1979; Aarestrup et al., 1997; Kelley et al., 1998). Much less is known about the ability of low concentrations of antibiotics to induce resistance in the environmental microbial population or to provide selective pressure for maintenance of antibiotic resistance genes among microorganisms, although the transfer of antibiotic-resistance from agricultural settings to humans has been reported (Oppegaard et al., 2001).

In addition to concerns over changes in microbial resistance, there are concerns over direct and indirect effects on non-target eukaryotic organisms in important habitats found across agricultural landscapes. In particular, small ponds and wetlands that serve as key breeding sites for amphibians and support invertebrate communities that provide food for migrating birds (Reynolds, 1987) can receive significant amounts of contaminated agricultural runoff, which could contain antibiotic residues (Maltby et al., 1995; Lahr, 1997). The aquatic risk of these chemicals has not been extensively characterized (Baguer et al., 2000; Wollenberger et al., 2000). Indirect effects on nontarget organisms could occur via alteration of the microbial component of the food web since algae and bacteria provide vital carbon sources that drive secondary production in many ponds and wetlands. Additionally, bacterial communities are particularly important throughout the life stages of invertebrates, both as nutrient sources and as
symbionts within the invertebrate gut (Dubilier et al., 1995; Thorp and Kovich, 2001; Waiser, 2001; Wetzel, 2001).

**Tylosin and Sulfamethazine**

Tylosin and sulfamethazine were listed in the top ten most frequently detected veterinary and human antibiotics in surface water in 1999 to 2000 (Kolpin et al., 2002). Although research has been conducted on the fate and effects of several antibiotics, the respective environmental fates of tylosin, erythromycin, and sulfamethazine are not well understood (Baguer et al., 2000; Tolls, 2001). It is believed that these drugs may have an affinity for clay particles in soil or sediment, which could affect their availability for degradation; this is also likely important for bioavailability and uptake for higher organisms (Rabølle and Spliid, 2000). Additionally, Huang and colleagues (2001) described tylosin and sulfamethazine as two of the most likely water contaminants from agricultural runoff. An understanding of the degradation and fate in environmental matrices is important because of widespread use of the compounds in livestock production in the U.S. and the concurrent application of manure to land with subsurface tile drain networks.

Tylosin is a macrolide antibiotic with activity against gram positive and certain gram-negative bacteria, including *Staphylococcus, Listeria, Legionella*, and *Enterococcus*. It has little activity against gram-negative enteric bacteria such as *E. coli*. Tylosin (TYL) is used exclusively in veterinary applications, and is closely related to erythromycin which has an important role in public health. Tylosin consists of four major factors: tylosin A, B, C, and D; each of the factors is biologically active, with
tylosin A being most active and most prevalent in medicinal and feed formulations (Fig. 1a; Teeter and Meyerhoff, 2003). Tylosin acts bacteriostatically in bacteria by binding to the 50S ribosome subunit which leads to inhibition of protein synthesis. Sensitive bacteria are inhibited by as little as 500 μg/l. Tylosin is used as a growth promoter applied in swine feed, and as a therapeutic product in swine and cattle. Tylosin is also commonly used internationally in swine, cattle, and poultry production as both a therapeutic and a prophylactic (Massé et al., 2000; Rabølle and Spliid, 2000). In swine production, tylosin is among the three antibiotics which accounted for the majority (78.8 %) of disease prevention. Tylosin was the most used antibiotic at 31.3 % of swine production facilities surveyed (Bush and Biehl, 2001). It has been shown that tylosin is transformed to several metabolites in the animal (e.g. tylosin D, dihydrodesmycosin, and cysteinyl tylosin A), but some residues may be converted back to tylosin A in excreta (EMEA, 1997; FAO/WHO, 1991). Concentrations of tylosin in swine feed range from 10 to 110 mg/kg tylosin in feed for growth promotion purposes (Elanco Animal Health Tylan® Premix product label). Erythromycin is also a macrolide antibiotic, with the same mode of action as tylosin, and is used widely in human medicine; concern has arisen regarding resistance development which could affect erythromycin use and efficacy.

Sulfamethazine, also known as sulfadimidine, is a sulfonamide antibiotic with activity primarily against gram-positive bacteria, including Enterococcus, and also against some enteric gram-negative bacteria (Fig. 1b; Prescott, 2000). The sulfonamides are structural analogs of p-aminobenzoic acid and act by preventing folic acid synthesis. Sulfamethazine (SMZ) is used in cattle and swine production as a therapeutic and as a
growth promoter. This antibiotic has been found in concentrations exceeding 100 μg/l in swine lagoon water, and in lower concentrations in tile drainage water and stream water (Lindsey et al., 2001; Campagnolo et al., 2002). Concentrations can exceed 5 mg/kg in swine manure (Haller et al., 2002). In addition to concerns over the presence of each of these compounds in the environment and the potential implications for their individual toxicities, mixtures of these contaminants are also of concern. For example, Elanco™ produces a formulation of tylosin and sulfamethazine known as Tylan®-Sulfa-G, which is manufactured for use in swine feed; so it is likely that both tylosin and sulfamethazine may be present in manure from a single hog, which brings about concerns over mixtures of antibiotics. The recommended dose of sulfamethazine in feed is 110 mg/kg in feed for growth promotion in swine (Elanco Animal Health Tylan® Sulfa-G product label).

![Figure 1a. Chemical structure of tylosin including factors: A (R₁=CHO, R₂=CH₃), B (TYL A minus mycinose), C (R₁=CHO, R₂=H), and D (R₁=CH₂OH, R₂=CH₃).](image)

![Figure 1b. Chemical structure of sulfamethazine.](image)

**Rationale and Significance**

The significance of low concentrations of veterinary antibiotics in lagoon water, soil, and surface water (5 to 500 μg/l) is not well understood, particularly when so few data are available regarding sorption and bioavailability of antibiotics. Simply detecting the compounds does not provide adequate information on the ecological significance.
These concentrations appear to be too low for significant antimicrobial activity; for instance Prescott (2000) showed the lowest MIC (minimum inhibitory concentration) of sulfonamides to be 8 mg/l, except for Brucella canis with an MIC of 2 mg/l; however MIC data exist for only a small number of microorganisms. Low or transient concentrations of antibiotics could have several effects; they may have secondary modes of action causing harm to non-target populations in aquatic and terrestrial habitats. For instance, diaminopyrimidines with some structural similarity to sulfonamide antibiotics are used for the treatment of protozoan diseases (Prescott, 2000). Antibiotic residues in the environment may also contribute to the development of antibiotic resistance or act to maintain resistance acquired elsewhere. Since antibiotic concentrations are greatest in the animal digestive tract, this appears to be the most likely site where resistance is acquired; a recent paper by Onan and LaPara (2003) reveals increased incidence of tylosin-resistance in bacteria cultured from agricultural soils receiving regular manure applications from livestock fed subtherapeutic doses of antibiotics. Low concentrations of antibiotics in the environment may cause the retention of the resistance genes or promote their transfer between bacterial species. Importantly, the bioavailability of antibiotics to non-target organisms has not been well-studied, and it is unknown whether these µg/l or µg/kg concentrations will have the ability to bioaccumulate in organism tissue. Some discrepancies still exist on how to define and assess bioavailability in the context of environmental toxicology. Traditional methods for assessing bioavailability include uptake studies using laboratory test organisms (e.g. Lumbriculus variegatus, an aquatic oligochaete); however, much effort has been put into the development of chemical-based methods of assessing bioavailability, including the use of biomimetic
devices and chemical extraction techniques (Ehlers and Loibner, 2006). While much research has focused on persistent hydrophobic contaminants such as PAHs, PCBs, and DDT analogs, little work has been performed evaluating more polar contaminants such as antibiotics, which have the potential for persistence, have relatively continual input into the environment, and have known biological potency. A more thorough review of the state of bioavailability science is described in Chapter 5 of this dissertation.

Studies on the fate, bioavailability, and effects of veterinary pharmaceuticals in terrestrial and aquatic systems will lead to improved management practices through the understanding of the potential for contamination of water resources. Results obtained from the studies presented in this dissertation provide information that is essential to understanding the environmental mobility and degradation of the two classes of veterinary antibiotics selected: macrolides and sulfonamides. The bioavailability studies serve as a crucial link to understanding the likelihood of environmental residues impacting species or microbial communities in a negative way. Published reports have confirmed that very low levels of residues are detected in water and sediment monitoring studies (Kolpin et al., 2002; Diaz-Cruz et al., 2003; Beausse, 2004; Kim and Carlson, 2007a and b), but the significance of those residues will depend, to a great extent, on their biological availability. The synthesis of these data will provide a more comprehensive evaluation of the fate and effects of these contaminants in the environment.

Goals and Objectives

Data are needed to understand the potential impacts of veterinary antibiotics once they enter the environment; methods and results described in this dissertation may be
useful for risk assessment and evaluation of manure management practices, and applied toward the protection of natural resources in agricultural landscapes.

Objectives:

1. Determine mobility and degradation of manure-applied tylosin, and survival and movement of enteric bacteria in soil column
2. Determine the fate of TYL and SMZ in surface water microcosms containing manure and sediment
3. Determine the persistence of antibiotic-resistant Enterococcus faecalis in relation to non-resistant strains in aquatic microcosms
4. Evaluate the bioavailability of SMZ in aquatic microcosms
   a. Develop a method to estimate bioavailability using a biomimetic device
   b. Compare bioaccumulation results obtained from bioassay with those obtained from biomimetic device
5. Develop a dose-response method to evaluate the potential effects of SMZ on gut microflora of invertebrates

Organization of Dissertation

This dissertation is organized into chapters, with each chapter serving as an individual manuscript to be published, or already published, in a peer-reviewed journal or book. Co-authors are also listed where applicable. Each chapter addresses one of the objectives listed in the preceding section. Chapter 1 consists of a general introduction to
the topic of veterinary antibiotics in the environment; a brief literature review and description of dissertation organization are included.

Chapter 2 details findings from Objective 1. An intact soil column study was performed to examine the mobility of tylosin and enteric bacteria applied to soil in a manure slurry. Data suggest a differential mobility and/or differential persistence of tylosin D compared to tylosin A. Higher than expected mobility of enteric bacteria was also noted; in many cases, the values for *E. coli* and *Enterococcus* exceeded EPA suggested water quality criteria.

Chapter 3 details methodology and results obtained from examination of the dissipation of tylosin in surface water (Obj. 2). The fate of environmentally relevant concentrations of tylosin was evaluated in surface water, and the potential impact of manure and plant life was assessed.

Chapter 4 describes the results obtained from an examination of the fate of sulfamethazine in surface water (Obj. 2). We investigated the degradation and fate of sulfamethazine in small pond water microcosms using $^{14}$C-phenyl-sulfamethazine, and found significant binding of sulfamethazine to sediment over a 63-day period. The main metabolites detected in both water and sediment were photoproducts. Greater than 40% of the applied radiolabel was bound to the sediment at the end of the study, indicating a potential route of exposure for benthic organisms.

Chapter 5 details studies on the bioavailability of sulfamethazine (Obj. 4). The bioavailability of radiolabeled sulfamethazine in surface water microcosms was evaluated using C8-Empore™ extraction disks as passive sampling devices and *Lumbriculus variegatus* in a bioassay. The disks and worms were incubated in treated water or
sediment-containing microcosms. At the end of the study, the disks and worms were extracted, and uptake of radioactivity was determined. Bioconcentration factors were calculated to allow for comparison between disks and worms. Sulfamethazine uptake by *Lumbriculus variegatus* was measured during the study.

Chapter 6 provides concluding remarks regarding the results obtained from the work described in this dissertation.

Finally, methods were developed for Objective 3 (assessing persistence of antibiotic-resistant organisms) and Objective 5 (assessing the potential impact of veterinary antibiotics on invertebrate gut microflora using a dose-response test). A brief description of those methods are outlined in the Appendix.

References


Chapter 2. Mobility of tylosin and enteric bacteria in soil columns

Keri L. Henderson¹, Thomas B. Moorman², and Joel R. Coats¹

¹ Department of Entomology, Iowa State University, Ames, IA
² USDA-ARS National Soil Tilth Laboratory, Ames, IA

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Introduction

The production of swine, cattle, and poultry raised for human consumption represents a significant portion of the U.S. agricultural economy. To maximize production, producers regularly use antibiotics as supplements in animal feed and water to increase weight gain and prevent diseases among their livestock. In swine, for example, it is estimated that antibiotics are used for disease prevention and growth promotion in more than 90% of starter feeds, 75% of grower feeds, 50% of finishing feeds, and 20% of sow feeds, and equally relevant numbers are seen in beef cattle production (Hayes et al., 1999; USDA APHIS Swine 2000; USDA APHIS COFE). It has been well documented that measurable quantities of these antibiotics are excreted, often in original form, in feces and urine of livestock (FAO/WHO, 1991). Livestock waste, containing antibiotics, is often used as fertilizer for farm fields or pastures and may result in nonpoint source pollution of ground or surface waters (Loke et al., 2000). Although antibiotic residues have been studied extensively in tissues and excrement, we are only
beginning to understand the environmental fate of antibiotics and their metabolites once the excreta reaches soil and water environments.

Recently, antibiotics, including the veterinary antibiotic tylosin which is described in this study, were found in 48% of 139 stream water tested in 30 states, according to the United States Geological Survey (Kolpin et al., 2002). Antibiotics entering the environment could potentially alter bacterial populations and their activity in sediment and water, thus affecting biodegradation, nutrient cycling, and water quality. In addition, there is concern that antibiotics in the environment may induce antibiotic resistance resulting in adverse human health effects. Certainly, there is significant evidence for development of antibiotic resistance within animals and in the excretion of antibiotic-resistant bacteria in manure (Beaucage et al., 1979; Aarestrup et al., 1997; Kelley et al., 1998). Much less is known about the ability of low concentrations of antibiotics to induce resistance in the environmental microbial population or to provide selective pressure for maintenance of antibiotic resistance genes among microorganisms, although the transfer of antibiotic-resistance from agricultural settings to humans has been reported (Oppegaard et al., 2001).

**Tylosin**

Tylosin is a macrolide antibiotic with activity against-gram positive and certain gram-negative bacteria, including *Staphylococcus, Listeria, Legionella* and *Enterococcus*. It has little activity against gram-negative enteric bacteria such as *E. coli*. Tylosin is used exclusively in veterinary applications, and is closely related to erythromycin which has an important role in public health. Tylosin consists of four major factors: tylosin A, B, C,
and D (Fig. 1); each of the factors is biologically active, with tylosin A being most active and most prevalent in medicinal and feed formulations (Teeter and Meyerhoff, 2003). Tylosin acts in bacteria by binding to the 50S ribosome subunit which leads to inhibition of protein synthesis. Sensitive bacteria are inhibited by as little as 500 μg/L. Tylosin is used as growth promoter applied in swine feed, and as a therapeutic product in swine and cattle. Tylosin is a common antibiotic used internationally in swine, cattle, and poultry production as both a therapeutic and a prophylactic (Massé et al., 2000; Rabølle and Spliid, 2000). In swine production, tylosin is among the three antibiotics which accounted for the majority (78.8 %) of disease prevention. Tylosin was the most used antibiotic at 31.3 % of swine production facilities surveyed (Bush and Biehl, 2001). It has been shown that tylosin is transformed in the animal from tylosin A to tylosin D, which is a change from an aldehyde to an alcohol on the macrolide ring. However, tylosin D may be converted back to its original form in excreta (FAO/WHO, 1991). Concentrations of tylosin in swine feed range from 10 to 100 g tylosin/ton feed for growth promotion purposes (Elanco Animal Health Tylan® Premix product label).

Tylosin was listed in the top ten most frequently detected antibiotics in surface water in 1999 to 2000 (Kolpin et al., 2002). Boxall et al. (2003) identified tylosin as a key pharmaceutical of interest in the environment. Several studies have shown that this antibiotic may have an affinity for clay particles and organic matter in soil, as well as the organic components of manure, which could affect its ability to degrade (Rabølle and Spliid, 2000; Kolz et al., 2005). Sorption to soil and manure components may affect its bioavailability. Huang et al. (2001) described tylosin as one of the most likely water contaminants from agricultural runoff. Due to its sorption characteristics, it is believed
that tylosin would be transported with sediment during a runoff event (Davis et al., 2006).

Very few studies have evaluated mobility and degradation in the environment in the presence of a manure substrate (Rabølle and Spliid, 2000; Kay et al., 2004; 2005), however these studies assessed only total tylosin residues, and did not quantify tylosin metabolites. Sorption of chemicals onto solid phases, such as soil, sediment, or manure, is extremely important because it could affect the fate and impact of these substances in that environment. An understanding of the degradation and fate of veterinary antibiotics in soil is important because of widespread use of the compounds in livestock production in the U.S., and the concurrent application of manure to land. Agricultural lands typically contain subsurface tile drain networks, which may drain directly into streams and other surface water bodies. Based on the lack of data regarding the leaching ability of tylosin factors in soil, one objective of the present study was to address these data gaps by quantifying tylosin residues (specifically tylosin A and tylosin D) in leachate from tylosin applied to soil columns in a manure slurry.

