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Pharmaceutical compounds in treatment wetlands: potential removal and effects on microbial processes

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PHARMACEUTICAL COMPOUNDS IN TREATMENT WETLANDS: POTENTIAL REMOVAL AND EFFECTS ON MICROBIAL PROCESSES

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DEDICATION

This work is dedicated to Mom, Dad and Uncle Booper, you have shaped my life, opened my eyes and provided me with the tools needed to be successful at whatever it is I decide to do with my life. For that I am gracious and could never thank you enough.

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I would like to thank everyone who has helped me along the way to shape my life and mold me into the person (whether good or bad) I am today. I am thankful for the huge family I am a part of and the broad spectrum of views to which I have been exposed.

To Dr. White, I can still remember vividly the first day we met and talked over lunch at Walk-On's. The analogies began that day with one about the pile of sand and continued during my entire time at LSU. Thank you for your patience; I couldn't imagine another professor allowing me to ask so many questions without barring me from their office. Over the last 4 years I have noticed that our dynamic has evolved from student-mentor to colleagues. You have provided me with the best example I could imagine of how to mentor and educate students. If I ever have students, I hope that I can provide them the effort and time you allotted me.

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ABSTRACT

Pharmaceutically active compounds (PhACs) have been detected in the aquatic environment as a result of loading from various sources. In Louisiana, USA, many municipalities treat wastewater using natural systems, such as lagoons and wetlands, rather than using conventional wastewater treatment technologies and may discharge PhACs into the environment. These treatment systems are not designed to remove PhACs from wastewater, nor is it currently a regulatory requirement. Research on the fate of PhACs in the environment is needed to understand impacts on Louisiana's important coastal system. Wastewater sampling for PhACs at the Mandeville, LA wastewater treatment plant (WWTP) determined that this system significantly reduces the concentrations of PhACs prior to discharge into the environment. Most of the concentration reduction occurred in the first phase of treatment, where research suggests sorption may have been the major removal pathway. A wetland soil similar to the forested wetland at the Mandeville WWTP was tested for sorption and desorption of ciprofloxacin, ofloxacin and norfloxacin. Two HPLC methods were developed for compound analyses in this experiment. It was determined that sorption to wetland soil is a major and potentially long-term removal pathway for these pharmaceutical compounds from wastewater. The presence of antibiotics in the environment may have the ability to alter the microbial community in soils. We observed the greatest effect of antibiotics on soil microbial respiration followed this trend: sulfamethoxazole>ciprofloxacin>tetracycline. Most antibiotic effects were seen in the mineral, not the peat soil. Suppression of microbial respiration was observed, at low or high concentrations, depending on the antibiotic and soil examined. This result indicates that antibiotics can have negative impacts on microbial functions in treatment wetlands at "environmentally relevant" concentrations. Based on the findings of these studies, WWTPs

systems similar to the Mandeville plant are effective at reducing the concentrations of many PhACs discharged into surface waters. Furthermore, sorption appears to be a major pathway for this concentration reduction. However, antibiotics showed the potential to exhibit a negative influence on microbial activity in wetland soil. These natural treatment systems appear to be ideal for effectively treating PhACs in surface waters.

CHAPTER 1: INTRODUCTION

1.1. Introduction

The presence of pharmaceuticals in the environment is an area of research that has recently garnered worldwide attention (Ternes, 1998; Daughton and Ternes, 1999; Kolpin et al., 2002; Ternes et al., 2002; Webb et al., 2003). Past and current research has focused on wastewater treatment plant (WWTP) treatment capacity, soil and aqueous concentrations, sorption, desorption and toxicology (Halling-Sørensen et al., 2002; Miao et al., 2004; Bendz et al., 2005; Kolz et al., 2005; Pan et al., 2009). Reduced cost, increased accuracy and decreased analysis time has spurred research in this field and lead to a greater understanding of the presence, transport and fate of pharmaceutically active compounds (PhACs) in the environment (Kolpin et al., 2002).

PhACs enter the environment through a variety of pathways (Figure 1.1), with human excretion being a major source (Hirsch et al., 1999). Most pharmaceuticals are not completely absorbed by our bodies, and the remaining parent compound and its associated metabolites are excreted though urine and feces. The same can be said for livestock treated with veterinary medicines. While agricultural pharmaceuticals are released in the environment via farms, stock yards and sludge application, human PhACs are generally released through septic systems or WWTP effluents (Hirsch et al., 1999).

As technology to monitor and detect PhACs has improved, the scope and scale of our understanding of the issue has grown. Research has branched out to examine toxicological effects of these compounds at low and high trophic levels (Stuer-Lauridsen et al., 2000; Webb et al., 2003; Schwaiger et al., 2004; Mimeault et al., 2005). A 2006 USGS report found that the sex

