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Fate of agrochemicals in wood chip denitrifying reactors and their impacts on wood chip microbial ecology

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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ABSTRACT

Subsurface tile drainage systems have contributed towards increasing agricultural production, but have also contributed towards water pollution by rapidly transporting excessive nutrient and agrochemicals to surface water and ground water. One of the pollution control strategies is to treat the tile drainage water or the contaminated subsurface water with denitrifying bioreactors. Wood chips have been used in denitrifying bioreactors, providing organic carbon and attachment surface area for denitrifiers. The focus of this research is to investigate fate of agrochemicals in wood chips from the in situ reactors and their potential effects on denitrification and the denitrifiers. The selected agrochemicals for study are atrazine, enrofloxacin, monensin and sulfamethazine.

Partition coefficients of atrazine, enrofloxacin, monensin and sulfamethazine were determined by single-point sorption experiments by using wood chips from an in situ reactor. Of the four chemicals tested, enrofloxacin had the highest partition coefficient (K_{ow}) while sulfamethazine had the lowest. Atrazine and monensin had moderate sorption coefficients. In addition, partition coefficients for the four chemicals for wood chips were larger than the partition coefficients for soils obtained close to the in situ reactor. Freundlich distribution coefficients (K_f) for isotherm studies for the four chemicals were in the order of (highest to lowest): enrofloxacin > monensin > atrazine > sulfamethazine. Desorption hysteresis were found for enrofloxacin, atrazine and sulfamethazine when the wood chips were desorbed by water. For monensin, the desorption aqueous phase concentrations were larger than the adsorption aqueous phase content. A possible reason for the larger desorption concentration was that the monensin adsorbed onto wood chips were on the eternal surface of the wood chips due to its larger molecular structure which allowed monensin to be easily desorbed. Only 5% of enrofloxacin, 14% of monensin, 23% of sulfamethazine and 25% of atrazine were recovered from the wood chips after two desorption and an acetonitrile-water extraction indicating the strong binding of the chemicals onto wood chips.

Degradation studies with atrazine, enrofloxacin, and sulfamethazine onto wood chips indicate that a large majority of the chemical mass was removed from the aqueous phase within the first 48 hours followed by a slow removal over time. Dissipation rates were estimated using the availability-adjusted first-order degradation model. Disappearance of

sulfamethazine was slower than disappearance of enrofloxacin and atrazine. No impact on denitrifiers as measured by the denitrification potential assays, most-probable-number (MPN) and *nosZ1* copy number was found for atrazine at an initial concentration of 5 mg L^{-1}. The MPN was reduced under enrofloxacin treatment after 2 days of the incubation; however, at the end of the experiment the denitrifier MPN was similar to control treatment MPN. Sulfamethazine was found to initially impact the denitrification (both MPN, *nosZ1* copy number and denitrification potential) but after 5 days the denitrification potential assays, most-probable-number (MPN) and *nosZ1* copy number were found to be similar to that of the control.

CHAPTER 1. INTRODUCTION

1.1 General Introduction

Subsurface tile drainage systems can improve agricultural production by draining and maintaining the subsurface water levels of the fertile lands. However, the tile drainage can negatively impact surface and subsurface water quality through contamination by nutrients such as nitrates and organic contaminants (i.e., pesticides and veterinary antibiotics). Nitrate (NO_3^--N) pollution is a concern as it can cause eutrophication in lakes and rivers and hypoxia conditions as in the Gulf of Mexico. Subsurface tile drainage water is one of the major contributors of NO_3^--N in the upper Midwest (Nangia et al., 2010). The sources of nitrogen in tile water are fertilizers (50%) and animal manure (15%) (Goolsby and Battaglin, 2000). Reduction in nitrogen-based fertilizer use may not be sufficient to decrease the NO_3^--N concentration to levels of minimum impact. As such, novel management and control strategies are needed. One of the strategies is the treatment of nitrate by in situ denitrifying reactors and denitrifying walls where nitrate is reduced when tile water flows through them under denitrifying conditions (Schipper and Vujdovic-Vukovic, 2000, Jaynes et al., 2008, Greenan et al., 2006). In situ reactors or denitrifying walls are typically constructed with a mixture of organic residues such as wood chips or saw dust, and sand to create the denitrifying conditions.

In addition to denitrification, the wood chips in the bioreactors can also remove organic contaminants such as herbicides and insecticides (Boudesocque et al., 2008), lipophilic organic compounds (Trapp et al., 2001) and phenolic compounds (Barrera – Garcia et al., 2008). Pesticides and herbicides use have improved crop yields but their presence in various media can be detrimental to the environment. Pesticides and herbicides application have increased rapidly since 1950 with a 1992 pesticides market value of US$ 25,200 million in North America, Latin America, and Eastern Asia countries and a 1992 herbicide market of US$ 11,440 million in the world (Yudelman et al., 1998). In recent years, attempts have been made to reduce consumption rates. Major concerns with pesticide and herbicides use include development of resistance in target species, injury in non target species and overall human and ecological damage such as decrease in the number of bird species in the United

States (Wheeler, 2002). Another concern is the endocrine disruptor activity of herbicides such as atrazine which can impact reproduction and growth development (Sass and Colangelo, 2006). For example, as a result of exposure to 2.5 $\mu g\ L^{-1}$ of atrazine, African clawed frogs (*Xenopus laevis*) breeding gland size of about 1.8 μm^2 was found to decrease, spermatogenesis and fertility were reduced and demasculinization of laryngeal development was observed (Hayes et al., 2010).

In addition to pesticides, veterinary pharmaceuticals and their metabolites can enter the surface and subsurface water after application of manure in the fields. Microorganisms can develop resistance to these veterinary pharmaceuticals which in turn can indirectly impact human health through the ineffective treatment of these microorganisms by the antibiotics (Casewell et al, 2003). Interactions of antibiotics with soil microorganisms include: impact on the degradation or detoxification of the anthropogenic chemicals by soil microorganisms, inhibition of growth of certain communities, changes in relative abundances of communities among each other, and development of resistance for survival (Kemper, 2008).

At this time, the fate of nitrate in denitrifying in situ bioreactors or denitrification walls has been evaluated, but the fate of agrochemicals and veterinary pharmaceuticals in wood chip reactors and the effect of these chemicals on microbial activity are unknown. Several factors such as the type and age of wood chips in the bioreactors, pH conditions, and flow rates through the bioreactors will affect the sorption and degradation of the agrochemicals chemicals while the concentration levels of the agrochemicals may affect their biodegradation in the bioreactors. The overall goal of this study is to understand the fate especially the partition behavior of these agrochemicals onto bioreactor wood chips and their potential impact on denitrification potential in the bioreactors. Chemicals selected for that study were atrazine, a widely used herbicide for corn; and three veterinary antibiotics commonly found in manure: enrofloxacin (a fluoroquinolone), monensin (an ionophore) and sulfamethazine (a sulfonamide). The specific objectives of this study were:

1) Investigate the sorption-desorption of the selected chemicals onto wood chips and compare the sorption results to the sorption onto soils obtained close to the wood chips bioreactor
2) Investigate the degradation/dissipation of the selected compounds in wood chips

3) Investigate the interference of the selected chemicals on the denitrification potential of the reactors and the denitrifier community.

1.2 Thesis Organization

The thesis contains five chapters with Chapter 1 providing a broad overview of the issues and the goals and objectives of the study. Chapter 2 consists of the literature review providing information on wood chip bioreactors, microbial processes occurring in the bioreactors, environmental concentrations, risks, and sorption and degradation of the selected chemicals. Chapter 3 discusses the batch sorption experiments of the selected chemicals onto wood chips and soils. Chapter 4 describes the degradation/disappearance of selected chemicals in wood chips and impacts of their presence on denitrifying communities. Finally, Chapter 5 provides the main conclusions of the batch sorption and degradation studies and implications and work for future research.

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CHAPTER 2. LITERATURE REVIEW

This chapter consists of two parts: a discussion on wood chip in situ bioreactors for nitrate removal and a discussion on potential agrochemicals that can be removed by the wood chip bioreactors and their interactions with wood chips and the microbial community established on them.

2.1 Denitrification and Wood Chip Bioreactors

In the upper parts of the Midwest, excessive water in soils are drained with subsurface tile drainage system to increase agricultural production. Dissolved nutrients, sediment and soil particles and pesticides can be transported rapidly from the field through the tile drainage system to water bodies and as a result impair surface and subsurface water quality (Randall et al., 1997). Nitrate (NO_3^--N) contamination of surface water is of concern especially in the Midwest. The total annual nitrogen load in the Gulf of Mexico between 1980 and 1996 was estimated to be 1,568,000 tonne yr^{-1}, where 61% of the N load was NO_3^--N. Most of the nitrogen in the Mississippi basin comes from agricultural lands in southern Minnesota, Iowa, Illinois, Indiana and Ohio (Goolsby et al., 2001) where tile drainage is a common practice.

There have been efforts to reduce the nitrogen in surface and ground waters, most commonly via denitrification. However, in subsurface environments, denitrification is limited due to the availability of organic carbon (Yeomans et al., 1992, Cambardella et al., 1999). Smith et al. (2001) proposed adding formate as an electron donor for the denitrifiers in the remediation of nitrate-contaminated groundwater where they found decrease of nitrate and formate concentrations by 80-100% and 60-70%, respectively. At the field scale, nitrate in groundwater can be reduced via denitrification by placing a porous media such as sawdust or wood chips in the flow path of the groundwater (Robertson and Cherry, 1995). Schipper and Vojdovic-Vukovic (2000) constructed a denitrification wall of a sawdust-sand mixture (30% sawdust) and observed a reduction in nitrate concentrations from 5-16 mg NO_3^--N L^{-1} to below 2 mg N L^{-1} in 1-10 days. Denitrifiers on the media are responsible for nitrate removal. In a wood chip bioreactor, the carbon source is provided by the wood chip or sawdust itself, and the media is kept submerged to keep the conditions anaerobic (Jaynes et al., 2008).

Long-term nitrate removal studies by denitrification walls indicates that nitrate loss was via denitrification (Robertson et al., 2000) and not by immobilization of nitrate on the media (Greenan et al., 2009). Jaynes et al. (2008) compared NO_3^--N losses in tile drainage over 5 years in field-scale conventional drainage system, deep tile system and denitrification wall (with wood chips) and found that NO_3^--N concentrations in the deep tile system (0.6 m deeper than the pipes in the conventional system) and the conventional system did not differ significantly, while the denitrification wall (0.6 m wide x 1.83 m deep) reduced nitrate load in tile water on average by 55% with a mass loss of 29 kg N ha^{-1}.

Blowes et al. (1994) used a fixed-bed reactor made of a mixture of coarse sand and tree bark, wood chips or leaf compost treated tile drainage water at a rate of 10-60 L day^{-1} containing 3-6 mg L^{-1} NO_3^--N. Greenan et al. (2006) used a mixture of various carbon sources (wood chips, wood chips amended with soybean oil, cornstalks, and cardboard fibers) and subsurface soil as the media for an in situ reactor and found that after 180 days of incubation, nitrate removal with wood chips (80.13%) was less efficient than removal with corn stalks (91.75%). However, removal rate of nitrate was found to be steady over a longer period with wood chips indicating that wood chips would be more effective in the field than corn stalks (Greenan et al., 2006). Saliling et al. (2007) evaluated wood chips and wheat straw as an alternative to plastic media for treatment of aquaculture wastewater with 200 mg NO_3^--N. They used 3.8-L reactors (40-cm packed height x 10-cm diameter) and removed 99% of nitrate in wastewater with a denitrification rate of 1330 g N $m^{-3}d^{-1}$ for plastic media (Kaldnes plastic) and 1360 g N $m^{-3}d^{-1}$ for wood chips and wheat straw media. For nitrate removal wood chips and wheat straw are low-cost media compared to plastic media, however, 16.2% and 37.7% of the masses of wood chips and wheat straw, respectively, were lost in 140 days. Van Driel et al. (2006) used lateral flow (13 m x 1.2 m x 1.1 m) and upflow (10 m x 2 m x 0.8 m) reactors consisting of coarse (1-50 mm) and fine (1-5 mm) wood mixture to treat nitrate in tile water at a cornfield and a golf course site. They monitored nitrate removal rates for 26 months. Annual NO_3^--N removal rate of 12 kg N yr^{-1} in the cornfield was maintained when the flow rate was 7.7 L min^{-1} and the nitrate load in influent was 11.8 mg N L^{-1}. For the golf course field reactor, 3.1 kg N yr^{-1} removal rate was achieved when the flow rate was 7.8 L min^{-1} and the nitrate concentration was 3.2 mg N L^{-1}.

Greenan et al. (2009) found a correlation between the water flow/nitrate loading rate and nitrate removal rate with a laboratory column study where nitrate removal efficiency of 100, 64, 52 and 30% was achieved for flow rates of 2.9, 6.6, 8.7, 13.6 cm d^{-1} respectively. Chun et al. (2009) evaluated the impact of water flow rate and retention time on nitrate removal of a wood chip subsurface bioreactor. At high retention times (low flow rate, i.e., 5.3-6.8 cm s^{-1}) and low retention times (high flow rates, i.e., 20-28 cm s^{-1}) nitrate removal rates of 100% and 10-40 %, respectively were observed in a laboratory-scale polyvinyl chloride (PVC) pipe (0.25 m in diameter x 6.1 m in length) column study. They suggested that the decrease in nitrate reduction rate at high flow rates may be due to wash off of the biofilm on the bioreactor.

Denitrification can be performed by a variety of microorganisms by using oxidized nitrogenous compounds as electron acceptors. This key process occurs in soils, sediments, wastewater treatment plants and wood chip denitrifying reactors. The rapid transformation of nitrite to nitrogen gas prevents accumulation of nitrite and nitric oxide in the environment which can influence environmental quality (Ka et al., 1997). In order to determine the activity of denitrifiers, two major approaches can be employed: by conducting short-term denitrification enzyme activity assays and by measuring the denitrification potential (Luo, 1996). Both denitrification enzyme assay (DEA) and denitrification potential determine the denitrification rate based on nitrous oxide (N_2O) production when conversion of N_2O to N_2 is blocked. The obstacles of measuring denitrification activity are rapid conversion of N_2O to N_2 and bias in measurements due to cell growth and synthesis of new N_2O reductase. Tiedje et al. (1989) reported that acetylene gas inhibits conversion of N_2O to nitrogen (N_2 gas) by inhibiting nitrous oxide reductase and can be used in denitrification rate estimation studies. DEA should be performed in short time periods to minimize interference of enzyme production of new organisms (Luo, et al., 1996). Tiedje et al. (1989) suggested measurement of denitrification activity in the presence of chloramphenicol to inhibit synthesis of new denitrifying enzymes. The measurements are generally considered as denitrification potential rather than denitrification activity because in laboratory experiments, the microorganisms are under optimum conditions (anaerobic and nitrate is not limiting at all times) and, therefore,

denitrification rate is at its maximum value, which may not be reflecting the actual activity in soils or natural environments.

On the other hand, when the soil denitrifier populations need to be measured, the most probable number (MPN) is used. Lensi et al. (1995) enumerated soil denitrifier bacteria by incubating 10-fold soil dilutions in potassium nitrate (5 mM) and cyclohexamide media at 28 °C for 2 weeks and checking the presence of nitrite and nitrate with Griess–Ilosway's and Morgan's reagents. Lensi et al. (1995) eliminated fungi, which were found to be important contributors to denitrification (Appleford et al., 2008).

Populations based on MPN may not reflect all of the denitrifier population (Martin et al., 1988), as not all microorganisms can be cultured in the selected media. More recently, molecular tools were developed to measure the abundance of denitrifiers. Quantitative polymerase chain reaction (qPCR) protocols were developed and applied by several researchers (Henry et al., 2006, Miller et al., 2009, Nogales et al., 2002, Smith et al., 2007, Siciliano et al., 2007) to determine the prevalence of genes encoding enzymes catalyzing denitrification reactions. Amplified functional genes involved in denitrification are: the nitrate reductase encoding gene *nar* (Lu et al., 2007) and the periplasmic nitrate reductase encoding gene *napA* (Flanagan et al., 1999), nitrite oxide reductase gene *nirS* (Braun and Zumft, 1992) and nitrous oxide reductase gene *nos*Z (Henry et al., 2006). N_2O reductase can be purified from only gram negative bacteria (Coyle et al., 2005, Synder et al., 1987) and is a key enzyme that is not present in all denitrifiers (Henry et al., 2006). For instance, *Agrobacterium tumefaciens* is able to synthesize periplasmic nitrate reductase, copper nitrite reductase, and nitric oxide reductase with genes encoding them, but is unable to synthesize nitrous oxide reductase and produce N_2 (Wood et al., 2001). Examples of primers used in qPCR include *nosZF* and *nosZR* primers designed by Kloos et al. (2001) and degenerate primers of *nosZ1* (259 bp) and *nosZ2* (267 bp) from *nos*Z sequence of *Pseudomonas fluorescens* designed by Henry et al., (2006).

2.2 Selected Organic Contaminants in the Environment

2.2.1 Consumption rates and usage

In the U.S., 556 million lb of herbicide active ingredient was applied which made herbicides the most widely used pesticides (Short and Colborn, 1999). Triazines are most commonly used for weed control in many crops including corn, sorghum, citrus orchards, olive groves, fruit trees, grapes, sugarcane and Christmas trees (Cabrera et al., 2008). The total use of triazines accounted for 43% of all herbicides in Europe in 2003 (Eurostat, 2007). Among all herbicides, atrazine was applied more than others with 68-73 million lb in 1995 in United States (Short and Colborn, 1999). In Quebec, Canada, 27% of all pesticide sales were atrazine in 1985 (Cossette et al., 1988). In 1988, 1,045,110 kg active ingredient of atrazine was applied, comprising of 15% of all pesticide application in Ontario, Canada (Moxley et al., 1989).

To conserve the necessary nutrients for agricultural production, manure is used in many farms. Although manure has the benefit of not using synthetic fertilizers, veterinary pharmaceuticals used to control bacterial diseases in livestock and promote meat production enter the environment via manure application to the field (Tolls, 2001). Typical classes of veterinary pharmaceuticals the most common used in farm animals are tetracyclines (tetracycline, chlorotetracycline and oxytetracycline), macrolides (tylosin and erythromycin), sulfonamides (sulfamethazine and sulfamethoxazole), fluoroquinolones (enrofloxacin and sarafloxacin) and ionophores (monensin and lasalocid).

In European Union countries and Switzerland, about 13,288 tons of antibiotics were used in 1999 (FEDESA, 2001). In industrialized countries, sulfonamides are one of the more widely used antibiotic classes (Campbell, 2002). In the U.S., sulfonamide use ranks fourth among all the antibiotics sold for animal husbandry (AHI, 2001). Annually in the U.S., 1.5 million kg of monensin is used for cattle and poultry production, accounting for 13% of the total subtherapeutic usage for animal husbandry (Mellon et al., 2001). Total quinolone production in the U.S., European Union, Japan and South Korea was about 120 tonnes in 1998 while the annual quinolone consumption in China is 470 tons for animal health purposes (WHO, 1998).

2.2.2 Characteristics of atrazine, enrofloxacin, sulfamethazine and monensin

Triazines are pre-emergent herbicides, applied directly to the soil or crop as aqueous sprays and are the most widely used herbicides (Cabrera et al., 2008). The most commonly used s-triazine is atrazine which was selected to represent triazines in this study. The chemical name of atrazine is 6-chloro-N-ethyl-N'-isopropyl-1,3,5-triazine-2,4-diamine (Formula: C8H14ClN5, CAS Number: 1912-24-9; molecular weight-215.69) and its chemical structure is illustrated in Figure 1a. It is moderately soluble in water with a solubility of 28 mg L-1 at 20 C° (Worthing and Walker, 1987) and a pKa of 1.7. In acidic waters (pH = 5) at 20 °C, degradation of atrazine occurs via hydrolysis and N-dealkylation while in neutral or alkaline waters, breakdown is relatively negligible (Cohen et al., 1984). In soils, it is persistent in temperate climates (Ashton, 1982). Due to its potential to contaminate ground water, it is classified as Restricted Use Pesticide (RUP) by EPA (EPA, 2008).

Fluoroquinolone is a class of pharmaceuticals, basically derived from nalidixic acid and polycyclic derivatives. Fluoroquinolones (FQs) were discovered in 1960s and were used to treat urinary infections in humans. The most widely used fluoroquinolone in human medicine is ciprofloxacin, a second generation FQ. They are also used for agriculture, and veterinary purposes (Picó and Andreu, 2007) due to their activity against a broad spectrum of microorganisms such as *E. coli* and *Pasteurella multocida* and Salmonella causing diseases in livestock (Prescott et al., 2000). For veterinary use, enrofloxacin is one of the most important fluoroquinolones (Picó and Andreu, 2007) used to control infections in chickens, cows and pigs. For cattle, sheep and goats, fluoroquinolones are used to treat acute respiratory diseases, and in the U.S. they have been only approved for treatment of pneumonia. For the swine industry, FQs are administered for *Mycoplasma hyopneumoniae* infections. For poultry, sarafloxacin and enrofloxacin are approved for the treatment of *E. coli* infections (Prescott et al., 2000).

Enrofloxacin (CAS Number: 93106–60–6, Formula: $C_{19}H_{22}FN_3O_3$, molecular weight: 359.4) has an ethyl group attached to the piperazine ring and various functional groups that can ionize (see Figure 1b). Dissociation constants of enrofloxacin are; pK_{a1} = 5.94 (carboxylic acid in 3–position) and pK_{a2} = 8.70 (piperazinyl group in the 7-position). Depending on the pH, enrofloxacin can be found as an acidic cation, a neutral un-ionized

form, an intermediate zwitterion and a basic ion. At low pH, protonation of the carboxyl and piperazinyl groups occur (Lizondo et al., 1997).

FQs are slightly soluble in water, but most of FQs are lipophilic (Picó and Andreu, 2007). Enrofloxacin is a broad–spectrum antibiotic, controlling mostly Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Enterobacteraceae* (Angulo et al., 2000). Its mode of action is to inhibit the bacterial DNA gyrase enzyme (Prescott et al., 2000). The cyclopropyl group at N-1 position enhances its activity on both Gram-positive and Gram-negative, while the ethyl group on piperazine ring enhances its adsorption and decreases its antipseudomonal activity (Walker et al., 1990; 1992). Minimum inhibition concentrations of enrofloxacin for target species vary between 0.03 and 2 µg mL^{-1} (Walker et al., 1990; 1992).

Sulfonamides are derived from sulfanilamide and are broad-spectrum antimicrobials. They are effective against both Gram-positive and Gram-negative bacteria including *Chlamydia spp.* (Baroni et al., 2008). Their mode of action is on folic acid biosynthesis in bacteria by competing for dihydropteroate synthetase which interferes with the incorporation of para–aminobenzoic acid (PABA) with the folic (pteroylglutamic) acid. The chemical structure of sulfamethazine is illustrated in Figure 1c. pK_a values for sulfonamides vary from 5.0 to 10.4 (Prescott et al., 2000). The essential part of the molecule is the para-NH_2 group with the amide NH_2 group substitutions changing the antimicrobial activity of the compound. Minimum inhibition concentrations (MIC_{90}) of sulfonamides can be as low as 2 µg L^{-1} and can be as high as 515 µg L^{-1} for Gram-negative aerobes (Prescott et al., 2000).