Enteric bacteria

Two genera of enteric bacteria were selected for use in the present study. *Escherichia coli* are gram-negative, rod-shaped members of γ-Proteobacteria. *Enterococcus sp.* are gram-positive cocci. Both *E. coli* and enterococcus inhabit the gastrointestinal tract of many mammals, including livestock, and are excreted from the animals and found in manure (Schaechter, 2000; Schleifer and Kilpper-Balz, 1987). Both organisms are potentially pathogenic, and can develop resistance to antibiotics and have the potential to transfer resistance genes to other bacteria (Ochman et al., 2000; Wegener
et al., 1999). These bacteria are also used as fecal indicator species for water quality assessments (Molina, 2005). Because of these characteristics, it is important to understand the survival and mobility of these microorganisms in the environment. Several researchers have reported *E. coli* surviving up to 8 weeks, and enterococcus survival ranging from 35 to >200 d in soils, depending on soil texture, amount and type of manure applied, temperature, and competition with indigenous soil microorganisms (Cools et al., 2001; Lau and Ingham, 2001; Andrews et al., 2004; Entry et al., 2005; Johannessen et al., 2005). A study examining the mobility of enteric bacteria in soil indicated 2-6% of the inoculated enterococcus leached through soil columns; however the bacteria were applied directly to the top of the soil, rather than in a manure slurry (Celico et al., 2004). Soupir et al. (2006) reported enterococcus as being highly mobile in runoff from a simulated heavy rainfall event; different types of manure were tested and counts ranged from 6000-187,000 cfu/100 mL.

As very little information is available on the fate of bacteria excreted in manure once the manure is applied to soil, particularly in the presence of drug residues, another objective of the present study was to determine the survival, movement, and antibiotic resistance of enteric bacteria in undisturbed soil columns.

**Materials and Methods**

*Preliminary trial*

Twenty intact soil cores of Tama series soil were collected from an agricultural field near Grinnell, IA, USA. The field had not received manure application for over 20 years, thereby reducing the likelihood of background contamination of antibiotics in the
present study. The soil was a loam, containing 46% sand, 36% silt, and 18% clay. Soil cores (10-cm diameter x 30-cm depth) were collected using a Giddings soil core apparatus (Giddings Machine Co., Windsor, CO). Soil columns were immediately taken to the lab and were saturated from the bottom with 5mM CaSO$_4$ for 48 hr. Soil columns were placed in shelving units and rested on funnels plugged with glass wool and filled with washed sea sand (Fisher Scientific, Pittsburgh, PA), so that the bottom of the soil column rested firmly on the sand. Soil columns were allowed to drain, and those with drainage times of 24-48 hr were chosen for the experiment and were randomly divided among treatment and control groups.

Fresh hog manure was collected from hogs on an antibiotic-free diet (Iowa State University Swine Nutrition Farm, Ames, IA). Twelve-gram aliquots of manure were prepared and 10 mL ultrapure water was added to each aliquot to make a field-representative manure slurry. The manure slurry was spiked with 60 μg tylosin tartrate in a methanol carrier to reach a tylosin concentration of 5 ppm in manure. Next, $2 \times 10^8$ gfp-labeled ampicillin-resistant E. coli 0157:H7 B6914 were added to the slurry. These organisms were selected for the preliminary trial because of their ease of detection, relevance, and availability. The slurry was then poured onto the surface of the columns. The five untreated (control) columns received 20 mL ultrapure water, equivalent to the moisture addition of the treated columns. After application, the top 2 cm of the columns were raked with a sterilized spatula to simulate the incorporation of manure into soil that would occur during manure application in the field. These methods are similar to those described by Saini et al. (2003).
Sand was wetted with ultra-pure water prior to leaching events. Forty-eight hours after application, a 5-cm “rainfall” in the form of 410 mL 5 mM CaSO\(_4\) was applied to the column drop-wise over 2.5 to 3.5 h. Leachate was collected from the bottom of the columns for 48 h, then immediately analyzed for tylosin and \textit{E. coli} O157:H7 B6914.

The concentration of tylosin in leachate was determined using enzyme-linked immunosorbent assay kits (ImmunoDiagnostic Reagents, San Diego, CA, USA) in which the concentration was correlated to absorbance at 405 nm using a THERMOMax microplate reader with SOFTmax Pro V3.0 software (Molecular Devices, Sunnyvale, CA).

The presence of \textit{gfp}-labeled \textit{E. coli} O157:H7 in leachate was measured using a most-probable number (MPN) technique (IDEXX, Westbrook, ME). Total coliform bacteria were also enumerated using MPN.

The remaining leachate was concentrated using solid-phase extraction cartridges (Waters Oasis® HLB, Milford, MA). Cartridges were conditioned with 5 mL acetonitrile followed by 5 mL of 10% acetonitrile. Following sample retention, cartridges were rinsed three times with 10 mL distilled water and 5 mL 10% acetonitrile. Tylosin was eluted from the cartridges with 2 mL of 98:2 acetonitrile:glacial acetic acid. Extracts were analyzed for total tylosin, tylosin A, and tylosin D using LC/MS/MS with a gradient of ammonium acetate pH 4.0 : acetonitrile over 35 min at 40 °C on a Zorbax SD-C18 4.6 \(\times\) 250mm column (Agilent Technologies, Santa Clara, CA). Mass spectrometry was used for analysis of tylosin factors based on mass, as reference standards for each factor were not available. Mass spectrometry methods were similar to those described by Kolz et al. (2005). The limit of detection was 0.5 \(\mu\)g/L.
Main study

Fifty-six soil columns were collected at the same site previously described. These columns were divided among 7 treatment groups: control, manure only, manure plus Enterococcus, manure plus tylosin, tylosin plus Enterococcus, and manure plus tylosin and Enterococcus. Enterococcus was chosen for the main study because of its greater susceptibility to tylosin compared to E. coli, as one of the objectives of the present study was to evaluate development of resistance by manure-associated bacteria in the soil columns. Similar methods were employed as those previously described, except the saturation and pre-leaching components were not performed and all soil columns were utilized, resulting in 8 replicates per treatment group. Additionally, we increased the concentration of tylosin and the amount of manure applied to better represent real-world applications. Thirty grams of fresh hog manure was spiked with tylosin in an acetone carrier to reach a concentration of 50 μg/g tylosin in the manure.

Both enterococcus (which was inoculated) and E. coli (from manure) were measured in leachate water after each of the four rain events, and results are expressed as cells per 100 mL of leachate water. A few samples were not sufficiently diluted, and thus saturated the MPN panel, resulting in a MPN value that underestimates the true concentration. These values were used in the data analysis. Control columns (no manure) and columns treated with manure and Enterococcus leached no E. coli.

Results and Discussion

Preliminary trial
Following a single rain event, analysis of leachate using LC/MS/MS revealed total tylosin residues up to 2.8 ng/mL, with a mean concentration of 0.8 ng/mL (se=0.3). We found similar results when using immunoassay; 0.6 ng/mL was detected in leachate. When examining specific tylosin factors using LC/MS/MS, we found that tylosin A accounted for approximately 22% of the total tylosin residues; this corresponds to a concentration of 0.2 ng/mL. Tylosin D, another major factor, was detected at 0.5 ng/mL, or 65% of the total residues. This result is quite interesting considering the composition of the tylosin applied to the top of the column; tylosin D only accounted for approximately 10% in the formulation applied. Finding such different proportions in the leachate implies a differential metabolism and/or a differential mobility between tylosin A and D. It is possible that tylosin D is more stable or more mobile than tylosin A. Further studies are needed to elucidate this phenomenon.

Additionally, tylosin D has only 35% of the antibacterial activity as tylosin A, so the differences could have implications for low-level effects on soil microbial communities (Teeter and Meyerhoff, 2003). Each of the other tylosin factors (B and C), and even metabolites, can possess antimicrobial activity, which contributes to a complex situation in soil and water with respect to biological activity. Likewise some analytical methods, e.g., ELISA residue quantification, also are differentially less sensitive to some factors or metabolites and more sensitive to others. The major advantage to the HPLC or LC/MS method is the specificity, but the ELISA method is faster, cheaper, and can detect very low concentrations in aqueous samples (Hu et al., 2006).

Following MPN testing of leachate, total coliform bacteria were highly variable in manure-treated columns, with a range of 1 to 644 CFU/mL in 3 of 5 treatments. One
control column had 1 cell/mL in the leachate, indicating external sources of bacteria, which could include wildlife from the area in which the columns were collected. *E. coli* O157:H7 B6914 were detected in the leachate of 2 of the 5 treated columns; they also ranged from 1-644 cells/mL (the limit of detection for the assay).

Although <0.1% of the gfp-labeled cells and tylosin residues applied to the top of the column were detected in the leachate in this study, these results do indicate the ability of tylosin and some bacteria to move in an agronomic soil.

*Main study*

After four rain events, less than one-third of the treated columns leached detectable amounts of tylosin, with the average concentration at <1 ng/mL in leachate. These results were similar to those found in the preliminary trial.

There were no apparent differences in the *E. coli* leaching from any of the manure treatments, therefore the *E. coli* were averaged over these treatments for each leaching period (Figure 2). *E. coli* (from manure) were detected in all leachates, but the maximum mean concentrations were in the second and third leachates. The decline in *E. coli* concentration seen in the fourth leachate is likely due to decreasing survival in the soil and washout from the column.

*Enterococcus* also leached from the soil columns, but in numbers far exceeding those observed for *E. coli* (Figure 3). In addition, the number of organisms was dependent upon treatment. Soil treated with manure leached no *Enterococcus* in the first rain event, but averaged 5,199 cells/100 mL in the second leaching, then declined to less than 400 cells/100 mL in the third and fourth rain events. The manure plus tylosin (MT)
treatment was similar in magnitude and pattern to the manure-only treatment in the leaching of Enterococcus, suggesting that the tylosin was not active towards the Enterococcus in this soil/manure environment. Enterococcus added to manure (MB) also resulted in bacteria being leached, as did the MTB treatment. Tylosin plus Enterococcus without manure (TB) treatment resulted in much fewer Enterococcus being leached, reaching a maximum average of 30 cells/100 mL at the third leaching. It is possible that the tylosin was more available to inhibit the microbes in this treatment compared to the similar manure-containing treatment (MTB). Enterococcus was detected in leachate from four of 23 untreated soil columns (controls) and in two of 12 leachates from columns treated only with tylosin, indicating minimal input of enterococcus from the soil.

Putative tylosin-resistant Enterococcus were not recovered in the controls or the tylosin treatment (no manure or Enterococcus added), which was expected. Only trace levels (2 cells/100 mL) of tylosin-resistant Enterococcus were recovered in the second leaching from the manure-treated columns. Resistant Enterococcus were recovered in both the first and second leachates from the MB treatment at levels of 1955 and 779 cells/100 mL. These detections were in the absence of exposure to tylosin or other antibiotics, and may be explained by a natural level of resistance in the population. Leaching of tylosin-resistant Enterococcus in the MT and MTB treatments was also observed (3228 cells/100 mL in MT second leaching and 137 cells/100 mL in MTB second leaching), but no tylosin-resistant bacteria were found in leachate from the TB columns. Thus, it can be concluded that tylosin-resistant bacteria were only leached from manure-treated columns, but that there was no obvious effect of tylosin. It is possible those resistant organisms were present in the manure, or that some organisms in the
manure developed resistance to tylosin during the study. The results of the tylosin plus Enterococcus treatment (TB) could be due to the poor survival of Enterococcus in the absence of manure, the effect of tylosin, or both these factors.

The pattern of leaching was the same for both bacteria (E. coli and Enterococcus) over time, with peak concentrations coming from the second and third leachings. Greater concentrations of Enterococcus were seen in column leachates compared to E. coli, particularly at the second leaching. There was no obvious effect of tylosin on the prevalence of tylosin-resistant Enterococcus, but manure treatment resulted in elevated levels of resistant Enterococcus. This could be due to the presence of indigenous tylosin-resistant Enterococcus already present in the swine manure. The movement of both the indicator bacteria and antibiotics is likely due to macropores in this well-structured soil. The transport of these agents illustrates their mobility.

Examination of current literature reveals a small number of comparable studies. Rabølle and Spliid (2000) performed a leaching study with tylosin in packed soil cores of two soil types: a sandy loam and a sandy type. The Kₐ values described for those soils were 128 and 10.8, respectively, and desorption was reported at 13% and 26%. After one “rain” event, approximately 70% of the tylosin was recovered in the top 20 cm of the 48-cm soil columns, and no tylosin was detected in the leachate from either soil type; however it should be noted that the limit of detection reported in the study was 7 μg/L, compared to 0.5 μg/L reported in this study. Using the sorption coefficient data from this study, Tolls (2001) stated tylosin would be low to slightly mobile in most soils, if comparing to pesticide sorption and mobility data; these results are similar to our findings in a loam soil.
Freundlich partition coefficients for tylosin in silty clay loam, sand, and manure ranged from 1000-2000 (Clay et al., 2005); desorption was found to be <0.2% in the same soils. The concentrations used in these batch sorption studies were 23-200 mg/kg, similar to the concentrations of tylosin in the manure applied to our soil columns. These sorption and desorption values may also be useful in a comparison to the conditions in our soil.

A field-scale study performed on tylosin mobility in a clay loam soil determined that up to 6% of applied tylosin was present in runoff water from an agricultural soil (Oswald et al., 2004). The same study also examined the effect of manure on tylosin mobility, and found increased runoff potential of tylosin (up to 23%) in manured treatments; this was likely due to the greatly decreased infiltration of the applied rainfall. Infiltration was reduced by approximately 85% in manured treatments. This information may be important in identifying factors that affected leaching of tylosin in our study.

Finally, Saini et al. (2003) found increased survival of an *E. coli* strain when manure in which they were residing was incorporated into the soil. Additionally, they reported that most bacteria leached from soil columns after the first rain event, and that increased time between application of manure and the rainfall event resulted in decreased leaching of the bacteria, which could be a result of decreased survival. These results are different from our finding of higher numbers of bacteria leaching from the second and third rain events. Saini et al.(2003) reported 3.4-4.5 log CFU/100 mL in the leachate from the rain event 16-d post-application; 1-10% of the applied inoculum was detected in the leachate, regardless of time between application and first rain event (Saini et al., 2003). Additionally, Recorbet et al. (1995) found that survival of bacteria in soil may be
attributed to colonization of clay fractions in soil, which could provide protection from stressors, including environmental contaminants, and that preferential flow in soil columns may be extremely important, which is in agreement with our results.

Conclusions

The goal of the present study was to evaluate the mobility and degradation of tylosin and the mobility of enteric bacteria in undisturbed agronomic soil columns. Results from the present study indicate a low amount of mobility of tylosin in a loam soil, with an average of 0.8 ng/mL total tylosin detected in the leachate from multiple simulated rainfall events. Tylosin D was the predominant factor present in the leachate. Microbiological analysis of the leachate revealed that enteric bacteria were frequently present in the leachate at numbers exceeding the suggested water quality criteria of 126 cells/100mL for E. coli, and 33 cells/100mL for enterococcus (U.S. EPA, 2003). It is likely that preferential flow played a role in the transport of tylosin and bacteria through the soil profile in the intact soil columns.

Numerous monitoring studies have now detected very low residues of antibiotics in surface water. The current study has endeavored to take some first steps toward understanding how the compounds move to surface water, how long they persist in soil, and what transformation processes and products are evident in environmental matrices. Many questions remain unanswered, of which the most intriguing one is “What is the significance of the low concentrations of antibiotic residues in the environment?” Much work is still needed to answer questions regarding the significance of pharmaceuticals in the environment.
Acknowledgements

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References


Figure 1. Chemical structure of tylosin including factors: A ($R_1=CHO$, $R_2=CH_3$); B (TYL A minus mycinose); C ($R_1=CHO$, $R_2=H$); and D ($R_1=CH_2OH$, $R_2=CH_3$).
Figure 2. Average *E. coli* in leachate water from intact soil columns treated with swine manure.
Figure 3. Average *Enterococcus* in leachate water from soil columns treated with swine manure alone (M), manure plus *Enterococcus* (MB), manure plus tylosin (MT), manure plus tylosin and *Enterococcus* (MTB), or tylosin and *Enterococcus* without manure (TB).
Chapter 3. Dissipation of tylosin in surface water

Keri L.D. Henderson¹, Todd A. Phillips¹, Thomas B. Moorman², and Joel R. Coats¹

¹ Department of Entomology, Iowa State University, Ames, IA
² USDA-ARS National Soil Tilth Laboratory, Ames, IA

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Abstract
Tylosin is a veterinary antibiotic commonly used as a livestock feed additive for growth promotion and disease prevention. Tylosin enters the environment via application of manure to soil, and has recently been detected in many surface water bodies at low µg l⁻¹ concentrations. The objective of this study was to determine the fate of tylosin in surface water at environmentally relevant concentrations, and the potential impact of manure and/or vegetation (Ceratophyllum demersum) on its fate. Dilute pond water, containing 7.5 µg l⁻¹ tylosin, was treated with 0.1% manure solution and vegetation, alone and in combination. Dissipation of tylosin was monitored over 24 days using enzyme-linked immunosorbent assay (ELISA). Water receiving manure treatments had significantly lower concentrations of tylosin concentration compared to manure-free treatments beginning at day 4; this decrease could be due to either increased microbial degradation or binding of tylosin to manure organic matter. Vegetation did not have a significant effect on dissipation during the 24-day test period. Despite an initial short dissipation period, tylosin residues persisted beyond 24 days in the surface water microcosms.
Introduction

Livestock producers regularly use antibiotics in animal feed and water for growth promotion and disease prevention. In swine, over 90% of starter feeds, 75% of grower feeds, and 50% of finisher feeds contain antibiotics. Tylosin, a macrolide antibiotic, has been ranked first among antibiotics used in swine production (31.3% of sites surveyed) (Bush and Biehl, 2001), and is also commonly used in cattle and poultry production. Tylosin is used solely in veterinary medicine; it is most active against gram-positive, but also some gram-negative bacteria. Tylosin is produced as a fermentation product by *Streptomyces spp.* (Seno et al., 1977). The tylosin formulation is composed of four factors; tylosin A (TYL A) is most prevalent in formulation at >80%, and is also the most biologically active factor. Tylosin D (TYL D) is 2nd most prevalent at approximately 10%, and tylosins B & C are less prevalent forms (Figure 1). These forms, or metabolites, also have biological activity (Teeter and Meyerhoff, 2003). As with other macrolides, including the human drug erythromycin, its mode of action is inhibition of transcription at 50S ribosomal subunit. Concern has been raised over the potential for cross-resistance between veterinary and human-use antibiotics.