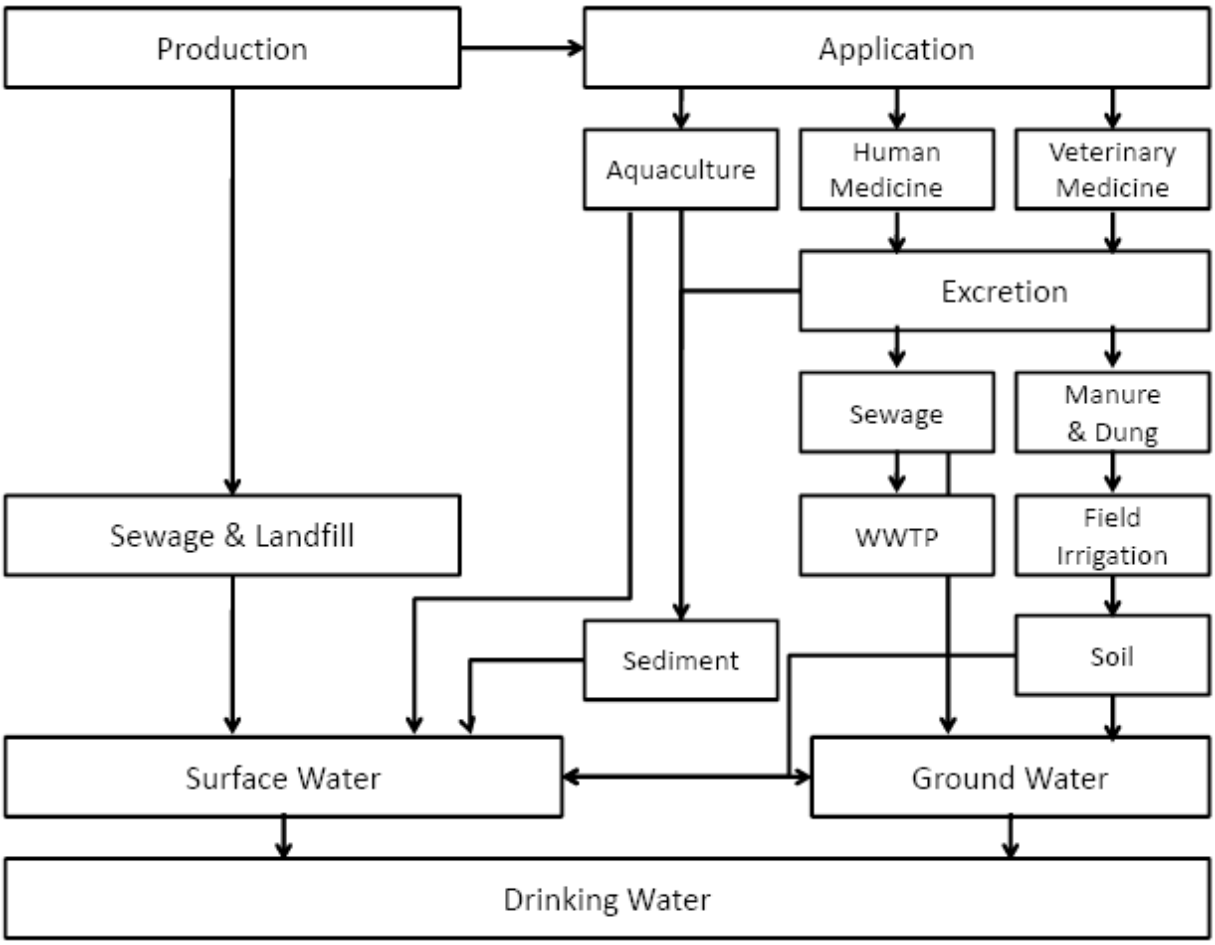


Figure 1.1. Potential pathways for PhACs to enter the environment. Adapted from Hirsch et al. 1999.

of small mouth bass was altered in rivers feeding the Potomac River, possibly as a result of endocrine disruptors (Chambers and Leiker, 2006). A study was also conducted that showed that the anti-inflammatory drug diclofenac was responsible for vulture population decline in Pakistan (Oaks and Gilbert, 2004). PhACs have also been found to affect algae and microbes (Halling-Sørensen, 2001; Isidori et al., 2005). While PhACs have been detected in drinking water, to the author's knowledge, there have been no studies demonstrating that exposure to pharmaceuticals in the environment and in drinking water has negatively impacted humans.

Hundreds of drugs are prescribed worldwide, and new compounds are constantly being developed for human, veterinary and agricultural uses. In Germany in 1995, 100 tons of

prescription drugs were purchased (Ternes, 1998). This number does not account for the number of over the counter and veterinary medicines that were used (Ternes, 1998). The average number of prescriptions and nonprescription drugs dispensed during doctor visits has risen from 189.8 per 100 people in the U.S. population during 1995-1996 to 226.4 from 2003-2004 (NCHS, 2006). The percentage of the US population using a prescription drug each month has risen from 39.1% between 1988-1994 to 45.3% from 1999-2002 (NCHS, 2006).

Due to the widespread usage of pharmaceuticals in the world, numerous drugs have been detected in the environment. Advances in analytical instrumentation have lead to the ability to extract and measure PhAC concentrations in the environment down to the parts per trillion (pptr) level. As detection limits decrease, there is an increase in the likelihood of detecting and quantifying compounds, which will ultimately lead to a greater understanding of the processes that influence the fate of PhACs. Many studies have focused on measuring compounds in the environment and removal or persistence with specific water treatment processes. A short list of these drugs, along with measured concentrations, removal rates, removal processes and drug classes are listed in Table 1.1. Data in the table also shows that the range of compound concentrations varies greatly from study to study.

In order to determine the existence and concentrations of pharmaceuticals in environmental samples, advanced techniques have been developed to extract, concentrate, and identify these compounds. Concentrations of PhACs are generally found in the environment in the parts per billion ($\mu g\ L^{-1}$) or pptr ($ng\ L^{-1}$) range. Therefore, detecting a PhAC in a water sample must involve extraction and concentration of the target compound. Two commonly used methods are solid phase extraction (SPE) and passive sampling. SPE methods have been developed to target the removal of individual compounds or groups of compounds from samples (Koutsouba et al., 2003; Moldovan, 2006; Vieno et al., 2006). For SPE, the PhAC compounds

Table 1.1. Select pharmaceutical compounds detected in the environment along with their percent reduction and water treatment method.