Sulfamethazine (4-amino-N-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide) (CAS Number: 57–68–1, Formula: $C_{12}H_{14}N_4O_2S$, molecular weight: 278,33) is the most widely used sulfonamide for animal husbandry (Huang et al., 2001). The water solubility of sulfamethazine is 1500 mg L^{-1} at 29 ºC (Merck Index, 2001). The chemical structure of sulfamethazine is presented in Figure 1c.

The ionophores are a relatively new class of antibiotics, most commonly used to increase feed efficiency and anticoccoidal activity. They are fermentation products of *Streptomyces* species. The mechanism of inhibiting bacteria by ionophores is to change cell membrane permeability by complexing with sodium anions on the membrane and driving passive extracellular potassium ion transport, which replaces hydrogen ions and lowering the

intracellular pH. They are more effective on Gram-positive bacteria than Gram-negative bacteria, which favor production of propionic acid rather than acetic acid and butyric acid in rumen flora (Prescott et al., 2000).

Monensin, a commonly used ionophore, is a polyether monocarboxylic acid, produced by *Streptomyces cinnamonensis.* Formula of monensin sodium salt is $C_{36}H_{61}NaO_{11}$ with a molecular weight of 692.85 and CAS Number of 22373–78–0. The water solubility of monensin is 4.8-8.9 mg L^{-1} (ELANCO, 2006) and the pK_a value is 10.5 (Hoogerheide and Popov, 1979). Monensin sodium salt comprises of 4 factors: A, B, C, and D. Factor A constitutes more than 90% of monensin while factors B, C and D form the rest of the molecule (ELANCO, 2006). The chemical structure of monensin A is shown in Figure 1d. Although ionophores are more active on gram positive bacteria, monensin is used to control some gram negative bacteria including *Campylobacter* spp., *Brachyspira (Serpulina) hyodysenteria*, coccidia and *Toxoplasma* (Prescott et al., 2000). Additionally, more than 90% of monensin is excreted together with the manure (Donoho, 1984).

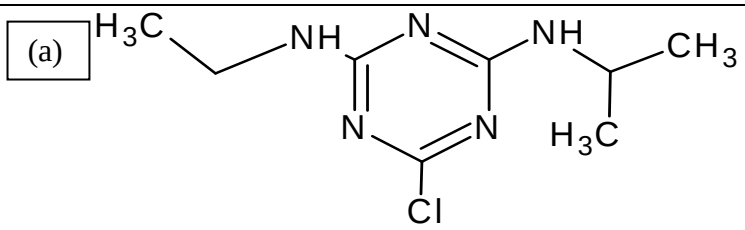
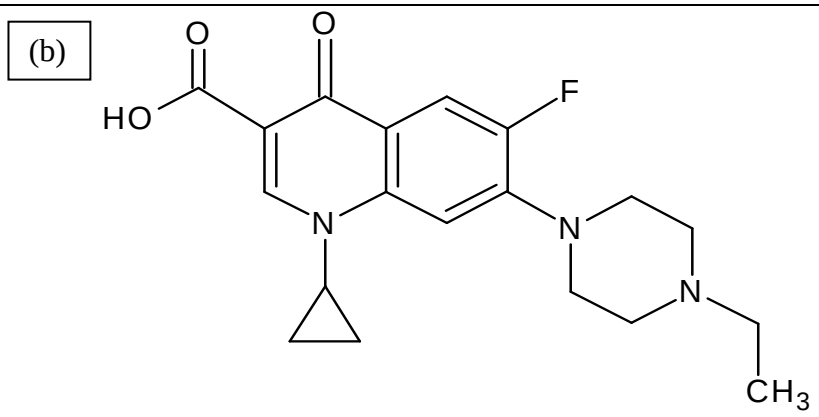
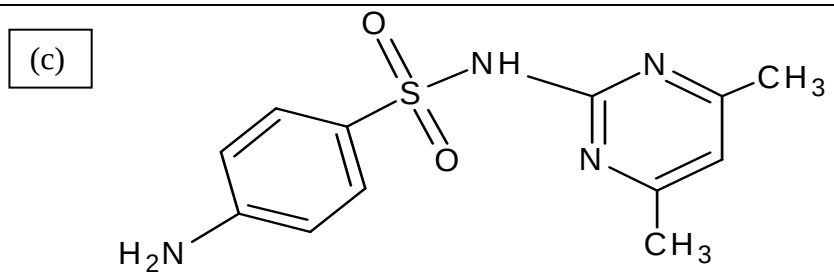
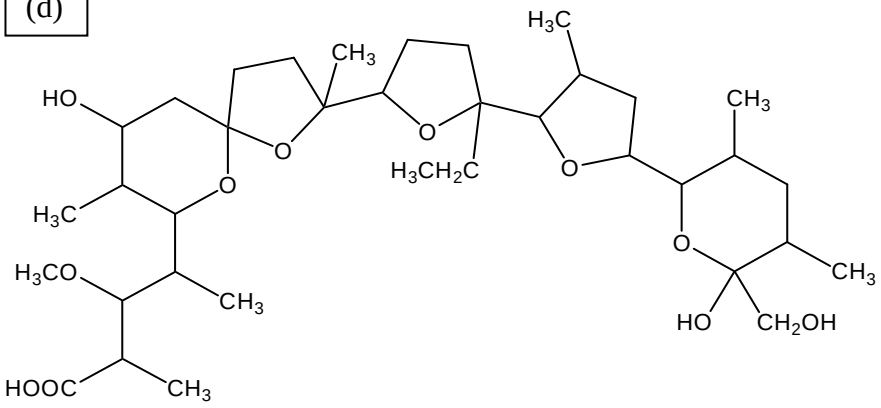
Chemical	Structure
Atrazine	
Enrofloxacin	
Sulfamethazine	
Monensin A	

Figure 1. Chemical structures of (a) atrazine, (b) enrofloxacin, (c) sulfamethazine, and (d) monensin A.

2.2.3 Sources and environmental concentrations

Atrazine concentrations in surface waters and ground waters have been monitored in most parts of the world. In St. Lawrence River (Canada), measured atrazine concentrations were 10.4 and 3.4 µg L^{-1} in 1990 and 1991, where concentrations in the tributaries of the river were 31.1 and 27.9 ng L^{-1} for the same years (Lemieux, et al., 1995). Likewise, mean atrazine concentrations in 147 Midwestern streams were monitored between 1989 and 1998 where the concentrations ranged between 4.27 and 10.9 µg L^{-1} (Scribner et al., 2000). The same study showed that atrazine concentrations were higher than the concentrations of other herbicides such as alachlor and cyanazine. A two-year monitoring study by Wu (1981) showed that atrazine concentrations in an estuary of the Chesapeake Bay were between 6 and 190 ng L^{-1} and were unexpectedly higher in rainwater with concentrations ranging from 3 to 2190 ng L^{-1}. In tile water from Waseca, MN, estimated concentration of atrazine was 1.24 µg L^{-1}in 1987 (Buhler et al., 1993).

Veterinary antibiotics on the other hand were found in water sources at concentrations usually lower than pesticide concentrations. Kolpin et al. (2002) reported mean concentrations of sulfamethazine of 0.02-0.22 µg L^{-1} in surface waters in a monitoring study of more than 100 rivers. Sulfonamides are highly water soluble and have low octanol-water partition coefficients (K_{ow}), and therefore are mobile in soils and pose risks in contaminated groundwater (Batt et al., 2006). A sulfonamide monitoring study in Idaho revealed wells of nearby confined animal feeding operations were contaminated with both sulfamethazine and sulfadimethoxine at concentrations of 0.076-0.22 µg L^{-1} and 0.046-0.068 µg L^{-1}, respectively (Batt et al., 2006). They also measured high nitrate concentrations (up to 39.1 mg L^{-1} nitrate) in the same wells. Besides surface and subsurface waters, sulfonamides have been detected in soils at concentrations as high as 11 ng g^{-1} (Höper et al., 2002).

2.2.3 Transport and fate in soils

Parent and daughter compounds of pesticides and veterinary medicine in manure enter the environment directly through their applications to cropland. After agrichemicals come in contact with soil, the compounds may partition into soil particles, may leach into ground water via soil and percolation water, and undergo biotic or abiotic degradation.

2.2.3.1 Sorption

Sorption is one of the major processes affecting the fate of organic compounds in the environment. In order to predict movement of agrichemicals in soils, partition coefficients are estimated. Atrazine sorption was found to be strongly correlated to the total organic carbon or matter content of the soils. In addition, the aromaticity of the organic matter was also found to influence atrazine sorption (Spark and Swift, 2002, Kulikova and Perminova, 2002). Work done by Novak et al. (1997) indicates that besides soil organic carbon, clay content and pH of the soil also have an impact on sorption of atrazine on soils. Moorman et al. (2001) reported Freundlich partition coefficients (K_f) of 0.43, 0.51 and 0.55 for atrazine sorption onto subsurface oxidized till, loess and alluvium, respectively. Dissolved organic matter addition to soil increased atrazine sorption coefficients (K_d) by a factor of 1.1 to 3.1 (Ling et al., 2006). Atrazine adsorption onto fluvo-aquic soil was found to increase from 24% to 77% by increasing the contact time from 24 hours to 72 hours (Deng et al., 2007).

While it is common to explain partitioning of pesticides in soils based on organic carbon content of the soils, this rule does not typically apply for all veterinary antibiotics (Kümmerer, 2004) where only weak correlations between organic carbon normalized sorption coefficients (K_{oc}) and octanol-water partition coefficients (K_{ow}) have been observed by Tolls (2001). Non-hydrophobic interactions including surface complexations, H-bonding, and ion exchange should be taken into consideration in predicting sorption behavior of both pharmaceuticals and pesticides (Tolls, 2001).

Lertpaitoonpan et al. (2009) found that organic carbon content of the soil and the pH influence sulfamethazine sorption onto soils with linear sorption coefficients (K_d) of 0.58 and 3.91 L kg^{-1} at pH 5.5 with organic carbon contents of 0.1% and 3.8%, respectively. When pH was at 9, the K_d values decreased to 0.23 and 1.16 L kg^{-1} for soils with organic carbons of 0.1% and 3.8%, respectively. When the pH of the soil was below the sulfamethazine pK_{a2}, hydrophobic sorption was the dominant sorption mechanism but when the pH was above pK_{a2}, surface sorption was also involved (Lertpaitoonpan et al., 2009). Higher sulfamethazine sorption was observed with an increase in humic acid on smectite clay minerals (HA:clay mass ratio of 1:5) due to the abundance of carboxyl moieties and aliphatic carbon content of the humic acid-clay complex (Gao and Pedersen, 2010).

Nowara et al. (1997) found that fluoroquinolone carboxylic acid derivatives (fluoroquinolones) sorb strongly onto clay particles causing an expansion of the spacing between the layers of montmorillonite clays. They estimated that sorption coefficients for enrofloxacin onto clay minerals ranged from 260 and 5610 L kg^{-1}. Freundlich sorption coefficients (K_f) of enrofloxacin onto loamy sand (OC - 2.27%) and sandy soil (OC - 0.59%) were found to be 0.66 and 0.32 L kg^{-1}, respectively (Ötker Uslu et al., 2008).
In contrast to sorption of fluoroquinolones, sorption of monensin onto soils was lower with a K_d of 9.3 L kg^{-1} (Kumar et al., 2005). Linear sorption coefficients of monensin onto soils were found to be between 0.915 (for a soil with CEC of 4.3 $cmol_{(+)}$ kg^{-1}) and 33.7 L kg^{-1} (for a soil with CEC of 26.5 $cmol_{(+)}$ kg^{-1}) which corresponded to K_{oc} values of 143 and 1160 L kg^{-1} (Sassman and Lee, 2007).

2.2.3.2 Degradation

Degradation of triazines in soils depends on the soil type and environmental conditions including soil temperature and soil pH (Bowman, 1989). Biodegradation of atrazine and its metabolite production in various environments and a variety of microorganisms are documented in Behki et al. (1986) and Giardina et al. (1980). Biodegradation of atrazine in wetland soils under anaerobic conditions was found to occur with the production of hydroxyatrazine, deethylatrazine and deethylatrazine. Atrazine was found to have a half life of 38 days in anaerobic wetland soils at 24 °C producing hydroxyatrazine and deethylatrazine (Seybold et al., 2001). The s-triazine ring which is a hexameric structure is shown in Figure 2 and the common atrazine metabolites found for aerobic and anaerobic conditions are listed in Table 1. Atrazine in subsurface soils has a half life of 5.2 yr (Arena subsurface soil) and 1.4 yr (Waunakee subsurface soil) (Rodriguez and Harkin, 1997)

Donnelly et al (1993), investigated atrazine (1-4 mM) degradation by 9 mycorrhizal fungi species in the presence of ammonium tartrate (0.0, 1.0, and 10.0 mM) as nitrogen source. At the end of 8-week incubation period, incorporation of atrazine molecules into biomass ranged between 0.59 and 11.38% for the fungal species. Shapir et al. (1998) took sediment samples from a shallow aquifer (from 210-230 cm) in a corn field receiving atrazine and amended it with atrazine solution (0.01 mg L^{-1} to 10 mg L^{-1}) and *Pseudomonas*

sp. strain ADP culture. They found that atrazine mineralization by this strain in 4 days varied between 75% and 48% at atrazine concentrations of 0.01 mg L^{-1} and 10 mg L^{-1}, respectively. The major degradation step of atrazine in soils by *Pseudomonas sp.* strain ADP is dechlorination (Shapir and Mandelbaum, 1997). *Delftia acidovorans* D24 strain isolated from Danube river water, Hungary was found to mineralize atrazine (100 µg L^{-1}) as both carbon and nitrogen source, producing primarily hydroxyatrazine as the intermediate product (Vargha et al., 2005).

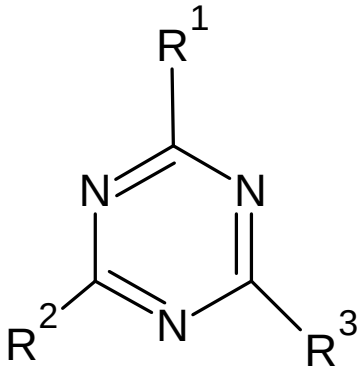


Figure 2. General structure of s-triazine ring

Table 1.Atrazine and its major metabolites in the environment (functional groups (R) adopted from Radosevich et al., 1995).

Common Name	Chemical Name	Functional Groups		
		R^1	R^2	R^3
Atrazine	2-Chloro-4-ethylamino-6-isopropylamino-striazine (CIET)	Cl	C_2H_5NH	C_3H_7NH
Deethylatrazine	2-Chloro-4-amino-6-isopropyl-amino-striazine (CIAT)	Cl	NH_2	C_3H_7NH
Deisopropylatrazine	2-Chloro-4-ethylamino-6-amino-*s*-triazine (CEAT)	Cl	C_2H_5NH	NH_2
Deethyldeisopropylatrazine	2-Chloro-4,6-diamino-*s*-triazine (CAAT)	Cl	NH_2	NH*2*
Hydroxyatrazine	2-Hydroxy-4-ethylamino-6-isopropylaminos-triazine (OIET)	OH	C_2H_5NH	C_3H_7NH
Deethylhydroxyatrazine	2-Hydroxy-4-amino-6-isopropyl-amino-striazine (OIAT)	OH	NH_2	C_3H_7NH

Sulfonamides can be degraded by photo catalytic activity. Half-lives of sulfamethoxazole in pond water and sediment under light conditions (7.3 days and 4.9 days) were shorter than under dark conditions (47.7 days and 10.1 days) (Lai and Hou, 2008). The same study showed that half lives of four sulfonamides (sulfadiazine, sulfadimethoxine, sulfamethazine and sulfamethoxazole) at 50 mg L^{-1} initial concentration in pond water under light conditions ranged from 8.0-48.9 days for sterile treatments and 1.7-7.3 days for non sterile treatments.In a similar manner, half lives of that sulfonamide compounds (sulfadiazine, sulfadimethoxine, sulfamethazine and sulfamethoxazole) in sterile and non-sterile sediment ranged between 6.5-47.3 days and 0.7-5.4 days, respectively, indicating microbial degradation was occurring. Another study emphasizing importance of microbial degradation of sulfonamides by Accinelli et al. (2007) showed sulfonamide persistence in soils was lower when soils were amended with liquid swine slurry which is due to the higher microbial activity.

Fluoroquinolones are resistant to hydrolysis and degradation at high temperature making them fairly stable in the environment. However, they can be photolyzed under UV

light (Thiele-Bruhn, 2003). Photodecomposition of FQs occurs via oxidation, dealkylation, and cleavage of the piperazine ring (Sukul and Spiteller, 2007). Burhenne et al. (1997) estimated the half life of enrofloxacin to be about 36.2 minutes under an irradiation intensity of 200 W/m^2, where the degradation was found to be occurring at the piperazine ring.

Knapp et al. (2005) investigated enrofloxacin degradation and ciprofloxacin formation in outdoor mesocosms and estimated enrofloxacin half lives to be between 0.8 days and 72 days based on different light conditions. By deethylation of the ethylpiperazine ring, enrofloxacin can be phototransformed to ciprofloxacin. Biodegradation of enrofloxacin was found to be limited due to its bioavailability since it binds strongly to soil or manure (Wetzstein et al., 1999). Indigenous agricultural soil isolates of *Basidomycetes* can degrade enrofloxacin by cleaving the fluoro–aromatic bond (Wetzstein et al., 2005). White and brown wood–rotting fungi species such as *Gloeophyllum striatum* were found to mineralize 53% of enrofloxacin in 8 weeks of incubation (Martens et al., 1996). Parshikov et al. (2000) found that *Mucor ramannianus* can degrade 78% of dosed enrofloxacin (253 µM) in 21 days and enrofloxacin metabolites identified were enrofloxacin N–oxide (62%), N-acetylciprofloxacin (8%) and desethylene-enrofloxacin (3.5%).

2.2.4 Human health risks

The effects of pesticides on human health can vary depending on the compounds but in general the potential effects can be carcinogenic, skin or eye irritation and effects on the nervous and endocrine system. The risk of pesticides on human health has been documented by EPA (EPA, 2008). Pharmaceuticals, on the other hand, pose risks to human health by developing microbes that are resistant to the pharmaceuticals.

A major route of exposure of agrochemicals to humans is via consumption of contaminated of drinking water. A study on drinking water quality for North Carolina, Pennsylvania, Kentucky, Illinios and Indiana showed the presence of two most common herbicides used in agricultural production, atrazine and simazine and the metabolites of atrazine: diaminochlorotriazine (CAAT), deisopropyl-atrazine (CEAT), and deethylatrazine (CIAT) at concentrations as high as 26.25 µg L^{-1} (EPA, 2008).

Pharmaceuticals such as sulfamethoxazole, erythromycin-H_2O and chloramphenicol have been detected in surface waters at concentrations of 0.06-1.70 µg L^{-1} where concentrations of sulfamethazine and sulfamethoxazole can be as high as 0.16 and 0.47 µg L^{-1} in ground waters in Germany (Hirsch et al., 1999). Potential effects of drinking water contaminated with pharmaceuticals include endocrine system disorders in humans and animals and a reduction in the efficiency of antibiotic treatment (Kümmerer, 2004).

2.2.5 Microbial ecology risks

Pesticides and pharmaceuticals may impact the quality and quantity of soil microbial communities when they are bioavailable. Unlike pesticides, not much is known about fate and effects of pharmaceuticals in the soils or water bodies. Antibiotics are designed to control harmful bacteria in humans or livestock animals, but excretion of unmetabolized compounds and their metabolites from livestock can continue to be bioactive in the environment (Sarmah et al., 2006). Impacts of these veterinary pharmaceuticals on soil and surface water microflora are not fully known and under current intensive study. One potential impact of pesticides and veterinary pharmaceuticals is the effect on the microbial activity of wood chip bioreactors.

A variety of pesticides and pharmaceuticals have been reported to inhibit microbial activities of several soil microorganisms (Sarmah et al., 2006, Cole, 1976, Moreno et al., 2007) When semiarid soils were treated with atrazine at concentrations of 0.2 to 1000 mg L^{-1} and incubated for 45 days, the amount of CO_2-C evolved per unit C_{mic} per hour was found to be significantly higher than atrazine-free control (0.11 C-CO_2 $g^{-1}C_{mic}h^{-1}$) in soils treated with 500 mg L^{-1} (0.38 C-CO_2 $g^{-1}C_{mic}h^{-1}$) and 1000 mg L^{-1} (0.50 C-CO_2 $g^{-1}C_{mic}h^{-}1$) atrazine (Moreno et al., 2007). Liu et al. (2009) reported that veterinary antibiotics (sulfamethazine, sulfamethoxazole, trimethoprim, tetracycline, chlortetracycline and tylosin) can reduce the rate of microbial respiration of soils at high antibiotic concentrations and are also dependent on incubation time. For instance, at the effective concentration (EC 10) for sulfamethazine for the application of manure containing sulfonamides was determined to be 13 mg kg^{-1}. A sulfonamide, sulfadiazine, was found to reduce by 10 times the *nirK* and *nirS* copy number of denitrifiers in earth worms' guts (Kotzerke et al., 2010). Despite the many different

studies, there is a lack of fundamental data and information in the literature on the impacts of these compounds on denitrifiers.

In the presence of antibiotics such as sulfonamides, microorganisms may develop resistance by replacing inhibited metabolic pathway with a by-pass mechanism and, for quinolones, chromosomal mutations and acquisition of genes can lead to alteration of target and efflux systems (Acar and Röstel, 2001). In general, resistance to sulfonamides may develop by a mutation in the chromosomal dihydropteroic acid synthetase (DHPS), where sulfonamides inhibit its synthesis (Acar and Röstel, 2001). The other mechanism is the acquisition of *sul* genes, drug resistance gene for DHPS (Guerra et al., 2004). Sulfanomide resistance genes have been isolated from several strains of *E. coli* and *Salmonella spp*. (Guerra et al., 2004). A wide range of bacteria species such as *Salmonella spp.*, *Campylobacter spp.*, and *Escherichia coli*, developed resistance to enrofloxacin (Turnidge, 2004), therefore their usage as human therapeutics have raised concerns of resistant bacteria.

Soil microorganisms may also benefit from the presence of agrochemicals in their surrounding environments due to the inhibition of their natural competitors. An increase in soil biomass was observed in 16 days when soil was treated with 0.2-1 mg kg^{-1} atrazine (Moreno et al., 2006). Thiele–Bruhn and Beck (2005) claimed that residual pharmaceutical concentrations of sulfapyridine residue at environmental concentrations can apply temporary selective pressure on microorganisms causing a reduction in soil bacteria numbers and an increase in fungal: bacteria ratio during 14 days of incubation.

2.3 Summary

Nitrate and agrochemicals are detected in surface waters and subsurface waters, mostly due to leaching of the chemicals and their metabolites from agricultural lands. The amount of water drained from agricultural lands with artificial systems such as tile drainage is typically higher than from lands without tile drainage which to contributes towards nitrate pollution of surface waters, especially in the Midwest. To reduce the nitrogen pollution, denitrfying bioreactors have been proposed where nitrates are denitrified in the bioreactors using the wood chips or saw dust in the bioreactors as a source of organic carbon.

In addition to nutrients, agrochemicals and their metabolites may be present in the tile drainage which went dispersed into the environment may have human health risks and impact on the ecology. The fate of agrichemicals in soils, lagoons, manure and sediments have been investigated and documented. Sorption and degradation of atrazine and sulfamethazine in soils have been widely studied since their occurence in soils and water sources is likely due to the application rates and the nature of the chemicals. Occurence of enrofloxacin in the environment is of concern even though it binds strongly to soil particles due to various evidences indicating antibiotic resistance development. The fate of monensin in the environment has not been investigated thoroughly.