It has been well-documented that antibiotics, including tylosin, are excreted in parent form or as biologically active metabolites in urine and feces; some metabolites may be converted back into parent compound during manure storage, or in the environment (FAO/WHO, 1991). Tylosin enters the environment when livestock manure is applied as fertilizer to agricultural fields. Numerous soil fate studies have been performed, and indicate tylosin has relatively low mobility and strong binding affinity for soil constituents, including clays, cation exchange sites, or manure or hydrophobic...
components of soil organic matter; these studies also indicate that tylosin may also persist for several weeks or months in soils (Rabølle and Spliid, 2000; Clay et al., 2005; Kay et al., 2005; Kolz et al., 2005; Henderson et al., 2007; Hu and Coats, 2007; Sassman et al., 2007). Despite its affinity for soil components and low mobility, water monitoring studies have shown tylosin has the ability to reach water bodies. Tylosin was one of the top five most frequently detected veterinary and human antibiotics in surface water (Kolpin et al., 2002). Kolpin and colleagues detected concentrations of tylosin in surface water bodies at <1 µg l⁻¹; however, very little is known about the bioavailability and potential significance of low levels of antibiotics in aquatic ecosystems. There is great need for investigation of environmental fate of veterinary antibiotics, such as tylosin, because of their high usage rates and concern over microbial resistance seen in human health.

The objectives of this study were to determine the fate of environmentally relevant levels of tylosin in surface water, and to evaluate the potential impact of manure and vegetation inputs on tylosin’s dissipation.

**Methods**

*Collection of surface water, vegetation, and manure*

Pond water (surface 15 cm) was collected from the Iowa State University Horticulture Farm Pond, Ames, IA, USA. The pH of the pond water was 8.1, the alkalinity was 103 mg ml⁻¹, and the total hardness was 150 mg ml⁻¹. *Ceratophyllum demersum* (coontail) was selected for this study because of its previously established ability to remediate agrichemical contamination in surface water (Rice et al., 1997).
Plants were collected by removing two randomly selected plant masses from the same area of the pond where surface water was collected (depth = 1 m).

Fresh manure was obtained from adult female hogs on a corn-based, antibiotic-free diet (>20 days withdrawal) from the Iowa State University Swine Nutrition Facility (Ames, IA). Pond water and manure were brought back to the laboratory and refrigerated at 4°C until use (<7 days). Plants were maintained at 25°C, with a 16:8 photoperiod for one day before trimming and placement in treatment containers.

_Treatment preparation_

Bristol’s algal media was used to provide a nitrogen-containing nutritive source for plants and microflora in the test systems. Bristol’s media was prepared as follows: 250 mg NaNO₃, 75 mg K₂HPO₄, 175 mg KH₂PO₄, 25 mg CaCl₂, 25 mg NaCl, 75 mg MgSO₄·7H₂O, 0.3 mg FeCl₃, 0.3 mg MnSO₄·4H₂O, 0.2 mg ZnSO₄·7H₂O, 0.2 mg H₃BO₃, and 0.06 mg CuSO₄·5H₂O in 1 l ultrapure water (pH 6.8; Millipore Corp., Billerica, MA).

Test systems were 250-ml French square glass jars, and were prepared as listed in Table 1. There were five replicate jars per treatment. The pH of the test systems was measured at day 0, and found to be 6.9; pH was monitored at each timepoint, and no significant change in pH was noted at any point during the 24-d study.

Treatments with vegetation were prepared by trimming 2.32 g (sd=0.07) wet weight of freshly collected coontail (*Ceratophyllum demersum*). Each 2.3-g section of plant material was carefully added to the appropriate treatment systems. Water displacement by the plant material was <10 ml.
A 33% manure slurry (w/v) was prepared by weighing 33 g fresh solid hog manure (22% dry wt) into 100 ml ultrapure water. The slurry was stirred vigorously on a stir plate for 1 h until a smooth slurry consistency was achieved. Each manure treatment jar received 0.454 ml of 33% slurry to achieve 0.1% manure treatment solution by mass.

After a 72-hr acclimation period, solutions were spiked to 7.5 µg l\(^{-1}\) tylosin by adding 0.375 ml of a 3 mg l\(^{-1}\) tylosin tartrate stock solution prepared in ultrapure water (Sigma-Aldrich, St. Louis, MO, USA).

Test systems were maintained at 25°C and 16:8 light:dark using fluorescent lamps specific for plant growth (Sylvania Gro-Lux®, Danvers, MA), and were covered loosely with clear plastic wrap to decrease evaporation, but still allow light penetration. Solution levels were checked daily and were maintained at 150 ml throughout the 24-day testing period. The length of the test period was chosen because a previous surface water microcosm study indicated a half-life of tylosin of 9-10 days (Brain et al., 2005).

**Tylosin residue quantification using enzyme immunoassay**

Tylosin residues were measured at 0, 4, 8, 12, 16, and 24 days in a repeated measures design. Jars were gently swirled prior to sampling to allow mixing. Triplicate sub-samples of 25 µl each were removed at each timepoint and immediately pipetted onto the 96-well enzyme immunoassay plate specific for tylosin (ImmunoDiagnostic Reagents, Vista, CA). Error between triplicate samples was monitored and was consistently less than between-replicate variability. Samples were re-run if >20% coefficient of variance was observed. A standard curve was prepared (0.5, 1.0, 2.5, 5.0, 10.0, and 50.0 µg l\(^{-1}\)) at each timepoint, and concentration of total tylosin residues was
correlated to absorbance at 405 nm using a THERMOMax microplate reader with SOFTmax Pro V3.0 software (Molecular Devices; Sunnyvale, CA).

Statistical analysis

Statistical analysis of the data included repeated measures testing using a mixed-model approach with the Kenward-Roger method for adjustment of degrees of freedom using SAS V8.0 (SAS Institute, Cary, NC; Kowalchuk et al., 2004).

Results and Discussion

The tylosin enzyme immunoassay used in this study measures all four tylosin factors (A-D) and two tylosin degradation products (isotylosin A alcohol and aldol) (Hu et al., 2008). Standard curves were prepared using tylosin tartrate, which contains each of the four tylosin factors, with tylosin A making up >80% of the formulation. The standard curves were linear between 0.5 and 50 µg l$^{-1}$ ($r^2$>0.97). Pond water and manure blanks were also analyzed at each timepoint; slight matrix effects were noted for 0.1% manure blanks, which registered a tylosin background concentration of 0.025 µg l$^{-1}$. However, these effects were below the limit of detection of the immunoassay (0.1 µg l$^{-1}$), so they were not considered relevant in our assessment.

All treatments began at the same initial concentration of 7.5 µg l$^{-1}$ (s=1; no significant differences at day 0; p>0.05), however variability was observed, and was attributed to incomplete diffusion of the tylosin spiking solution throughout the solutions at T=0. Treatments that included manure (manure only, and manure + vegetation) had significantly lower concentrations of tylosin detectable beginning at day 4 (p<0.001),
with the manure only treatment mean at 2.3 µg l$^{-1}$ (s=0.5), and the manure + vegetation treatment at 3.1 µg l$^{-1}$ (s=0.7). The non-manured treatments and control means were not significantly different, and their mean was 5.6 µg l$^{-1}$ (day 4-24). Vegetation did not appear to have an effect on tylosin dissipation during the 24-day study (two-sided p-value = 0.0809; Figure 2).

The means for the manured treatments did not change at day 8; however concentrations appeared to increase slightly over the remainder of the experiment. This phenomenon may be explained by the cross-reactivity of several tylosin metabolites recently described by Hu and colleagues (2008). It was noted that tylosin D and a tylosin photopродuct, isotylosin A alcohol, had stronger specificity for antibody recognition in the immunoassay than tylosin A (Hu et al., 2008). In the present study, it is possible that tylosin A was metabolized to tylosin D, or a photopродuct such as isotylosin A alcohol may have formed during the course of the present study, as noted by the increase in concentration detected at day 12 of the study (Figure 2). The immunoassay is a competitive binding assay, therefore tylosin residues are competing with enzyme conjugate for antibody sites. If a metabolite has stronger affinity for an antibody than the conjugate, it is possible that an increase in concentration would be noted. Alternatively, it is possible that a change in manure (i.e. via degradation) may occur over time, which could allow for desorption of tylosin residues from the matrix, thus corresponding to the noted increase in concentration around day 12 of the study.

The rapid initial decline in concentration noted in manure-containing treatments at day 4 is likely due to binding of tylosin residues to particulate matter, or to microbial degradation of tylosin, however previous studies have indicated a significant lag time (3
weeks) in initiation of microbial degradation of tylosin (Sassman et al., 2007). Treatments that included manure had significantly increased dissipation of tylosin during the test period. One possible explanation for this observation is that tylosin sorbed to the particulate matter in the manure-treated systems. This explanation seems most plausible when considering the $K_{oc}$ of tylosin is 500-8000. Sassman and colleagues (2007) reported sorption as the main mechanism for dissipation of tylosin in 7 different soil types. Several other studies have reported similar tylosin sorption characteristics in soil matrices (Rabølle and Spliid, 2000; Clay et al., 2005; Hu and Coats, 2007).

In a study of tylosin sorption to manure, Kolz and colleagues (2005) determined sorption coefficients for tylosin on manure slurry at environmentally relevant concentrations of 1 to 30 mg l$^{-1}$; they found that tylosin was strongly sorbed to manure with $K_{oc}$ values from 570 to 818 l kg$^{-1}$, similar to those described by Loke and coworkers (2002). Although the manure contribution of organic carbon in the present study was likely <0.05% OC in the pond water solution (based on 42% organic carbon in manure; Loke et al., 2002), it is possible that enough organic surface area was added to allow for tylosin binding. To determine the impact of binding in the present study, the mean concentration for the manured treatments at equilibrium was calculated (days 4-24; mean= 3.72). If it is assumed that dissipation was based solely on binding, then nearly 50% of the tylosin in the system would have to be bound to manure particles. This result corresponds to a $K_{oc}$ of 2419 l kg$^{-1}$, which is three-fold higher than that reported by Kolz and colleagues (2005), but still within the $K_{oc}$ range reported in soil by Rabølle and Spliid (2000); it seems that binding to manure-derived organic carbon may be an important mechanism involved in tylosin dissipation in the present study.
A few studies are available on tylosin dissipation in water, however most of these studies used artificial test solutions such as reagent water or buffers, and/or used significantly higher concentrations. Loftin and colleagues (2008) examined the effect of temperature, pH, and ionic strength on tylosin degradation in sterile buffer systems. No degradation was observed in environmentally relevant ranges of pH (pH 5 to 9) during the 3-week study; neither temperature nor ionic strength had any significant impact. It was concluded that hydrolysis is not the main mechanism for tylosin degradation (Loftin et al., 2008); it is important to note that the study was performed in amber containers, thus minimizing any potential photodegradation, which has been established as an important degradation mechanism (Brain et al., 2005; Hu and Coats, 2007).

In another aquatic study, Brain and colleagues (2005) examined the effect of tylosin on macrophytes in a mesocosm setting, and dissipation of tylosin was also monitored during the study for the 0.6 to 3 mg l\(^{-1}\) exposure concentrations. Pseudo-first order dissipation was noted during the first three weeks of the study, and half-lives were calculated to be 9-10 d. However, this relatively rapid decline was followed by a second phase with a slower rate of dissipation, which was thought to be related to low biodegradability and lack of mineralization of tylosin. The DT\(_{50}\) calculated by Brain and colleagues only reflects the loss of the first 50% of tylosin in their system; the biphasic nature of tylosin kinetics in their system is apparent, and indicates the potential for part-per-billion concentrations of tylosin to persist for long periods of time, similar to the results presented in the current study. Additionally, model-based predictions indicated photodegradation as the primary mechanism for tylosin degradation. The authors also
mention that tylosin may have partitioned into the sediment and later desorbed during the course of the study, though no sediment monitoring was performed (Brain et al., 2005).

The present study is among the first to examine the fate of tylosin at environmentally relevant concentrations using surface water microcosms containing manure inputs and vegetation. The vegetation used in this study did not significantly remediate tylosin concentrations, and no overt toxicity of tylosin to the plants was noted. In the presence of manure-derived organic carbon, tylosin residues rapidly declined, but then persisted and even increased over the 24-day period, indicating the potential for desorption of tylosin residues from these matrices. Additionally, the analyses using immunoassay may also measure tylosin-related compounds, including metabolites (Hu et al., 2008); however considering the known biological activity of tylosin metabolites, enzyme immunoassay remains an appropriate tool for measuring toxicologically relevant tylosin residues in environmental matrices. The results presented here indicate potential persistence of tylosin residues at even low µg l⁻¹ concentrations, which implies that even low levels of antibiotics detected in monitoring studies may have significance due to their persistence over time, and unknown potential biological significance.

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References


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<th>Treatment name</th>
<th>Preparation (150 ml)</th>
<th>Purpose</th>
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<td>Bristol’s control</td>
<td>2:4 Bristol’s algal medium:ultrapure water</td>
<td>Abiotic control</td>
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<tr>
<td>Pond water control</td>
<td>1:1:4 Bristol’s:pond water:ultrapure water</td>
<td>Impact of pond water (biota and dissolved OC) on TYL fate</td>
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<td>Manure</td>
<td>0.1 % w/v fresh hog manure slurry in 1:1:4 Bristol’s:pond water:ultrapure water</td>
<td>Impact of manure and increased organic matter on TYL fate</td>
</tr>
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<td>Vegetation</td>
<td>2.3 g fresh <em>Ceratophyllum demersum</em> in 1:1:4 Bristol’s:pond water:ultrapure water</td>
<td>Effect of vegetation on TYL fate</td>
</tr>
<tr>
<td>Manure + Vegetation</td>
<td>0.1% manure and 2.3 g <em>Ceratophyllum demersum</em> in 1:1:4 Bristol’s:pond water:ultrapure water</td>
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Figure 1. Chemical structure of tylosin including factors: A ($R_1$=CHO, $R_2$=CH$_3$); B (TYL A minus mycinose); C ($R_1$=CHO, $R_2$=H); and D ($R_1$=CH$_2$OH, $R_2$=CH$_3$).
Figure 2. Dissipation of tylosin residues in surface water microcosms. Data points are means for treatments ± standard error.
Chapter 4. Fate of sulfamethazine in a freshwater sediment microcosm

Keri L.D. Henderson¹, Thomas B. Moorman², and Joel R. Coats¹

¹ Department of Entomology, Iowa State University, Ames, IA
² USDA-ARS National Soil Tilth Laboratory, Ames, IA

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Abstract

The antibiotic sulfamethazine can be transported from manured fields to surface water bodies. We investigated the degradation and fate of sulfamethazine in surface water using ¹⁴C-phenyl-sulfamethazine in small pond water microcosms containing intact sediment and pond water. We found a 2.7-d half-life in pond water and 4.2-d half-life when sulfamethazine was added to the water with a dilute manure solution. Sulfamethazine dissipated exponentially from the water column, with the majority of loss occurring via movement into the sediment phase. Parent sulfamethazine in sediment accounted for 10-15% of the applied antibiotic within 14 days, and then declined thereafter. Sulfamethazine was transformed mainly into non-extractable sediment-bound residue (40 to 60% of applied radioactivity) and smaller amounts of photoproducts. Biodegradation, as indicated by metabolite formation and ¹⁴CO₂ evolution, was less significant than photodegradation. Two photoproducts were detected, and accounted for 15-30% of radioactivity in the water column at the end of the 63-day study; the photoproducts were the major metabolites in the aqueous and sediment phases. Other unidentified
metabolites individually accounted for <7% of radioactivity in the water or sediment. Less than 3% of applied radioactivity was mineralized to $^{14}\text{C}O_2$. Manure input significantly increased sorption and binding of sulfamethazine residues to the sediment. These results indicate sediment is a potential sink for sulfamethazine and sulfamethazine-related residues; this fate may have important implications for benthic organisms.

Introduction

Recently, veterinary antibiotics were detected in 48% of 139 stream waters tested in 30 states, according to the United States Geological Survey (Kolpin et al., 2002). Recent sediment monitoring studies by Kim and Carlson (2007a and b) detected extractable antibiotic residues up to 0.1 mg/kg in sediment from an impacted river; typical mean concentrations were 0.001 to 0.03 mg/kg. Antibiotics entering the environment could potentially alter bacterial populations and their activity in soil, sediment, and water, thus affecting biodegradation, nutrient cycling, and water quality. In addition to concerns over changes in microbial resistance, there are concerns over direct and indirect effects on non-target eukaryotic organisms in important habitats found across agricultural landscapes. In particular, small ponds and wetlands that serve as key breeding sites for amphibians and support invertebrate communities can receive significant amounts of contaminated agricultural runoff, which could contain antibiotic residues (Davis et al., 2006; Lahr, 1997). It is believed that these drugs may have an affinity for clay particles in soil or sediment, which could affect their availability for degradation; this is also likely important for bioavailability and uptake for higher organisms (Rabølle and Spliid, 2000). An understanding of the degradation and fate in
small ponds is important because of widespread use of the compounds in livestock production in the U.S., concurrent application of manure to agricultural lands dotted with small ponds and wetlands, and detection of the compounds in surface waters throughout the world (Managaki et al., 2007; Kim and Carlson, 2007; Kolpin et al., 2002).