Select PhACs in the Environment

Drug	Reduction	Environmental Concentration	Treatment Method	Drug Use	Study
Acetylsalicylic acid	81%	0.220 μg/L	STP removal	Aspirin	(Ternes, 1998)
Bezafibrate	83%	2.200 μg/L	STP removal	Lipid Regulator	(Ternes, 1998)
Caffeine	94%		STP Removal	Stimulant	(Bendz et al., 2005)
Caffeine	>99%	0.190 μg/L	STP removal	Stimulant	(Ternes et al., 2001)
Carbamazepine	30%	1.680 μg/L	STP Removal	Anti-epileptic, psychiatric drug	(Bendz et al., 2005)
Carbamazepine		0.251 μg/L		Anti-epileptic, psychiatric drug	(Miao et al., 2005)
Clarithromycin		0.240 μg/L		Antibiotic	(Hirsch et al., 1999)
Clofibric acid	51%	0.320 μg/L	STP Removal	Lipid Regulator	(Ternes, 1998)
Clofibric acid		0.003 μg/L		Lipid Regulator	(Webb et al., 2003)
Diclofenac	69%	0.810 μg/L	STP Removal	Analgesic/Anti-inflammatory	(Ternes, 1998)
Diclofenac	22%	0.160 μg/L	STP Removal	Analgesic/Anti-inflammatory	(Bendz et al., 2005)
Fenofibric acid	64%	0.380 μg/L	STP Removal	Lipid Regulator	(Ternes, 1998)
Gemfibrozil	67±51%	0.000-0.180 μg/L	Santa Anna River	Lipid Regulator	(Gross et al., 2004)
Ibuprofen	90%	0.370 μg/L	STP Removal	Analgesic/Anti-inflammatory	(Ternes, 1998)
Ibuprofen	47±37%	0.001-0.023 μg/L	Santa Anna Wetland	Analgesic/Anti-inflammatory	(Gross et al., 2004)
Naproxen	66%	0.300 μg/L	STP Removal	Analgesic/Anti-inflammatory	(Ternes, 1998)
Naproxen	100%	0.000-0.105 μg/L	Santa Anna River	Analgesic/Anti-inflammatory	(Gross et al., 2004)
Propranolol	32%	0.050 μg/L	STP Removal	Anti-epileptic	(Bendz et al., 2005)
Propranolol	96%	0.170 μg/L	STP Removal	Anti-epileptic	(Ternes, 1998)
Triclosan	97-99%		Activated Sludge Treatment	Antibacterial Agent	(Waltman et al., 2006)

STP = Sewage Treatment Plant

are extracted and concentrated from a pre-determined volume of sample. Passive sampling involves the use of a sorption medium that is deployed in the environment for a pre-determined amount of time. As water moves across the medium, target PhACs accumulate on sorbents in the sampler, which are later extracted for analysis (Petty et al., 2004; Alvarez et al., 2008). The instrumentation used to analyze these compounds is usually a gas (GC) or liquid (LC) chromatograph. These instruments are usually coupled with mass spectrometers (single or tandem), ultra-violet/visible spectrometers and/or fluorescence detectors.

1.2. Mississippi River and Davis Pond Pilot Study

Pharmaceuticals and personal care products have been detected in the Mississippi River (Zhang et al., 2007). A pilot study was conducted in February of 2007 to determine concentrations of a suite of pharmaceuticals in the Mississippi River at the locations north and south of Baton Rouge and at the inflow to both the Davis Pond and Caernarvon freshwater diversions (Figure 1.2). Samples were also taken at the midpoint of the Davis Pond diversion ponding area and the outfall into Lake Cataouatche to determine if the diversion wetland could be a potential tool for removal of pharmaceuticals from river water.

Water samples were filtered to remove particulates and the compounds of interest were extracted using solid phase extraction, which is discussed more extensively in Chapter 2. Samples taken from the Mississippi River, both north and south of Baton Rouge and New Orleans also contained trace amounts of PhACs. Sample concentrations were averaged for all sampling sites and potential compound discharge into the Gulf of Mexico was calculated using the average river discharge rate of 450,000 $ft^3\ s^{-1}$ (12,750 $m^3\ s^{-1}$). The calculations demonstrate that even when only a trace amount of a contaminant is detected, due to the volume and discharge rate of the Mississippi River, the total mass of contaminant equates to several metric tons per year being transported down the river and into coastal waters of Louisiana (Table 1.2).

Figure 1.2. Sampling locations in the Mississippi River during February of 2007.

Table 1.2. Mississippi River concentrations and discharge into the Gulf of Mexico based on average annual discharge.

Drug	Mississippi River Concentration $\mu g\ L^{-1}$	Potential Mass Discharge metric ton yr^{-1}
Cotinine	0.014	5.5
Caffeine	0.071	28.7
Carbamazepine	0.014	5.8
Fluoxetine	0.006	2.6
Naproxen	0.007	2.8
Ibuprofen	0.010	4.0
Sulfamethoxazole	0.028	11.4

Caffeine, contained in many common drinks and medicines, had the highest annual loading at nearly 29 metric tons a year. These samples were tested for 15 compounds, but only 7 were detected. The compound that was detected at the lowest concentration (fluoxetine) had a potential annual discharge rate of 2.6 metric tons to the Gulf of Mexico.

Of the 15 compounds that were under investigation, only 5 were detected in the inflow to Davis Pond, while 3 other drugs were detected at either the midpoint in Davis Pond or the outfall

into Lake Cataouatche (Table 1.3). All concentrations of detected compounds were less than 0.1 ppb ($\mu g\ L^{-1}$). Based on the concentrations observed, the Davis Pond diversion could potentially reduce loading to coastal waters by 73 kg annually. The diversion wetland potentially has the ability to reduce the PhAC compound concentrations, based on the estimates of percent removal near 100%. The designed use of the diversion is to control salinity in the Barataria Basin, but this initial screening demonstrates that it could also function to reduce some contaminants, specifically pharmaceuticals, in the Mississippi River water that is diverted into coastal Louisiana.

Table 1.3. Pharmaceutical concentrations measured for the Davis Pond freshwater diversion in February of 2007 and their loading and concentration reduction rates.

	DP Inflow	DP Midpoint	Lake Cataouatche	Inflow 2007	Discharge 2007	% Reduction
	$\mu g\ L^{-1}$			$kg\ yr^{-1}$		
Cotinine	0.013	0.013	b.d.	19	0	>99%
Caffeine	0.075	0.081	0.024	108	35	68%
Carbamazepine	0.021	0.023	b.d	30	0	>99%
Fluoxetine	0.006	b.d	b.d	9	0	>99%
Acetaminophen	b.d	0.045	b.d	66	0	>99%
Naproxen	b.d	0.007	b.d	10	0	>99%
Ibuprofen	0.010	b.d	b.d	14	0	>99%
Sulfamethoxazole	b.d	b.d	0.010	0	15	0%

b.d.: below detection

The Mississippi River contains a significant amount of contaminants that are discharged into the Gulf of Mexico (Boyd et al., 2003; Zhang et al., 2007). The use of recycled wastewater for drinking water is a controversial subject. Many municipalities directly or indirectly discharge treated wastewater into the Mississippi River, which likely contains pharmaceuticals and personal care products. New Orleans, LA, which utilizes the Mississippi River for some portion of its drinking water. The water is treated prior to consumption but the treatment is not designed to remove these compounds. The use of a diversion, such as Davis Pond, to reduce aqueous pharmaceutical concentrations would be a large scale effort to reduce loadings from the

Mississippi River into the Gulf of Mexico. However, communities along the river might also decrease their discharge of these compounds through the implementation of more efficient wastewater treatment systems or a tertiary treatment system such as a natural or constructed wetland This would decrease the potential for drinking water contamination in cities that use the Mississippi River as a drinking water source.