However, of interest here is the sorption and degradation of agrochemicals in the wood chip bioreactors. Not much work has been done or known about the partition of agrochemicals onto wood chips. The physical characteristics of wood chips are different from that of soils where macropores may be present in the wood chips. In addition, the agrochemicals flowing through the wood chip bioreactors or sorbed onto wood chips may have an impact on the denitrification of nitrates. Not much is known about the impact of agrochemicals on the denitrfying communities in wood chip bioreactors.

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CHAPTER 3. SORPTION OF VETERINARY ANTIBIOTICS AND A HERBICIDE (SULFAMETHAZINE, ENROFLOXACIN, MONENSIN A AND ATRAZINE) ONTO WOOD CHIPS OF A BIOREACTOR

A paper to be submitted to Chemosphere

3.1 Abstract

Sorption and desorption of atrazine, enrofloxacin, monensin and sulfamethazine onto wood chips from a wood chip bioreactor was studied with batch experiments to evaluate retention of agrichemicals on wood chip bioreactors. Based on the Freundlich distribution coefficients (K_f), the order of sorption from highest to lowest was enrofloxacin > monensin > atrazine > sulfamethazine. Of the four chemicals tested, enrofloxacin desorbed the least while monensin desorption was greater than atrazine, sulfamethazine and enrofloxacin. The sorption of atrazine and sulfamethazine to wood chips were higher than the sorption of surface and subsurface soils obtained next to the wood chips while enrofloxacin and monensin sorbed less to wood chips than to the surface soils at depth 5-15 cm. The apparent hysteresis index (AHI) value for atrazine was lower than for enrofloxacin and sulfamethazine indicating hysteresis was more for atrazine than enrofloxacin and sulfamethazine. Desorption hysteresis increased with decreased initial amounts of atrazine and enrofloxacin, while for sulfamethazine no trend was observed. Following two consecutive steps of desorption and organic solvent extraction, more than 65 % of adsorbed atrazine, 70% of sulfamethazine, 90% of enrofloxacin and 80% of monensin were retained in wood chips. The results of that study showed wood chip biofilters can retain atrazine, sulfamethazine, enrofloxacin and monensin and therefore reduce their concentrations in tile water.

Keywords: biofilters, wood chips, atrazine, sulfamethazine, enrofloxacin, monensin, sorption, desorption

3.2 Introduction

In the late 19th and early 20th century, much of the wetlands or lands with shallow groundwater in Midwest were converted to agricultural lands using tile drainage systems (Dahl and Allord, 1997). Although tile drainage has improved agricultural production, it has negatively impacted valuable water resources. Many Midwest states suffer from nitrogen and phosphorus pollution in surface and ground waters due to heavy use of fertilizers and manure which are rapidly conveyed by the tile drainage system to open bodies of water. For example, a four-year nitrate monitoring study in Iowa by Cambardella et al. (1999) revealed that nitrate-N concentrations in subsurface drainage waters ranged between 6 and 9 mg L^{-1} for three quarters of the annual monitoring period but exceeded 10 mg L^{-1} for the rest of the period.

Various management strategies have been applied to reduce nitrate in surface and ground waters. One of the approaches to reduce nitrate concentration in drainage water is to build a 'denitrification wall' where nitrate in water is reduced by denitrifiers. Robertson and Cherry (1995) monitored nitrate removal from ground water with a 0.6 m wide denitrification wall consisting of an 80/20 (vol/vol) mixture of soil and sawdust or wood chips and found that NO_3^--N concentrations were reduced from 57-62 mg L^{-1} to 2-25 mg L^{-1}. Schipper and Vojvodić-Vuković, (1998) constructed a 35-m long, 1.5-m deep and 1.5-m wide denitrification wall consisting of soil and saw-dust (30% vol/vol) and treated groundwater for a year. They reduced NO_3^--N from 5-16 mg L^{-1} to below 2 mg L^{-1}. Removal of nitrate by wood chips was attributed to a denitrification process rather than immobilization of NO_3^--N; with the wood chips providing the organic carbon for the denitrification process (Greenan et al., 2009).

In addition to fertilizers, other chemicals applied to the fields may also leach into the subsurface drainage water and pollute surface and subsurface waters. These chemicals include herbicides and insecticides, antibiotics and estrogens in manure. At many sites, groundwater or tile water around agricultural lands may be contaminated by multiple contaminants. For example, Kalita et al. (2006) monitored both atrazine and NO_3^--N concentrations in a watershed at four different locations in east central Illinois from 1991 to 2003 range from 0.87 to 1.22 µg $L^{-1}$and 15-20 mg L^{-1}, respectively. Atrazine concentrations

in tile drainage water was found to range from 1.3 to 5.1 µg L^{-1} by Jayachandran et al. (1994), which were close to atrazine concentrations detected (3 µg L^{-1} and 10 µg L^{-1}) in surface runoffs of Midwest states (Battaglin et al., 2003). Drain tiles may speed the movement of pesticides and veterinary antibiotics into surface waters. Besides pesticides, antibiotics present in manure such as sulfonamides, fluoroquinolones and ionophores have been found in groundwater and surface waters (Boxall et al., 2003).

Wood chips used in denitrification walls or wood-chip bioreactors can act as a potential sorbent for various pollutants including pesticides and veterinary antibiotics. Boudesocque et al. (2008) found that sorption of terbumeton, desethyl terbumeton, dimetomorph and isoproturon by wood components and lignocellulosic materials was a fast process where less than four hours was required to reach steady state, and the amount of pesticides adsorbed varied between 1-8 µg g^{-1} of wood chip. Bras et al. (1999) found about 97% of heptachlor, aldrin, endrin, dieldrin, DDD, DDT and DDE were sorbed when 1 to 10 µg L^{-1} solutions of the pesticides were exposed to pine bark. Rodriguez-Cruz et al. (2009) investigated sorption of ionic and non-ionic pesticides onto hydrophilic (cellulose) and hydrophobic (lignin) wood components. The K_f for linuron ranged from 121 to 165 L kg^{-1} for lignin and 2.22 L kg^{-1} for cellulose indicating cellulose, and lignin content of wood residues affect its sorption potential. Sharma et al. (2008) found that about 74.7 to 80.5% of atrazine were removed by sorption onto sawdust (42.3% C) which was treated with 0.1 N H_2SO_4 and kept at 200 C° for 4 hours. There are no studies on the sorption of antibiotics onto wood, but there are previous reports on the sorption of these pharmaceuticals onto soil particles.

Enrofloxacin, a fluoroquinolone carboxylic acid derivative was strongly sorb to various soil types with linear sorption coefficients (K_d) ranging between 260 and 5612 L kg^{-1} (Nowara et al., 1997). On the other hand, sulfamethazine, a sulfanomide was found to be mobile in the soil due to its weak sorption (0.9-1.8 L kg^{-1}) onto soil particles (Boxall et al., 2002). For the same approximate pH, sulfamethazine sorption on to soils was reported to increase with higher organic carbon contents (Lertpaitoonpan et al., 2009). Likewise, sorption of atrazine, a typical co-contaminant with nitrate, onto soil was proportional to soil organic carbon (Moorman et al., 2001). Monensin, an ionophore and growth promoter sorbed

to soils with organic carbon sorption coefficients (K_{oc}) ranging from 2.1 and 3.8 (Sassman and Lee, 2007).

Pesticide and veterinary antibiotics contamination of ground and surface waters are of great concern due to their potential impacts on both aquatic and terrestrial ecosystems (Garner et al., 1986, Halling-Sørensen et al., 1998, Kolpin et al., 2002) and microbial resistance development (Teuber, 2001). There are currently very few studies on the fate of pesticides and veterinary antibiotics in wood chip bioreactors or denitrification walls. The primary objective of this study was to investigate the sorption and desorption of herbicides and veterinary antibiotics onto wood chips of denitrifying in situ bioreactor designed to treat nitrate in tile water. For the study, atrazine, sulfamethazine, enrofloxacin and monensin were selected as representative chemicals of the main groupings of compounds: triazines, sulfonamides, fluoroquinolones, and ionophores, respectively. Sorption studies using soils sampled from same field as the wood chip bioreactor were conducted as a comparison with the sorption results for wood chips. The effect of wood chips particle sizes on atrazine sorption was also evaluated.

3.3 Materials and Methods

3.3.1 Chemicals

Enrofloxacin (CAS number: 93106- 60- 6, 99% purity), monensin sodium salt (CAS Number: 22373-78- 0, 99% purity), sulfamethazine (4-amino-N-(4, 6-dimethyl-2-pyrimidinyl)-benzenesulfonamide, CAS number: 57-68-1, 99% purity) were purchased from Sigma–Aldrich (St. Louis, MO) while atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), (CAS number: 1912- 24- 9 , 99% purity) was purchased from Chem Service (West Chester, PA). Selected physical and chemical properties of the four compounds are presented in Table 1. Stock solutions of enrofloxacin (1000 mg L^{-1}) and atrazine (1000 mg L^{-1}) were prepared in analytical grade acetonitrile. Monensin sodium salt stock solution of 100 mg L^{-1} was prepared in analytical grade methanol, and a sulfamethazine stock solution of 100 mg L^{-1} was prepared in high performance liquid chromatography (HPLC) grade water. All standards for HPLC and liquid chromatography mass spectrometry (LC-MS) calibration curves were prepared by diluting stock solutions into 10 mM $CaCl_2$ and were stored at 4 °C under dark conditions.

3.3.2 Stability of monensin A

The stability of monensin was studied using 1 mg L^{-1} of monensin sodium salt in MilliQ (purified and deionized) water, 10 mM $CaCl_2$, 500 mg L^{-1} KNO_3 and a mixture of 10 mM $CaCl_2$ and 500 mg L^{-1} KNO_3, at 4°C and 22°C. The stability tests were conducted in 20-mL glass tubes with 10 mL of the above solutions. The samples were mixed for 48 hours. Two mL of solution was removed with a syringe from each tube and analyzed for monensin A sodium salt using LC-MS. Separate calibration curves were prepared for monensin for the above four solution matrices. The stability of monensin sodium salt A in the four solution matrices at two different temperatures, represented by % recovery, is presented in Table 2. Percent recoveries after two days of incubation ranged between 84 and 111 % depending on the temperature and the solution matrix.

In addition to monensin stability tests in the above matrixes, sodium azide (NaN_3) interference with monensin analysis was evaluated for a 1-mg L^{-1} monensin solution treated with 5000 mg L^{-1} of NaN_3 prepared in MilliQ water. The presence of NaN_3 interfered with LC- MS signal, and monensin could not be detected. As a result monensin sorption experiments were performed in 10 mM $CaCl_2$ at 22±1°C for 48 hours with a recovery rate of 99 %.

3.3.3 Sorbents

Wood chips were collected in 2004 at a depth of 170 cm from a denitrifying reactor located at Iowa State University agricultural research farm in Ames, Iowa. Denitrification walls were placed ten years ago on both sides of drainage tiles in a field cropped with corn and soybean (Jaynes et al., 2008). The wood chips used for the denitrification wall were mainly oak (*Quercus sp.)* containing 46.54% organic C and 0.15% N. Over the 10 years, the field was not treated with manure and pesticides. Wood chips with a length or width larger than 5 cm were mechanically chopped with a blender to a size of less than 2 cm in width or length. The wood chips were air dried and kept in a sealed bag at 4°C until they were used.

Besides the wood chips, soils samples were collected from the same site at 5-15cm, 80cm, and 168 cm depth. The soil samples from each depth were sieved through a 2-mm sieve, mixed, air dried and stored in plastic bags at 4 °C until they were used. Selected physical and chemical properties of the soil samples are presented in Table 3.

3.3.4 Sorption and desorption of chemicals onto woodchips and soils

Single-point sorption experiments of the selected chemicals onto wood chips and soils was performed in order to compare sorption behavior of selected chemicals onto wood chips with soils before sorption-desorption isotherms were generated. The study was repeated two times in triplicate and the results averaged for atrazine, monensin and sulfamethazine. For the initial trial, the concentration of enrofloxacin in the aqueous phase after 48 hours of mixing was below detection limit (<0.005 mg L^{-1}), therefore only the result of the second trial for enrofloxacin (was also performed in triplicate) was evaluated. The solid-to-liquid ratio was adjusted for the second trial. Single point sorption experiments were conducted in 30 mL fluorinated ethylene propylene (FEP) tubes (Nalgene, Oak Ridge). In each tube, one g of wood chips were added along with 10 mL of 10 mM $CaCl_2$ containing 0.94 mg L^{-1}, 0.79 mg L^{-1}, and 0.83 mg L^{-1} of atrazine, monensin A sodium salt or sulfamethazine, respectively. For the enrofloxacin experiment, only 0.5 g of wood chips was used and the initial aqueous concentration was 0.96 mg L^{-1}. Wood chips in each tube were soaked in 10 mM $CaCl_2$ for 48 hours and then drained before the chemical was added. This step is required to saturate the wood chips to reduce its effect on sorption equilibrium time. Sorption experiments with atrazine, enrofloxacin and sulfamethazine also contained 5000 mg L^{-1} of NaN_3 to inhibit microbial growth. Sorption experiments with monensin were prepared in 10 mM $CaCl_2$ solution only. The contents of the tubes were gently mixed for 48 hours in a reciprocating shaker and then centrifuged at 6574 x g for 20 minutes. Two mL of the supernatant were removed and filtered through 2-µm Whatman glass fiber filters for HPLC analysis.

Similar single-point sorption experiments were conducted using the soil samples. Three grams of soils (1 g of soil for enrofloxacin treatment) were placed into the 30-mL FEP tubes along with 10 mL of 10 mM $CaCl_2$ solution with 1 mg L^{-1}atrazine, enrofloxacin or sulfamethazine. The tubes were mixed in a reciprocating shaker for 48 hours and then centrifuged at 6574 x g for 20 minutes. Two mL of the supernatant were removed and filtered through 2-µm Whatman glass fiber filters for HPLC analysis.

Sorption and desorption isotherm experiments were conducted in a similar manner as the single point sorption experiments. For each sorption isotherm experiment, a total of 6

amber 30-mL vials were used with 1-2 g of wood chips. Wood chips were soaked in 10 mM $CaCl_2$ for 168 hours and then drained prior to addition of chemical for the sorption experiment. The initial concentrations in the vials ranged from 0.5 to 8.0 mg L^{-1}. For each concentration, triplicate vials were prepared. The experiments were conducted at 22±1°C. After equilibration, 2 mL of the supernatant were removed, filtered, and analyzed using the HPLC. For the desorption experiments, a further 6 mL of the supernatant was removed from the tube and 8mL of fresh 10 mM $CaCl_2$ solution added. The contents were mixed for 48 hours and then centrifuged. Two mL of supernatant were removed and filtered for HPLC analysis. The desorption procedure was then repeated.

Solid-phase concentrations (mg kg^{-1}) were calculated based on the difference between initial aqueous phase amount (weight) and equilibrium aqueous phase amount (weight) of the chemical. Sorption-desorption isotherms were determined by linear regression and fitting the data to Freundlich equation using SigmaPlot 10 Software (San Jose, CA). Partition coefficient (K_d) and distribution coefficient (K_f) were calculated using equation 1 and 2, respectively,

$$C_s = K_d \times C_w \quad (1)$$

$$C_s = K_f \times C_w^{\,n}, \quad (2)$$

where C_s and C_w are solid phase and aqueous phase concentrations of analyte at equilibrium, respectively and n is Freundlich linearity parameter.

3.3.5 Wood chip particle size sorption experiments for atrazine

An experiment was conducted to assess the effect of wood chip particle size on atrazine sorption. The wood chips were separated into 3 different sizes using 4 mm, 2 mm and 150 µm sieves. An additional test size was attained by mixing equal amounts by weight of the three sizes. Two grams of woodchips from each group were placed in 30 mL FEP tubes along with 10 mL of 1.75 mg L^{-1}atrazine in 10 mM $CaCl_2$ solution after wood chips were soaked in 10 mM $CaCl_2$ for 168 hours. The tubes were mixed for 48 hours on a reciprocal shaker at 22±1°C. Three mL of supernatant was drawn from each tube and filtered through a 2-µm Whatman glass fiber filter. The filtered supernatant was then analyzed with a HPLC. The linear sorption coefficients (K_d) were calculated based on mass sorbed and the equilibrium concentrations in the aqueous phases.

3.3.6 Extraction of chemicals from wood chips

Following the desorption tests, the solutions in the tubes were drained and 8 mL of 4:1 (v/v) mixture of acetonitrile–MilliQ water were added to the wood chips in the tubes to extract atrazine, sulfamethazine or monensin. The extraction pH was 6.3±0.7 for atrazine, 6.9±0.3 for sulfamethazine and 6.8±0.4 for monensin. Extraction solvent for enrofloxacin was prepared in a similar manner to Nowara et al. (1997). Eight mL of 100% methanol (MeOH), ammonium acetate (10 mM) and MilliQ water in a ratio of 1:1:1 (v:v:v) was added to the wood chips. The extraction pH was 6.3±0.9. The tubes were mixed for one hour with a reciprocating shaker and then equilibrated for 24 hours. The tubes were centrifuged at 6586 x g for 20 minutes and the supernatant in the tubes was poured out and collected. The wood chips were then extracted a second time using the same volume of the mix solution. The acetonitrile of the combined volume (16 mL) of acetonitrile-water mixture was then evaporated using nitrogen gas in an analytical evaporator. The remaining solution was then cleaned and concentrated using Waters OASIS HLB cartridges. The sample was percolated through the cartridge at around 0.5 mL min^{-1} and the flow rate for the conditioning or washing solution was 0.1 mL min^{-1}.

To prepare the supernatant for atrazine analysis, the manufacturers' instructions were followed with a few modifications. The HLB cartridges were conditioned with 3 mL of 100 % MeOH followed by 3 mL of MilliQ water. The cartridges were then loaded with the concentrated solution, and the cartridges washed with 3 mL of 5% MeOH followed by elution with 3 mL MeOH. Elutes were mixed with 3 mL of MilliQ water and the MeOH evaporated and the final volume was brought to 3 mL before the eluent was analyzed with HPLC.

For sulfamethazine, the extraction followed the procedure developed by Henderson (2008). The cartridges were conditioned with 3 mL of 100% MeOH followed by 3 mL of 0.5 N HCl. The HLB cartridges were loaded with the concentrated extracts and then washed using 3 mL of MilliQ water. Elution was completed with 3 mL of methanol. The MeOH in the eluent was evaporated and 3 mL of MilliQ water added before HPLC analysis.

A method established by Gölet et al. (2001) for enrofloxacin extraction with mixed phase cation exchange disk cartridges was modified for enrofloxacin extraction using SPE-HLB cartridges. The cartridge was conditioned (3 mL of 100% MeOH and 3 mL of MilliQ and 0.5 N HCl at pH 3) before the samples were loaded, and the cartridges were vacuum dried for 5 minutes. Compound was eluted with 2.5 mL of 5% ammonium hydroxide in 100% MeOH. The eluent was neutralized by adding 0.5 mL of 50 mM H_3PO_4 solution.

The method developed by Watanabe et al. (2008), was used with minor modifications for monensin extraction. The cartridges were conditioned with 6 mL of MeOH followed by 6 mL of 0.5 N HCl, and 6 mL of MilliQ water. After the extracts were loaded through the cartridges, the cartridges were washed with 6 mL of MilliQ water loaded with 60 µL of 1.0 mg L^{-1} simeton as an internal standard. The cartridges were then eluted with 5 mL of MeOH. The MeOH in the extracts were evaporated and the volumes brought back to a volume of 1.2 mL by adding 500 µL of MeOH and 700 µL of MilliQ water. The extracts were then analyzed immediately with LC-MS.

3.3.7 Chemical analysis

Atrazine, enrofloxacin and sulfamethazine were analyzed using an Agilent HPLC Series 1100 (Eagan, MN) with diode array and fluorescence detection. The HPLC eluent flow rate was set at 0.5 mL min^{-1} for atrazine and enrofloxacin analyses with the following solvents and times: 3 min with 10% acetonitrile and 90% HPLC grade water (4% glacial acetic acid and 1 mM ammonium acetate) followed by 70% acetonitrile and 30% water for 9 minutes and 10% acetonitrile and 90% water for the last 3 minutes. Retention times for atrazine and enrofloxacin were 12.1 and 8.1 minutes, respectively. The eluent flow rate for sulfamethazine was at 0.3 mL min^{-1} with 25% acetonitrile and 75% water for 8 minutes, increasing the acetonitrile to 45% for the next three minutes, followed by 100% acetonitrile for 2 minutes and finally the acetonitrile reduced to 10% for last 5 minutes. Injection volumes were 20 µL for enrofloxacin, 30 µL for atrazine and 50 µL for sulfamethazine Detection wavelengths for atrazine and sulfamethazine were 254 nm while the wavelength for enrofloxacin was 278 nm. Excitation and emission for enrofloxacin analysis were 278 and 445, respectively. HPLC column temperature was set at 60 °C for atrazine and enrofloxacin,

and 40 °C for sulfamethazine. Quantification was performed using external standards. Recoveries exceeded 99%.

Monensin was analyzed with a LC-MS equipped with SBC-18 Zorbax, Agilent column (part 830990-02 2.1 x 150 mm with 3.5-µm particle size) based on the method developed by Watanabe et al. (2008) with modifications. A gradient method was followed where simeton was used as an internal standard and injection volume was set at 5 µL. The gradient ramp used was; 30% HPLC grade water (5% acetonitrile and 0.1% formic acid) and 70% acetonitrile for 5 minutes followed by 5% water and 95% acetonitrile for 12.5 minutes and 30% water and 70% acetonitrile for 2 minutes. Quantification was based on external standards using the monensin sodium adduct.

3.4 Results and Discussion

3.4.1 Single point sorption study-wood chips and soils

Single-point partition coefficients estimated for the sorption of atrazine, enrofloxacin, monensin and sulfamethazine onto wood chips and soils for the batch sorption experiments are summarized in Table 4. The initial aqueous phase atrazine concentration in the single-point sorption experiment with wood chips was 1 mg L^{-1} which was two orders of magnitude higher than the concentrations found in tile drainage water in Canada (13.9µg L^{-1}) (Lakshminarayana et al., 1992). Atrazine partition coefficient, K_d, for wood chips was 24.1 L kg^{-1} while the organic carbon-normalized distribution coefficient, K_{oc}, was 49.2 L kg^{-1}. K_{oc}'s estimated for atrazine onto various organic plant residues (dewaxed cuticle, nonsaponifiable residue, nonhydrolyzable residue) with organic carbon contents ranging from 42.55 to 61.99% were between 44.1 and 644.0 L kg^{-1} (Chefetz et al., 2003) which was higher than the wood chip K_{oc} in this study. K_d values for soils ranged between 0.8 and 4.2 kg L^{-1} and were within the same range of partition coefficients reported by Moorman et al. (2001) ranging between 5.8-0.4 L $kg^{-1}$for soils with organic carbon contents of 2.54-0.08% . Atrazine K_d, for wood chips were about one order of magnitude larger than that for soils samples from the 3 depths. Atrazine K_{oc} values for wood chips were found to be much lower than the K_{oc} values for the soils at three depths. Ling et al. (2008) observed a positive correlation between K_d and

soil organic matter content and higher K_{oc} values for soils with less organic carbon content, and they suggested that the soil organic carbon content is not the only factor influencing sorption of atrazine onto soils. On the other hand, Mackay and Gschwend (2000) suggested that for wood particles, values are poorly correlated to the organic C of the wood. Therefore, it is more accurate to use K_d values when comparing sorption of hydrophobic compounds onto wood chips and soils.