The sulfonamides are a class of antibiotics frequently detected in the aforementioned monitoring studies. These drugs have been used extensively in human and veterinary medicine applications for decades. The sulfonamides are mostly active against gram-positive, but also some gram-negative bacteria; their mode of action is prevention of folic acid synthesis in bacteria by acting as a structural analog of p-aminobenzoic acid. Sulfamethazine was chosen as a representative sulfonamide for the present study because of its wide use in livestock production for growth promotion purposes, and because of its frequent detection in aquatic systems (Kolpin et al., 2002; Batt et al., 2006; Kim and Carlson, 2007; Managaki et al., 2007). Sulfamethazine (Figure 1), as with other sulfonamides, is bacteriostatic. Sulfamethazine (SMZ) is used in cattle and swine production as a therapeutic and as a growth promoter. The recommended dose of sulfamethazine in feed is 110 mg/kg in feed for growth promotion in swine (Elanco Animal Health Tylan® Sulfa-G product label). This antibiotic has been found in concentrations exceeding 100 μg/L in swine lagoon water, and in lower concentrations in tile drainage water and stream water (Lindsey et al., 2001; Campagnolo et al., 2002). Concentrations can exceed 5 mg/kg in swine manure (Haller et al., 2002).

Sulfamethazine has a log \( K_{ow} \) of 0.89, is soluble in water (S=1.5 g/l), and is capable of ionization (pK\(_{a1}\) 2.65, pK\(_{a2}\) 7.65; Tolls, 2001). Previous soil fate studies have indicated that SMZ is not strongly sorbed to soils and is likely to be highly mobile in the
aqueous portion of runoff, thus being likely to reach surface water bodies, e.g. small ponds (Davis et al., 2006; Boxall et al., 2002; Thiele-Bruhn et al., 2004; Coats et al., 1976). Though its fate has been extensively examined in soil, SMZ fate in surface water, and sediment in particular, has not been extensively studied. The objective of the present study was to investigate the fate of sulfamethazine in microcosms simulating pond water systems. Specific objectives were to determine the effects of sediment and swine manure inputs on persistence of SMZ in a surface water system, and to evaluate potential mechanisms of SMZ dissipation in surface water, including sorption to sediment and degradation processes.

**Materials and Methods**

*Chemicals*

$^{14}$C-U-phenyl-sulfamethazine and non-labeled analytical grade sulfamethazine (SMZ) were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, HCl, and NaOH were purchased from Fisher Scientific (Waltham, MA).

*Matrix collection*

Pond water (surface 20 cm) and sediment were collected from the Iowa State University Horticulture Research Station pond (Gilbert, IA); 10-15 cm (depth) sediment samples were manually collected using a soil auger. Sediment and water were transported to the laboratory and stored in the dark at 4°C. Prior to use, sediment was thoroughly mixed, and moisture content was determined to be 46.2%. The sediment was a sandy loam (60% sand, 28% silt, 12% clay) with 2.0% organic matter, and a pH of 8.1.
The pH of the pond water was 8.1, the alkalinity was 103 mg/ml, and the total hardness was 150 mg/ml.

Fresh manure was obtained from adult female hogs on a corn-based, antibiotic-free diet (>20 days withdrawal) from the Iowa State University Swine Nutrition Facility (Ames, IA). Manure was refrigerated at 4°C until use (< 7 days).

**Treatment preparation**

Seventy-three grams of sediment (50 g dry wt) was measured into wide-mouth pint jars (Ball Corp., Broomfield, CO), and topped with 177 ml pond water, to equal 200 ml water per jar. Each jar served as a replicate. There were 4 replicates at each timepoint per treatment group. The experimental design is listed in Table 1. For the autoclaved pond water and sediment treatment (autoclaved PWS), four 1L-samples of pond water were autoclaved for 20 min each, and sediment was autoclaved in 1 hr cycles three times over the course of 1 week, with a day of rest between each cycle.

Sediment was allowed to settle 1 hr prior to treatment with $^{14}$C-SMZ solution. Stock solutions of labeled and non-labeled sulfamethazine were prepared to make a final treatment solution to be added to each microcosm. Eighty-five milligrams of non-labeled sulfamethazine were dissolved in 100 ml of ultrapure water, and 0.188 ml of a 90.47 $\mu$Ci/ml $^{14}$C-SMZ (in methanol) solution was added; the solution was then diluted with methanol and ultrapure water to a final volume of 200 ml in 10% methanol. The final treatment solution was 0.425 mg/ml and 0.085 $\mu$Ci/ml. Each replicate jar received 2.35 ml treatment solution, so the final concentration of SMZ in pond water was 5 mg/l, and
0.2 µCi/jar. After treatment solution was added, water was gently stirred with a sterile spatula to allow for mixing without disturbing the sediment.

For the manure treatment (PWS+M), a manure slurry was added using a syringe, to obtain 0.1% manure in pond water. This was enough manure to make the pond water slightly murky in appearance. The slurry consisted of a 33% w/v solution of fresh manure from adult hogs on an antibiotic-free diet (33 g wet wt (22% dry mass) in 100 ml distilled water). Slurry was stirred for 40 min to break up large chunks of manure, and 0.6 ml of 33% slurry was added to each replicate. For the autoclaved PWS treatment (sterile system), all work was completed using sterilize equipment in a laminar flow hood. Microcosms were maintained in environmental chambers at 22ºC in a 12:12 photoperiod for the 63-day period; pH was monitored weekly in the surface water of the microcosms, and did not significantly change during the test period.

Mineralization

To track mineralization of $^{14}$C-SMZ added to the microcosms, NaOH traps for CO$_2$ were included in the systems. Traps consisted of a 25-ml high-density polyethylene vial glued to the inner surface of the jar, and filled with 10 ml 0.5M NaOH. Traps were changed at Day 3, 7, 14, 21, 28, 35, 42, 49, and 56; at each trap timepoint, 3 ml of NaOH solution were mixed with 12 ml Ultima Gold XR cocktail and counted for radioactivity using a Packard Tri-Carb 1900 (Perkin-Elmer, Waltham, MA).

Water extraction
Replicates were sacrificed at the specified timepoints (Table 1). On Day 7, for example, pond water was siphoned from the top of the sediment and a 2-ml aliquot was mixed with 12 ml Ultima Gold XR cocktail and counted for radioactivity. The remaining water was filtered through glass fiber filters and counted again to determine any loss of radiolabel during filtering; minimal loss occurred (<2%). Water was stored in amber jars at 4°C until extracted using HLB solid phase extraction cartridges (6 cc, Oasis HLB®, Waters Corporation, Milford, MA); cartridges were conditioned with 3 ml each of methanol, 0.5N HCl, and ultrapure water prior to sample loading. After water samples were passed through, cartridges were washed with 3 ml ultrapure water, and eluted with 3 ml methanol. Eluates were brought to a final volume of 5.0 ml using ultrapure water, and stored in a freezer until analysis. Efficiency of the solid phase extraction technique was >95%.

Sediment extraction

Sediment was transferred to a Teflon centrifuge bottle using 100 ml of a 70% methanol solution. The bottles were then shaken 80 min and allowed to settle overnight at room temperature. Bottles were then balanced and centrifuged at 3000-3500 rpm for 15 min, then decanted into preweighed 500 ml amber jars. A second 100-ml aliquot of 70% methanol was added to each centrifuge bottle, and shaken for 15 min. The bottles were again centrifuged and decanted. The amber jars were weighed to estimate the amount of extract. Extracts were then refrigerated until they could be concentrated by evaporation under nitrogen flow (15 psi, 40°C), and enriched using the SPE method previously described.
Extracted sediments were dried in a fume hood overnight. Next, each replicate was manually processed to remove rocks and debris larger than 5 mm, and ground using mortar and pestle to ensure thorough mixing. To determine the amount or radioactivity remaining bound in the sediment following extraction, 0.5-gram aliquots were combusted using an OX-600 biological oxidizer (RJ Harvey Instrument Co., Hillsdale, NJ), using a 4-min combustion cycle. The $^{14}$C-CO$_2$ produced by combustion was trapped and radioactivity was determined by LSC as previously described.

HPLC

A reverse-phase high-performance liquid chromatography (HPLC) method was used to quantify parent sulfamethazine and to identify related metabolites. Analysis of the samples was performed using a Hewlett-Packard (Palo Alto, CA, USA) series 1100 HPLC system with a quaternary pump, an autosampler, a thermostatted column compartment, and UV and β-RAM detectors. A Waters Atlantis$^{TM}$ (Milford, MA, USA) dC18 column (4.6 × 250 mm, 5-μm particle size) was used. Detection was conducted at 254 nm, with a flow rate of 1.0 ml/min at 30º C, and 200µl injection volume. The mobile phase consisted of 30% methanol. Radiolabeled and non-labeled standards were used to quantify parent sulfamethazine in samples. To ensure consistency, radiolabeled SMZ standards were run at the beginning and end of each set of samples. Data were collected and analyzed using HP Chemstation system software (REV. A.04.01) A Beta-RAM radiodetector (IN/US Systems, Tampa, FL) was also used to detect radioactivity in the sample, using a 30-second residence time and IN-FLOW® cocktail in a 1:1 ratio and LauraLite 3$^{®}$ software for integration (IN/US Systems).
Statistical analysis

Statistical analysis of the data included general linear models and least squares means to assess differences among treatments and timepoints for the various endpoints (e.g. % bound, SMZ concentration in sediment, % mineralized, etc.), with the Tukey method for adjustment of multiple comparisons using SAS V9.1 (SAS Institute, Cary, NC).

Exponential decay models were also used to describe SMZ dissipation from the pond water, and are outlined in Table 2. First-order exponential decay models fit the data for the PWS, autoclaved PWS, and PW treatments (Equation 1).

Equation 1: \( C_t = C_0 e^{-kt} \)

where \( C_t \) is concentration remaining in pond water at time \( t \), \( C_0 \) is the initial concentration of SMZ in the pond water, \( t \) is days after spiking, and \( k \) is the rate constant.

An exponential decay model with two compartments (fast and slow) fit the data for the PWS+M treatment (Equation 2).

Equation 2: \( C_t = C_1 e^{-k_1 t} + C_2 e^{-k_2 t} \)

where \( C_1 + C_2 = C_0 \) and \( k_1 \) and \( k_2 \) are rate constants for the fast and slow dissipation pools, respectively.

First-order single-compartment accumulation models were used to estimate total bound residues (% of applied) at equilibrium (\( P_{beq} \)) from the measured percent bound at time \( t, (P_{m,t}) \), where \( \alpha \) is the accumulation rate constant (Equation 3).

Equation 3: \( P_m = P_{beq} (1 - e^{-\alpha t}) \)
Partition coefficients ($K_d$s and $K_{oc}s$) were calculated from the data on SMZ concentration in water and sediment at day 63. Day 63 was chosen because it is closest to equilibrium for bound residues within the system. $K_d$ (l/kg) was calculated as shown in Equation 4

Equation 4: $K_d = C_s/C_w$

where $C_s$ is the concentration of SMZ in sediment (mg/kg), as determined through extraction, and $C_w$ is the concentration of SMZ in the pond water (mg/l).

$K_{oc}$ was calculated by standardizing to the organic carbon fraction of the sediment matrix, $f_{oc}$ (2% OC for PWS and autoclaved PWS, 2.16% OC for PWS+M; see equation 5).

Equation 5: $K_{oc} = K_d/f_{oc}$

Analyses and plots were created using SigmaPlot 10.0 (SyStat Software, Inc., San Jose, CA).

Results and Discussion

Mass balance

Mean $^{14}$C balances for pond water (PW) systems were >90% for each timepoint. All sediment containing systems (PWS, PWS+M, and Autoclaved PWS) exceeded 95%, with the exception of the PWS+M treatment at day 63, with a mean $^{14}$C mass balance of 84±2%. Table 3 displays the distribution of $^{14}$C in pond water and sediment at day 7 and
day 63. Clear differences exist between day 7 and day 63 for sediment binding and amount of radiolabel remaining in water (p<0.001 for all treatments).

* **Dissipation kinetics**

Parent sulfamethazine dissipated from surface water rather rapidly in all treatments, with the most rapid loss occurring in the sediment-containing microcosms. The slowest dissipation occurred in the autoclaved sediment-water treatment. The PWS+M treatments followed a two-compartment exponential decay model, while the PWS, autoclaved PWS, and PW only treatments followed a single exponential decay model (Figure 2). Table 2 describes the decay equations and statistics for the exponential models. The input of manure organic matter into the system appears to add a second phase of dissipation to sulfamethazine decay; this may be due to sorption-desorption cycling, alteration of indigenous microbial communities, or decreased availability of SMZ residues for photo- or biodegradation.

* **Sulfamethazine residues in sediment**

Sulfamethazine moved from the water column into the sediment within the first 14 days of the study. Parent SMZ detected in the sediment peaked at 7-14 days, and then showed a slow decline, which corresponded with an increase in bound residue detected in all sediment-containing treatments. Figure 3 displays sediment residues.

$^{14}$C-bound (unextractable) residues increased exponentially, and appeared to plateau toward the end of the 63-day study. First-order rate constants for accumulation of bound residues were 0.052, 0.067, and 0.055 and equilibrium was 40.5, 61.7, and 39.2%
of applied $^{14}$C as bound residues for the PWS, PWS+M, and autoclaved PWS treatments, respectively. In the manure-containing treatment, >60% of applied radioactivity was bound to sediment at the end of the study, nearly twice the amount of binding as the other two treatments. Beginning at day 7, PWS+M had significantly more bound residues than PWS or the autoclaved treatment; mean differences were 8.6 and 9.6% of applied radioactivity ($p=0.0428$ and 0.0161, respectively). At all other timepoints, PWS+M had significantly more bound residues than PWS or the autoclaved treatment ($p<0.0001$), ending with 61.2% bound residue at day 63 (95% CI 57.7, 64.7) (Fig. 3). A separate batch desorption study was performed with bound residues, and <1% of bound residues desorbed (data not shown), demonstrating the strong degree of binding of these residues in the sediment. Similar bound/unextractable residue amounts have been reported for sulfonamides in soil (Heise et al., 2006).

Although the manure contribution of organic carbon in the present study was <8% of the total OC in the test system (based on 42% organic carbon in manure; Loke et al., 2002), it is possible that enough organic surface area was added to allow for increased binding. $K_d$s and $K_{oc}$s were calculated for each treatment based on mean concentration of parent SMZ in water and extracted from sediment at day 63, 2% OC in sediment, and 42% OC in manure, corresponding to an addition of 0.08 g OC in the manure-containing test systems. It was assumed that the fraction of SMZ in the sediment pore water would be minimal compared to the adsorbed fraction. For the PWS systems, $K_d$ was calculated to be 1.68 and $K_{oc}$ was 83.8 l/kg, for PWS+M 2.87 l/kg and 132.98 l/kg, and for the autoclaved PWS, the $K_d$ was calculated to be 1.09 l/kg and $K_{oc}$ was 54.7 l/kg. In a study of the effect of hog manure slurry on sulfonamide sorption in soil, Thiele-Bruhn and Aust
(2004) found an increase in the nondesorbable (bound) fraction for SMZ in systems with hog manure input; however, this involved a much higher amount of manure input into the system to achieve increased binding compared to the amount of manure used in the present study. At lower concentrations of manure in soil, they saw a decrease in sorption compared to non-manured treatments, which is not in agreement with what was observed in our sediment microcosms. Importantly, Thiele-Bruhn and Aust also found that adsorption to 100% manure slurry was significantly greater compared to soil sorption; it is possible that SMZ sorbed to manure in the pond water phase in our study, and then settled onto the sediment. We did not differentiate between manure and sediment in our extractions and calculations.

The amount of binding observed in this study is relatively consistent with $K_d$ values previously reported ($K_{oc}$ 48.2 l/kg, Sukul and Spiteller, 2006; $K_d$ 0.9-3.1 l/kg, Davis et al., 2006; Thiele-Bruhn et al., 2004; Boxall et al., 2002; Tolls, 2001). However, Gao and Pedersen (2005) examined sulfonamide sorption to clays and found $K_d$ of 12 for SMZ at neutral pH in montmorillonite clays, while protonated species had $K_d$ values two orders of magnitude higher than the neutral SMZ (e.g. 2620-4270). Gao and Pedersen also reported that cation exchange appeared to be a main sorption mechanism for protonated SMZ species; however their experimental conditions involved testing sorption to clays only, and did not include whole sediment and organic matter, as in the present study. Boxall and colleagues (2002) found similar pH-dependent sorption, further indicating the importance of pH and $pK_a$ in the fate of sulfonamide antibiotics. Results from these studies indicate that association with organic matter is likely not the only mechanism for sulfonamide sorption. Alternatively, Thiele-Bruhn and colleagues
(2004) have suggested that some sulfonamide sorption might also be related to cation exchange or bridging, surface complexes, or hydrogen bonding. Though these forces were in play for several sulfonamide antibiotics in their study, SMZ adsorption was predicted to be based on diffusion into soil organic matter voids. Bialk and colleagues (2005) also report that bound residues may result from possible covalent binding of SMZ or SMZ degradation products with humic substances present in soil; these mechanisms are also likely applicable to sediments. Our results showing increased binding in the treatment with highest organic carbon content (PWS+M) are in agreement with both Bialk et al. (2005) and Thiel-Bruhn et al. (2004); however, at pH 8.1 in pond water and sediment, a significant portion of SMZ is likely to be ionized, and some of the alternative sorption mechanisms may come into play.