1.3. Conventional and Natural Wastewater Treatment

Communities in Louisiana use natural treatment systems to treat wastewater along with natural wetlands to polish and remove excess nutrients prior to discharge into rivers and lakes. While wetland treatment systems are excellent for nutrient and biological oxygen demand (BOD) reduction (Day et al., 2004), it has recently been shown that they are also effective at reducing aqueous concentrations of pharmaceuticals in wastewater (White et al., 2006; Matamoros et al., 2008).

In Mandeville, LA, located on the north shore of Lake Pontchartrain, the wastewater treatment systems consists of three earthen detention basins with aeration fountains, a constructed wetland with native vegetation and UV treatment. The treated wastewater then flows into Bayou Chinchuba before discharge into Lake Pontchartrain. This constructed system treats wastewater for a period of ~14 days before discharge into the Bayou for further removal of nutrients and BOD. This system contains several wastewater treatment technologies that are utilized by conventional plants, but has a longer retention time and the potential for greater treatment at the soil/water interface. Natural systems are desirable to communities because the construction costs are a fraction of conventional plants and they can be as efficient at achieving regulatory requirements for wastewater discharge. However, these systems generally require more land area, have less treatment capacity and take longer to achieve treatment levels of conventional treatment plants.

Many conventional treatment plants utilize constructed concrete detention basins which combine physical mixing, aeration, particulate precipitation and some form of disinfection technology to sterilize the water. Treatment of wastewater in these plants generally takes less than a few days, compared to the 2 weeks in the Mandeville system.

1.4. Aqueous Environmental Removal Mechanisms

In the environment, there are various mechanisms that impact the removal or degradation of PhACs. The effectiveness of these mechanisms varies with the physical properties of each individual compound and environmental conditions. While research has not been conducted to determine the physical properties that affect the fate of every PhAC in the environment, studies have shown that many processes play roles in treatment and degradation of these compounds in the environment. Some of these mechanisms are sorption, desorption, reduction, oxidation, temperature, pH, photolysis and complexation with minerals such as magnesium and iron (Tolls, 2001; Zhang and Huang, 2003; Vogna et al., 2004; Scheytt et al., 2005; ter Laak et al., 2006; Zhang and Huang, 2007). These varied mechanisms and soil properties collectively play a large role in aqueous concentration reductions within wastewater treatment plants and wetlands.

Sorption of pharmaceuticals to environmental medium, such as soil (mineral and organic) and suspended particulates is known to be a significant removal pathway for many compounds from the aqueous phase (Bonin and Simpson, 2007; Gu et al., 2007; Pan et al., 2009). However, there are compounds (i.e. carbamazepine) that appear to be more persistent in the environment, due to their low sorption potential (Andreozzi et al., 2002; Miao et al., 2005; Williams et al., 2006).

Within the Mandeville WWTP and wetland, there are significant amounts of organic materials (fecal matter) within the plant to sorb pharmaceuticals. However, the use of earthen detention ponds and a constructed and natural wetland to treat wastewater provides another

sorption medium, soil, not found in conventional concrete treatment plants. Therefore, the Mandeville treatment system provides a wider range of sorption sites (mineral and organic) than can be found in conventional treatment systems. This range of sorption sites, coupled with longer hydrologic retention times may make natural treatment systems more effective at reducing the aqueous concentrations of pharmaceuticals.

Each pharmaceutical has unique properties that influence its sorption potential, which will also vary with soil properties. Therefore, when measuring a single compound's sorption potential, the distribution coefficient (K_D) will only apply to that medium at that particular concentration. In order to gain a more generalized range of sorption potentials, a compounds K_D must be measured over a wide range of concentrations. The two most widely used models for describing compound behavior are the Langmuir and Freundlich isotherms. The following assumptions must be met to use the Langmuir isotherm; 1) adsorption must be a monolayer, 2) homogenous sorption surface, and 3) the sorption of a molecule is not influenced by the occupation of neighboring sorption sites. The assumptions of the Langmuir isotherm are not likely to apply to soils and sediment found in a wetland and treatment plant due to the heterogeneity of the sorption medium. Therefore, the Freundlich isotherm should be used when examining wetland soils. The Freundlich isotherm is an empirically derived coefficient obtained by plotting the Log of the amount sorbed to the soil, by the Log of the concentration in solution at equilibrium. The points are then fitted with a linear trendline and the y-intercept serves as the Freundlich coefficient (K_F). Higher values indicate higher sorption potential and generally values >3.5 are considered highly sorptive.

There has been a significant amount of research on the sorption of many pharmaceutical compounds (Pouliquen and LeBris, 1996; Kan et al., 2000; Thiele-Bruhn, 2003; Hildebrand et al., 2006; Bonin and Simpson, 2007). However, many studies address sorption to standard

substances (Gu and Karthikeyan, 2005; Zhang and Dong, 2008) and competition between compounds for binding has only been examined for traditional pollutants such as pesticides and herbicides (Pignatello et al., 2006; Shechter et al., 2006). In wastewater it has been demonstrated that many compounds are usually present, with the likelihood that many more are not detected (Bedner and MacCrehan, 2006; Gros et al., 2007; Kummerer, 2009b). While soils possess numerous sorption sites, the presence of multiple compounds in wastewater may lead to competition between compounds for specific binding sites.