Similar to the atrazine sorption results, K_d of sulfamethazine for wood chips was about one order of magnitude larger than the K_d for the three soils at pH 6.1±0.4 (Table 4). Lertpaitoonpan (2009) suggested that partitioning of sulfamethazine below pH 7.4 (pK_{a2} for sulfamethazine) may be due to hydrophobic sorption since sulfamethazine was in the unionized form. The K_d values for the soils in this study were similar to that of Boxall et al. (2002) and Lertpaitoonpan et al. (2009) who reported K_d values between 0.9-1.8 L kg^{-1} and 0.6-2.8 L kg^{-1}, respectively. The K_{oc} value for sulfamethazine for wood chips was comparable to the K_{oc} for the surface soils from depth 0-15 cm.

Partition coefficients of enrofloxacin for wood chips were 281.9 L kg^{-1} (K_d) and 570.9 L kg^{-1} (K_{oc}). In comparison to other fluoroquinolones, enrofloxacin is the most lipophilic compound and its movement from water phase to solid phase occurs fairly rapid (Picó and Andreu, 2007). In addition, formation of cation bridges in soil is another likely mechanism to explain enrofloxacin sorption on to soils (Tolls, 2001). Boxall et al. (2006) reported K_{oc} values of 15,800 L kg^{-1} for enrofloxacin which is about 31 times larger than K_{oc} calculated for wood chips.

Enrofloxacin partition coefficients for soils at 15 cm, 80 cm and 168 cm depth ranged from 1357-2746 L kg^{-1}, which is within the range of K_d values found in the literature. For loamy sand with 2.27 % organic carbon content, the K_d was reported to be 970 L kg^{-1}, which was higher than for sandy soil (0.59 % OC) and sandy loam(1.24 OC %) (Ötker-Uslu et. al, 2008). For clay minerals, the K_d was between 260 and 5610 L kg^{-1} (Nowara et al., 1997). Sorption of enrofloxacin onto surface soils was found to be higher than that of wood chips with partition coefficients for wood chips about one order of magnitude smaller than that for surface soils (0-15 cm) However, Rodriguez et al., (2007) stated that there was no correlation between total carbon content of wood residues and Freundlich partition coefficients (K_f) of

ionic and non-ionic pesticides. It is probable that, sorption to the clay content and ionic interactions can be the major mechanism of enrofloxacin sorption. Additionally, solid (sorbent) to liquid (aqueous phase) ratio should be taken into account as the solid to liquid ratio for the wood chips experiments were about 2 times smaller than that for soils.

Monensin A sodium salt partition coefficients for wood chips was 24.1 L kg $^{-1}$ (K_d) and 49.1 L kg^{-1} (K_{oc}). The environmental fate of monensin is poorly understood (Dolliver et al., 2007); especially its sorption behavior. Carslon and Mabury (2006) suggested that monensin is immobile in soils, despite the fact, that ionophores are highly lipophilic (Hansen et al., 2009). Monensin was found to sorb more strongly to subsurface soils than surface soils and wood chips (See Table 4). Sassman and Lee (2007) reported monensin organic carbon normalized partition coefficients (Log K_{oc}) as 2.1 to 3.8, which corresponds to 125.8 to 6309 L kg^{-1}and higher than the K_{oc} calculated for monensin in this study. They also reported reducing pH of the soil from 6.2 to 4.9 increased K_d from 6.6 to 19.3 L kg^{-1} which is likely below the pK_a of monensin. However, in this study the lowest K_d of monensin was found for the soil with the lowest pH.

In summary batch linear sorption results indicated that atrazine and sulfamethazine were sorbed more strongly to wood chips than soils. Enrofloxacin sorbed 3 orders of magnitude less onto woodchips than soils even though enrofloxacin partition coefficient was one order of magnitude larger than partition coefficients of atrazine and sulfamethazine for wood chips. Monensin A sodium salt appeared to sorb less onto wood chips than subsurface soil, but the partition coefficient of monensin was in the same range as the partition coefficient of atrazine for wood chips.

3.4.2 Sorption and desorption experiments

Sorption-desorption isotherms for all four chemicals are shown in Figure 1. The Freundlich distribution coefficients, K_f, and linearity constants, n, along with their 95% confidence intervals for sorption and desorption of atrazine, sulfamethazine, enrofloxacin and monensin onto wood chips were determined by non–linear regression analysis using the SigmaPlot 10.0 software (Systat Software Inc., San Jose, CA). Results of the non-linear regression analysis are presented in Table 5. The Freundlich equation gave a better fit of the

wood chip sorption data with n values less than 0.85 as compared to the linear isotherm equation. According to Pignatello et al. (2006), n constants for Freundlich isotherms in the range of 0.95 and 1.05 are presumed to be linear isotherms.

The K_d values estimated from the isotherms were significantly different than the K_d values calculated from single-point sorption experiments (See Table 4). The wood chip-chemical solution pH, equilibration time, solid-to-liquid ratio were similar for both experiments. However, some of the major differences in the two experiments were the size of wood chips and saturation time of wood chips prior to the equilibration. For the single-point sorption experiments, size of wood chip particles were larger (> 0.5 x 0.5 x 0.5 cm) while a more homogeneous wood chip mixture was used for isotherm study. For the single-point experiments, wood chips in tubes were soaked in 10 mM $CaCl_2$ for 48 hours, whereas for the isotherm study they were soaked in the same solution for 168 hours. Mackay and Gschwend (2000) indicated that uptake of sorbate by wood residues may be slower if the wood particles are not fully saturated. It is possible that 48 hours of pre-treatment soaking of wood chips may not be enough for water to penetrate through wood particles which may reduce magnitude of sorption.

The atrazine distribution coefficient (K_f) for this study was similar to the sorption of atrazine onto plant residues (cuticle) with K_f of 120.8-137.37 L kg^{-1} (Chefetz et al., 2003), but was much higher than sorption onto sugarcane mulch with K_f and K_d of 20.3 L kg^{-1} and 17.22 L kg^{-1}, respectively (Selim and Zhu, 2005). However K_f for atrazine sorption onto wood chips in this study was lower than K_f estimated by Boudesocque et al (2008) for terbumeton sorption onto lignocellulosic material (1090 L kg^{-1}). Sorption of linuron, alachlor and metalaxyl onto cellulose ranged between 1.36 and 9.15 L kg^{-1} (Rodriguez-Cruz et al., 2009) which were lower than Kf estimated for atrazine onto wood chips in this present study. The K_f and K_d values for wood chips were also comparable with K_f and K_d of 44.3 L kg^{-1} and 43.1 L kg^{-1} for soil with 38.3% organic carbon (Park et al., 2004). Atrazine distribution coefficients on soils with conventional and no-till agricultural management systems were 3.7 and 3.8 L kg^{-1} (Prata et al., 2003) which were lower than sorption coefficients for woodchips (65.8 L kg^{-1}). Aromatic carbon and carboxylic acid unit content of the material play a significant role in atrazine binding onto organic materials where aromatic carbon content

increases hydrophobic interactions in atrazine binding and carboxylic acids enhances hydrogen bonding between atrazine and organic matter (Lima et al., 2010). Therefore, strong binding of atrazine onto woodchips can be attributed to abundance of carboxylic units and aromatic groups in wood chips. Strong binding can retain atrazine to wood chips and reduce its transport into tile water.

The estimated Freundlich distribution coefficient (K_f) and the partition coefficient, K_d, of sulfamethazine were of similar values (see Table 5). Sulfonamide sorption to organic matter is also related to presence of phenolic and carboxylic groups, N-heterocyclic compounds and lignin decomposition products (Thiele-Bruhn et al., 2004). They also suggested sulfonamide would be binding onto organic matter (soil) via hydrogen bonds and van der Waals interactions. Sulfamethazine adsorbed the least in comparison to other chemicals tested. Sulfamethazine has the highest water solubility (1500 mg L^{-1}) which reflects the ionic nature of the compound and may explain the lower K_f value compared to the other three compounds.

The enrofloxacin Freundlich distribution coefficient was 232.4 L kg^{-1} and the partition coefficient was 372.1 L kg^{-1}. Based on its lipophilic property it was expected that enrofloxacin sorbed more onto wood chips than atrazine and sulfamethazine. Monensin A distribution coefficients were in the same range with enrofloxacin which was expected due to its low water solubility and large molecular size. Sorption isotherm study indicates chemicals with different molecular sizes, and chemical properties partition differently onto woodchips. Lignin (hydrophobic) and cellulose (polar) content of the wood also control sorption of hydrophobic compounds onto wood (Mackay and Gschwend, 2000). They also suggested normalization of sorption parameters based on organic carbon content of the material is a poor estimate for wood which is commonly used for soils. Soil organic matter (SOM) and wood chips are chemically different where C content of wood is less stable than of soils. However, the overall sorption results indicate that wood chips are good sorbents to retain agrichemicals and wood chips can reduce agrichemical concentrations in tile water.

Desorption isotherms for atrazine, enrofloxacin, and sulfamethazine after two desorption steps are shown in Figure 1 (a), (b) and (c), respectively. The desorption results indicate that the three compounds did not desorb readily which are reflected by the increase

K_{fdes1} and K_{fdes2} values after each desorption step. The lack of desorption indicates the lack of mobility and bioavailability of the compounds (Wu et al., 2009) in the wood chip bioreactor which, in turn, may affect their degradation.

Desorption behavior of monensin was different from desorption behavior of the other three compounds where a lower K_{fdes1} value was obtained as compared to the K_f value. However, the K_{fdes2} value was statistically similar to that of K_f. This lower K_{fdes1} value shows the binding of monensin to wood chips may be due to external surface binding. Monensin is a larger molecule than the other three molecules tested and the penetration of the molecule into the micropores and the wood fibers may be limited. As such, for the first desorption, about 12% of sorbed monensin was desorbed readily.

To assess the extent of desorption, the desorption apparent hysteresis index (AHI), defined as the ratio of n_{des}/n_{sorp} may be estimated for each compound (Huang et al., 1998) and are presented in Table 6. The n_{des} in the AHI were estimated differently from n_{des1} and n_{des2} values in Table 5. The n_{des} was estimated by using the Freundlich equation, but the data used were sorption, first and second desorption data for each initial concentration. AHI values indicate the degree of difficulty to desorb a chemical from a matrix (Drori et al., 2005, Chefetz et al., 2004). In general, higher AHI values were observed for atrazine, and enrofloxacin, which ranged from 0.043 to 0.073, 0.064-0.179, respectively (Table 6). For sulfamethazine, AHI values ranged between 0.05 and 0.169, and a different trend was observed. For enrofloxacin, the trend between initial concentration and AHI values was found to be linear ($R^2 = 0.95$), whereas for sulfamethazine and atrazine the initial concentration and AHI values were not related with R^2 of 0.0615 and 0.0623, respectively. Unlike enrofloxacin and atrazine, the AHI values decreased at higher solute concentrations for sulfamethazine. This can be explained by gradient phenomenon suggested by Chefetz et al. (2004). At higher solute concentrations, sulfamethazine may be forced to move into micropores of wood chips and penetrate through the deepest sites where it cannot desorb easily.

Hysteresis was well documented for atrazine sorption-desorption onto various soils or organic residues Lima et al. (2010), Bhandari and Lesan, (2003), Chefetz et al. (2004) but, there is a lack of information on hysteresis of sulfamethazine, enrofloxacin and monensin

from soil matrices or from wood. However, Drillia et al. (2005) and Sukul et al. (2008) observed sorption-desorption hysteresis for ofloxacin and sulfadiazine, respectively in soil.

3.4.3 Effect of wood chips particle size on atrazine sorption

Single-point partition coefficients for atrazine and each wood chip particle size with and without NaN_3, are presented in Table 7. The K_d values increase with decreasing wood chip particle size. Atrazine K_d value for wood chips smaller than 2 mm x 2 mm (128.8 L kg^{-1}) was significantly higher than the K_d for wood chips larger than 4 mm x 4 mm (64.1 L kg^{-1}) ($F = 3.63$, $P<0.05$). Mackay and Gschwend (2000) compared sorption of toluene onto wood sticks (1 x 0.16 x 0.16 cm), shavings and chips (1 x 2 x 0.16 cm) and concluded time required to reach steady state was higher for chips (~2000 min) and sticks than for shavings (~10 min). But for an exposure time of 33 hours the exhibited K_d's for all wood sizes were similar, ranging from 11 to 13 L kg^{-1} (Mackay and Gschwend, 2000). The presence of NaN_3 (5,000 mg L^{-1}) did not interfere with the sorption of atrazine onto wood chips. The K_d for the mixture of the three sizes was similar to the smallest particle size fraction between 150 μm and 2 mm. This may be due to larger surface area provided by the smaller size particle which may control the sorption.

3.4.4 Extraction of adsorbed chemicals from wood chips

Recovery of the chemicals after adsorption and the two desorption steps are presented in Figure 3. Solvent extraction was performed to determine the unextractable fractions of the chemicals. The amount extracted from wood chips after desorption steps ranged from 12.2-21.3% for atrazine, 10.6-15.4% for sulfamethazine, 0.05-2.4% for enrofloxacin, and 0.1-0.8% for monensin. These values equate to a total of 75% of atrazine, 77% of sulfamethazine, 95% of enrofloxacin and 86% of monensin that adsorbed onto wood chips but were not extracted through the four consecutive extractions of two water desorption steps and two organic solvent extractions. In comparison, extraction of atrazine from sugarcane mulch with 100% MeOH resulted in a recovery of 5.49% (Selim and Zhu, 2005) which is lower than the extractable percentage of atrazine from wood chips in this study. However, total incubation time should be taken into account in evaluating the extractable portions of

the chemicals. Lesan and Bhandari (2003) reported the amount of atrazine recovered with water extraction (Log K_{fd} = 0.516) within one hour of exposure was significantly different than atrazine extracted at after 84 days of exposure (Log K_{fd} = 0.965). For this study, chemicals were extracted on the 8 days after addition.

3.5 Conclusion

Dissociation constant indexes (K_d), and (K_{oc}) for atrazine and sulfamethazine adsorbed to wood chips as the sorbents were found to be higher than that for soils from 3 different depths from the same site as the in situ wood chip bioreactor, indicating that both chemicals have a strong tendency to be sorbed onto wood chips of denitrification walls. On the other hand, partition coefficients of enrofloxacin for soils were found to be about 3 orders of magnitude larger than the wood chips. The higher sorption of chemicals by wood chips as compared to soils may be attributed to higher organic C content and the available macro and micropores of the wood chips. Sorption isotherms indicated that sorption of sulfamethazine onto wood chips was less than the other three chemicals, possibly due to its high water solubility. Desorption hysteresis was observed for sulfamethazine, and enrofloxacin, indicating that desorption increased with higher initial concentrations. The estimated AHI indicated that chemicals readily desorbed more at higher initial concentrations compared to lower initial concentrations. After two water desorptions and two solvent extractions of the 66-80% of the adsorbed atrazine, 77-79% of sulfamethazine, 92-96% of enrofloxacin and 83-91% of monensin were retained in wood chips. The results indicate that wood chip bioreactors can reduce the concentrations of atrazine, sulfamethazine, enrofloxacin or monensin present in tile water.

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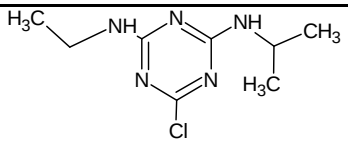
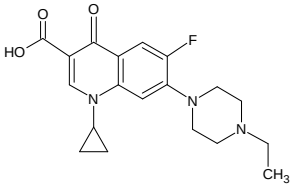
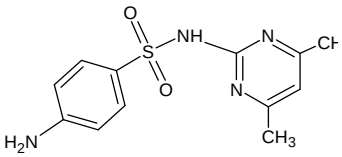
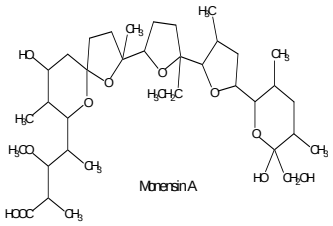
Table 1. Selected physical chemical properties of atrazine, enrofloxacin, sulfamethazine and monensin.

Common Name & Chemical Formula	Chemical structure	Molecular weight (mg mol^{-1})	Water solubility (mg L^{-1})	pKa
Atrazine $C_8H_{14}ClN_5$		215.69	28	1.7
Enrofloxacin $C_{19}H_{22}FN_3O_3$		359.40	10.4[a]	6.27[b], 8.3[c]
Sulfamethazine $C_{12}H_{14}N_4O_2S$		278.33	1500	2.65[d], 7.65[e]
Monensin Sodium Salt A $C_{36}H_{61}NaO_{11}$		692.9	4.8-8.9	10.30[f]

([a]Lizondo et al., 1997; [b]Hamscher et al., 2000; [c]Kolpin et al., 2000; [d]Maxin and Kögel-Knabner, 1995; [e]Lai et al., 1995; [f] Hoogerheide and Popov, 1979).

Table 2. Concentration of monensin A sodium salt after 48 hours in various media at 4 °C and 22 °C.

	4°C			22°C		
	[a]C_i	[b]C_{eq}	Recovery	Ci	Ceq	Recovery
Matrix	(mg L^{-1})	(mg L^{-1})	(%)	(mg L^{-1})	(mg L^{-1})	(%)
MilliQ water	0.89	0.95	106	0.89	0.99	111
10 mM $CaCl_2$	0.91	0.88	97	0.95	0.94	99
500 mg $L^{-1}KNO_3$	0.99	0.85	86	1.02	0.86	84
10 mM $CaCl_2$ and 500 mg $L^{-1}KNO_3$	0.98	0.94	95	0.98	0.92	94
5000 mg L^{-1} of NaN_3	*	*	*	*	*	*

* = No peak was observed

[a]C_i = Initial aqueous phase concentration (mg L^{-1})

[b]Ceq = Equilibrium (48 hours) aqueous phase concentration (mg L^{-1})

Table 3. Selected physical-chemical properties of soils

Sample Identification	Depth (cm)	Organic Carbon (%)	pH	CEC (meq100 g^{-1})	Sand (%)	Silt (%)	Clay (%)	Soil Texture
Surface soil	0-15	2.15	7.7	23.1	37	36	27	Loam
Subsurface soil	80-120	0.64	7.6	12.7	57	23	20	Sandy Clay Loam
Subsurface soil	168	0.23	8.2	15.7	47	30	23	Loam

Table 4. Partition coefficients (Kd±95% CI) and Koc of atrazine, enrofloxacin, monensin and sulfamethazine onto soils from various depths and wood chips

Sorbent	Atrazine		Sulfamethazine		Enrofloxacin		Monensin	
	K_d	K_{oc}	K_d	K_{oc}	K_d	K_{oc}	K_d	K_{oc}
Soil (0-15 cm)	4.2 ± 0.2	197	5.5 ± 10.8	256	2747 ± 1936	127765	26 ± 24	1233
Soil (80-120 cm)	2.2±0.5	80	9.8 ± 3.8	587	[a]<DL	[b]N/D	N/D	N/D
Soil (168 cm)	0.8 ± 0.2	354	0.6 ± 0.3	262	1357 ± 602	59003	101 ± 14	43965
Wood chips	24.1± 8.4	49	61 ±12	124	282 ± 169	571	24.2 ±8.8	49

[a]<DL = Below detection limit
[b]N/D = Not determined

Table 5. Sorption and desorption linear partition coefficients (Kd), Freundlich partition coefficients (Kf) and constants, n, of atrazine, enrofloxacin, monensin and sulfamethazine for wood chips. (parameters reported with 95% confidence limit)

Chemical	K_f[a]	n_{sorp}	r^2	K_d	r^2	K_{fdes1}[b]	n_{des1}	r^2	K_{fdes2}	n_{des2}	r^2
Atrazine	65.5 (6.5)	0.82	0.98	66 (12)	0.98	176 (246)	0.82	0.96	240 (66)	1.35	0.82
Sulfamethazine	36.4 (3.1)	0.79	0.98	35.0 (7.9)	0.98	64 (23)	0.74	0.95	127 (56)	1.02	0.95
Enrofloxacin	232 (77)	0.53	0.98	372 (31)	0.99	209 (75)	0.43	0.89	305 (37)	0.49	0.99
Monensin	161 (191)	0.73	0.66	226 (12)	0.63	44 (13)	0.53	0.83	58 (37)	0.36	0.58

[a]Sorption experiments following 48 hours of equilibration
[b]Desorption measurements 48 hours after sorption or first desorption experiments

Table 6. Apparent hysteresis index (AHI) values for atrazine, sulfamethazine and enrofloxacin.

Chemical	C_i	[a]$K_{fdes,AHI}$	[b]n_{des}	r^2	[c]AHI
	mg L^{-1}				
Atrazine	5.64	49.9	0.060	1.0	0.073
	2.81	26.4	0.054	0.81	0.066
	2.30	21.8	0.059	0.87	0.072
	1.72	16.4	0.074	0.45	0.090
	1.17	11.3	0.035	0.45	0.043
Sulfamethazine	4.20	32.1	0.134	0.99	0.169
	2.30	20.5	0.106	0.92	0.133
	1.90	17.2	0.126	0.93	0.158
	1.40	16.4	0.195	0.99	0.245
	0.90	7.8	0.040	0.15	0.050
Enrofloxacin	7.80	123.4	0.095	0.66	0.179
	4.50	94.5	0.056	1.0	0.106
	3.80	77.2	0.043	0.99	0.081
	2.80	59.0	0.034	0.98	0.064
	2.00	44.4	0.036	0.70	0.068

[a]$K_{fdes,AHI}$ = Freundlich coefficient calculated based on adsorption, and two desorption data points for each concentration

[b]n_{desorp} = linearity parameter for desorption estimated by non-linear regression

[c]AHI =Ratio of n_{desorp} / n_{sorp}

Table 7. Atrazine partitioning coefficients, [a]K_d, for various wood chip particle sizes and in medium with and without NaN_3.