Current monitoring data reveal that sulfonamides are frequently and widely detected in the water column and in sediments of surface water bodies throughout the world; sources of these residues include human and livestock origins. Ingerslev and Halling-Sørensen (2000) indicated that sulfonamides in activated sludge may be persistent enough to pass through sewage treatment plants and enter receiving waters. Managaki and colleagues (2007) reported SMZ concentrations in surface waters approaching 20 \( \mu \text{g/l} \) in Vietnamese agricultural regions. Although detection of sulfonamide residues in the water column is important, sediment may serve as a sink for these residues. For example, in a sediment monitoring study, Kim and Carlson (2007) detected SMZ in 25% of river sediment samples, with mean of 4.7 \( \mu \text{g/kg} \) and maximum concentration detected of 13.7 \( \mu \text{g/kg} \); overlying water samples from that study were typically <0.1 \( \mu \text{g/l} \), pointing to the significance of sediment in the fate of SMZ in aquatic
ecosystems. The sediment sorption data presented here further support Kim and Carlson’s work relating sediment as a potential sink for SMZ residues; our results indicated that SMZ or metabolite residues may be transported from the aqueous phase into the sediment via diffusion, residues become adsorbed, and a fraction of those residues become bound over time. Heise and coworkers (2006) report similar sulfonamide affinity for soil, with non-extractable residues exceeding 90% in their study. Thiele-Bruhn and colleagues (2004) suggest that SMZ is likely adsorbed or bound to non-humified organic matter in soil, and that the SMZ residues might desorb or become bioavailable during humification or mineralization of the non-humified fraction. The potential implications of sediment-associated residues for benthic-dwelling organisms need to be further explored.

Degradation

Chromatographic separation of filtered water samples revealed unidentified metabolites with retention times of 5.3 and 7.6 minutes, detected using a diode array detector at 254 nm. An Agilent 1100 HPLC with a 0.7 ml/min flow rate through a Zorbax SB-C18 column (60 °C; 4.6 x 250 mm, 5µm; Agilent Technologies, Inc., Santa Clara, CA) was used for separation. The mobile phase and timetable are described in Table 4. Fractions corresponding to the retention times were collected and counted for radioactivity; presence of radiolabel indicates that at least a portion of the phenyl ring of SMZ is present in these metabolites. Interestingly, neither compound was retained on the Oasis HLB cartridge during the solid phase extraction step, pointing to the polarity of the compounds. Importantly, both compounds (RT=5.3 min and 7.6 min) were seen in all
treatments, indicating that they were likely products of a physical or chemical
degradation process, such as hydrolysis or photodegradation. Correspondingly, a separate
non-radiolabeled light/dark study was performed (data not shown) in sterile conditions
using the same environmental chamber conditions described in the Methods section.
Deionized water was filter-sterilized and spiked to 5 mg/l SMZ; the dark treatment was
achieved by wrapping the sealed vessels in aluminum foil (n=4). Replicates were
extracted and analyzed using HPLC/MS. Results from a 14-d study revealed a
photoproduct (RT=5.3 min) with a mass (M+H) of 215 (results to be described in a later
publication).

A search of existing literature found a study indicating a SMZ photoproduct
(Boreen et al., 2005). Boreen and colleagues reported significant photolysis of several
sulfonamides in surface water, particularly in anaerobic conditions; predicted half-lives
from that study were 1.2-7.5 d, and calculations included photodegradation and reactions
with dissolved organic matter as possible dissipation mechanisms. Those half-lives
correspond well with half-lives observed in this study, however their conditions are quite
different from those described here. We have not definitively identified the
photoproducts detected in this study, but Boreen et al. (2005) suggested a possible
identity of the photoproduct, with a 215 mass by mass spectrometry. Figure 4 indicates
possible identities of the photoproducts detected in this study, based on mass of 215.

No statistically significant differences in concentrations of photoproducts were
noted among treatments until day 28 of the study. At day 28, the PW treatment had
significantly higher amounts of photoproducts in the water compared to PWS+M and
autoclaved PWS treatments (p=0.0257 and 0.0083). Figure 5 displays the amount of
photoproduct detected in the water as a fraction of radioactivity in the water at each
timepoint. It is important to note that the water in the PWS+M treatment was visibly
cloudy, thus inhibiting light penetration of the water. The higher levels of photoproducts
in PW compared to sediment-containing treatments at day 28 may indicate that
photoproducts were more easily formed in the pond water only treatment, possibly due to
increased light penetration. Alternatively, adsorption of SMZ residues to sediment could
limit the amount of SMZ available in the water column for photodegradation.

By day 63, unidentified photoproducts were 31.0% (95% CI 24.2, 37.9%) of total
radioactivity in the PW treatment, and were the dominant metabolites. For the PWS and
PWS+M treatments, the photoproducts accounted for 23.2% of applied radioactivity
(95% CI 16.4, 30.0%) and 19.9% (95% CI 13.1, 26.7%) of detected compounds in the
aqueous phase at day 63, respectively. The autoclaved PWS treatment had slightly less
photoproduct at day 63 compared to the other treatments, with a mean 14.6% (95% CI
7.8, 21.5%; p=0.0832).

To evaluate the timeline for photoproduct formation, comparisons of amount of
photoproduct at each timepoint were made within individual treatments. Comparisons
within the sediment-containing treatments (PWS, PWS+M, and autoclaved PWS)
revealed no significant differences in photoproduct concentrations across time (Fig. 5).
However, the PW treatment showed significant increases in amount of photoproduct
when comparing days 7 and 14 to days 28 and 63 (p<0.005), indicating that the majority
of the photoproduct formation occurred between days 14 and 28 in the study.
Additionally, a relative plateau in concentration is visible when comparing data for days
28 and 63 for the PW treatment (Fig. 5); no differences were detected between concentrations at days 28 and 63 for the PW treatment.

Analysis of sediment extracts revealed that photoproducts were the major metabolites in all treatments, accounting for approximately 15% of radioactivity in the extracts. At day 63, the mean amount of photoproducts in sediment extracts was 16.6% of radioactivity in sediment for PWS (95% CI 11.2, 22.0%), 15.8% for PWS+M (95% CI 10.4, 21.2%), and 13.1% (95% CI 7.7, 18.5%) for the autoclaved treatment. No differences were noted between treatments, however an overall time effect was noted (p=0.0054), indicating an increase in the photoproducts partitioning from the water column into the sediment over time. The photoproducts were the major metabolites detected in both the aqueous and sediment fractions in our microcosm study.

Other unidentified radiolabeled metabolites were detected in pond water and sediment extracts. Cumulatively, the non-photoproduct degradates accounted for a significant fraction (up to 60% of radioactivity within an extract). However individual unidentified metabolites accounted for less than 8% of radioactivity in pond water or sediment extracts, while photoproducts often exceeded 15%, thus indicating the significance of photodegradation in the environmental fate of SMZ. These unidentified compounds may be products of incomplete biodegradation or chemical degradation processes. Other possible chemical degradation routes for sulfamethazine have been suggested, including hydrolysis or oxidation with ferrate or hydroxyl radical (Mezyk et al., 2007; Sharma et al., 2006). Sulfamethazine biodegradation products have been studied extensively, with the major metabolite being N^4-acetyl-sulfamethazine; N-methylation and hydroxylations also occur (Hou et al., 2003; Coats et al., 1976). The
slower rate of SMZ dissipation in the autoclaved treatment in the current study points to the potential importance of biodegradation in the environmental fate of SMZ.

Less than 3% of the applied $^{14}$C-SMZ was mineralized to CO$_2$, however differences in mineralization were noted among the treatments (Fig. 6). Interestingly, the treatment containing pond water alone had the highest mineralization rate, while the manure-containing and autoclaved treatments had nearly identical mineralization rates. This could be due to differences in availability of the SMZ and related residues for complete degradation by microbes in the systems. Though mineralization was not extensive during this study, some evidence points to the impact of biodegradation on SMZ fate in surface water. The longer half-life calculated for the autoclaved treatment compared to the other biota-containing treatments (Table 2), higher concentrations of parent SMZ in water and sediment compared to other treatments, and the slower rate constant for SMZ dissipation in the autoclaved treatment all point to the contribution of biodegradation in the dissipation of SMZ in surface water systems.

Conclusions

The relatively low sorption coefficients calculated for SMZ indicate that it is likely to be mobile in the aqueous component of runoff following application to soil, and these predictions have been validated in previous field-scale studies. In sediments these low K$_d$ values, however, do not predict the degree of sorption observed in this study. This observation raises concern for use of K$_d$ values calculated from batch sorption studies in models for prediction of environmental fate, as adsorption and binding of SMZ residues was highly significant for the fate of the drug in this study. Additionally, these
batch studies do not typically include manure inputs; based on our findings of increased sorption and binding in the manure-containing treatment, the environmental fate of SMZ may not be well-predicted using models relying on $K_{ds}$.

This study is among the first to mechanistically examine the fate and dissipation of a sulfonamide antibiotic in surface water. The results presented here indicate that SMZ dissipates exponentially from the water column, with the majority of loss occurring via movement into the sediment phase. Importantly, manure or other particulate organic matter, as is likely to be present in surface water ecosystems, leads to increased adsorption, which may have important implications for availability of SMZ and related residues to be more completely degraded or bioavailable. Exposure assessment and environmental fate models should incorporate a manure parameter to more adequately assess potential risk.

Biodegradation, though important in the environmental fate of SMZ, was not a major contributor to the dissipation of SMZ in our microcosms. Photoproducts were the major metabolites detected in the microcosms throughout the entire study; these compounds developed in the water phase over the course of the study, with peak formation occurring at 2-4 weeks. The photoproducts partitioned into sediment, similarly to SMZ, and were detected as the most prevalent degradate in sediment as well. Sediment is a potential sink for SMZ and SMZ-related residues; this fate has important implications for benthic organisms, such as sediment-dwelling invertebrates or bacteria. The toxicity of these unidentified photoproducts remains to be determined, and the uncharacterized bound residues may contain SMZ or toxicologically significant metabolites that could become bioavailable over time. Future studies are needed to
determine the bioavailability and toxicity of SMZ photoproducts and bound residues to aquatic and benthic organisms.

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**References**


Antimicrobial residues in animal waste and water resources proximal to animal feeding operations. Sci. Total Environ. 299:89-95.


Table 1. Experimental design

<table>
<thead>
<tr>
<th>Treatment name</th>
<th>Description</th>
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<tr>
<td>PWS</td>
<td>SMZ in pond water and sediment</td>
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<td>Impact of sediment on system and degradation</td>
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<tr>
<td>PWS+M</td>
<td>SMZ in pond water-sediment amended with diluted manure to simulate manure runoff event (0.1%)</td>
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<td></td>
<td>Impact of manure</td>
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<tr>
<td>Autoclaved PWS</td>
<td>SMZ in autoclaved pond water and autoclaved sediment</td>
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<td></td>
<td>Sorption and non-biotic processes</td>
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<tr>
<td>PW</td>
<td>SMZ in pond water only (200 ml)</td>
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<td></td>
<td>Photodegradation</td>
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Table 2. Dissipation kinetics for sulfamethazine in pond water phase.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dissipation model</th>
<th>k ( (\text{se})^a )</th>
<th>( r^2 )</th>
<th>p-value</th>
<th>Half-life ( (d) )</th>
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</thead>
<tbody>
<tr>
<td>PWS</td>
<td>( C = C_0 e^{kt} )</td>
<td>0.26 (0.03)</td>
<td>0.9941</td>
<td>0.0002</td>
<td>2.7</td>
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<tr>
<td>PWS+M</td>
<td>( C_t = C_1 e^{k_1 t} + C_2 e^{k_2 t} )</td>
<td>( k_1 ): 0.20 (0.04) ( k_2 ): 0.03 (0.028)</td>
<td>0.9994</td>
<td>0.0307</td>
<td>4.2(^b)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Autoclaved PWS</td>
<td>( C = C_0 e^{kt} )</td>
<td>0.04 (0.004)</td>
<td>0.9881</td>
<td>0.0006</td>
<td>17.8</td>
</tr>
<tr>
<td>PW</td>
<td>( C = C_0 e^{kt} )</td>
<td>0.08 (0.009)</td>
<td>0.9859</td>
<td>0.0007</td>
<td>8.9</td>
</tr>
</tbody>
</table>

\(^a\) Standard error (se)

\(^b\) Half-life calculated as DT\(_{50}\)
Table 3. Mass balance of SMZ-residues in each treatment at day 7 and day 63.\(^1\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Remaining in pond water (%)</th>
<th>CO(_2) (%)</th>
<th>Extracted from sediment (%)</th>
<th>Bound in sediment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWS</td>
<td>80.03 (0.43)</td>
<td>0.21 (0.01)</td>
<td>4.38 (0.03)</td>
<td>16.14 (0.36)</td>
</tr>
<tr>
<td>PWS+M</td>
<td>72.7 (0.59)</td>
<td>0.05 (0.003)</td>
<td>4.96 (0.22)</td>
<td>24.81 (0.40)</td>
</tr>
<tr>
<td>Autoclaved PWS</td>
<td>80.17 (1.85)</td>
<td>0.02 (0.004)</td>
<td>6.79 (0.32)</td>
<td>15.21 (0.48)</td>
</tr>
<tr>
<td>PW</td>
<td>92.14 (0.69)</td>
<td>0.07 (0.005)</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Remaining in pond water (%)</th>
<th>CO(_2) (%)</th>
<th>Extracted from sediment (%)</th>
<th>Bound in sediment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWS</td>
<td>49.22 (1.31)</td>
<td>0.91 (0.15)</td>
<td>5.32 (0.11)</td>
<td>40.42 (0.92)</td>
</tr>
<tr>
<td>PWS+M</td>
<td>18.45 (3.42)</td>
<td>0.25 (0.03)</td>
<td>4.28 (0.29)</td>
<td>61.21 (1.08)</td>
</tr>
<tr>
<td>Autoclaved PWS</td>
<td>52.06 (2.04)</td>
<td>0.25 (0.05)</td>
<td>8.33 (0.49)</td>
<td>38.25 (1.89)</td>
</tr>
<tr>
<td>PW</td>
<td>88.03 (0.48)</td>
<td>2.45 (0.11)</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

\(^1\) All values are shown as mean % of applied radioactivity with standard error listed in parenthesis.

Note: Data for Days 14 and 28 are provided in the Appendix of this dissertation (Table 3A).
Table 4. Timetable for HPLC method for separation of SMZ photoproducts

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-8</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>8-12</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>12-15</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>15-16</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>16-17</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>17-19</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>19-24</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Solvent A: 1 mM ammonium acetate with 0.1% glacial acetic acid
Solvent B: Acetonitrile with 0.1% glacial acetic acid
Figure 1. Chemical structure of sulfamethazine.
Figure 2. Dissipation of parent sulfamethazine in pond water microcosms. The manure-containing treatment (PWS+M) treatment followed a two-compartment exponential decay model. The pond water plus sediment (PWS), the autoclaved PWS, and pond water only (PW) treatments followed a single exponential decay model. Standard error bars are shown.
Figure 3. Movement of parent sulfamethazine (SMZ) into sediment (top) and exponential increase in bound SMZ-residues in sediment (bottom) over time. Standard error bars are shown.
Figure 4. Possible identities of SMZ photoproducts with estimated mass units of 215 (M+H).
Figure 5. Amount photoproduct(s) in water over time, displayed as percent of radioactivity in the water column detected as photoproduct. Standard error bars are shown.
Figure 6. Mineralization of $^{14}$C-sulfamethazine in surface water microcosms.
Chapter 5. Estimating bioavailability of veterinary antibiotics: comparing passive samplers to traditional aquatic invertebrate bioassays

Keri L.D. Henderson¹, Thomas B. Moorman², and Joel R. Coats¹

¹ Department of Entomology, Iowa State University, Ames, IA
² USDA-ARS National Soil Tilth Laboratory, Ames, IA

A paper to be submitted to *Environmental Toxicology and Chemistry*

Abstract
Veterinary antibiotics are commonly used as feed additives in livestock production for growth promotion and disease prevention. These pharmaceuticals are often excreted by the livestock in urine and feces, and enter the environment via manure application. Understanding the bioavailability of pharmaceuticals in environmental matrices is particularly important considering they are often in an active form. In this study, the bioavailability of radiolabeled sulfamethazine in surface water microcosms was evaluated using C8-Empore ™ extraction disks as passive sampling devices and *Lumbriculus variegatus* in a bioassay. The disks and worms were incubated in treated water for several timepoints. At the end of the study, the disks and worms were extracted and uptake of radioactivity was determined. Bioconcentration factors (BCFs) were calculated to allow for comparison between disks and worms. A significant inverse relationship between exposure concentration and BCF was observed for worms, with log BCF of 2.17
at 0.05 mg/l during aquatic exposure, which is on the order of persistent hydrophobic contaminants such as polycyclic aromatic hydrocarbons. Disk accumulation factors were of similar magnitude, indicating their potential usefulness as biomimetic predictors of pharmaceutical bioavailability in aquatic ecosystems.

**Introduction**

Detection of a compound in the environment must be taken into context. Is the contaminant actually available to an organism to be absorbed into tissue to elicit a potential effect? Bioavailability is the bridge between the fate and/or amount of a contaminant in the environment and the potential hazard(s) of the contaminant to non-target organisms. It seems intuitive that bioavailability would be intimately incorporated into risk assessment schemes; however little progress has been made to date toward consensus-forming or adoption of ideas or techniques related to bioavailability for risk assessment purposes. Bioavailability has been an active area of research in ecotoxicology for several decades, and countless studies and review articles have been published. Bioavailability studies serve as a crucial link to understanding the likelihood of environmental residues impacting a species in a negative way.

**Bioavailability defined**

In pharmacology, the bioavailable fraction of a compound is the portion of parent compound that reaches circulation, as detected in blood plasma for example.