1.5. Pharmaceutical Impacts on the Environment

While a significant amount of research has been conducted to detect and begin to understand the fate of PhACs in the environment, more is needed to address their potential impacts. There are hundreds of compounds and each has its own unique properties, resulting in varying reactions to environmental conditions and potential biological effects to organisms. Research has shown that discharged wastewater usually contains a suite of compounds (Kolpin et al., 2002; Watkinson et al., 2007), which may also lead to impacts as a result of multiple drug interactions. Many studies have examined the toxicological effects of these compounds on plants (Halling-Sorensen, 2000; Andreozzi et al., 2002), animals (Oaks and Gilbert, 2004; Chambers and Leiker, 2006) and microbes (Kummerer et al., 2000; Gu et al., 2002; Jiao et al., 2008). Microbial antibiotic resistance has garnered significant attention and it has been shown that microbes in the environment have developed resistance to many antibiotic compounds (Tendencia and de la Pena, 2001; Chelossi et al., 2003; Baker-Austin et al., 2008). However, the impacts of pharmaceuticals on microbial community activity is less well studied (Fountoulakis, 2004; Costanzo et al., 2005).

Wetland soils support a wide range of microbial communities, which are responsible for many ecosystem processes, including organic matter decomposition and nutrient cycling (Wright

and Reddy, 2001). The microbial community structure and activity is tied to the efficiency of nutrient cycling and ecosystem function (Yao et al., 2000). Many environmental factors, such as pH, organic matter composition, and nutrients, influence the microbial community structure (Ye et al., 2009). Physical (Wright et al., 2007) and chemical (Girvan et al., 2004) disturbances are known to alter the microbial community structure in soils. While there has been significant research on the effects of pesticides and herbicides on microbial activity (Chatterjee et al., 2010; Wang et al., 2010), relatively little has examined pharmaceutical effects, especially in wetlands.

Antibiotics, which are used to treat and prevent bacterial infections in humans and livestock, have been routinely detected in the environment (Hirsch et al., 1999; Haggard and Bartsch, 2009). Therefore, there is reason to believe that antibiotics may impact microbial function and community structure in environmental systems. A simple metric for determining an antibiotic impact in wetland soils is to measure the soil respiration. Gas evolution is a result of microbial respiration or activity, which produces CO_2, CH_4 and N_2 gas. If the addition of an antibiotic alters the soils respiration rate, then it has altered the microbial activity, which may impact organic matter decomposition and nutrient cycling. While, there has been little research on the effects of antibiotics on microbial activity, research has determined that many bacteria found in the environment posses resistant genes to commonly used human and agricultural antibiotics (Boon and Cattanach, 1999; Baker-Austin et al., 2008). However, it remains to be studied how long it may take resistance to develop in the environment or for the community structure to change once an antibiotic is added to the system.

1.6. Hypothesis

The research presented in this dissertation sought to examine the fate, transport and microbial effects of pharmaceutical compounds in a treatment wetland. Studies focused on compounds entering wetlands and surface waters in Southeast Louisiana and their removal by

wetland processes. Research also includes laboratory studies to understand the fate of these compounds through sorption and desorption and the effects of antibiotics on wetland soil microbial respiration (CO_2, CH_4 and N_2 production).

Natural wetland treatment systems will remove pharmaceuticals from domestic wastewater in amounts that are equal or greater than conventional wastewater treatment systems, and the major mechanism for removal (for most compounds) is sorption to particulates and sediments. The presence of multiple antibiotic compounds in a wetland can also result in competition for preferred binding sites between the compounds studied. Antibiotics will reduce the microbial activity in treatment wetlands. However, sorption may decrease the impact of antibiotics on wetland soil microbial communities. In addition, wetland soils that have previously sorbed pharmaceuticals may act as a source of pharmaceuticals if loading concentrations are decreased or stopped.

1.7. Synopsis of Chapters

In Chapter 2, the effectiveness of a “natural” wastewater treatment system in Mandeville, LA that uses earthen retention basins, constructed and natural wetlands along with aeration and UV exposure to treat wastewater over a ~14 day period is examined. Comparisons were made between this system and more conventional systems with regards to the reduction of aqueous concentrations of pharmaceuticals in wastewater. Individual treatment phases were also studied to determine their effectiveness, especially the natural treatment wetland. Estimations of the total annual loading and removal were calculated based on the concentrations observed.

After examining pharmaceutical removal in a full-scale wastewater treatment system in Chapter 2, it was decided to further examine sorption as a potential aqueous removal pathway for Chapter 3 and 4. While several analytical methods were available in the literature to detect the three fluoroquinolone (ciprofloxacin, norfloxacin, ofloxacin) antibiotic compounds, new

methods were needed in order to analyze samples and efficiently complete the experiment. In Chapter 3, HPLC methods were developed to analyze the three compounds both individually and simultaneously for use in determining sorption potentials. Chapter 4 presents the sorption and desorption potential of three antibiotics over a concentration range of 20 to 80 ppm, which simulates the long-term loading that a treatment wetland may experience over decades. Competition was examined between a three-compound mixture at 20 ppm of each compound and compared it the individual sorption potentials at 60 and 20 ppm. These comparisons shed light on sorption interactions when multiple compounds are present in the aqueous phase, which is common in wastewater effluent.

After examining the presence of pharmaceuticals in a wastewater treatment system and studying sorption as a major removal pathway, potential antibiotic effects on the microbial activity in a treatment wetland soil were determined in Chapter 5. Three widely used antibiotics (ciprofloxacin, tetracycline, sulfamethoxazole) were added to two wetland soils (peat, mineral) at "environmentally" relevant concentrations to examine effects on microbial activity. The evolution of CH_4, CO_2 and N_2 was measured for basal and substrate induced respiration (non carbon limited and also non nitrogen limited for N_2). Implications of all the research chapters are discussed in Chapter 6.