Medium	Particle Size			
	≥ 4 mm	2-4 mm	150 µm-2 mm	Homogeneous Mixture
10 mM $CaCl_2$	63±23	97±12	129±14	134±40
10 mM $CaCl_2$ & 5000 mg L^{-1} NaN_3	73±39	[b]N/D	133±48	[b]N/D

[a]K_d = Mean of three replications of K_d ± 95% confidence interval
[b]N/D = not determined

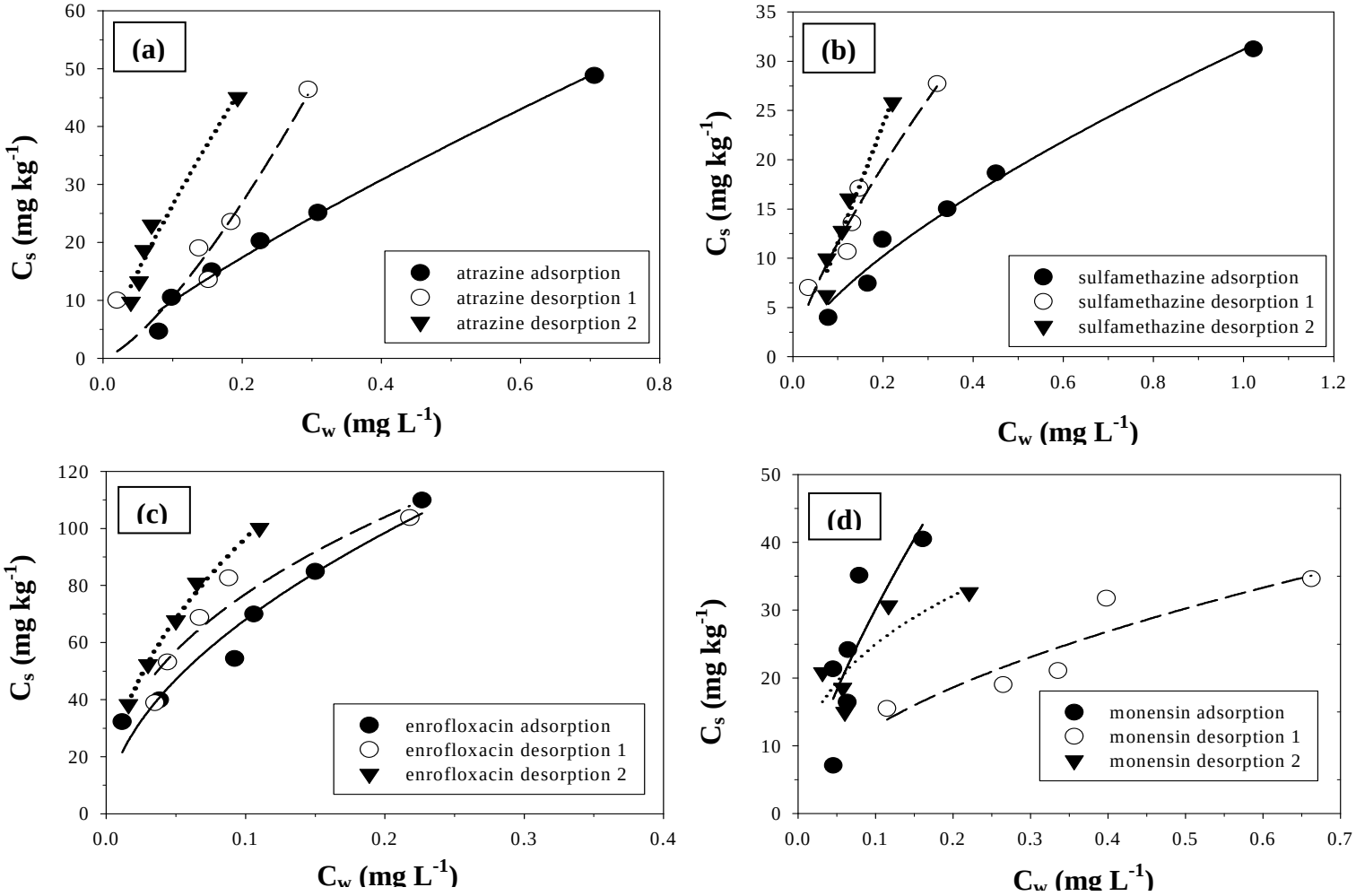


Figure 1. Sorption-desorption isotherms of (a) atrazine, (b) sulfamethazine, (c) enrofloxacin and (d) monensin A sodium salt to wood chips. Symbols represent measured values (means, n=3). Solid lines show the isotherm predicted by non-linear regression using the Freundlich model.

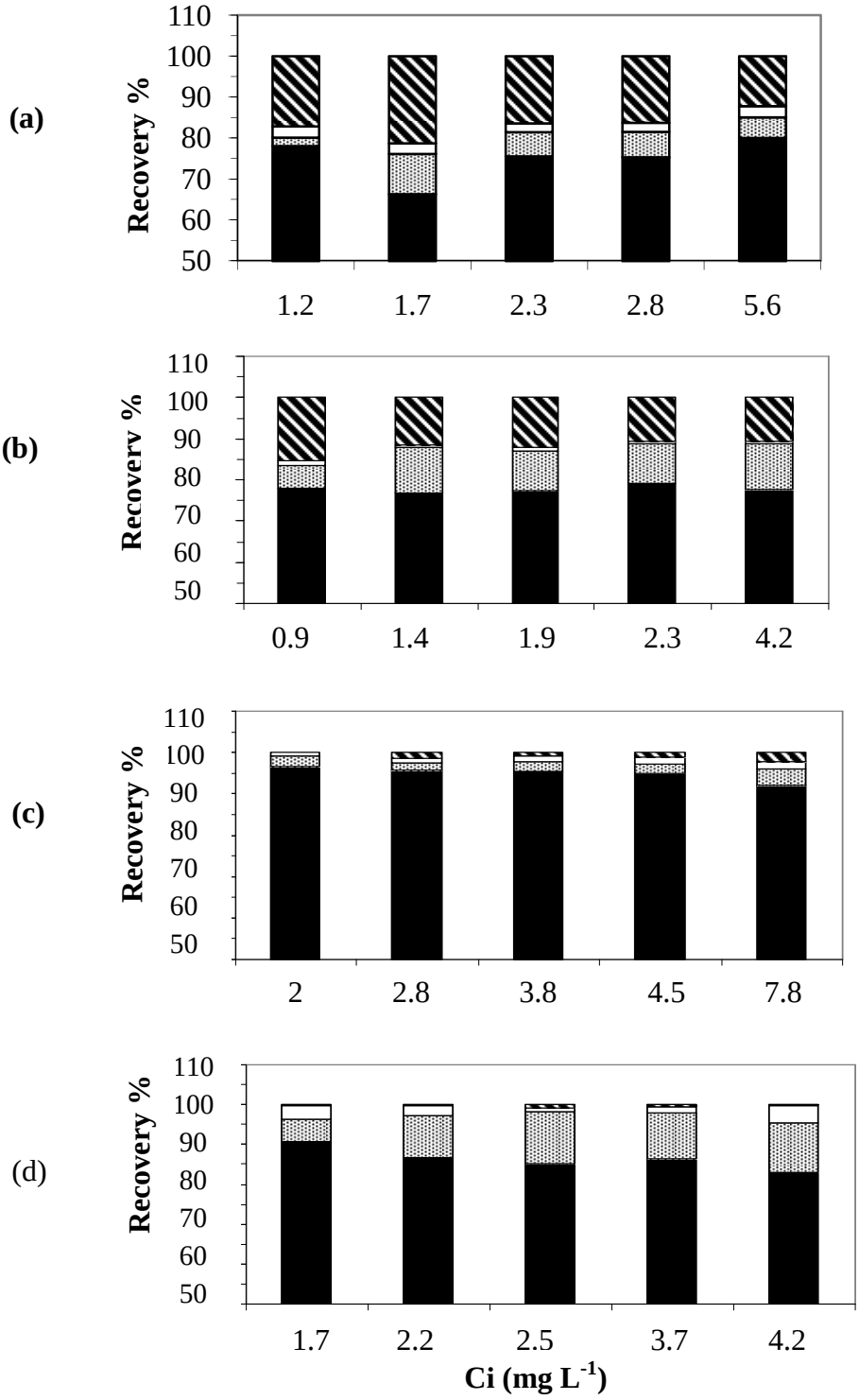


Figure 2. Recovery of adsorbed (%) (a) atrazine, (b) sulfamethazine, (c) enrofloxacin, and (d) monensin. Desorption was performed with 10 mM $CaCl_2$ followed by extraction with 80% acetonitrile. (Unextractable fraction = ■ , desorbed fraction 1= ▦ , desorbed fraction 2 = □ , solvent extraction fraction = ▨)

CHAPTER 4. DISSIPATION OF ATRAZINE, ENROFLOXACIN AND SULFAMETHAZINE ON WOOD CHIP BIOFILTERS AND IMPACT IN WOOD CHIP DENITRIFIERS

4.1 Abstract

Wood chip bioreactors are receiving increasing attention as a means of reducing nitrate in subsurface tile drainage systems. Agrochemicals in tile drainage water entering wood chip bioreactors can be degraded and may impact denitrification in the bioreactor. The degradation of atrazine, enrofloxacin and sulfamethazine under denitrifying conditions using wood chips from an in situ reactor was studied. The impact of atrazine, enrofloxacin and sulfamethazine on denitrifying microorganisms was assessed using the denitrification enzyme potential assay (DEA), most probable number (MPN) and quantitative polymerase chain reaction targeting *nosZ1* gene of the denitrifiers. Both enrofloxacin and atrazine disappeared rapidly within 48 hours from the aqueous phase with availability-adjusted rate constants of 0.8 d^{-1} and 4.5 d^{-1}, respectively. The similar disappearance during the first 48 hours in autoclaved and non-sterile wood chip solution suggested sorption as the dominant mechanism. For sulfamethazine, disappearance was slower with an availability-adjusted rate constant of 0.13 d^{-1}. The presence of atrazine did not impair denitrification as shown by comparing the nitrous oxide (N_2O) production rate for the DEA, the MPN and *nosZ1* gene copy number with the control. For wood chips treated with enrofloxacin, MPN decreased at 48 hours of incubation, whereas DEA and *nosZ1* copy number were not affected. A significant difference in the MPN and the N_2O production rate was observed on day 5 for sulfamethazine treatment compared to the untreated control. However, after 45 days, the N_2O production rate, MPN and *nosZ1* gene copy numbers for sulfamethazine were similar to that of the control, indicating that acclimation of the denitrifier population to the sulfamethazine or reduced bioavailability of sulfamethazine over time allowed recovery of the denitrifier population.

Keywords: enrofloxacin, atrazine, sulfamethazine, denitrifiers, degradation, wood chips, *nosZ1*, nitrous oxide reductase

4.2 Introduction

Tile drainage is practiced in many parts of the world, including the Midwest of United States, to enhance drainage of water-logged land for agricultural production (Kladivko et al., 1999). However; suspended matter, excessive nitrate and various agricultural chemicals can be transported rapidly by tile water to surface waters. A major concern in the Midwest is the elevated of nitrogen concentrations in surface and subsurface drainage waters from agricultural systems (Jaynes et al., 2001, Jaynes et al., 2008, Kladivko et al., 1999).

One possible approach in reducing the NO_3^--N in tile water is to treat water with denitrification walls or in situ bioreactors. Several organic materials such as wood chips, cornstalks, sawdust and cardboard fibers have been used as a carbon source for microorganisms in denitrification walls. Denitrification with external carbon sources studies by Greenan et al. (2006) showed that higher nitrate removal rate was achieved with cornstalks as compared to wood chips, but wood chips provided a more stable percent removal and lasted longer in the fields than cornstalks. Greenan et al. (2009) also reported that NO_3^--N removal as high as 100% with a denitrification wall can be achieved. Several factors can affect the denitrification in the denitrification walls or in situ bioreactors. These factors include oxygen concentration, pH, temperature, and amount of nitrogen and carbon (Hofstra and Bouwman, 2005) with water flow rate as a significant factor (Greenan et al., 2009). Interestingly NO_3^--N removal per gram of wood increased with increasing flow rate, but the percent of nitrate removal decreased from 100% to 30% when the flow rate increased from 2.9 to 13.6 cm d^{-1}.

In addition to NO_3^--N, agrichemicals such as pesticides and antibiotics can be transported to surface waters via the tile drainage systems. Concentrations of pesticides in subsurface drainage water were found to be an order of magnitude lower than the concentrations in surface waters (Kladivko et al., 1999). For instance, atrazine concentrations ranging from 1.3-5.1 µg L^{-1} and 0.5-20.5 µg L^{-1} were detected in tile drain water and in lysimeter water, respectively along with atrazine metabolites, deethylatrazine and deisopropylatrazine at concentrations of 0.1-2.2 µg L^{-1} and 0.9-3.2 µg L^{-1}, respectively (Jayachandran et al., 1994). Similarly, veterinary pharmaceuticals in manure when applied to

farm lands may be present in tile drainage water. For example, sulfamethazine and flubendazole were found at concentrations of 16 µg L^{-1} and 0.3 µg L^{-1} in soil seepage water when manure was treated with 600-1700 µg L^{-1} of sulfamethazine and 25-56 µg L^{-1} of flubendazole (Weiss et al., 2008).

Persistent residues of sulfonamides and fluoroquinolones were found in the soil environment (Wang et al., 2006, Gölet et al., 2003, Yang et al., 2009) and there is limited information on the biological degradation of veterinary antibiotics in the subsurface environment. However biodegradation of enrofloxacin under aerobic and anaerobic conditions by two wood rotting fungi (*Phanerochaete chrysosporium* and *Gloeophyllum striatum*) in agricultural soils was observed (Wetzstein et al., 1997). In a similar manner, under aerobic conditions Accinelli et al. (2007) reported a half life of 18.6 days for sulfamethazine in soils treated with 10 µg L^{-1} indicating biological degradation is present. There are no prior studies indicating that enrofloxacin and sulfamethazine can be degraded under denitrification conditions. However, atrazine can be degraded under denitrifying conditions. Radosevich et al. (1995) showed that under denitrifying conditions atrazine can be mineralized by soil microorganism by cleaving the *s*-triazine ring. More recently Katz et al. (2000) characterized atrazine degradation and nitrate reduction by an isolated *Pseudomonas sp.* strain (Katz et al., 2001). Degradation of atrazine in wood chip biofilters is still unknown.

The presence of antibiotics in tile water and ground water may negatively impact the aerobic and anaerobic microbial activity such as nitrification and denitrification (Costanzo et al., 2005). Antibiotics and pesticides entering a wood chip bioreactor could reduce the nitrate removal by interfering with wood chip denitrifier organisms. Soil microbiology assays can be adopted to study wood chip bioreactor microorganisms. Methods to investigate interactions of agrichemicals with soil microbial biomass include measurement of biomass respiration over time or the monitoring degradation of atrazine and changes in microbial biomass size (Ghani et al., 1996), and determination of metabolic activity such as monitoring N_2O production and relating it with population counts (Martin et al., 1988). More recently, molecular biomarkers have been employed to study microbial communities. Several quantitative polymerase chain reaction (qPCR) assays were developed and evaluated (Henry

et al., 2006; Smith et al., 2006; Siciliano et al., 2007) in order to quantify and qualify denitrification gene prevalence in environmental samples. Kloos et al. (2001) and Henry et al. (2006) designed *nosZ* and *nosZ1* primers which amplify gene fragments responsible for synthesis of nitrous oxide reductase (N_2O R) which catalyzes the last step of denitrification.

The fate of selected agrichemicals in soils has been well established; however there is a lack of information on degradation of these chemicals in wood chip reactors and the influence of these chemicals on microbial community established in the bioreactors. The objectives of this study are to study the degradation of atrazine, enrofloxacin and sulfamethazine on wood chips obtained from in situ reactors. The influence of these chemicals on the denitrifier community was measured by quantifying the denitrification activity, the denitrifier populations using the most probable numbers, and specific denitrification genes measured using qPCR.

4.3 Materials and Methods

4.3.1 Materials and chemicals

Woodchips were collected June of 2009 at a depth of 120 cm from an in situ bioreactor at Iowa State University Research Farm, Ames, IA and stored in plastic bags at 4 °C until use. Details of bioreactor performance and construction are given by Jaynes et al. (2008). Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) was purchased from Chem Service (West Chester, PA). Enrofloxacin (1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1, 4-dihydro-4-oxo-3-quinolinecarboxylic acid) and sulfamethazine (4-amino-N-(4, 6-dimethyl-2-pyrimidinyl)-benzenesulfonamide) were obtained from Sigma-Aldrich (St Louis, MO). Working solutions of 26-100 mg L^{-1} of atrazine, enrofloxacin and sulfamethazine were prepared in MilliQ water from 1000 mg L^{-1} stocks solutions of sulfamethazine in MilliQ water and atrazine and enrofloxacin in acetonitrile. All standards were stored at 4 °C in amber bottles.

4.3.2 Degradation studies

For the biodegradation studies, 50-mL amber vials were filled with 20 mL of basal minimum salt (BMS) (1.6 g L^{-1} of K_2HPO_4, 0.4 gL^{-1} of KH_2PO_4, 0.2 gL^{-1} of $MgSO_4.7H_2O$,

0.1 g L^{-1} of NaCl, 0.025 g L^{-1} of $CaCl_2$) solution at pH 7.2 containing 40 mg L^{-1} KNO_3-N and autoclaved for 15 minutes at 121 °C. The vials lost about 1-2 mL of the volume due to autoclaving and the volume lost was replaced with sterile BMS. Wood chips were mixed thoroughly and 2 g of wood chips with 256 % moisture were weighed and added into each vial with sterile tweezers. All vials were sealed with Teflon® caps and evacuated with a vacuum manifold 3 times with a one-minute cycle using helium gas. The final helium relative pressure in the head space of vials was 5 kPa. A total of 21 vials were prepared for each chemical treatment and 3 of them were without wood chips and 3 of them were with sterile wood chips. The sterile treatments were not sacrificed until last sampling day. For the sterile control, 3 vials were immediately autoclaved on 3 consecutive days to sterilize and minimize microbial growth in the vials.

For the degradation studies, wood chips were pre-incubated in BMS for 7 days for acclimation since they had been kept at 4 °C for 3 months before the experiment, which may result in a reduction of microbial activity. At the end of the 7-day acclimation period each set of vials were spiked separately with 5 mL of 26 mg L^{-1} of atrazine, enrofloxacin or sulfamethazine to obtain an initial concentration of 5 mg L^{-1} in each vial. For the atrazine and enrofloxacin treated vials, the total volume of acetonitrile did not exceed 0.5% of the total solution volume. The final wood chips-to-solution ratio was 2:25 (weight: weight) in each vial. All vials were incubated under dark conditions in an incubator at a temperature of 19±0.2 °C throughout the experiment. The pH of the solution was stable at 6.1±0.7 for atrazine vials, 6.0 ± 0.9 for enrofloxacin vials, and 6.2±0.4 for sulfamethazine vials. Nitrate was monitored using colorimetric test strips and ion chromatography. Additional KNO_3 was added to each vial before nitrate was depleted in the vials.

After 0, 2, 5, 20 and 45 days of incubation, triplicate vials of each non-sterile treatment were sacrificed. An aliquot was removed with a sterile syringe from the vial and filtered with a 2-µm glass fiber filter. A 1.5-mL filtered aliquot was stored in a 2-mL HPLC amber glass vial with Teflon cap tip at 4 C° until they were analyzed for atrazine, enrofloxacin or sulfamethazine with protocols described below.

The degradation or disappearance of the selected chemicals was modeled using the availability-adjusted first-order model that has been used previously for degradation of

sulfadimethoxine (Wang et al., 2006) and sulfamethazine (Lertpaitoonpan, 2008). The model is given:

$$\frac{dC}{dt} = k'' \, Ce^{-(at)} \quad (1)$$

$$ln\,(C_t/C_0) = k''\,(1-e^{-(at)}\,a^{-1}) \quad (2)$$

where C_0 and Ct are the concentrations of the chemicals at time *t*(d) and t(0); *k″* is the availability-adjusted rate constant; and *a* is the first-order coefficient describing change in the non-adsorbed fraction of the chemicals. The first-order rate coefficient (k″) and the availability-adjusted rate constant were estimated using aqueous phase concentrations of the chemicals at different time points by non-linear least-squares regression.

4.3.3 Extraction of atrazine, enrofloxacin, sulfamethazine

At the end of 45 days, one g of wood chips from the sacrificed vials of the sterile and non-sterile treatment with atrazine, enrofloxacin and sulfamethazine were extracted for atrazine, enrofloxacin or sulfamethazine to determine the extractable mass available after 45 days of incubation. The extraction solution used was 10 mL of 80:20 (v: v) acetonitrile and water and the pH was adjusted to 7.9±0.2 with ammonium acetate for enrofloxacin extraction. No pH adjustment of the extraction solution for atrazine and sulfamethazine extraction was made. The wood chips were shaken for one hour and mixed for 24 hours with the extraction solution. pH of the extraction solution was 7.5±0.2 for atrazine and 7.3±0.6 for sulfamethazine after 24 hours of equilibration. The supernatant was transferred into FEP tubes (30 mL) and centrifuged at 6586 x g for 20 minutes and then the supernatant transferred to 50-mL amber vials. Eighty percent of the total volume (acetonitrile in the supernatant) was evaporated using an analytical evaporator. The remaining extracted aliquots were then cleaned and concentrated with solid-phase extraction hydrophilic-lipophilic balance (HLB) cartridges. For atrazine extraction, manufacturer's (Waters, Milford, MN) instructions were followed where the cartridges were conditioned with 3 mL of 100% methanol (MeOH) and 3 mL of deionized water; followed by loading the cartridges with the

sample; and then washing the cartridges with 3 mL of 5% MeOH and eluting with 3 mL 100% MeOH. For the extraction and concentration of sulfamethazine the method by Henderson (2008) was applied where the cartridges were conditioned with 3 mL of 100% MeOH and 3 mL of 0.5 N HCl; the cartridges were loaded with the sample, the cartridges were then washed with 3 mL MilliQ water. Elution was conducted with 3 mL of 100% MeOH. The method by Gölet (2003) was used for enrofloxacin extraction and concentration. The cartridges were conditioned with 3mL of 100% MeOH and 3 mL of MilliQ water with the pH adjusted to 3 with 0.5 N HCl. The cartridges were loaded with the extracted samples followed by vacuum drying for 5 minutes. The cartridges were eluted with 2.5 mL of 5% ammonium hydroxide in 100% MeOH and 0.5 mL 50 mM H_3PO_4 was added. MeOH in all elutes was evaporated using an analytical evaporator and the remaining solution brought to 2 mL by adding MilliQ water. The final solution was analyzed using HPLC.

Atrazine, enrofloxacin and sulfamethazine were analyzed with an Agilent HPLC Series 1100 (Eagan, MN) with an Eclipse XDB-C18 column (3.5 µM diameter, 2.1x150 mm) and the detectors were diode array detector and a fluorescence detector. The HPLC eluent flow rate was 0.5 mL min^{-1} for atrazine and enrofloxacin analyses with the following solvents and times; 3 min of10% acetonitrile and 90% HPLC grade water (containing 4% glacial acetic acid and 1 mM ammonium acetate) followed by 70% acetonitrile and 30% HPLC grade water for 9 minutes and 10% acetonitrile and 90% HPLC grade water for the last 3 minutes. Retention times for atrazine and enrofloxacin were 12.1 and 8.1 minutes, respectively. The eluent flow rate for sulfamethazine was at 0.3 mL min^{-1} with 25% acetonitrile and 75% HPLC grade water for 8 minutes, increasing the acetonitrile to 45% for the next 3 minutes, followed by 100% acetonitrile for 2 minutes and finally the acetonitrile was reduced to 10% for last 5 minutes. Injection volumes used were 20 µL for enrofloxacin, 30 µL for atrazine and 50 µL for sulfamethazine. Detection wavelength was 254 nm for atrazine and sulfamethazine, and for enrofloxacin emission and excitation were 278 and 445, respectively. Column temperature was 60 °C for atrazine and enrofloxacin, and 40 °C for sulfamethazine.