Bioavailability in ecotoxicology is defined as the fraction of a compound that is immediately available to an organism, as indicated by the compound’s ability to transfer
from the habitat (soil, sediment, or water) and subsequently cross the organism’s cellular membrane. Therefore, the bioavailable portion is the amount taken up from the substrate (Semple et al., 2004). Transformation products are included in total residues within the organism, because any transformation products that form are the result of uptake of parent (in the case of a laboratory exposure to pure parent compound). Quantifying bioavailability, according to Semple and coworkers, involves measuring uptake by an organism; uptake, and therefore bioavailability, will be organism-specific. In bacteria, uptake would occur across the cellular membrane; for aquatic worms, uptake would occur via the epidermis and gastrointestinal tract.

Semple and colleagues (2004) go on to further define bioaccessible contaminants as those “which are available to cross an organism’s cellular membrane from the environment, if the organism has access to the chemical.” This differs from immediately available compound (bioavailable) because the chemical may be physically or spatially removed from the organism, in the case of soil-borne bacteria or zooplankton with limited mobility, or the chemical might become bioavailable after a period of time, e.g. desorbed compounds. Calculating bioaccessible residues would include those levels immediately bioavailable, and those residues which may become available at a later time. Under Semple’s definition, chemical extraction-based techniques for determining bioavailability would actually be estimating bioaccessibility.

Equilibrium partitioning theory (EPT) is commonly used in assessing bioavailability of hydrophobic contaminants and is based on distribution coefficients between pore water, sediment organic carbon, and lipids in organism tissues. However, EPT assumes that the processes involved in governing bioavailability, such as desorption
and uptake, are capable of reaching equilibrium, and are passive diffusion-based processes. EPT does not account for active processes such as feeding or biotransformation, and assumes that contaminants are evenly distributed among sediment components, such as pore water, organic matter, and clay particles. Additionally, the theory is not appropriate for charged chemical species, such as some pharmaceuticals (Sijm et al., 2000). While EPT has performed reasonably well for very hydrophobic and persistent contaminants (e.g. PCBs, DDT and metabolites, dieldrin, and PAHs; Ehlers and Loibner, 2006; Trimble et al., 2008), the theory seems inappropriate for the study of veterinary antibiotics and other contaminants that are less persistent, and may be in flux in a system due to periodic inputs from agricultural or waste water sources.

Another proposed method for evaluating bioavailability is the sediment (or soil) availability ratio (SARA). SARA uses organisms to measure bioavailable concentrations as total amount of contaminant taken up by an organism, regardless of route of uptake or biotransformation. Bioconcentration factors (BCFs) are calculated for aged and fresh residues; the ratio between the BCF from the aged residues and from the fresh residue reveals the true bioavailable fraction of the aged residue. We propose that SARA could be applied to compare a system with all residues being bioavailable (aquatic only exposure in ultrapure laboratory water) to a system with decreased availability, such as one that includes dissolved organic material and sediment.

*Veterinary antibiotics*

Recently, antibiotics, including the veterinary antibiotics tylosin and sulfamethazine, were found in 48% of 139 stream water tested in 30 states, according to
the United States Geological Survey (Kolpin et al., 2002). A recent sediment monitoring study detected antibiotic residues throughout an agriculturally impacted watershed (Kim and Carlson, 2007). Antibiotics entering the environment could potentially alter bacterial populations and their activity in soil, sediment, and water, thus affecting biodegradation, nutrient cycling, and water quality. In addition, there is concern that antibiotics in the environment may induce antibiotic resistance, resulting in adverse human health effects.

Published reports have confirmed that very low levels (e.g. <5 µg/l) of antibiotic residues are detected in water and sediment monitoring studies (Kolpin et al., 2002; Kim and Carlson, 2007), but the significance of those residues will depend, to a great extent, on their biological availability. Although acute effects in traditional test organisms are unlikely (Baguer et al., 2000; Wollenberger et al., 2000; Kim et al., 2007), the potential biological significance of antibiotics lies in their potential to induce resistance in bacterial populations, maintain resistant populations or allow for easier transfer of resistance, or potentially alter beneficial bacterial populations such as gut symbionts, or those bacteria responsible for nutrient cycles or biodegradation of contaminants.

The sulfonamides are a class of antibiotics frequently detected in the aforementioned monitoring studies. These drugs have been used extensively in human and veterinary medicine applications for decades. The sulfonamides are mostly active against gram-positive, but also some gram-negative bacteria; their mode of action is prevention of folic acid synthesis in bacteria by acting as a structural analog of p-aminobenzoic acid. Sulfamethazine is a member of the sulfonamide class, and was chosen as the representative for the present study because of its wide use in livestock production for growth promotion purposes, and because of its frequent detection in
aquatic systems (Kolpin et al., 2002; Batt et al., 2006; Kim and Carlson, 2007; Managaki et al., 2007). Sulfamethazine (SMZ; Figure 1), as with other sulfonamides, is bacteriostatic; a typical minimum inhibitory concentration is 5 mg/l for susceptible bacteria. Interestingly, SMZ has a log $K_{ow}$ of 0.89, is soluble in water ($S=1.5$ g/l), and is capable of ionization ($pK_{a1}=2.65$, $pK_{a2}=7.65$; Tolls, 2001). These characteristics are strikingly different from the compounds typically studied for bioavailability; for example PCBs or PAHs, which have log $K_{ows}$ ranging from 4.5-8.1, relatively low water solubility (e.g. <1 µg/l), and are typically not ionized (Kraaij et al., 2003). Because of their known biological activity, frequent input in the environment, and subsequent persistence as contaminants, veterinary antibiotics such as sulfamethazine should be studied for their bioavailability and potential to bioaccumulate.

*Estimating bioavailability*

Bioavailability has traditionally been studied using bioassays by examining the amount of a chemical taken up into an organism’s tissues following exposure to a known concentration. A detailed description of EPT is provided by Di Toro and colleagues (1991), and only a brief summary will be included here. Di Toro et al. indicated that EPT is based partly on the assumption that organic chemicals sorbed to sediment are in equilibrium with the pore water and with the organisms present in the system; further, bioaccumulation factors were described as “direct” measures of a compound’s bioavailability. Simplistically, a bioconcentration factor (BCF) or bioaccumulation factor (BAF) is calculated by determining the ratio between the concentration of a compound detected in an organism ($C_{biota}$) and the concentration in the matrix ($C_{substrate}$).
Equation 1: \[ \text{BCF or BAF} = \frac{C_{\text{biota}}}{C_{\text{substrate}}} \]

Bioconcentration factors are the result of accumulation of a compound in an organism’s tissues during aquatic exposure. For example, in a laboratory study, the compound would be freely dissolved at varying concentrations in laboratory-grade water, and it would be assumed that 100% of the compound was available to the organism for potential uptake. Other routes of exposure (i.e. food or sediment) would be excluded. Bioaccumulation factors are calculated from exposure via food, water, and sediment, and it is assumed that some of the compound will become less available as a result of partitioning into the sediment and/or dissolved organic matter. In the case of EPT, biota-to-sediment accumulation factors (BSAFs) describe the relationship between the organism and the sediment; however, the authors indicate that EPT does not provide a means for establishing the actual route of exposure, i.e. pore water versus sediment (Di Toro et al., 1991).

Traditional bioassays, such as the *Lumbriculus variegatus* sediment bioaccumulation test (USEPA, 2000), are time-consuming and expensive. Recently, significant effort has been put into developing a more cost-effective, chemical-based method of estimating bioavailability (Kelsey et al., 1997; Friedig et al., 1998; Tang et al., 1999; Wells and Lanno, 2000; Krauss and Wilecke, 2001; Vinturella et al., 2004; Gourlay et al., 2005; Yang et al., 2006; Trimble et al, 2008).

Ehlers and Loibner (2006) provide a solid review of chemical extraction- and biomimetic device-based methods as alternatives to bioassays. They suggest that chemical extraction techniques might be most appropriate for assessing the potentially bioaccessible residues, which may include slower desorbing residues. However, they
acknowledge that chemical extraction techniques overestimated bioavailability for various hydrophobic contaminants (e.g. PAHs, DDT and metabolites, dieldrin), even when relatively mild, non-exhaustive extraction techniques were employed (Ehlers and Loibner, 2006).

Biomimetic devices, such as solid-phase extractants Tenax, XAD, or C\textsubscript{18} disks, simulate uptake and bioavailability based on the analyte in the aqueous phase selectively partitioning onto the sampler, thus measuring the freely dissolved residues in a system. These residues may be considered to be the bioavailable fraction (Sijm et al., 2000; Ehlers and Loibner, 2006). Reid and colleagues (2000) describe use of passive sampling devices (PSDs) for assessing bioavailability, in which the PSD measures uptake from the aqueous phase, which includes the rapidly desorbed fraction of the contaminant. This rapidly desorbed fraction corresponds to the fraction that is available to microorganisms in the soil or sediment. Ehlers and Loibner (2006) suggest the more depletive, SPE-based techniques for measuring the freely dissolved and rapidly desorbing fractions. Solid-phase microextraction (SPME) techniques have also been employed as non-depletive methods for measuring the freely dissolved fraction and mimicking bioaccumulation; however, these techniques are based on the assumption of equilibrium within the system, and that each process is passive diffusion-based, ca. EPT (Ehlers and Loibner, 2006). As previously discussed, bioavailability of pharmaceuticals such as sulfamethazine may not be accurately assessed by EPT because of their polar characteristics and flux within a system. The objective of the present study was to assess the uptake and bioaccumulation of SMZ residues by \textit{L. variegatus}, a common sediment-dwelling oligochaete, and
compare those results to a biomimetic device. Further, we evaluate EPT and SARA as methods for predicting bioavailability of sulfamethazine in aquatic ecosystems.

**Methods**

**Chemicals**

$^{14}$C-U-phenyl-sulfamethazine and non-labeled analytical grade sulfamethazine (SMZ) were purchased from Sigma-Aldrich (St. Louis, MO). Spiking solutions (labeled and non-labeled) were made in 50% methanol, and final concentration of carrier solvent in test chambers was <0.5% (v/v).

**Lumbriculus variegatus**

The test species, *L. variegatus*, was obtained from existing cultures in the Department of Entomology at Iowa State University (Ames, IA). The worms were reared in aquaria containing distilled water with an unbleached shredded paper towel substrate at 24 ± 0.5°C and a 16:8 photoperiod. Adult worms (8 ± 1 mg fresh wt.) were selected and used in the study.

**C$_8$ disks**

We selected C$_8$ Empore™ disks (3M™ Corporation, St. Paul, MN) as the biomimetic devices in our study. The disk technology incorporates solid-phase sorbent material in a PTFE fibril framework. We tested C$_8$, C$_{18}$, and SDB-RPS (a styrenedivinylbenzene polymer with sulfonic acid modification), and found C$_8$ to be most
applicable for pharmaceutical adsorption and subsequent extraction and quantification (data not shown). The disks are 47 mm diameter and 15.5% carbon.

Aquatic exposure—bioassay

Test chambers consisted of 200 ml glass jars filled with 50 ml ultrapure water spiked with a mixture of $^{14}$C- and non-labeled sulfamethazine to achieve the following concentrations: 0.05, 0.5, and 5 mg/l in test water. Specific activity within test chambers was 0.72 $\mu$Ci/mg for the 0.05 mg/l treatment, and 0.52 $\mu$Ci/mg for the 0.5 and 5 mg/l treatments. At Day 0, five adult Lumbriculus variegatus, weighing approximately 8 mg each, were placed in test chambers. Test chambers were maintained in the dark at 23 ± 1º C. Worms were not fed during the study.

Worms were sacrificed at day 1, 3, 7, and 14, and worm tissue from a single replicate was pooled for analysis, with 4 replicates per treatment and timepoint. Worms were removed from treatment water and placed in 5 ml fresh ultrapure water for 6 hours to allow for gut clearance. Clearance water was removed for quantification of $^{14}$C residues using liquid scintillation counting (LSC; Beckman Coulter 6500, Fullerton, CA). Next, worms were homogenized in 5 ml methanol for 10 min; extracts including homogenized tissue were shaken at 300 rpm for 15 min on an orbital shaker and then centrifuged at 2000 rpm for 5 min.

Treatment water was extracted using solid-phase extraction (SPE) cartridges (6 cc, Oasis HLB®, Waters Corporation, Milford, MA). Cartridges were conditioned with 3 ml methanol, 3 ml 0.5 M HCl, and 3 ml ultrapure water (Millipore Corporation, Billerica, MA). Samples were loaded onto cartridges at a flow rate of 2 ml/min. Cartridges were
allowed to air dry for 5 min and were eluted with 3 ml methanol at 1 ml/min. Extracts were brought to a 5 ml final volume with ultrapure water. Aliquots of worm extract supernatant or water eluate were immediately counted for radioactivity using LSC, and remaining extract was stored at -20º C until analysis using high pressure liquid chromatography with radiodetection (β-RAM®, IN/US Systems, Tampa, FL).

Aquatic exposure—biomimetic device

C8 disks were washed and conditioned with 10 ml each of acetonitrile, methanol, water. Moist disks were transferred to treated water containing 0.05, 0.5, or 5 mg/l 14C-sulfamethazine as described for the *L. variegatus* bioassay, with one disk per replicate, and four replicates per treatment and timepoint. Treatment jars were then placed on an orbital shaker at 150 rpm to simulate worm movement. Test chambers were maintained in the dark at 23 ± 1ºC. Disks were removed from treatment water at day 1, 3, 7, and 14, and were eluted twice with 10 ml of 100% methanol. Next, disks were soaked overnight in 20 ml 100% methanol to remove any remaining 14C-residues. Disk eluate and soak samples were pooled for analysis of radioactivity, which is described for worm extracts. Next, the pooled sample was concentrated to 3 ml final volume under N2 gas at 40ºC for HPLC analysis. Treatment water was extracted using SPE as previously described.

Sediment exposure—bioassay

The sediment exposure studies were run in parallel with a sulfamethazine environmental fate experiment of identical design (Chapter 4 of this dissertation). Pond water and sediment were collected from the Iowa State University Horticulture Research
Station pond (Gilbert, IA); 10-15 cm (depth) sediment samples were manually collected using a soil auger. Sediment and water were transported to the laboratory and stored in the dark at 4°C. Prior to use, sediment was thoroughly mixed, and moisture content was determined to be 46.2%. The sediment was a sandy loam (60% sand, 28% silt, 12% clay) with 2.0 % organic matter, and a pH of 8.1.

Seventy-three grams (50 g dry wt) was measured into wide-mouth pint jars, and topped with 177 ml pond water, to equal 200 ml water per jar. Each jar served as a replicate (n=4). Sediment was allowed to settle for 1 h, and 10 adult L. variegatus (approximately 8 mg each) were added to each replicate test chamber. Worms were allowed to acclimate for 1 h, prior to addition of the 14C-SMZ spiking solution. The final spiking solution was 0.425 mg/ml non-labeled SMZ and 0.085 µCi/ml 14C-SMZ in 10% methanol. Each replicate jar received 2.35 ml spiking solution, so the final concentration of SMZ in pond water was 5 mg/l, and 0.2 µCi/jar. Microcosms were maintained at 22°C in a 12:12 photoperiod for the 7-day study period.

At the end of the 7-day period, pond water was removed from the top of the sediment and the remaining water and sediment were carefully sieved through a 2 mm mesh to remove worms. Worms were then rinsed with ultrapure water, and allowed the 6-h clearance period described for the aquatic exposure study. Worms were ground and extracted, and extracts analyzed as previously described.

Sediment exposure—biomimetic devices

Conditioned disks were partially buried in the sediment of the pond water microcosms previously described, with one disk per replicate (n=4). One-third of the
disk was buried 3 mm in sediment, and the remaining two-thirds was allowed to float in the water; this was to simulate the behavior of *L. variegatus* in surface water. The worms place their head region in the sediment, but allow their tail end to float upward in the water column to allow for gas exchange. At end of test period, disks were removed from the treatment jars and placed in 30 ml ultrapure water for 30 minutes to remove any excess sediment. Following this rinse step, disks were eluted and soaked in methanol, as described in the aquatic exposure section. Eluate and soak extracts were pooled and analyzed by LSC and HPLC.

**Sample analysis**

For the sediment exposure assays, concentrations of sulfamethazine in water and sediment were determined using the fate data gathered in the parallel study. A brief description will follow. On Day 7, pond water was siphoned from the top of the sediment, filtered and extracted using Waters Oasis HLB solid phase extraction cartridges as previously described. Sediment was shaken for 80 min in 100 ml of a 70% methanol solution, allowed to settle overnight at room temperature, then centrifuged and decanted into amber jars. A second 100-ml aliquot of 70% methanol was added to the sediment, and shaken for 15 min. The bottles were again centrifuged and decanted. Extracts were pooled, concentrated under nitrogen flow (15 psi, 40°C), and enriched using the SPE method previously described.

Extracted sediments were dried, sieved (<5 mm), and ground using mortar and pestle to ensure thorough mixing. To determine remaining bound residue in sediment,
0.5-gram aliquots were combusted using an OX-600 biological oxidizer (RJ Harvey Instrument Co., Hillsdale, NJ).

A reverse-phase high-performance liquid chromatography (HPLC) method was used to quantify parent sulfamethazine and to identify related metabolites. Analysis of the samples was performed using a Hewlett-Packard (Palo Alto, CA, USA) series 1100 HPLC system with a quaternary pump, an autosampler, a thermostatted column compartment, and UV and β-RAM detectors. A Waters Atlantis™ (Milford, MA, USA) dC18 column (4.6 × 250 mm, 5-μm particle size) was used. Detection was conducted at 254 nm, with a flow rate of 1.0 ml/min at 30°C, and 200μl injection volume. The mobile phase consisted of 30% methanol. Radiolabeled and non-labeled standards were used to quantify parent sulfamethazine in samples. Data were collected and analyzed using HP Chemstation system software (REV. A.04.01) and Laura-Lite® software (for radiodetection, IN/US Systems).