CHAPTER 2: REDUCTION OF PHARMACEUTICALLY ACTIVE COMPOUNDS BY A LAGOON-WETLAND WASTEWATER TREATMENT SYSTEM IN SOUTHEAST LOUISIANA[1]

2.1. Introduction

The fate of pharmaceutically active compounds (PhACs) in the aquatic environment is an emerging area of research (Ternes, 1998; Daughton and Ternes, 1999; Kolpin et al., 2002; Ternes et al., 2002; Webb et al., 2003; Kolpin et al., 2004; Jones et al., 2005a). A significant portion of this research has focused on the capacity of wastewater treatment plants (WWTPs) to remove PhACs from wastewater, and the contribution of WWTPs to PhAC loadings to receiving waters (Heberer et al., 2002; Boyd et al., 2003; Miao et al., 2004; Bendz et al., 2005; Joss et al., 2006; Lishman et al., 2006; Gobel et al., 2007). However, there are very few data on the ability of constructed wetlands and natural wetland systems to reduce the concentrations of pharmaceuticals before the release of wastewater into aquatic systems (White et al., 2006; Matamoros et al., 2008).

PhACs enter the environment through a variety of pathways, and the human body plays a major role. A portion (varies by drug and individual) of each pharmaceutical dose is retained in the human body, but residual parent compound and its metabolites are excreted in urine and feces (Daughton and Ternes, 1999; Khetan and Collins, 2007; Lienert et al., 2007). The PhACs are then released either through septic systems or in wastewater effluents (Jones et al., 2005b). Within wastewater treatment plants and in the natural environment, the rates of degradation of PhACs vary, depending on the chemical and physical properties of each compound, and environmental conditions (Jones et al., 2005b). Physical, chemical and biological parameters that influence degradation include; sorption/desorption, redox potential, temperature, pH, photolysis,

[1] Re-print with permission from Elsevier, Appendix A

microbial activity, and select minerals (Tolls, 2001; Zhang and Huang, 2003; Vogna et al., 2004; Scheytt et al., 2005; ter Laak et al., 2006; Zhang and Huang, 2007).

It has been predicted that the discharge of pharmaceuticals into the environment will increase over time (Jones et al., 2005b). For example, in Germany in 1995, 100 tons of prescription drugs were purchased, which does not account for sales of non-prescription drugs and veterinary medicines that were used (Ternes, 1998). As a result of greater reliance on pharmaceuticals and an aging population, the number of prescription and nonprescription drugs dispensed during doctor visits has risen from 190 per 100 people in the USA during 1995-1996 to 226 during 2003-2004 (NCHS, 2006). Monthly prescription drug usage as a percentage of the USA population has risen from 39.1% during 1988-1994 to 45.3% during 1999-2002 (NCHS, 2006).

Southeast Louisiana has been shaped by deltaic processes over several hundreds of years by the meandering of the Mississippi River. The majority of this region is near, at or below sea level. Due to the low elevation, much of the area is covered by freshwater wetlands and marsh. The Mississippi River, which receives treated wastewater from urban centers within the watershed, has the potential to carry significant amounts of contaminants, including PhACs into the coastal waters of Louisiana and the Gulf of Mexico. There is an opportunity in this region to utilize the natural wetlands to provide a final level of treatment (i.e. “polishing”) to wastewater, prior to release into surface waters. These wetlands can be used to naturally remove nutrients, organic loads and contaminants, while other areas of the USA must use advanced treatment technologies to achieve the same level of removal. The drawback is that significantly longer retention times are required for natural systems versus conventional wastewater treatment systems due to slower treatment processes.

Constructed wetlands are effective at removing or reducing the concentrations of nutrients (Braskerud, 2002), pathogens (Karim et al., 2004) and microcontaminants, such as endocrine disruptors, PhACs and personal care products (Belmont and Metcalfe, 2003; Matamoros et al., 2005; Matamoros and Bayona, 2006; Waltman et al., 2006; White et al., 2006). Natural wetlands are known to mitigate the effects of both point and non-point source pollution (Johnston et al., 1990), but their capacity for removing PhACs has not been previously assessed. Evaluating the benefits and services provided by natural wetlands, and employing these systems, requires an understanding of the system processes, as well as the responses of the systems to point and non-point source pollution. For example, wetlands that are subject to pollution can become impaired as a result of inputs of wastewater, resulting in contamination of local wildlife (Barber et al., 2006; Pelley, 2006).

Two recent publications have identified PhACs and other "down the drain" chemicals in surface waters in southeastern Louisiana. As reported by Boyd et al. (2003) and Zhang et al. (2007), these compounds have been detected in the Mississippi River at New Orleans, in Lake Pontchartrain bordering New Orleans to the north, within the discharge from the Jefferson Parrish East WWTP and at the influent of the drinking water treatment plant. Of the 9 target analytes in the Boyd et al. (2003) study, two were detected (naproxen, triclosan) in WWTP effluent at ng L^{-1}. The Zhang et al. (2007) study found 10 of the 12 compounds of interest including; naproxen, ibuprofen, carbamazepine, clofibric acid, caffeine, triclosan, acetaminophen, bisphenol A, estrone, 17 α-ethinylestradiol and the natural estrogen, 17 β-estradiol. It is possible that a range of other PhACs were also present in these samples.

The goal of this study was to evaluate the removal of PhACs from untreated municipal wastewater in a lagoon-constructed wetland treatment system, and within a receiving forested wetland located in southeastern Louisiana. The study objectives included: 1) determining

concentrations of PhACs loaded to and within the wastewater treatment system, 2) estimating the loading of PhACs to the forested wetland, and to the final receiving waters of Lake Pontchartrain, and 3) comparing reduction rates for PhACs in this natural treatment system to removal rates for more conventional wastewater treatment plants.

2.2. Methods

2.2.1. Study Area

Mandeville, LA, USA is located on the north shore of Lake Pontchartrain. The Mandeville WWTP is a nontraditional plant that treats the water in a constructed wetland, followed by a natural wetland. Untreated wastewater flows into three 61 x 183 x 3 m aerated lagoons in series, (Figure 2.1). Each basin has a retention time of four and a half days, for a total of ~13 days of treatment. After retention in the aeration lagoons (basins), the water flows through a surface flow constructed wetland. Water is evenly distributed across the width of the constructed wetland by percolation through a crushed gravel bed. The water flows through the herbaceous marsh containing several wetland plant species, including *Hydrocottle spp*. and *Phragmites australis*. After a 1 day retention time in the constructed wetland, water is collected in two rock basins where 60% of the water is recycled back to the crushed gravel bed and pumped through a series of sprinklers to further aerate the wastewater. The remaining 40% of water is pumped through an 8 x 1.2 m ultra-violet irradiation channel with 176 UV bulbs for disinfection. The water is then pumped out of a standpipe and into the adjacent forested wetland (i.e. Bayou Chinchuba) for polishing before discharge into Lake Pontchartrain (Figure 2.1). The plant has historically discharged into Bayou Chinchuba (since 1989) at a rate of ~7200 m^3 day^{-1}. The retention time in the WWTP constructed system is ~14 days and Bayou Chinchuba flow varies by season due to precipitation and evapotranspiration rates.