4.3.4 Denitrifier population count - Most Probable Number

To estimate the denitrifier most probable number (MPN), a 1-mL aliquot was withdrawn from the same sacrificed vials used in the biodegradation studies after 0, 2, 5, 20 and 45days of incubation. In addition to the vials from the biodegradation study, 15 vials with 25 mL of BMS and 2 g of wood chips were prepared like the vials in the degradation study and also incubated at 19 C° as the untreated control, and 3 vials were sacrificed at each sampling day for MPN analysis. The 1 mL aliquot withdrawn from vials at each sampling day was transferred to a sterile 20-mL screw cap dilution tube containing sterile 9 mL of potassium buffer (0.0125 M, pH 7.1). A serial 10-fold dilution was then performed by transferring 1 mL to the next dilution tube containing 9 mL of buffer solution giving a serial dilution ranging from $1x10^{-5}$ to $1x10^{-9}$. However, dilutions for day 45 samples were extended to give a dilution ranging from $1x10^{-6}$ to $1x10^{-10}$ in order to adjust the detection limits to provide a more sensitive estimation. From each dilution tube, 0.2 mL of aliquot was transferred into 5 MPN tubes. The tubes were vortexed and incubated at 22±1 C° for 15 days. The MPN tubes were prepared based on a method developed by Tiedje et al. (1989) as follows: nutrient broth powder (8 g L^{-1}) and KNO_3 (5 g L^{-1}) were dissolved in MilliQ water and the solution was sparged with N_2 gas for 20 minutes in the presence of anti-foaming agent to remove oxygen. Approximately, 5 mL of medium was dispensed into a 28-mL Balch tube, and the tubes were sealed with gray butyl septa and aluminum crimp rings. The tubes were then evacuated 3 times for 1 minute and filled with 5 kPa helium. The tubes were vented to atmospheric pressure after they were autoclaved for 15 minutes at 121 °C.

At the end of the 15^{th} day incubation period, 0.3 mL of the supernatant was taken from each MPN tube with sterile syringes and tested for the presence of nitrate with diphenylamine solution. The diphenylamine solution was prepared by dissolving 2 g of diphenylamine $[(C_6H_5)_2NH]$ in 100 mL of concentrated sulfuric acid (H_2SO_4). For tubes which showed presence of nitrate, 2 mL of acetylene was added to each MPN tubes and then vented to atmospheric pressure before incubating for an additional one week. At the end of the week, 3 mL of helium was added to each MPN tube, and gas in the headspace of tubes was analyzed with a gas chromatograph to monitor presence of nitrous oxide.

4.3.5 Determination of nosZ1 abundance by qPCR

The qPCR assays were performed with an MJ Research Thermal Cycler (Hercules, CA). The 25-µL reaction mixture was prepared as follows: 12.5 µL of 2X SYBR Green PCR Master Mix (QuantiTect SYBR green PCR kit; QIAGEN, France), 5.0 µL of 6.25 µM of each *nosZ1* primer, 2.5 µL of template DNA. qPCR protocol for *nosZ1* primers was adopted from Henry et al. (2006) with few modifications. Thermal cycling conditions were, an initial Taq polymerase (thermostable DNA polymerase) activation of 95°C for 15 min and 40 cycles of 95°C for 15 s, 53°C for 15 s (annealing step), 72°C for 30 s, and 80°C for 15 s (acquisition data step) followed by melting curve analysis from 50 °C to 90 °C.

The primers used for PCR amplification were a pair of 259-bp gene fragments, *nosZ 1 F* and *nosZ 1R*. The fragments were designed by Kloos et al. (2001) and modified by Henry et al (2006). Selected properties of the primers (oligos) are shown in Table 1. Primer concentrations per reaction was optimized by running combinations of 0.5, 0.75, 1.00, 1.25 and 1.5 µM of both forward and reverse primer final concentrations. The higher amplification was achieved at 1.5 µM of each primer, although 1.25 µM gave a similar amplification rate. Annealing temperature for selected primers was determined by running a temperature gradient and evaluating the resulting qPCR products on an agarose gel (see Figure 1). The sharpest band was formed at annealing temperatures of 53 C° and 57 C° with the highest qPCR efficiency at 53 °C (data not shown).

DNA for qPCR standard curves were prepared using *Pseudomonas stutzeri* ATCC 14405 and *Escherichia coli* ATCC 43651 strains grown over night at 26 °C in marine broth (50 mL) and nutrient broth (50 mL), respectively and harvested with 10 mL of sterile potassium phosphate buffer (PPB). The cell numbers were estimated using the micro-drop technique on agar plates. A total of 2×10^9 cells were extracted according to the manufacturer's (MOBIO Soil Power, CA) manual, and the DNA concentrations were estimated by spectrophotometry (BioPhotometer, Eppendorf, Germany). Extracted *P. stutzeri* DNA was diluted for preparation of standards which contained copy numbers between 10^5 and 10^{10} per reaction assuming one copy per cell. qPCR standard curves (Figure 2)were

obtained by amplification of the 10-fold dilutions of *P. stutzeri* double stranded DNA (dsDNA) templates with *nosZ1 F* and *nosZ1 R* fragments. A set of templates that had initial copy numbers of 10^5 to 10^{10} cells of *P. stutzeri* was run each time with samples as standards. The amplification efficiency, E, was determined using the equation $E = (10^{-1/slope}) - 1$, where the slope is the slope of the standard curve (Henry, et al., 2006). Linearity of standard curves ($R^2 \geq 99\%$) was observed each time with amplification efficiencies (E) approximately 83% with slopes of about -3.7. Amplification of *E. coli* DNA was not observed during the 40 cycles indicating only fragments of *nosZ1*.

For each sacrificed vial from the degradation study, wood chips were taken from the vials and stored at -20C° until the end of experiments. From a total of 14 vials for each treatment (control, atrazine, enrofloxacin or sulfamethazine treatments) two sets of 0.25g of wood chips were extracted for microbial DNA. Microbial DNA on wood chips was extracted with MOBIO Power Soil DNA Isolation Kit (CA, USA) following the supplier's users protocol with several additional steps to increase DNA recovery. These additional steps include incubating the samples at 70 °C for 10 minutes and centrifuging the wood chips before the first step of extraction to remove excessive water. The amount of DNA extracted was increased by performing two consecutive lysis steps, but this approach reduced the qPCR efficiency by increasing contaminants in the DNA extract. To overcome variations as a result of material's heterogeneity, wood chips from the same source were extracted two times and the extracts pooled. DNA concentrations were measured by using a spectrophotometer (Eppendorf, Germany) at a wavelength of 260 nm.

Serial dilutions of microbial DNA extracted from wood chips were quantified and copy numbers of lower than 10^1 were spiked with *P. stutzeri* DNA template to detect for the presence of PCR inhibitors (data not shown). For each DNA sample, qPCR was performed in triplicate and results were pooled and 95% confidence intervals estimated. Melting curve analysis was used to check for the purity of *P. stutzeri* which appeared around 84 °C. The melting curve analysis revealed that the annealing temperature for environmental samples was not significantly different (see Figure 3).

4.3.6 Denitrification potential assays

Denitrification potential assays were performed to determine the effect of atrazine, enrofloxacin and sulfamethazine on the denitrification process. Denitrification potential assays were performed based on a method developed by Tiedje (1994). For each set of treatment (atrazine, enrofloxacin, sulfamethazine or untreated control), a total of 15 amber bottles (250 mL) were prepared. For each bottle, 8 g of wood chips with 156% moisture content and 90 mL of BMS buffer containing 0.101 g L^{-1} KNO_3 were added. The bottles were sealed with caps, evacuated 5 times with a 2-minute cycle and filled with helium gas to a final pressure of 5kPa. The bottles were incubated for 7 days at 19 °C with the vials for the degradation study, and at the end of 7^{th} day 5 mL of 100 mg L-1 of atrazine, enrofloxacin or sulfamethazine were added in each set of the bottles except the control treatment. For the control treatments 5 mL of MilliQ water was added to bottles. Then 5 mL of 40 mg L^{-1} of KNO_3-N was added to all bottles giving a final volume of 100 mL. The bottles were incubated under dark conditions in an incubator at a temperature of 19 ± 0.2 °C.

Triplicate bottles were sacrificed for treatments for the denitrification potential assay on 0, 2, 5, 20 and 45 days after the chemicals were added to each bottle. Denitrification potential was measured by quantifying the amount of N_2O produced under acetylene block (Tiedje, 1994). Each bottle was spiked with 10 mL of a solution containing 1.01 g L^{-1} of KNO_3 and 2.5 g L^{-1} chloramphenicol giving a final concentration of at least 0.101 g L^{-1} of KNO_3 and 0.25 g L^{-1} of chloramphenicol. Then the bottles were evacuated with vacuum manifold and acetylene was added to each bottle with a syringe to produce a 10% v/v concentration in the headspace, and all bottles were vented to atmospheric pressure. Bottles were placed on a reciprocating shaker. Ten mL of the gas in the bottles were collected after 4, 8, 24 and 48 hours of addition of acetylene and stored in evacuated 6-mL glass vials sealed with gray butyl septa and aluminum crimp rings. Nitrous oxide concentrations in the vials were measured with gas chromatography (Model GC17A, Shimadzu, Kyoto, Japan) equipped with a ^{63}Ni electron-capture detector and a stainless steel column (0.3175 cm diameter 3 and 74.54 cm long) with PorapakQ (80-100 mesh). Sample volume used was 10

mL. The analytical run was 9 minutes long, and the retention time of N_2O was approximately 4.4 minutes.

During the 45-day incubation nitrate concentrations in the bottles were monitored by withdrawing 1 mL of the supernatant with a sterile syringe from the bottles and testing the presence of NO_3^--N using nitrate/nitrite test strips. One mL of 40 mg L^{-1} KNO_3-N was added to each bottle or each tube (degradation study tubes) when nitrate was about to be depleted. In order to estimate the amount of nitrate consumed per each bottle, 5 mL of supernatant was removed before and after addition of KNO_3 solution, filtered with a 2-µm glass fiber filter and analyzed with ion chromatography with the method described below.

NO_3^--N concentration measurement followed the EPA method 300.1 and Dionex Application Note 154 where NO_3^--N was measured with a Dionex ICS- 2000 Reagent-free Ion Chromatography (RFIC) System (Sunnyvale, CA). The column used was an Ion Pac AS18 (4 x 250 mm) analytical column while the detector was a digital conductivity detector. Eluent used for the ion chromatography was 22 mM KOH with a flow rate of 1.0 mL min^{-1}. Injection volume of the sample was 25 µL. Data acquisition was performed with a Chromeleon 6.5 software. Stock solutions of 1000 mg L^{-1} of nitrate-N, nitrite-N, phosphate-P, chloride, fluoride, sulfate and bromide were purchased from Dionex and were used to prepare standards.

4.4 Results and Discussion

4.4.1 Degradation of atrazine, enrofloxacin and sulfamethazine

The results of the degradation experiments for atrazine, enrofloxacin and sulfamethazine are presented in Figure 4. The control without wood showed that the chemicals were stable throughout the experiment. More than 90% of aqueous concentration of atrazine and enrofloxacin were found to rapidly disappear within the first 48 hours. For sulfamethazine, there was a rapid disappearance to about half of the initial concentration within the first 48 hours which was followed by a slower disappearance over 30 days. For atrazine experiments, about 8.4% and 3.6% of initial concentration were measured for the sterile and non-sterile experiments, respectively at the end of 45 days in aqueous phase. Likewise, following 45 days of incubation, about 11.4% and 8.6% of sulfamethazine were

measured in sterile and non-sterile experiments, respectively. For enrofloxacin, about 5.5% and 1.4% of initial concentration was measured in the aqueous phase in sterile and non-sterile experiments, respectively. At the end of degradation experiments, the amount of atrazine, enrofloxacin and sulfamethazine extracted from wood are presented in Table 2. For all three chemicals, there were no statistical difference between (α = 5%) the sterile and the non-sterile experiments. The percent of chemicals extracted after 45 days were 16.8% for atrazine, 14.2% and for sulfamethazine, 5.3% for enrofloxacin. The extracted fractions of adsorbed chemicals after 8 days of incubation in Chapter 3 were generally higher than the extracted recoveries for Chapter 4 such as 24.3% for atrazine, 22.4% for sulfamethazine, and 7.8% for enrofloxacin. In general lower recoveries were attained for atrazine, enrofloxacin or sulfamethazine compared to the sorption experiments. The difference in extracted recoveries of the adsorbed chemicals between sorption and degradation experiments may be due to the longer equilibration time in the degradation experiments, which may have resulted in greater transfer of the chemicals into the inner pores of the wood. In general 70-90% of the chemical was accounted for by sorption and degradation.

The loss of chemicals from solution was described with the adjusted first-order model. The availability-adjusted dissipation rate constants of atrazine, enrofloxacin and sulfamethazine for sterile and non-sterile wood chips are shown in Table 3. The rate constant for atrazine was 0.80 d^{-1}. While the rate constant for atrazine was higher than rate constant for sulfamethazine (0.13 d^{-1}), it was lower than the rate constant for enrofloxacin (4.52 d^{-1}). Similar to rate constants, the highest unavailability coefficient (*a*) was estimated for enrofloxacin (1.02 d^{-1}) and the lowest for sulfamethazine (0.05 d^{-1}) and *a* was less than the value for enrofloxacin and higher than sulfamethazine for atrazine (0.25 d^{-1}).

A comparison of sterilized and non-sterilized treatments shows little difference for all chemicals, except for atrazine which showed greater loss of chemicals from water at 2 days in the non-sterile treatment than sterile treatment. One possible reason for the similar results in sterile and non-sterile treatments was that the sterile vials which were autoclaved three times did not fully sterilize the wood chips (Wolf et al., 1989). Aqueous phase samples from sterile vials at the end of the experiment inoculated onto agar plates showed microbial growth which indicated that autoclaving did not sterilize the wood chips. Based on the disappearance

of the aqueous concentration of the chemicals, it was probable that sorption was the main mechanism of loss but biological degradation cannot be entirely eliminated.

Previous studies have shown that atrazine can be degraded by microorganisms under denitrifying conditions (Katz et al., 2000, Herzberg et al., 2004, Shapir et al., 1998). In a recent study by Hunter and Shaner (2010), a denitrifier barrier was shown to remove 98% of nitrate and 30% of atrazine in contaminated ground water, although the removal of atrazine may be due to sorption. These studies suggested that, atrazine degradation is possible under anoxic conditions and disappearance of atrazine in wood chip bioreactors may be due to not only sorption but also biodegradation.

The rate of sulfamethazine disappearance was lower than atrazine, probably due to less sorption of sulfamethazine. Yang et al. (2009) showed sulfadiazine degradation in anoxic soils with degradation rate constants of 0.0026-0.0121 d^{-1}, for non-sterile soils and 0.0029-0.0104 $d^{-1}$for sterile soils, where after 24 hours of equilibration K_d values ranged between 0.09-0.24 L kg^{-1}suggesting sulfadiazine was persistent in anoxic soils. There are not many studies on sulfamethazine degradation under anoxic conditions. However, degradation of sulfamethazine under aerobic conditions and anaerobic conditions were shown by Henderson et al. (2008) and Lerpaitoonpan (2008) where the main mechanism was irreversible binding to soil.

Fluoroquinolones on the other hand, bind to soils or organic matter strongly and their bioavailability is relatively lower than sulfonamides which may delay their biodegradation (Hektoen et al., 1995). The higher availability-adjusted dissipation rate coefficient for enrofloxacin than for atrazine and sulfamethazine may be due to this strong binding to organic matter property of enrofloxacin. Degradation of fluoroquinolones has not been showed under anoxic conditions but, biodegradation of enrofloxacin was shown under aerobic conditions by wood rotting fungus *Gloephyllum striatum* (Martens et al., 1996, Wetzstein et al., 1997).

Assuming that all the atrazine loss from the aqueous phase was due to sorption, the estimated K_d values for atrazine after 48 hours of equilibration were 690±240 L kg^{-1} and 246±117 L kg^{-1} for non-sterile and sterile wood chips, respectively. This K_d value for sterile wood chips in the degradation experiment was lower than the K_d previously estimated for the

sorption experiments (66±12 L kg^{-1}), whereas K_d for atrazine non-sterile treatment was comparatively higher (see Chapter 3). K_d values for sulfamethazine in the degradation experiments after 48 hours of equilibration were found to be 26.3±6.8 L kg^{-1} and 19.2±8.2 L kg^{-1}, for sterile and non-sterile experiments, respectively which were in the same range of K_d calculated during the sorption experiment (35.7±7.9 L kg^{-1}). K_d values for enrofloxacin estimated from degradation experiments, for sterile and non-sterile wood chips were 594±99 L kg^{-1} and 899±453 L kg^{-1} which were higher than the K_d value (232 L kg^{-1}) previously estimated in Chapter 3.

The differences in the K_d values from the previous sorption experiments and these experiments can be attributed to the differences in the experimental protocols. Wood chips used in degradation and sorption experiments were taken from the bioreactor in 2009 and 2006, respectively. The wood chips used in the sorption experiments were also prepared differently than wood chips in the degradation experiment. The wood chips were air dried for the sorption experiment, while the wood chips used in degradation experiment were relatively fresh and wet. The wood chips were sterilized with NaN_3 in sorption experiments while they were autoclaved 3 times for the degradation experiment. During sorption experiments the tubes were mixed with a reciprocal shaker for 48 hours, whereas in degradation studies the tubes were not mixed.

Although sorption may be the main cause for the disappearance of the chemicals tested, the possibility of biodegradation cannot be ruled out based on the estimated higher K_d values for the degradation experiments as compared to the sorption experiments. Overall, it is probable that both biotic/abiotic degradation and sorption of atrazine, enrofloxacin and sulfamethazine may be occurring under denitrifying conditions with sorption the major mechanism.

4.4.2 Denitrifying enzyme assays

Potential denitrification was determined by measuring N_2O in the presence of acetylene. N_2O production rate over the 45-day period for each chemical and control are presented in Figure 5a. The rates were calculated based on the dry weight of the wood chips. The N_2O production rate for the control reached a maximum at 20 days at 4.57µg N $g^{-1}h^{-1}$

with a fairly similar production rate for the 45th day sampling event. The observed rate for the control was comparable to the rates reported by Greenan et al. (2006) at about 17.79 µg N g^{-1}h^{-1} for ground cornstalks and about 2.75 µg N g^{-1}h^{-1} for wood chips over 180-day incubation period. The denitrification rate observed for the control was also less than the rate of 27.5-36.4 µg N g^{-1}h^{-1} for shredded newspaper reported by Volokita et al. (1996).

After treatment with atrazine, the maximum N_2O production rate was reached within the 5th day, with the 45th day N_2O production rate statistically similar to that of the control (2.74±0.25 µg N g^{-1}h^{-1} for control and 4.52±5.13 µg N g^{-1}h^{-1}for atrazine) indicating denitrification in wood chips was not inhibited by the presence of 5 mg L^{-1} atrazine. Work done by others showed that a pure culture of *Pseudomonas* sp strain ADP was capable of both atrazine degradation and denitrification at a rate of 90.8 mg NO_3^--N g^{-1}cell^{-1}h^{-1} (Katz et al., 2000). However, denitrification activity of *X. autotrophicus* CECT 7064 was found to be inhibited in the presence of 10 mg L^{-1} of atrazine (Sáez et al., 2006). In this study, an aqueous concentration of 325 µg L^{-1} of atrazine was measured after 48 hours of equilibration, but the denitrification activity was not inhibited in the wood chips.

An initial concentration of 5 mg L^{-1} enrofloxacin did not appear to have an effect on denitrification throughout the 45 days of incubation. This may be due to the reduced availability of enrofloxacin since there was a rapid decrease of enrofloxacin in solution (see Figure 4c).

Sulfamethazine reduced the N_2O production rate 2 days after treatment, ($P = 0.0405$) compared to the control, but N_2O production gradually increased with time with the N_2O production rates at 20 days and 45 days statistically similar to that of the control ($P=0.354$ for day 20 and $P=0.847$ for day 45). There are no prior studies on the effect of sulfamethazine and enrofloxacin on denitrification and therefore their effects were unknown. However, this study showed that there was an initial inhibition of denitrification by sulfamethazine, followed by recovery of both potential activities compared to the control. It is possible that with time, sulfamethazine becomes less may available due to sorption onto the wood, reflected by the slow decreases of sulfamethazine (see Figure 4b).

4.4.3 Most probable number-denitrifiers

Denitrifier populations on wood, measured by MPN are presented in Figure 5b. The MPN for the untreated control showed a similar trend as that for the N_2O production rate with an increase in MPN to a maximum in 5 days and then remaining a fairly constant value of $1x10^9$ cells per g wood chips up to 45 days. The MPN for the vials with atrazine showed a slightly lower MPN than the control for the first 5 days, but showed a similar trend as the control with MPN statistically similar for the 20 and 45-day sampling event.

Both vials for sulfamethazine and enrofloxacin showed a statistically significant decrease (P=0.0002) in the MPN on day 2, with an increase in MPN on day 5, followed by MPNs statistically similar to the control for 20 days (P=0.827). At day 45, the MPN for sulfamethazine and the control were statistically similar (P =0.599), while MPN for enrofloxacin was higher than the control (P=0.0023). One possible reason for the reduction in MPN on day 2 and similar MPN for sulfamethazine and control treatments at day 45 was the change in sulfamethazine bioavailability over time. Schauss et al. (2009) reported that by the presence of sulfadiazine in the manure, number of denitrifiers declined while with the reduction in the bioavailability of sulfadiazine, the abundance of denitrifiers increased. There are no studies indicating impact of enrofloxacin on denitrifiers.

4.4.4 nosZ1 gene abundance

The *nosZ1* copy numbers are shown in Figure 5c. Over the 45 days of incubation a gradual increase in copy number was observed in control group, but was not statistically significant (P=0.760). A similar increment over time was observed for both MPN and *nosZ1* gene copy number, although the numbers obtained from the *nosZ1* copy number were an order of magnitude higher than the MPN of denitrifiers. MPN populations were lower than the qPCR assay, possibly due to the limitation of cultural methods. Additionally, the lower and upper confidence limits (LCL and UCL) of the calculated MPN value cover a wide range. For example, at day 0, MPN was 2×10^8, while LCL and UCL were 7×10^7 and 3×10^8, respectively. On the other hand, non-specific amplification with SYBR Green can cause over estimation of *nosZ1* copy number by qPCR. In this study, various concentrations of *E. coli* DNA were also run at each assay and no amplification was observed.

In atrazine-treated wood chips, the *nosZ1* gene copy number increased over time with the copy number on day 2 not significantly different from the copy number in the control (P = 0.543) while it was significantly higher than the control on day 5 (P=0.01) and day 20 (P=0.012). At the end of the experiment at day 45, the copy number in the atrazine experiment and control experiment were not different (P=0.06).

For enrofloxacin-treated wood chips, copy numbers of *nosZ1* was similar to the control over 45 days of incubation. The significant reduction in MPN at 2 days for enrofloxacin was not observed with qPCR. However, N_2O production rates over time under enrofloxacin treatment supported the change in *nosZ1*copy number.

In contrast to enrofloxacin treatment, the number of denitrifiers at 5 days was reduced in the presence of sulfamethazine in comparison to the control (P = 0.0049). Eventually, after day 20, the copy numbers in sulfamethazine treatment, atrazine, enrofloxacin and control were similar; suggesting the impact of antibiotics on denitrifiers was alleviated within 20 days of chemical addition. Sulfonamides were found to be impacting denitrifier copy number by Kotzerke et al. (2010) and Schauss et al. (2009) suggesting sulfamethazine may have inhibited denitrifiers during the 5 days of incubation.