Statistical analyses

Bioconcentration factors were calculated based on quantification of total radioactivity (SMZ equivalents) in worm or disk extracts, and parent sulfamethazine in water ($C_w$) for the aquatic exposure study, and in pond water and sediment for the sediment exposure ($C_{sedOC}$). Concentrations in worm tissue ($C_{lipid}$) were calculated as ng SMZ equivalents/g lipid, and concentrations on disks ($C_{disk}$) were calculated as ng SMZ equivalents/g disk organic carbon (15.5% OC, 3M Corp., St. Paul, MN). Sediment concentrations were also normalized by 2% organic carbon (mg SMZ/kg OC).
Bioconcentration factors were calculated using the normalized data, as described in Equations 2 and 3.

Equation 2: worm BCF = $C_{\text{lipid}}/C_w$

worm BAF = $C_{\text{lipid}}/C_{\text{sedOC}}$

Equation 3: disk BCF = $C_{\text{disk}}/C_w$

disk BAF = $C_{\text{disk}}/C_{\text{sedOC}}$

In addition to calculating BCFs and making comparisons with EPT methods, we used a modification to the SARA model in which we compared bioavailability in the aquatic-only, dissolved exposure (~100% available residues) to the more realistic sediment exposure model, with theoretically decreased availability.

Statistical analysis of the data included use of general linear models and least squares means to assess differences among treatments for the aquatic and sediment exposures, with time and exposure concentration as variables within the models for total uptake (µg) and percent uptake. Similarly, general linear models and least squares means were used for evaluating differences between bioconcentration factors for disks and worms. The Tukey-Kramer method for adjustment for pairwise comparisons was used, and significance was defined as $p<0.05$. If significant interactions between variables (e.g. time*treatment) were noted, interactions were explored using least squares means slicing methods; all analyses were performed with SAS V9.1 (SAS Institute, Cary, NC). First-order, single-compartment uptake models for tissue and disk concentrations were used to estimate $C_{\text{lipid}}$ or $C_{\text{disk}}$ at equilibrium ($C_{\text{eq}}$) from the measured concentrations at time
where $k$ is the uptake rate constant (Equation 4) using SigmaPlot 10.0 (SyStat Software, Inc., San Jose, CA).

Equation 4: $C_m = C_{eq} (1-e^{-kt})$

**Results and Discussion**

*Aquatic exposure—bioassay*

No toxicity of sulfamethazine to *L. variegatus* was noted during the study. The lipid content of adult *L. variegatus* was 12.7% as determined by acid hydrolysis (AOAC Method 4.5.02 16th ed; Midwest Laboratories, Inc., Omaha, NE). Mean total uptake of SMZ-equivalents was 0.05, 0.02, and 0.10 micrograms for the 0.05, 0.5, and 5 mg/l exposure concentrations, respectively. This corresponds to $\leq 2\%$ uptake of the total available $^{14}$C-residue (Table 1). Significant treatment effects were noted ($p<0.001$), indicating a relationship between amount of µg uptake and exposure concentration in the water; however, there was no significant time or time-treatment interaction effect ($p>0.10$).

The first-order uptake models were a reasonable fit at each exposure concentration (0.05 mg/l: $r^2=0.755$, $p=0.0558$; 0.5 mg/l: $r^2=0.733$, $p=0.064$; 5 mg/l: $r^2=0.759$, $p=0.0543$). Equilibrium was reached at approximately day 7 of the study (Figure 2). Rate constants for uptake were 0.35, 0.59, and 1.23 and equilibrium concentrations were 14.5, 4.1, and 21.7 ng/g lipid for the 0.05, 0.5, and 5 mg/l exposure concentrations, respectively. Tissue concentrations were approaching or exceeding published MICs in all three treatment concentrations, which may have implications for *L. variegatus* gut symbionts. Log BCFs were calculated for each treatment across times,
and a significant inverse relationship between BCF and exposure concentration was noted (Figure 3). At 0.05 mg/l exposure concentration, the mean log BCF was 2.17.

Aquatic exposure—biomimetic device

The C8 disks absorbed >55% of the total dissolved $^{14}$C-residue regardless of aquatic exposure concentration (Table 2). Significant treatment (exposure concentration) effects were noted for total uptake, indicating that the more SMZ available in the water, the higher the uptake by the disk ($p<0.0001$). Time effects were also noted for the highest exposure concentration (5.0 mg/l; $p=0.0148$), with more uptake occurring at each timepoint. First-order uptake models were a good fit at each exposure concentration (0.05 mg/l: $r^2=0.881$, $p=0.0182$; 0.5 mg/l: $r^2=0.998$, $p<0.0001$; 5 mg/l: $r^2=0.942$, $p=0.0061$). For the more environmentally relevant exposure concentrations (0.05 and 0.5 mg/l), the system appeared to be at equilibrium within 24 h (Figure 4), however the 5 mg/l treatment did not appear to reach equilibrium until >7 d. Rate constants for uptake were 2.32, 172.6, and 0.61 and equilibrium concentrations were 16.5, 175, and 1828 ng/g disk OC for the 0.05, 0.5, and 5 mg/l exposure concentrations, respectively. Log BCFs were calculated for each treatment across times, and no difference was noted between exposure concentrations, with the overall mean log BCF of 2.49.

Sediment exposure—bioassay

At the end of the 7-day study, 68% of worms were recovered from the four replicates, and all calculations were based off of recovered tissue mass. Less than 0.5% of total $^{14}$C was taken up by the worms, however the mean log BCF (or BSAF) was
calculated to be 1.89 (Table 1). Significantly more uptake (in µg) was observed in the sediment assay compared to all of the aquatic exposure concentrations, with a mean difference of approximately 1.5 µg (p<0.0001). Interestingly, there was no significant difference between log BCFs for the sediment exposure and the lowest aquatic exposure concentration (0.05 mg/l; p=0.8145), though the exposure concentrations differ by nearly two orders of magnitude. This observation may indicate the significance of feeding behavior as a route of uptake resulting from sediment exposure for *L. variegatus*.

*Sediment exposure—biomimetic device*

Disks absorbed 25.5% of the $^{14}$C residue in the test system within 7 days, and mean uptake was 111.5 µg (Table 2). Log BCFs calculated for the sediment exposure were not different than those calculated from the aquatic exposure (2.46-2.59; p>0.990), indicating no impact of sediment on BCF prediction, which shows the normalization for sediment OC, i.e. EPT, as described by Di Toro et al. (1991) works well for the disks. However, a significant sediment effect was observed when examining mean % uptake; the sediment-exposed disks took up proportionally less than disks exposed through the aquatic route only, which may be indicative of decreased availability of SMZ residues to the disks in the sediment exposure scenario (Table 3).

*Bioassay versus biomimetic device*

Disk uptake was >55% in the aquatic exposure with freely available SMZ residues, while worms took up <3%. In sediment, there was >50% reduction in uptake by disks. Contrastingly, in the worm bioassay, an increase in % uptake was noted in the
sediment system compared to the higher aquatic exposure concentrations, which could indicate that presence of sediment may increase *L. variegatus* exposure to SMZ residues; this is likely due to increased exposure from feeding behavior, which is not modeled in the disks.

Examination of the bioconcentration factors reveals high similarity between the biomimetic device method and the bioassay, particularly when considering the sediment exposure and the most environmentally relevant aquatic exposure concentration (0.05 mg/l). There was no significant difference between disk BCFs and worm BCFs for the 0.05 mg/l aquatic exposure (Table 4), indicating the potential usefulness for employing the disks as biomimetic devices in environmental situation. However, there were some significant differences between the disks and worm sediment exposure, though this exposure concentration (3.03 mg/l) is likely higher than levels found in the environment. The disk method is considerably quicker than the bioassay, since disks reach equilibrium within 1 day at environmentally relevant aquatic exposure concentrations.

*Testing EPT and SARA*

The equilibrium partitioning theory is based on organic contaminants being distributed between pore water, sediment organic carbon, and organisms’ lipid content, with partition coefficients acting as predictors (Di Toro et al., 1991). Further, BAFs (or BSAFs) can be predicted from log $K_{ow}$, and may be highly correlated, e.g. nearly 1:1 log BCF:log $K_{ow}$ for PAHs (Kraaij et al., 2003). However, the log $K_{ow}$ for SMZ is 0.89, and the log BCFs for the environmentally relevant samples were 1.89 to 2.17, so this 1:1 comparison does not hold in the case of SMZ. Additionally, Trimble and colleagues
describe using the linear relationship (nearly 1:1, slope 1.03) between lipid-normalized biota concentration and organic carbon-normalized exposure concentrations as an indicator for the use of EPT to predict PCB bioaccumulation. However, using a general linear model to check correlation between lipid-normalized SMZ tissue concentration and organic carbon-normalized exposure concentration for our results, a lack of relationship was shown ($r^2=0.055$, $p=0.1451$, slope=7.95), indicating that EPT does not adequately predict SMZ bioavailability to *L. variegatus* in our system.

Using the SARA method for the *L. variegatus* data, we computed a ratio of 0.87 between the sediment exposure log BCF and the most relevant aquatic exposure log BCF (1.89/2.17); this indicates a possible 13% reduction in bioavailability in the sediment-containing system, however statistical comparison of the two log BCFs reveals no significant difference (95% CI: -0.31, 0.87; $p=0.8145$). Further comparison of % uptake in the sediment versus aquatic systems reveals a decrease in the proportion available in the sediment system (Table 1). Modeling SARA with the C₈ disk data shows no decrease in bioavailability between the two exposure systems (ratio=1).

*Conclusions*

Sulfamethazine residues bioaccumulate in *L. variegatus* tissues, and the accumulation is not adequately predicted by the equilibrium partitioning theory. This is likely due to several factors, including the ionization potential of SMZ and feeding behavior of *L. variegatus*. At pH less than pKₐ₂ (7.65) as in the water phase of our exposures, protonation may occur to some degree on at least one site for SMZ; however, in the sediment used in this study (pH 8.1), we would expect more anionic compound
than neutral, therefore uptake from sediment may not follow a passive diffusion type model.

Addition of sediment resulted in only slightly reduced BCFs compared to aquatic-only exposures, in the case of *L. variegatus*; however, the trend was not statistically significant. This result was not consistent with the assumption that partitioning in sediment would result in decreased accumulation, as in EPT. Bioaccumulation in C8 disks was not impacted by sediment in the system; log BCFs remained at approximately 2.5. Bioaccumulation in C8 disks serves as a reasonable estimator of SMZ bioaccumulation in *L. variegatus* at environmentally relevant concentrations of SMZ in water. Further studies are needed to assess whether the disks are good predictors of bioaccumulation as a result of low level exposure in sediment-containing systems.

The mean log BCF for the 0.05 mg/l exposure concentration was 2.17, which is of the same magnitude as BCFs expected for persistent hydrophobic compounds such as PCBs (mixture of Aroclors 1242 and 1254; Trimble et al., 2008) or PAHs (e.g. phenanthrene and fluoranthene; Krauss and Wilcke, 2001; Kraaij et al., 2003), which was not expected based on the relatively low *K*\(_{ow}\) for SMZ. The inverse concentration-dependent accumulation appears to be unique for an organic compound; a brief review of the literature found similar relationships in metal bioaccumulation studies (McGreer et al., 2003; DeForest et al., 2007). Reasons for an inverse relationship in metals are proposed to be related to saturated uptake conditions at higher exposure concentrations (Simkiss and Taylor, 1989) and active regulation of metal uptake. Since SMZ may be ionized at environmental pHs, as in the present study, it is possible that it was similarly regulated. The inverse relationship points to a need for further study of bioavailability of
sublethal levels of pharmaceuticals in the environment. Based on the results presented here, chronic tests may need to be performed for greater than 7 days using low exposure concentrations (µg/l range) to allow tissue concentrations to reach equilibrium. Tissue levels of antibiotics may approach toxic concentrations even at low exposure levels; e.g. gut microflora could be impacted if tissue levels approach minimum inhibitory concentrations, as demonstrated in this study. Additionally, much remains unknown regarding toxicity of SMZ metabolites, therefore further toxicity evaluations may be warranted. Finally, risk assessments of pharmaceuticals in the environment should include bioavailability assessments at environmentally relevant concentrations to determine if bioaccumulation is significant at µg/l concentrations to allow for adequate hazard assessment.

Acknowledgements

The authors would like to thank Beth Douglass and Ashley Jessick for their technical assistance. Funding for this study was provided by a U.S. EPA STAR Fellowship and USDA-CSREES-NRI Grant #IOW05091.

References


Table 1. Worm bioaccumulation of sulfamethazine.

<table>
<thead>
<tr>
<th>Treatment exposure concentration (mg/l)</th>
<th>Mean uptake(^1) (µg) (sd)</th>
<th>Mean uptake (% of applied (^{14})C) (sd)</th>
<th>Log BCF(^2) (g OC/g lipid) (sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aquatic exposure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.052 (0.050)</td>
<td>2.07 (2.02)</td>
<td>2.17 (0.45)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.016 (0.016)</td>
<td>0.065 (0.065)</td>
<td>0.55 (0.64)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.101 (0.045)</td>
<td>0.040 (0.018)</td>
<td>0.56 (0.21)</td>
</tr>
<tr>
<td><strong>Sediment exposure(^3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.03</td>
<td>1.614 (0.489)</td>
<td>0.369 (0.112)</td>
<td>1.89 (0.12)</td>
</tr>
</tbody>
</table>

\(^1\) Uptake was calculated as SMZ equivalents based on total radioactivity in worms.

\(^2\) Log bioconcentration factors normalized by worm lipid content (12.7% wet wt.) and sediment organic carbon content (2.0%). BCFs for sediment exposure are the same as BSAFs.

\(^3\) Weighted sediment exposure concentration = 0.9(mean SMZ concentration in overlying water) + 0.1(mean SMZ concentration in sediment organic carbon). Based on 90% of \(^{14}\)C residues in overlying water during days 0-7, and observed worm behavior.
Table 2. Bioaccumulation of sulfamethazine by C₈ disks.

<table>
<thead>
<tr>
<th>Treatment exposure concentration (mg/l)</th>
<th>Mean uptake¹ (µg) (sd)</th>
<th>Mean uptake² (% of applied ¹⁴C) (sd)</th>
<th>Log BCF² (g OC/g OC) (sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Aquatic exposure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.5 (1.5)</td>
<td>59.9 (18.6)</td>
<td>2.48 (0.16)</td>
</tr>
<tr>
<td>0.5</td>
<td>16.3 (1.5)</td>
<td>65.0 (6.2)</td>
<td>2.54 (0.04)</td>
</tr>
<tr>
<td>5.0</td>
<td>141.4 (45.5)</td>
<td>56.6 (18.2)</td>
<td>2.46 (0.16)</td>
</tr>
<tr>
<td><strong>Sediment exposure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.03</td>
<td>111.5 (19.5)</td>
<td>25.5 (4.5)</td>
<td>2.59 (0.07)</td>
</tr>
</tbody>
</table>

¹ Uptake was calculated as SMZ equivalents based on total radioactivity.

² Log bioconcentration factors normalized by C8 disk carbon content (15.5% wet wt.) and sediment organic carbon content (2.0%). BCFs for sediment exposure are the same as BSAFs.