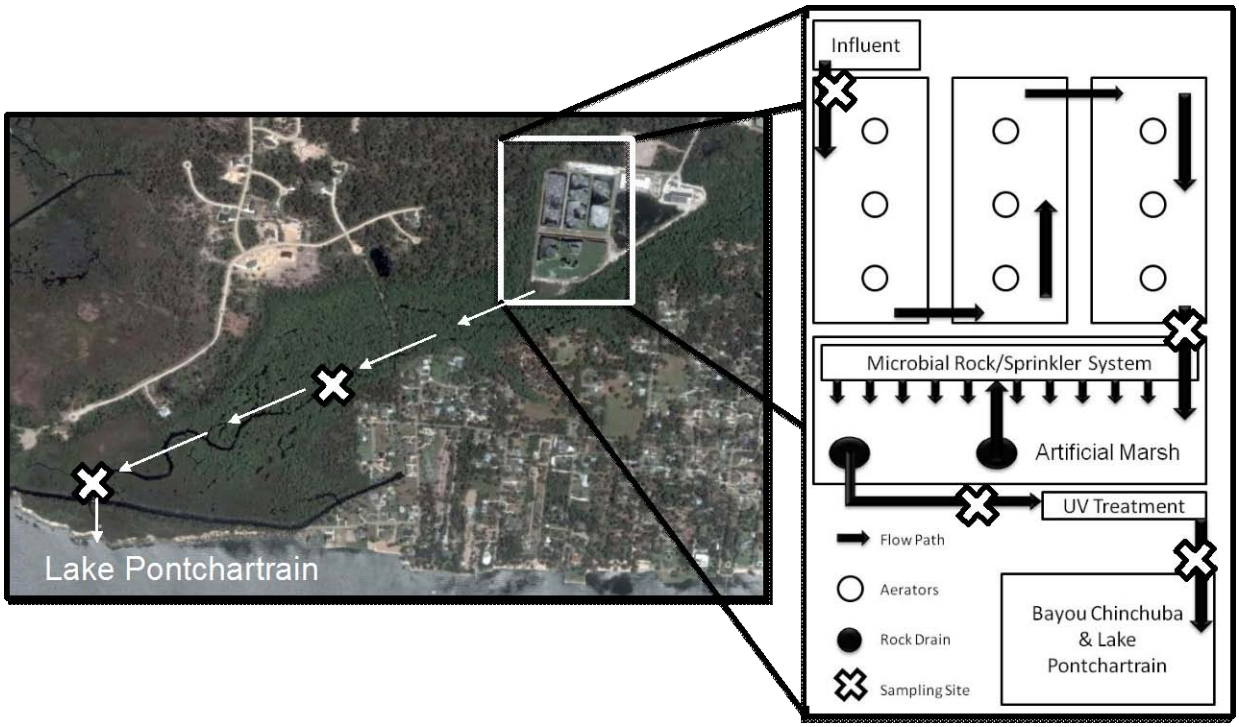


Figure 2.1. Schematic of the Mandeville wastewater treatment plant and aerial image of the adjacent forested wetland (Bayou Chinchuba). Arrows depict the direction of surface water flow from the plant, through the forested wetland and into Lake Pontchartrain.

Water entering the WWTP immediately mixes with the roughly 33,500 m^3 of water already in the first basin. Water is then continuously mixed by fountains and aeration hose along the bottom of the basins. Therefore, sewage entering the WWTP is quickly mixed and any pharmaceutical concentrations are normalized over the 4.5 day retention time within each basin. During the total 13 day retention time in the basins water is continuously mixed and concentrations should be homogenized within the basins. Therefore variation, whether daily or weekly, of pharmaceutical compounds within the WWTP is minimized in this system.

2.2.2. Field Sampling

All sampling containers were pre-cleaned by washing with soap and water, rinsing with deionized water and then washing with acetone, followed by hexane. The pre-cleaned 4 L amber bottles were used to collect water samples at various locations in the treatment plant and receiving wetland. The sampling locations within the WWTP and constructed wetland (Figure

2.1) were selected in order to assess the removal capacity of the various treatment phases of the plant. Samples were collected May 18, 2007 and chilled to 4 ^{o}C during transport to Louisiana State University for extraction.

2.2.3. Analysis of PhACs

All water samples were stored at 4^{o}C and processed within 48 hours of collection. Samples (250 ml) were filtered with hexane-washed Whatman GF/F filters (Fisher Scientific) to remove all particulate matter and extracted using HLB solid phase extraction (SPE) cartridges purchased from Waters (Millford, Mass). The methods have been previously described for solid phase extraction of acidic drugs (Miao et al., 2002), neutral drugs (Zhao and Metcalfe, 2008), sulfonamide antibiotics (Miao et al., 2004) and beta-blocker drugs (Topp et al., 2008). Briefly, the pH of the sample was adjusted according to the class of compound to be extracted, and stable-isotope labeled surrogates were spiked into the samples at nominal concentrations of 50 ng L^{-1} as internal standards. The four classes of PhACs were extracted by SPE using either HPLC Oasis or MCX cartridges. Extraction efficiencies for all analytes have previously been shown to exceed 75%. All samples were extracted in triplicate.