4.5 Conclusion

Disappearance of atrazine, enrofloxacin and sulfamethazine in wood chips taken from an in situ reactor may be primarily due to sorption and to limited degradation. The rapid disappearance observed for atrazine and enrofloxacin during the first 48 hours of incubation was mainly due to sorption which reduces the bioavailability of the chemical. Sulfamethazine disappearance was slower than atrazine and enrofloxacin, which could be due to less sorption onto wood chips. Denitrification rates on wood chips and denitrifier numbers estimated by MPN and qPCR were not inhibited by the concentration of 5 mg L^{-1} of atrazine. At the same concentration, enrofloxacin only inhibited MPN at 48 hours but did not impair N_2O production rate or *nosZ1* copy number. However that concentration of sulfamethazine impaired both N_2O production rate and growth of denitrifiers during the first 5 days of incubation. With a reduction in the aqueous concentrations of sulfamethazine over time, N_2O production rate and denitrifier populations recovered and were similar to the control at the

end of 45 days of incubation. The results of this study suggested wood chip bioreactors may remove agrochemicals by sorption and degradation. The presence of agrochemicals may impact denitrification potential of wood chip bioreactors by temporarily inhibiting denitrifier populations.

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Table 1. Selected properties of synthesized oligos: *nosZ* 1F and *nosZ 1* R.

Oligo Name	*nosZ 1 F*	*nos Z 1 R*
Sequence	5’ WCS YTG TTC MTC GAC AGC CAG 3’	5’ ATG TCG ATC ARC TGV KCR TTY TC 3’
Oligo Length	21	23
Concentration per well	1.25 μM	1.25 μM

Table 2. Recoveries of atrazine, enrofloxacin and sulfamethazine in aqueous phase and the recoveries of adsorbed chemicals with solvent extraction from sterile and non–sterile wood chips incubated at 19 °C under denitrifying conditions at the end of 45 days.

Chemicals	Initial Aqueous Concentrations (mg L^{-1})	% Recovery (± 95% *CI)			
		Aqueous phase		Wood chips	
		Sterile	Non–Sterile	Sterile	Non–Sterile
Atrazine	4.1-4.8	8.4	3.6	22.5 ±5.3	16.8±1.9
Enrofloxacin	5.2-6.0	5.5	1.4	8.2 ±2.6	5.3±4.6
Sulfamethazine	5.0-5.4	11.4	8.6	16.9±2.1	14.2±3.6

* CI = Confidence Interval

Table 3. Degradation rate constants and availability coefficients of atrazine, enrofloxacin and sulfamethazine (means reported with 95% CI).

Chemical / Treatment Type	Rate Constant (k'')	Availability Coefficient (a)	r^2
Atrazine			
Sterile	0.81±0.27	0.25±0.09	0.97
Non-sterile	0.80±0.25	0.25±0.09	0.98
Enrofloxacin			
Sterile	3.95±2.72	1.25±0.90	0.98
Non-sterile	4.52±1.12	1.02±0.09	0.99
Sulfamethazine			
Sterile	0.24±0.12	0.12±0.06	0.97
Non-sterile	0.13±0.08	0.05±0.05	0.94

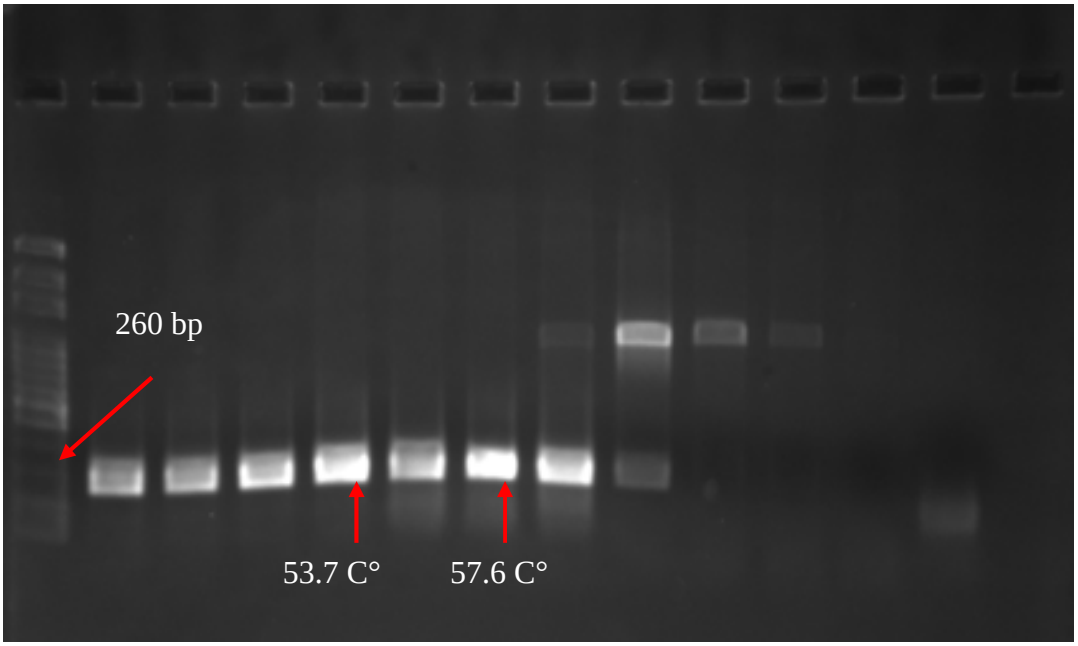


Figure 1. Gel eletrophoresis of gradient qPCR products of nosZ1 gene amplifications, annealing temperatures varying between 50 C° and 75 C°.

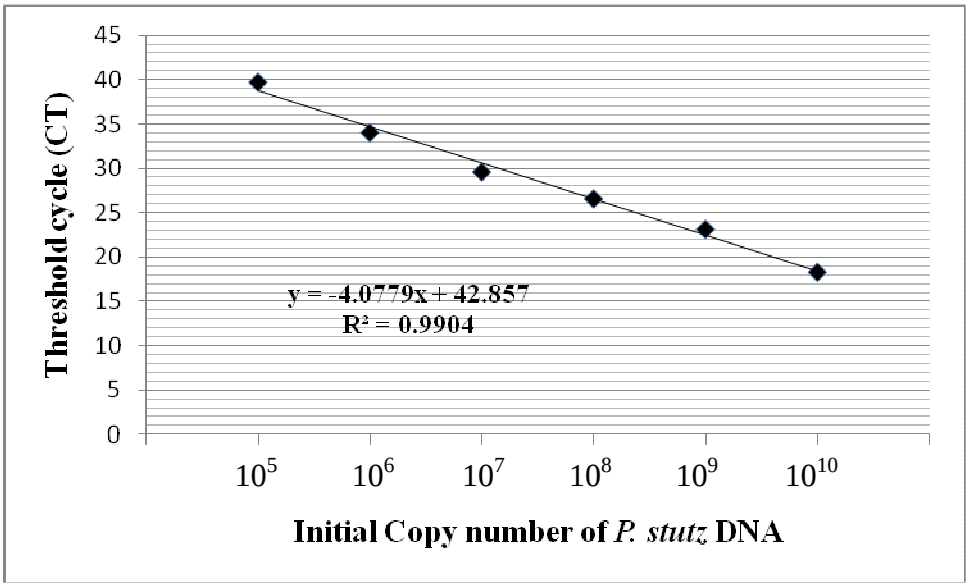


Figure 2. Standard curve for nosZ1 showing initial copy number of P. stutzeri against threshold cycle number (CT).

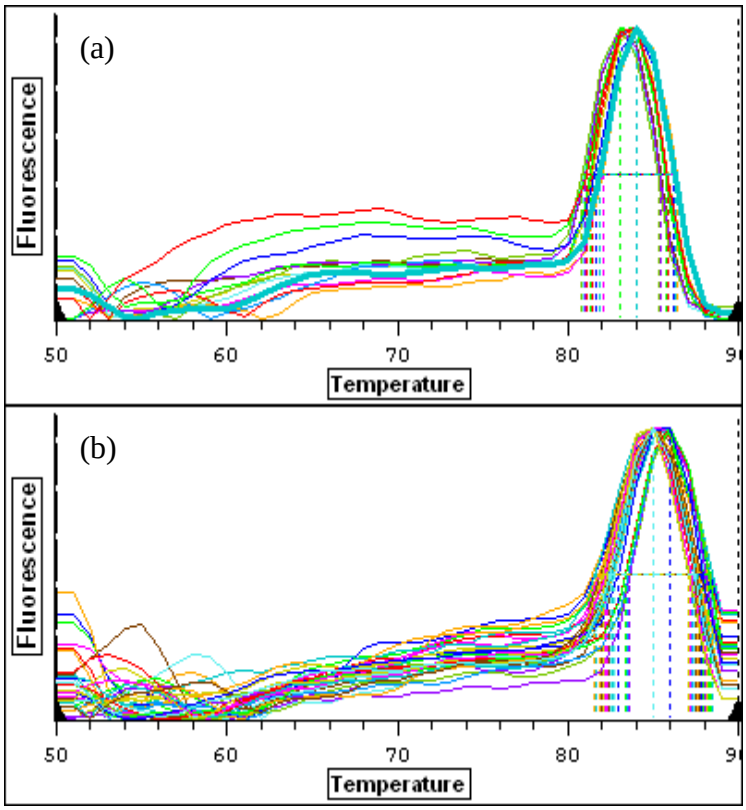
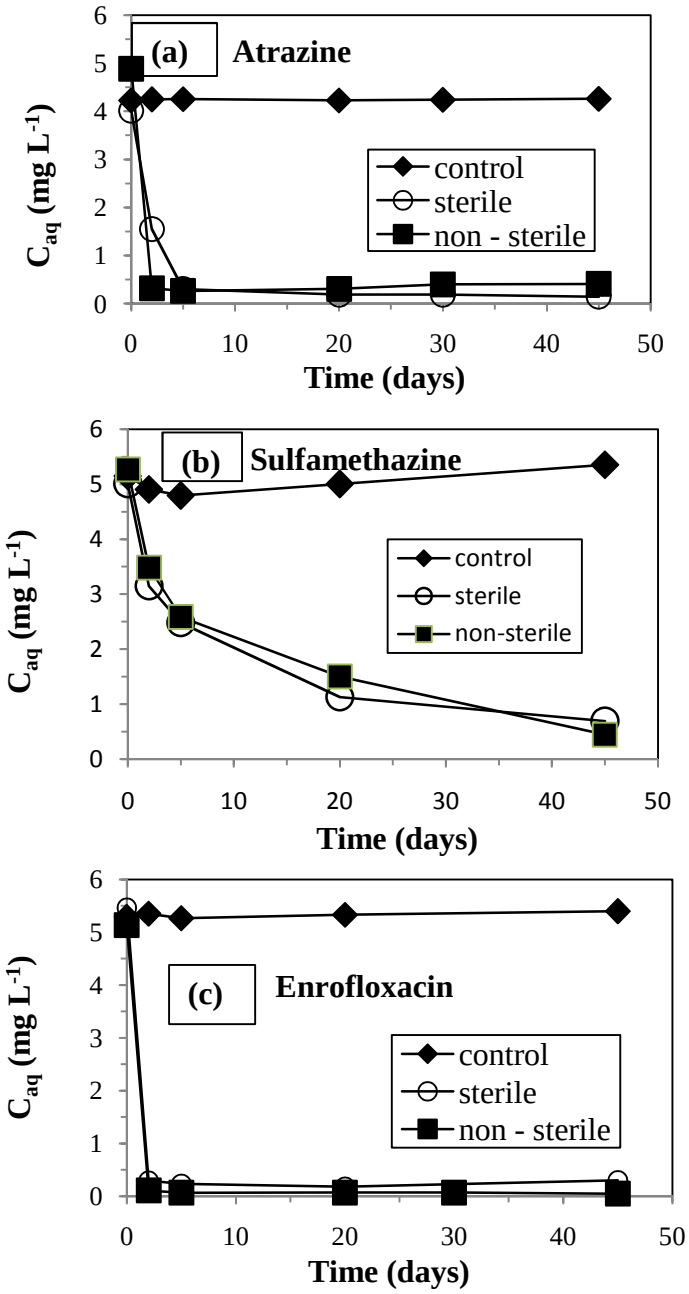


Figure 3. Melting curve analysis for amplicons of P. stutzeri (a) and wood chips DNA templates (b) obtained by nosZ1 primers.



Figur 4. Loss of (a) atrazine, (b) sulfamethazine, (c) enrofloxacin from water incubated with wood chips. The control treatment is water amended with chemicals without wood chips.

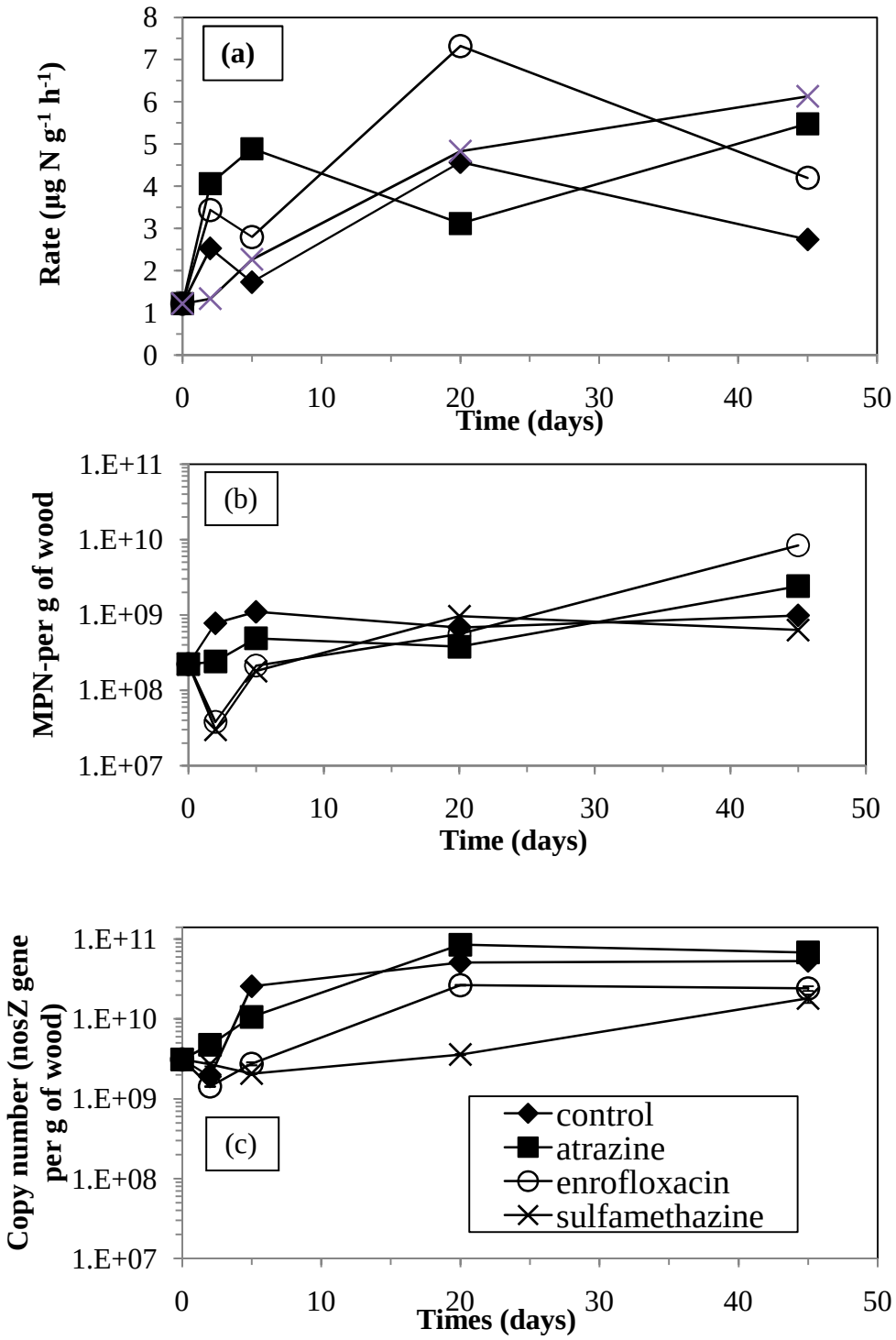


Figure 5. (a) Denitrification rate, (b) most-probable-number, (c) *nosZ1* gene copy number for control, atrazine, enrofloxacin and sulfamethazine. Denitrification activity was measured as N_2O production in the presence of acetylene.

CHAPTER 5 CONCLUSION

Agrochemicals entering the environment via direct application of pesticides and manure amendments (mostly veterinary antibiotics) may impact and interfere with the environmental quality. Contamination of groundwater with pesticides and veterinary antibiotics is of concern due to their potential health effects and potential development of resistant microorganisms. Wood chip reactors are used to remove nitrate from tile drainage water and these reactors may also reduce the transport of pesticides and pharmaceuticals.

Sorption-desorption of atrazine, enrofloxacin, monensin and sulfamethazine were investigated in Chapter 3, and sorption of atrazine, enrofloxacin and sulfamethazine to wood chips was found to be higher than their sorption to soils. This may be due to macro porosity, hydrophobic groups and organic C content of wood chips as compared to the soils used in this research. Freundlich isotherms were better for modeling the sorption-desorption data of the four chemicals than a linear model since partitioning of the selected chemicals onto woodchips was not linear. Of the four chemicals tested, sulfamethazine, with the highest water solubility, partitioned less onto the wood chips than the other three chemicals, whereas enrofloxacin partitioned the most. Desorption hysteresis was more notable for atrazine and sulfamethazine than enrofloxacin and suggests less tendency to desorb. Sorption-desorption hysteresis also increased for higher solute loads for sulfamethazine, whereas it decreased for atrazine and enrofloxacin. On the other hand, the first desorption experiments for monensin with water suggests that a fraction of monensin was sorbed onto the wood chips due to its large molecular structure.

For the degradation studies, disappearance of atrazine, enrofloxacin and sulfamethazine in wood chips over 45 days was described in Chapter 4. Monitoring with HPLC showed that the disappearance of all three chemicals was due to mainly sorption and limited degradation. During the first 2 days of incubation about more than 90% of atrazine and enrofloxacin disappeared, while about half of the sulfamethazine added remained in the vials, indicating bioavailability of sulfamethazine was higher than atrazine and enrofloxacin. At the end of the 45-day incubation period extractable percentages for atrazine, enrofloxacin and sulfamethazine were lower than extractable percentages at the end of 8 days for the

sorption experiment which suggested that long-term incubations caused the selected chemicals to be degraded or to become unavailable. Disappearance of sulfamethazine was slower than disappearance of atrazine and enrofloxacin.

Chapter 4 provides information on the effect of atrazine, enrofloxacin and sulfamethazine on denitrification potential and denitrifier community size on wood chips. Atrazine added at an initial concentration of 5 mg L^{-1} did not inhibit denitrification enzyme activity or the denitrifier population on wood chips; however sulfamethazine at same concentration reduced denitrification rate and population of denitrifiers estimated by MPN and qPCR during the first 5 days of incubation. However, after 45 days the denitrification rate and denitrifier populations were similar to rates and populations of the untreated control. In a similar manner enrofloxacin did not interfere with denitrification rate, but the MPN was reduced compared to the first two days of incubation although *nosZ1* copy number did not decline. The results of this study indicate that these antibiotics are unlikely to impact the performance of wood-chip reactors used for nitrate removal from drainage water.

APPENDIX A.WOOD CHIP DENITRIFYING REACTOR

Wood chips were collected from Iowa State University Research Farm, Ames, IA and the collection site is shown in Figure 1a, 1b, and 1c. The reactor is located in a field with corn-soybean rotation. It was set up 10 years ago.

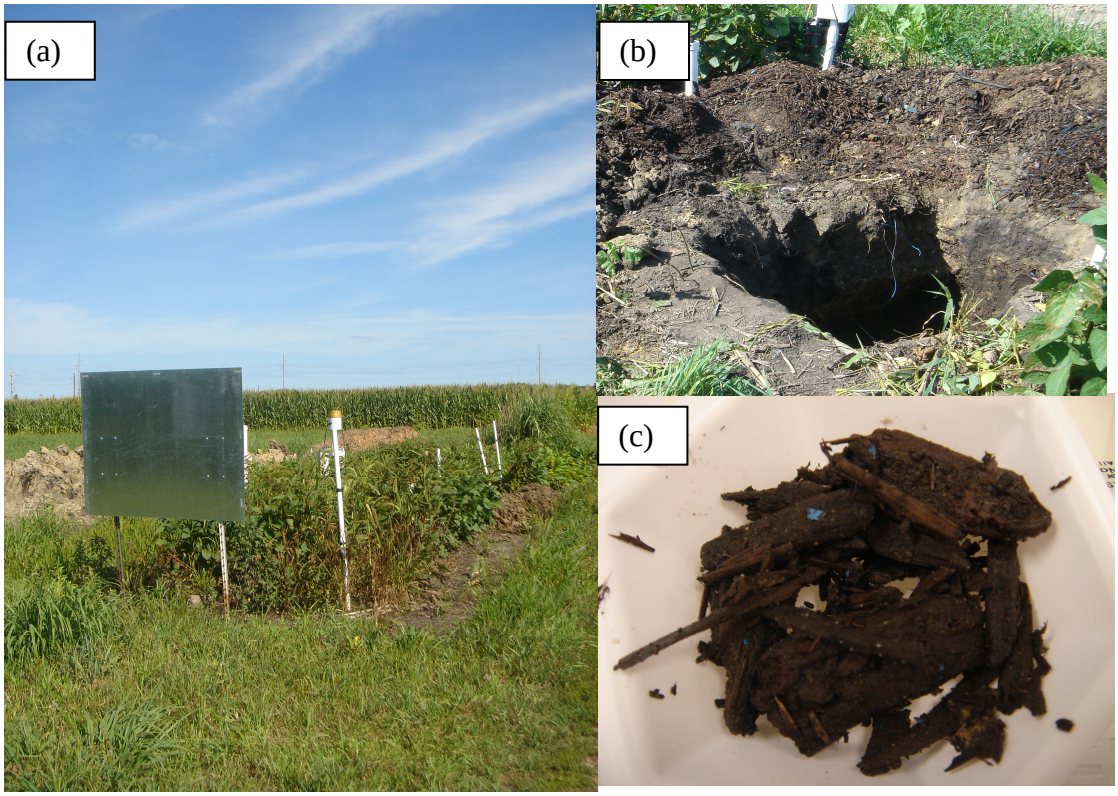


Figure A.1.(a) Wood chips collection site, (b) excavating wood chips and (c) collected wood chips.

APPENDIX B. ORGANIC SOLVENT EXTRACTION SET UP

Selected chemicals were extracted from wood chips as described in Chapter 3 and Chapter 4. Prior to extraction of the chemicals from wood chips with HLB SPE cartridges, the organic solvent was evaporated with an analytical evaporator (Figure B.1.), and then cleaned up and concentrated with HLB SPE cartridges using the vacuum manifold (Figure B.2.)

Figure B.1. Evaporation of organic solvent (acetonitrile) from samples under N2 gas flow at 40 C°.