³ Weighted sediment exposure concentration = 0.9(mean SMZ concentration in overlying water) + 0.1(mean SMZ concentration in sediment organic carbon). Based on 90% of ¹⁴C residues in overlying water during days 0-7.
Table 3. Sediment effect on % uptake by C₈ disks.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean difference (% uptake)</th>
<th>95% CI (LCL, UCL)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquatic 0.05 mg/l – Sediment 3.03 mg/l</td>
<td>34.4</td>
<td>11.3, 57.5</td>
<td>0.0016</td>
</tr>
<tr>
<td>Aquatic 0.5 mg/l – Sediment 3.03 mg/l</td>
<td>39.5</td>
<td>16.4, 62.6</td>
<td>0.0003</td>
</tr>
<tr>
<td>Aquatic 5.0 mg/l – Sediment 3.03 mg/l</td>
<td>31.0</td>
<td>7.9, 54.1</td>
<td>0.0048</td>
</tr>
</tbody>
</table>
Table 4. Comparison of biomimetic device method and *L. variegatus* bioassay for bioaccumulation of SMZ.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean difference (log BCF)</th>
<th>95% CI (LCL, UCL)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disk aquatic 0.05 mg/l – Worm aquatic 0.05 mg/l</td>
<td>0.31</td>
<td>-0.11, 0.73</td>
<td>0.2939</td>
</tr>
<tr>
<td>Disk aquatic 0.5 mg/l – Worm aquatic 0.05 mg/l</td>
<td>0.37</td>
<td>-0.05, 0.79</td>
<td>0.1233</td>
</tr>
<tr>
<td>Disk aquatic 5.0 mg/l – Worm aquatic 0.05 mg/l</td>
<td>0.29</td>
<td>-0.13, 0.71</td>
<td>0.4003</td>
</tr>
<tr>
<td>Disk sediment 3.03 mg/l – Worm aquatic 0.05 mg/l</td>
<td>0.42</td>
<td>-0.17, 1.01</td>
<td>0.3398</td>
</tr>
<tr>
<td>Disk aquatic 0.05 mg/l – Worm sediment 3.03 mg/l</td>
<td>0.59</td>
<td>0.01, 1.17</td>
<td>0.043</td>
</tr>
<tr>
<td>Disk aquatic 0.5 mg/l – Worm sediment 3.03 mg/l</td>
<td>0.65</td>
<td>0.07, 1.23</td>
<td>0.018</td>
</tr>
<tr>
<td>Disk aquatic 5.0 mg/l – Worm sediment 3.03 mg/l</td>
<td>0.57</td>
<td>-0.01, 1.15</td>
<td>0.0613</td>
</tr>
<tr>
<td>Disk sediment 3.03 mg/l – Worm sediment 3.03 mg/l</td>
<td>0.70</td>
<td>-0.01, 1.41</td>
<td>0.0568</td>
</tr>
</tbody>
</table>
Figure 1. Chemical structure of sulfamethazine.
Figure 2. Concentrations of sulfamethazine equivalents in *L. variegatus* tissue normalized by lipid content.
Figure 3. Inverse relationship of BCF and exposure concentration for *L. variegatus* in the aquatic exposure study. Linear regression $y=0.86 + -0.80x$, $r^2 = 0.5585$ (p<0.0001). 95% confidence intervals are also shown.
Figure 4. Concentrations of sulfamethazine equivalents absorbed by C₈ disks normalized by carbon content.
Chapter 6. General conclusions

Each chapter contains an individual conclusions section, therefore a summary conclusion is included in this chapter. The major objectives of this dissertation were centered around the assessment of the environmental fate and impact of veterinary antibiotics:

I. Determine mobility and degradation of manure-applied tylosin, and survival and movement of enteric bacteria in soil column

II. Determine the fate of two antibiotics from different classes in surface water microcosms containing manure and sediment
   a. Tylosin—representative of the macrolide class
   b. Sulfamethazine—representative of the sulfonamide class

III. Evaluate the bioavailability of SMZ in aquatic microcosms
   a. Develop a method to estimate bioavailability using a biomimetic device
   b. Compare bioaccumulation results obtained from bioassay with those obtained from biomimetic device

Results from the studies conducted within this dissertation indicate the potential significance of veterinary antibiotics in the environment. In our examination of tylosin and enteric bacteria in soil, we found tylosin has relatively low mobility through the soil column, and binding may prevent much leaching; less than 1% of applied tylosin leached from intact soil columns. Examination of data on individual tylosin factors suggests
increased mobility and/or persistence of tylosin D compared to tylosin A, as
demonstrated by the higher ratio of tylosin D to tylosin A detected in leachate. Tylosin D
was approximately 65% of the total tylosin detected in leachate, while tylosin A was only
22%. This is in contrast to the proportion of tylosin A in the formulation (>80%) that
was applied to the soil columns; tylosin D makes up approximately 10% of the total
tylosin residues in the formulation. Importantly, higher than expected mobility of enteric
bacteria was also noted; in many cases, the values for *Escherichia coli* and *Enterococcus*
readily exceeded EPA suggested water quality criteria. Additionally, tylosin-resistant
bacteria were leached from the columns that received manure treatments, likely due to
existing resistant organisms in the manure. These results highlight the importance of
understanding the context in which antibiotic residues and pathogenic bacteria enter the
environment; the manure matrix proved to be important in the mobility of antibiotic-
resistant organisms.

Further, in a study of tylosin persistence in surface water, we found that in
treatments receiving a manure input (0.1% w/v) had an immediate reduction in tylosin
residues, likely due to binding of the drug to the manure organic carbon; however, over
time we saw possible desorption of the tylosin residues from the manure matrix. Tylosin
persisted in surface water at low part-per-billion concentrations during the course of the
24-day study. These results indicate that, though low levels of the drug are detected in
the environment, the residues may persist long enough to allow for some build up within
the system, or to allow for extended or chronic exposures to non-target organisms.

A thorough study of sulfamethazine in surface water microcosms also yielded
results pointing to the importance of studying the effect of manure on fate of antibiotics.
We found that treatments receiving manure inputs had significantly more bound or unextractable sulfamethazine residues in sediment than treatments without manure inputs. Bound residues are not easily characterized, therefore it is unknown if these residues may be of toxicological importance, i.e. whether the residues are parent sulfamethazine or a biologically active metabolite, or whether the residues are bioavailable. Additionally, we detected the presence of two photoproducts in the overlying surface water in the microcosms; we also detected their presence in sediment, indicating their ability to partition similarly to parent sulfamethazine. The photoproducts have yet to be conclusively identified, and their toxicity is currently unknown. In summary, sulfamethazine exhibited biphasic dissipation in the manure-containing treatment, indicating the rapid initial dissipation to sediment, followed by a prolonged period of persistence of sulfamethazine in surface water systems.

The finding of relative persistence of sulfamethazine and binding of residues in sediment led to the examination of the bioavailability of sulfamethazine. Sediment and aquatic-only exposures were performed at three concentrations to *Lumbriculus variegatus*, an aquatic oligochaete. Interestingly, we found that sulfamethazine bioaccumulates in the worms, particularly at low exposure concentrations. Bioaccumulation factors (BAFs) were calculated based on sulfamethazine equivalents detected in worm tissue, lipid content of worms, and organic carbon content of sediment or water; log BAFs at the lowest exposure concentration (50 µg/l) were found to be 2.17, which is on the order of PCBs and PAHs, highly persistent hydrophobic contaminants. This result was not expected based on $K_{ow}$ of sulfamethazine and its likelihood to be metabolized. Finally, the inverse relationship between exposure concentration and BAF
has been previously demonstrated in metals uptake, but is not common for uptake and accumulation of organic contaminants. This relationship is highly significant considering the persistence of low concentrations of antibiotics in the environment, the subsequent exposure of aquatic and benthic organisms, and accumulation of residues within their tissues.

Due to cost and time inputs for bioassays, much effort has been put into development of chemically based bioavailability models. We chose to examine the potential efficacy of C8 Empore™ disks to serve as a reasonable model for *Lumbriculus* bioaccumulation of sulfamethazine. We found that the disks could potentially serve as a conservative model for uptake in aquatic systems, however sediment uptake and feeding behavior was not similarly modeled, therefore further research is needed before disks could be employed for that purpose.

Finally, we began method development for evaluation of the potential effects of veterinary antibiotics on gut microflora of *L. variegatus*. Methods were also developed to evaluate the persistence of antibiotic-resistance traits in pathogen populations. Those methods are described in the Appendix of this dissertation. Current data indicate the potential to develop a dose-response test for alteration of gut flora in aquatic invertebrates exposed to antibiotics via food or sediment. More work is needed to further develop these methodologies.

The results obtained will be useful toward ecological risk assessments of antibiotics. Perhaps most importantly, our results indicate that antibiotics should not be studied alone; manure inputs had a significant effect on fate in soil and sediment, and sediment may serve as a sink for antibiotic residues in surface water ecosystems. Our
data also indicate that the low concentrations of antibiotics detected in the environment may have biological significance, based on the bioaccumulation seen in organisms exposed to sulfamethazine. Finally, techniques and methodology developed in this dissertation will be useful for future evaluations of impacts of other antimicrobial agents, particularly when considering the importance of bacterial endpoints and sediment-dwelling invertebrates.
Appendix

Additional objectives were pursued during the course of my dissertation research, and the methodologies developed are briefly described here.

**Objective 3. Determine the persistence of antibiotic-resistant *Enterococcus faecalis* in relation to non-resistant strains in aquatic microcosms**

The rationale behind this objective stems from the knowledge that livestock manure contains antibiotic-resistant and -susceptible microorganisms, and these microbes, along with antibiotic residues, are detected in surface water bodies throughout the U.S. Considering the expected environmental concentrations of the antibiotics are typically well below their minimum inhibitory concentrations (MICs), it is not expected that the antibiotic residues would induce resistance. However, it is not known whether the presence of antibiotic residues in these environments would provide any selective advantage to microbes already containing resistance traits. To determine the potential impact of antibiotic residues on maintenance of resistance, we developed an assay to determine the persistence of antibiotic-resistant *Enterococcus faecalis* in relation to non-resistant strains in aquatic microcosms in the presence or absence of antibiotic residues.

Resistant and susceptible *Enterococcus* strains were selected from environmental isolates collected at several established sampling locations along the South Fork of the Iowa River in north central Iowa (South Fork, Beaver Creek, and Tipton Creek). The South Fork of the Iowa River has been listed as an impaired water body, and has been
extensively studied by scientists at the USDA-ARS National Soil Tilth Laboratory (Ames, IA).

Isolation of *Enterococcus* from stream water samples used standard membrane filtration and isolation methods, as described by U.S. Environmental Protection Agency Method 1106.1. Isolates were plated on mE agar and incubated for 48 h at 35 °C. Dark pigmented colonies were selected from mE agar and were streak plated onto BHI agar and incubated 48 h at 35 °C. Creamy white colonies were selected and were re-grown on mE agar, as previously described. Dark pigmented colonies were plated on iron esculin agar and incubated at 37 °C. Colonies were also selected and grown in BHI broth with 6.5% NaCl for verification of *Enterococcus* identification on an orbital shaker at 37 °C for 72 h. Confirmed colonies received identification names of SF400A, BC350B, SF450A and B, and TC3525A and B. Cells were enumerated and harvested from the broth into phosphate buffer (pH 7.1). Cell counts were $3.9 \times 10^7$ to $1.4 \times 10^9$ CFU/ml.

To confirm antibiotic susceptibility or resistance, BHI agar was produced with a range of 0, 0.5, 1, 5, 10, 20, 50, 100, and 500 mg/l sulfamethazine (SMZ); agar was poured into Nunc Omni™ trays (86 x 128 mm) so plates could be inoculated using a stainless steel 96-pin inoculation stamp. Cell cultures were diluted to $10^7$ cells/ml to allow for enumeration, and a 96-well plate was prepared with 80 µl of culture per well, with 4 replicate wells per isolate. The stamp was flame-sterilized with 100% ethanol between all isolates. Trays were incubated at 37 °C for 48 h, then growth of isolates was recorded for each concentration of SMZ. Based on lack of growth at the 100 and 500 mg/l concentrations, TC325A and B were determined to be susceptible isolates. All other isolates were SMZ-resistant.
Prior to initiation of the microcosm tests, cells were plated on mE agar to confirm culture purity, and were grown in BHI broth for harvesting. Pond water (surface 15 cm) was collected from the Iowa State University Horticulture Farm Pond and was transported to the laboratory and stored at 4 °C for 48 h until use. Pond water microcosms consisted of 100 ml pond water in 250 ml Erlenmeyer flasks. Treatments included autoclaved pond water spiked to 0, 0.01, 1, or 100 mg/l SMZ, or non-autoclaved pond water at 0 or 1 mg/l SMZ. There were two replicates per treatment. The carrier solvent was 20% methanol, and final solvent concentration in microcosms was <0.5%. Microcosms were inoculated with a total of $10^5$ cells (TC325A = susceptible or SF450A = resistant), resulting in a concentration of 1000 cells/ml. Jars were incubated at 110 rpm at 30°C for 14 d. Aliquots (0.1 ml) of inoculated pond water were removed at 72 and 336 h, and were diluted and spread plated on BHI agar (0 mg/l SMZ), then incubated for 36 h at 35 °C. After 36 hr growth, a replica plating technique was performed to transfer colonies from control BHI agar onto BHI agar containing 0.5 or 500 mg/l SMZ to identify resistant colonies. Colony counts were taken to compare growth on the control BHI versus growth on SMZ agar.

A check of microcosm colony numbers at 24 h revealed that all replicates had $10^5$ cells/ml, thus indicating growth in the pond water. At 72 h, the susceptible TC235A strain had some resistance to SMZ, as demonstrated by growth on 500 mg/l SMZ agar. There were no differences in cell counts or resistance when comparing isolates grown in autoclaved pond water controls or pond water containing SMZ. At 14 days (336 h), cell counts in the susceptible strain were back to the original inoculation amount of $10^3$ cells/ml, while the resistant strain showed maintenance of growth, with cell counts at $10^5$. 
cells/ml. Replica plating showed that none of the susceptible strain colonies were able to
grow on the 500 mg/l SMZ agar after 14 days, but all colonies were able to grow on 0.5
mg/l SMZ agar. There was a slight, but non-statistically significant, decrease in cell
counts for the susceptible isolate growing in autoclaved pond water spiked to 100 mg/l
SMZ. There were no differences between treatments when examining susceptibility to
SMZ at 500 ppm (Figure 1); isolates incubated in 0 mg SMZ /l pond water were equally
susceptible as those isolates grown in 100 mg SMZ /l pond water. Resistant isolates grew
consistently better than susceptible isolates, and maintained their resistant traits during
the course of the 14-day study.
Figure 1. Percent of colonies able to grow on 500 ppm SMZ agar following incubation in pond water treated with SMZ.
Objective 5. Develop a dose-response method to evaluate the potential effects of sulfamethazine on gut microflora of invertebrates

Based on sulfamethazine’s bacteriostatic activity, and the bioaccumulation of sulfamethazine detected in our bioavailability studies, we thought it relevant to develop a dose-response method for evaluating potential effects of antibiotics on invertebrate gut microflora. Additionally, our fate data indicating that sulfamethazine was likely to adsorb to sediment, we chose the benthic-dwelling oligochaete *Lumbriculus variegatus* as our test organism. A brief description of experimental design follows.

The test species, *L. variegatus*, was obtained from existing cultures in the Department of Entomology at Iowa State University (Ames, IA). The worms were reared on shrimp pellets (H.B.H Pet Products, Springvill, UT) in aquaria containing distilled water with an unbleached shredded paper towel substrate at 24 ± 0.5°C and a 16:8 photoperiod. Adult worms (8 ± 1 mg fresh wt.) were selected and used in the study.

Individual shrimp pellets (1 cm in length) were treated with 10 µl of sulfamethazine spiking solution (acetone carrier) to achieve 0, 0.5, or 500 µg sulfamethazine /g food pellet. Acetone was allowed to evaporate in a fume hood for 2 h before adding the pellets to test chambers. Test chambers consisted of 60 ml jars filled with 50 ml ultrapure water. Ten worms were placed in each jar, and there were 4 replicate jars per treatment. Test chambers were maintained at 24 ± 0.5°C with a 16:8 photoperiod.

After a 3-day exposure period, worms were removed from the test chambers, rinsed twice with sterilized ultrapure water to remove any adhering bacteria or biomass,
and placed in sterilized 8 ml vials. Worms were manually homogenized using sterilized
glass rods for 5 min per replicate in 1 ml autoclaved ultrapure water. Next, DNA was
extracted from worm homogenates using a MoBio® Soil DNA Kit (MoBio Laboratories,
Carlsbad, CA), and following manufacturer’s protocols. This method extracts all DNA
from the homogenates, including worm DNA and bacterial DNA. Next, PCR was
performed to amplify bacterial DNA to allow for electrophoresis and community
fingerprinting. First, universal primers 1390r and 007f for 16SrRNA were used to
amplify bacterial DNA. Next, primers specific for the V3 region of 16s rRNA (357f -GC
and 519r) were used to further amplify bacterial DNA and prepare for analysis using
denaturing gradient gel electrophoresis (DGGE). PCR products were purified and
separated using DGGE, with a 35-50% urea-formamide denaturing gradient on 10%
acrylamide gels at 85 V for 16 h in 0.5 x TAE buffer at a constant temperature of 60°C.
Gels were stained with SYBR Gold and photographed with a Kodak Imaging Station.
Figure 2 displays the image of a gel obtained from the study described. There are four
lanes per treatment, with each lane corresponding to one replicate. The box surrounds the
region of interest for this particular combination of PCR product and denaturing gradient.
Variability in banding patterns indicates differences in microbial communities within the
worm homogenates. Few differences are seen in the image depicted, however a longer
exposure period would likely yield improved results; since sulfamethazine is
bacteriostatic, it would likely take more time to inhibit bacterial communities to a
noticeable degree (e.g. a typical treatment duration in livestock might be 7 days). Future
studies should be lengthened to at least 7-day exposures, and should also include
sediment as an exposure route.
Figure 2. Image of gel following DGGE of amplification products of bacterial DNA from *L. variegatus* homogenates following worm exposure to 0, 0.5, or 500 µg SMZ/g food pellet.
From Chapter 4. Fate of sulfamethazine in a freshwater sediment microcosm.

Table 3A. Mass balance of SMZ-residues in each treatment at day 14 and day 28.¹

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Remaining in pond water (%)</th>
<th>CO₂ (%)</th>
<th>Extracted from sediment (%)</th>
<th>Bound in sediment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWS</td>
<td>76.45 (1.10)</td>
<td>0.37 (0.09)</td>
<td>5.58 (0.75)</td>
<td>20.77 (0.98)</td>
</tr>
<tr>
<td>PWS+M</td>
<td>61.26 (2.07)</td>
<td>0.09 (0.02)</td>
<td>6.95 (0.97)</td>
<td>36.32 (1.96)</td>
</tr>
<tr>
<td>Autoclaved PWS</td>
<td>75.10 (1.15)</td>
<td>0.05 (0.006)</td>
<td>7.77 (0.24)</td>
<td>19.10 (0.83)</td>
</tr>
<tr>
<td>PW</td>
<td>92.29 (1.27)</td>
<td>0.19 (0.02)</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 28</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PWS</td>
<td>71.53 (2.10)</td>
<td>0.61 (0.02)</td>
<td>4.93 (0.38)</td>
<td>27.77 (1.00)</td>
</tr>
<tr>
<td>PWS+M</td>
<td>41.71 (1.12)</td>
<td>0.21 (0.04)</td>
<td>5.59 (0.25)</td>
<td>51.57 (0.89)</td>
</tr>
<tr>
<td>Autoclaved PWS</td>
<td>62.28 (3.22)</td>
<td>0.14 (0.04)</td>
<td>8.41 (0.81)</td>
<td>30.91 (2.01)</td>
</tr>
<tr>
<td>PW</td>
<td>91.65 (0.94)</td>
<td>0.66 (0.01)</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

¹ All values are shown as mean % of applied radioactivity with standard deviation listed in parenthesis.
Acknowledgements

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