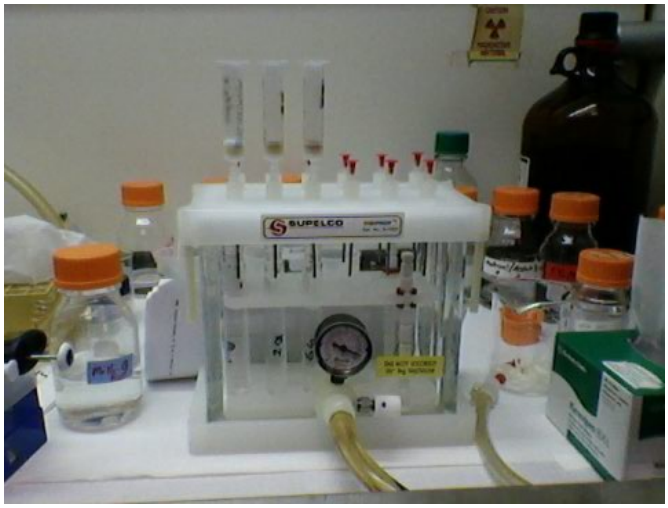


Figure B2. Cleaning and concentrating extracted chemicals with HLB cartridges with selected SPE methods

APPENDIX C. HPLC CHROMATOGRAMS

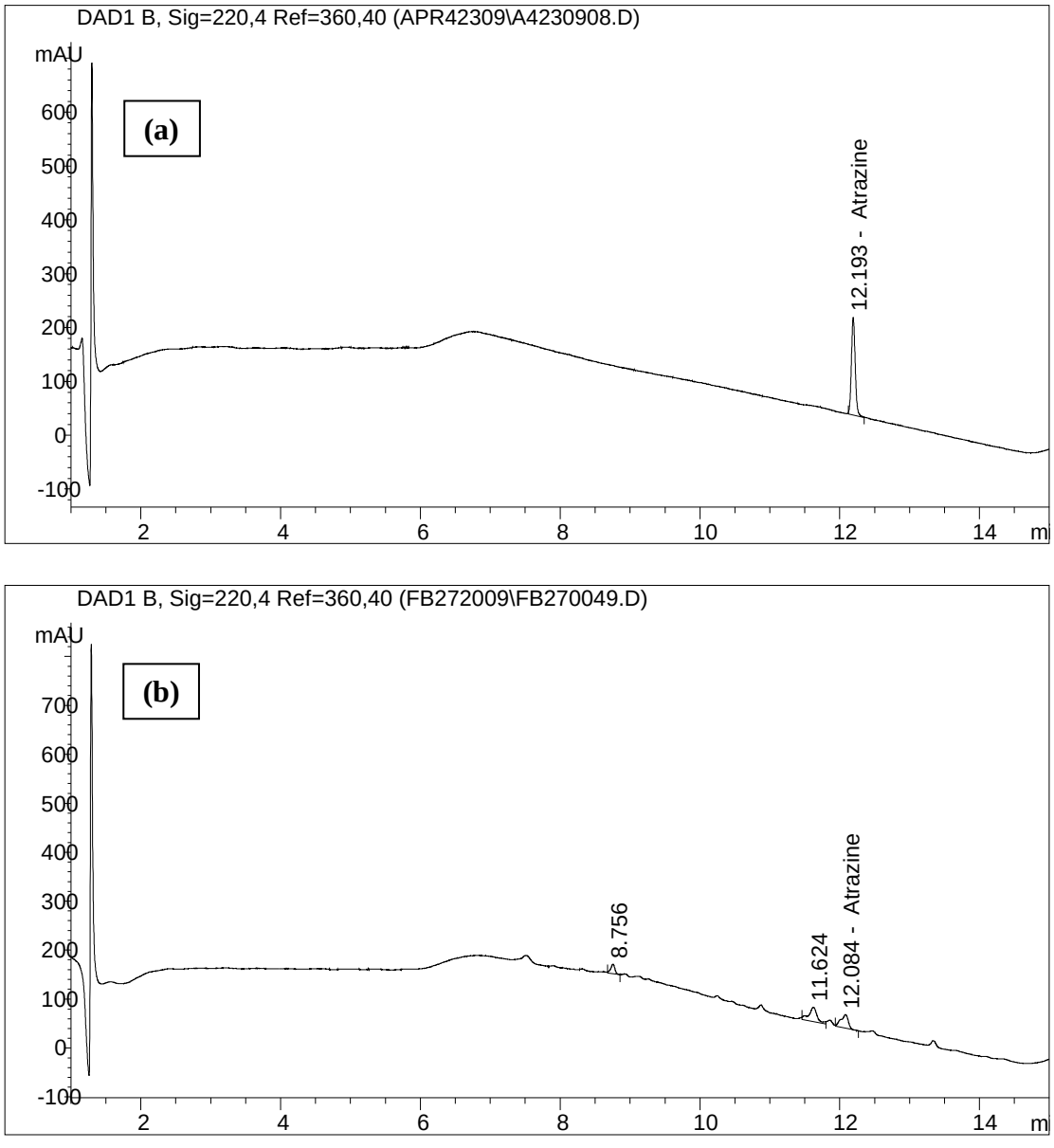


Figure C.1. Chromatogram of (a) atrazine standard (1 mg L^{-1}), (b) supernatant 48 hours after wood chips spiked with 1 mg L^{-1}atrazine.

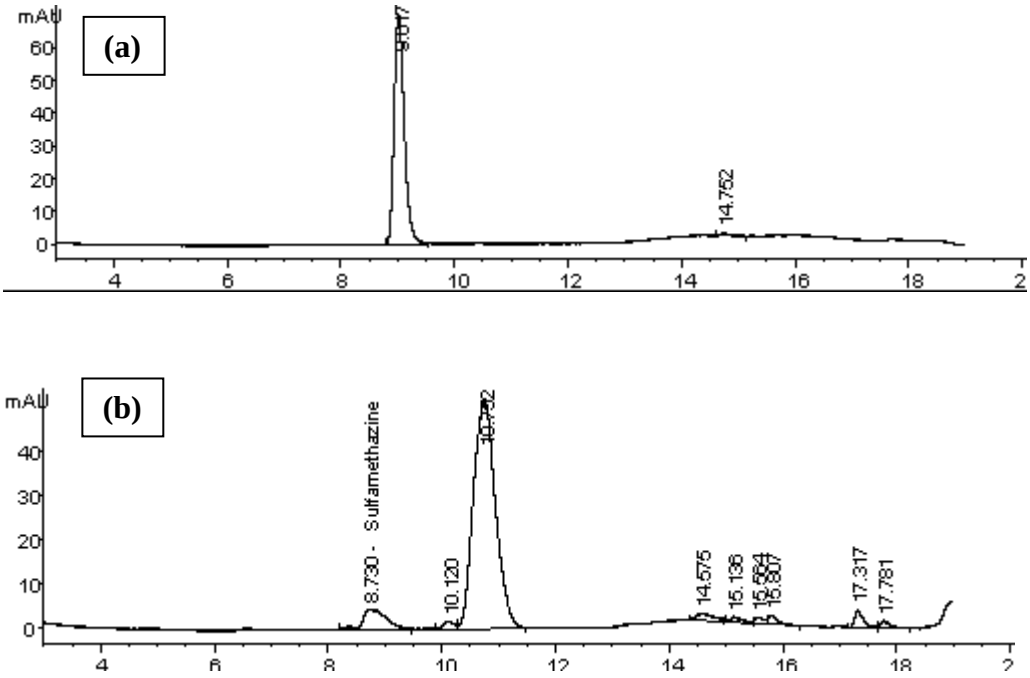


Figure C.2. (a) Sulfamethazine standard (5 mg L^{-1}), (b) wood chips spiked with sulfamethazine (5 mg L^{-1})at day 5.

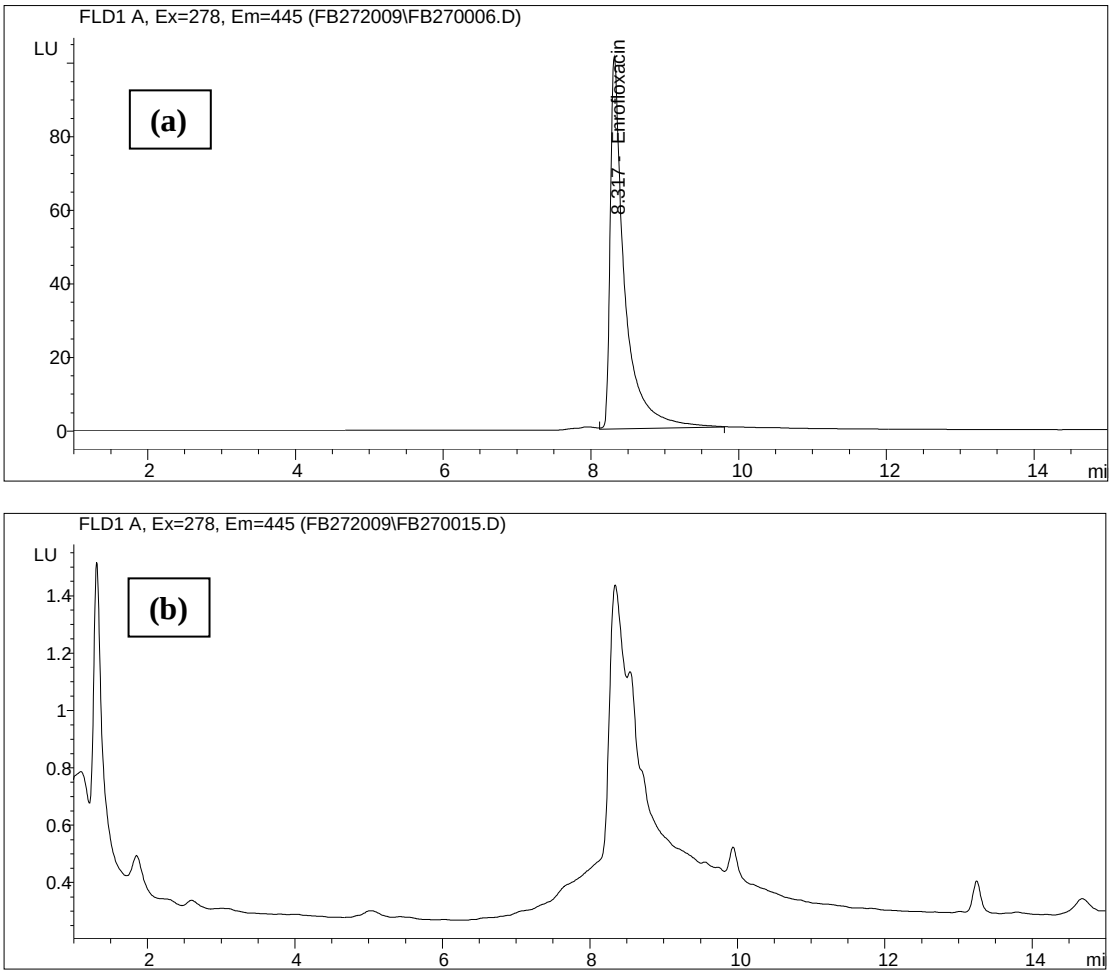


Figure C.3. (a) enrofloxacin standard (1 mg L^{-1}) (b) enrofloxacin chromatogram after 48 hours of application onto woodchips

APPENDIX D. DATA FOR CHAPTER 3

Table D.1. Atrazine Sorption – Desorption Isotherms Data

Chem.	$C_{initial}$	$C_{aq\text{-}sorp}$	$C_{aq\text{-}desorb1}$	$C_{aq\text{-}desorb2}$	$C_{wood\text{-}sorp}$	$C_{wood\text{-}desorb1}$	$C_{wood\text{-}desorb2}$
	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Atrazine	0.55	0.08	-	-	4.66	-	-
	1.17	0.10	0.02	0.04	10.54	10.22	9.95
	1.71	0.16	0.09	0.06	15.07	13.57	13.24
	2.30	0.23	0.13	0.08	20.31	19.03	18.55
	2.88	0.31	0.18	0.10	25.19	23.61	22.96
	5.65	0.71	0.30	0.19	48.87	46.31	45.03
Enrofloxacin	1.17	0.01			32.28		
	2.08	0.04	0.04	0.02	39.89	38.89	38.30
	2.90	0.09	0.04	0.03	54.41	53.21	52.35
	3.70	0.11	0.07	0.05	69.98	68.81	67.70
	4.52	0.15	0.09	0.07	84.92	82.68	80.98
	7.84	0.23	0.22	0.11	109.9	103.78	100.15
Monensin	0.77	0.05	-	-	7.09	-	-
	1.74	0.06	0.12	0.06	16.42	15.48	14.96
	2.22	0.04	0.26	0.06	21.32	19.01	18.52
	2.52	0.06	0.34	0.03	24.17	21.08	20.80
	3.66	0.08	0.40	0.12	35.12	31.74	30.67
	4.23	0.16.	0.66	0.22	40.48	34.62	32.62
Sulfamethazine	0.52	0.08	-		4.38	-	-
	0.93	0.17	0.03	0.07	7.45	7.04	6.22
	1.42	0.20	0.12	0.08	12.00	10.69	9.96
	1.93	0.34	0.13	0.11	15.01	13.64	12.77
	2.36	0.43	0.15	0.12	18.72	17.12	16.04
	4.20	0.82	0.32	0.22	21.17	27.76	25.80

Table D.2. Desorption and Organic Solvent Extractions

Chemical	[a]M_i	Desorb 1	Desorb 2	Solvent Extraction	Chemical	M_i	Desorb 1	Desorb 2	Solvent Extraction
	mg	[b]%	%	%		mg	%	%	%
ATRAZ	11.57	2.06	2.76	-	ENRO	20.44	0.04	2.96	1.27
ATRAZ	11.41	-	-	17.09	ENRO	20.46	0.05	2.35	0.27
ATRAZ	11.58	-	-	-	ENRO	20.31	0.07	1.56	1.12
ATRAZ	16.71	5.47	1.99	16.11	ENRO	28.12	0.38	1.62	1.33
ATRAZ	16.44	18.78	2.82	-	ENRO	28.45	0.84	1.41	1.53
ATRAZ	16.61	5.10	2.99	26.41	ENRO	28.44	2.86	2.30	0.62
ATRAZ	22.39	4.70	2.53	15.98	ENRO	33.63	0.38	2.43	1.59
ATRAZ	22.63	-	-	-	ENRO	36.61	2.01	-	-
ATRAZ	22.66	7.20	1.71	16.73	ENRO	36.22	0.24	2.08	1.24
ATRAZ	28.17	8.87	2.71	13.80	ENRO	44.12	0.89	2.64	1.44
ATRAZ	28.24	5.21	2.11	-	ENRO	44.68	1.38	2.07	1.43
ATRAZ	28.24	4.54	1.78	18.61	ENRO	44.46	1.42	2.08	1.63
ATRAZ	55.22	6.29	3.18	8.46	ENRO	77.49	2.38	2.97	1.83
ATRAZ	56.55	3.36	2.67	-	ENRO	68.96	2.10	3.93	1.52
ATRAZ	55.77	5.48	2.36	15.80	ENRO	75.13	2.56	4.82	1.43
SMZ	9.14	1.30	1.11	15.39	MON	17.09	7.24	1.54	0.67
SMZ	9.13	4.90	-	-	MON	17.13	4.99	2.63	0.01
SMZ	9.19	10.56	-	-	MON	17.14	4.84	5.50	
SMZ	14.03	9.49	0.71	11.41	MON	21.81	8.81	2.93	0.13
SMZ	13.94	14.08	0.74	11.32	MON	21.89	10.70	1.58	0.44
SMZ	13.94	9.67	0.68	10.12	MON	21.86	12.99	2.42	0.36
SMZ	17.02	10.56	0.96	12.80	MON	24.87	13.00	2.73	1.46
SMZ	18.84	9.56	0.76	12.11	MON	24.98	11.43	0.94	0.13
SMZ	19.10	8.44	0.75	19.12	MON	24.89	13.96	-0.22	0.73
SMZ	23.19	9.84	0.71	12.12	MON	36.27	11.03	2.15	0.19
SMZ	23.33	6.26	0.74	12.43	MON	35.92	9.38	2.41	1.12
SMZ	23.19	10.04	0.67	8.77	MON	36.18	8.47	4.54	
SMZ	41.13	12.05	0.85	10.28	MON	44.05	13.31	3.88	0.14
SMZ	41.48	10.13	0.71	10.92	MON	41.06	14.85	5.36	0.07

[a]M_i = Initial mass in the aqueous phase

[b]**%** = Recovery percentage of adsorbed chemical from wood chips

APPENDIX E DATA FOR CHAPTER 4

Table E.1. Dissipation Assay. Atrazine, Enrofloxacin, Sulfamethazine in aqueous phase and solid phase over 45 days.

Chemical	Time	Control C_{aq}	Sterile wood chips C_{aq}	Non - Sterile wood chips C_{aq}
	days	mg L^{-1}	mg L^{-1}	mgL^{-1}
Atrazine	0	4.23	4.01	4.89
Atrazine	2	4.25	1.55	0.32
Atrazine	5	4.25	0.30	0.26
Atrazine	20	4.23	0.18	0.31
Atrazine	30	4.24	0.19	0.40
Atrazine	45	4.26	0.14	0.41
Enrofloxacin	0	5.29	5.46	5.14
Enrofloxacin	2	5.35	0.30	0.11
Enrofloxacin	5	5.27	0.24	0.07
Enrofloxacin	20	5.33	0.18	0.07
Enrofloxacin	30	-	0.30	0.07
Enrofloxacin	45	5.40	0.30	0.05
Sulfamethazine	0	5.15	6.00	5.26
Sulfamethazine	2	4.91	3.12	3.48
Sulfamethazine	5	4.79	2.48	2.59
Sulfamethazine	20	5.00	1.13	1.50
Sulfamethazine	30	-	-	0.57
Sulfamethazine	45	5.35	0.69	0.63

Table E2. Nitrous oxide production rate calculations

Treatment Type	Description	Sampling Time	N_2O prod. rate	Mean of triplicates	STDEV
		days	ug N $g^{-1}hr^{-1}$	ug N $g^{-1}hr^{-1}$	
Control	no chemical	0	1.34		
Control	no chemical	0	0.86	1.22	0.32
Control	no chemical	0	1.47		
Control	no chemical	2	2.91	2.53	-
Control	no chemical	2	2.16		
Control	no chemical	5	2.42		
Control	no chemical	5	1.28	1.73	0.60
Control	no chemical	5	1.50		
Control	no chemical	20	8.04		
Control	no chemical	20	2.61	4.57	3.01
Control	no chemical	20	3.06		
Control	no chemical	45	2.48		
Control	no chemical	45	2.90	2.74	0.23
Control	no chemical	45	2.84		
Atrazine	5 mg/L	0	1.34		
Atrazine	5 mg/L	0	0.86	1.22	0.32
Atrazine	5 mg/L	0	1.47		
Atrazine	5 mg/L	2	4.07	4.07	0.32
Atrazine	5 mg/L	2	3.31		
Atrazine	5 mg/L	5	1.64		
Atrazine	5 mg/L	5	7.55	4.90	3.00
Atrazine	5 mg/L	5	5.50		
Atrazine	5 mg/L	20	2.01		
Atrazine	5 mg/L	20	3.30	3.12	1.03
Atrazine	5 mg/L	20	4.04		
Atrazine	5 mg/L	45	10.65		
Atrazine	5 mg/L	45	3.62	5.49	4.53
Atrazine	5 mg/L	45	2.19		

Table E.3. Most-probable-number enumarations for denitrifiers under atrazine, enrofloxacin and sulfamethazine treatments

Sample	Day	MPN	UCL	LCL
Control	0	3.5E+08	3.11E+08	1.E+08
Control	0	9.5E+07	3.14E+08	3.E+07
Control	2	7.5E+08	3.11E+08	2.E+08
Control	2	8E+08	*2.63E+09*	*2.E+08*
Control	5	2.1E+09	3.11E+08	6.E+08
Control	5	1E+08	1.01E+08	3.E+07
Control	20	2.1E+08	6.97E+08	6.E+07
Control	20	1.2E+09	3.8E+09	3.E+08
Control	45	*9.8E+08*	*3.5E+09*	*2.9E+08*
Atrazine	0	3.5E+08	3.11E+08	1.E+08
Atrazine	0	9.5E+07	3.14E+08	3.E+07
Atrazine	2	7E+09	*2.29E+10*	*2.E+09*
Atrazine	2	2.4E+08	7.96E+08	7.E+07
Atrazine	5	6.3E+08	2.08E+09	2.E+08
Atrazine	5	3.5E+08	1.15E+09	1.E+08
Atrazine	20	2.2E+08	7.31E+08	7.E+07
Atrazine	20	5.4E+08	1.77E+09	2.E+08
Atrazine	45	2.4E+09	7.9E+09	7.3E+08
Enrofloxacin	0	3.5E+08	3.11E+08	1.E+08
Enrofloxacin	0	9.5E+07	3.14E+08	3.E+07
Enrofloxacin	2	5.8E+07	1.91E+08	2.E+07
Enrofloxacin	2	1.8E+07	60253354	6.E+06
Enrofloxacin	5	2E+08	6.75E+08	6.E+07
Enrofloxacin	5	2.2E+08	7.31E+08	7.E+07
Enrofloxacin	20	9.9E+07	3.26E+08	3.E+07
Enrofloxacin	20	1E+09	3.37E+09	3.E+08
Enrofloxacin	45	8.3E+09	2.7E+10	2.5E+09
Sulfamethazine	0	3.5E+08	3.11E+08	1.E+08
Sulfamethazine	0	9.5E+07	3.14E+08	3.E+07
Sulfamethazine	2	3E+07	98983456	9.E+06
Sulfamethazine	2	7.6E+08	2.51E+09	2.E+08
Sulfamethazine	5	1.4E+08	4.77E+08	4.E+07
Sulfamethazine	5	2.2E+08	7.1E+08	7.E+07
Sulfamethazine	20	3.6E+08	1.19E+09	1.E+08
Sulfamethazine	20	1.6E+09	5.17E+09	5.E+08
Sulfamethazine	45	6.3E+08	5.2E+08	4.3E+07

Table E4. *noz1* copy numbers based on qPCR threshold cycle numbers

Chemical	Time	*nosZ1* copy no	Chemical	Time	*nosZ1* copy no
	Days			Days	
Enrofloxacin	2	1.04E+08	Control	0	2.29E+08
Enrofloxacin	2	1.02E+08	Control	2	1.84E+08
Enrofloxacin	5	3.14E+08	Control	2	1.06E+08
Enrofloxacin	5	1.94E+08	Control	5	5.24E+08
Enrofloxacin	5	1.11E+08	Control	5	3.34E+09
Enrofloxacin	20	1.9E+09	Control	20	2.48E+09
Enrofloxacin	20	1.88E+09	Control	20	8.4E+09
Enrofloxacin	20	2.32E+09	Control	20	5.11E+08
Enrofloxacin	45	5.88E+08	Control	45	1.06E+10
Enrofloxacin	45	1.66E+09	Control	45	1.78E+08
Enrofloxacin	45	3.34E+09	Control	45	56098017
Sulfamethazine	2	2.92E+08	Atrazine	2	1.32E+08
Sulfamethazine	2	1.37E+08	Atrazine	2	5.57E+08
Sulfamethazine	5	1.66E+08	Atrazine	5	1.35E+09
Sulfamethazine	5	1.7E+08	Atrazine	5	6.91E+08
Sulfamethazine	5	1.3E+08	Atrazine	5	3.28E+08
Sulfamethazine	20	2.37E+08	Atrazine	20	9.85E+09
Sulfamethazine	20	2.5E+08	Atrazine	20	4.74E+10
Sulfamethazine	20	3.37E+08	Atrazine	45	3.21E+09
Sulfamethazine	45	3.6E+08	Atrazine	45	5.83E+09
Sulfamethazine	45	3.36E+08	Atrazine	45	4.86E+09
Sulfamethazine	45	3.71E+09			