Extracts were shipped in chilled containers from Louisiana State University, Baton Rouge, Louisiana to Trent University, Ontario, Canada for analysis. The extracts were analyzed for the four classes of target compounds summarized in Table 2.1. Analysis was by liquid chromatography with tandem mass spectrometry (LC-MS/MS), conducted as described previously by Miao et al. (2002) for acidic drugs, Miao et al (2004) for sulfonamide antibiotics, Zhao and Metcalfe (2008) for neutral drugs, and Topp et al. (2008) for beta-blockers. The analytes were detected by monitoring in either negative or positive ion mode by multiple reaction monitoring (MRM). The acidic pharmaceuticals and beta-blockers were analyzed with a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) fitted with an

electrospray interface (ESI). The neutral pharmaceuticals and sulfonamide antibiotics were analyzed with a QTrap mass spectrometer (MDS Sciex, Toronto, ON) equipped with an atmospheric pressure chemical ionization (APCI) ion source.

Table 2.1. Target PhACs analyzed in samples of wastewater and water collected at the WWTP for Mandeville, LA, USA and the Bayou Chinchuba wetland, including data on the stable isotope surrogates used as internal standards and the limits of quantitation (LOQs).

Class	Compound	Surrogate	LOQ (µg/L)	Use
Neutrals	Cotinine	cotinine-D3	0.002	Nicotine metabolite
	Caffeine	caffeine-^{13}C3	0.002	Stimulant
	Carbamazepine	carbamazepine-D10	0.003	Anti-epileptic, psychiatric drug
	Fluoxetine	fluoxetine-D5	0.010	Psychiatric drug (Prozac)
Beta Blockers	Atenolol	atenolol-D7	0.004	Hypertension
	Nadolol	-	0.002	Blood pressure, Migraines
	Propranolol	propranolol-d7	0.003	Hypertension
	Metoprolol	metoprolol-d7	0.005	Hypertension
	Sotalol	sotalol-d6	0.005	Hypertension, Arrhythmias
Sulfonamides	Sulfapyridine	sulfamethazine-^{13}C6	0.011	Antibiotic
	Sulfamethoxazole	sulfamethoxazole-^{13}C6	0.007	Antibiotic
Acidics	Acetaminophen	acetaminophen-D3	0.017	Analgesic/anti-inflammatory
	Naproxen	naproxen-$^{13}C_1$D3	0.007	Analgesic/anti-inflammatory
	Ibuprofen	ibuprofen^{13}C3	0.011	Analgesic/anti-inflammatory
	Gemfibrozil	gemfibrozil-D6	0.008	Lipid Regulator

A series of external standards were prepared with different concentrations of the target analytes and fixed concentrations of stable isotope surrogates (Table 2.1). The concentrations of the analytes were determined by comparing the response to each analyte in the samples to the responses to each analyte in the external standards over the range of a calibration curve. These

response data were adjusted according to the relative ratios of the responses to the stable isotope surrogates in the sample and external standard. Note that this approach adjusts the quantitative data to compensate for efficiencies of extraction <100% and enhancement or inhibition of the signal due to the effects of the sample matrix. Blanks were prepared by spiking Milli-Q water with labeled surrogates, and extracting and analyzing them as described previously. The limits of quantitation (LOQs), which are listed in Table 2.1 were estimated as the second lowest point in the linear calibration curve prepared by analysis of the external standards, for which the signal to noise ratio for the analytes in the native samples was >10.

2.3. Results and Discussion

Nearly all of the target compounds were detected in the untreated wastewater entering the treatment plant, with the exception of fluoxetine and propranolol (Table 2.2). Nadolol, sotalol, and sulfapyridine were the only compounds that were detected in the untreated wastewater but not at the outfall into Lake Pontchartrain, indicating near complete aqueous concentration reduction. The data indicate that the WWTP constructed wetland significantly decreased the concentrations of most target compounds, but not to below the LOQs. The Bayou Chinchuba forested wetland was found to further reduce the concentrations of the target compounds.

The total reduction rates of the compounds within the entire treatment system were greater than 90% for all compounds, except carbamazepine at 51% and sotalol at 82% (Table 2.3). Several other PhACs (i.e. cotinine, caffeine, atenolol, nadolol, metoprolol, sulfapyridine, acetaminophen, naproxen, ibuprofen) were removed in amounts of 99% or greater by the entire system. A few compounds also showed reduction rates in the forested wetland between 20-31% (i.e. sulfapyridine, nadolol and gemfibrozil). The highest proportion of concentration reduction of the compounds from the aqueous phase occurred in the aeration basin of the WWTP (Figure 2.2), which had a 13 day retention time.

Table 2.2. Mean concentrations of target PhACs (μg L^{-1}; n=3) at sampling locations at the WWTP for Mandeville, LA, USA and the Bayou Chinchuba wetland. Standard deviations represent error associated with extraction and analytical precision.

Drug Class	Compound	WWTP Inflow [μg L^{-1}]	Cell 3 [μg L^{-1}]	Pre-UV [μg L^{-1}]	Outfall [μg L^{-1}]	WL mid [μg L^{-1}]	WL end [μg L^{-1}]
Neutrals	Cotinine	1.097 ± 0.060	0.030 ± 0.001	0.014 ± 0.001	0.019 ± 0.002	0.012 ± 0.004	0.015 ± 0.000
	Caffeine	25.567 ± 5.710	0.029 ± 0.006	0.033 ± 0.003	0.028 ± 0.006	0.065 ± 0.020	ND
	CBZ	0.057 ± 0.004	0.082 ± 0.006	0.087 ± 0.001	0.11 ± 0.007	0.028 ± 0.009	0.034 ± 0.001
	Fluoxetine	ND	ND	ND	ND	ND	ND
Acidics	Acetaminophen	39.300 ± 0.685	0.008 ± 0.001	0.015 ± 0.003	0.01 ± 0.001	ND	ND
	Naproxen	10.418 ± 1.530	0.064 ± 0.009	0.193 ± 0.033	0.090 ± 0.010	0.031 ± 0.003	0.020 ± 0.004
	Ibuprofen	9.922 ± 1.177	0.039 ± 0.003	0.080 ± 0.009	0.038 ± 0.002	0.017 ± 0.001	0.013 ± 0.000
	Gemfibrozil	1.652 ± 0.112	0.645 ± 0.031	1.819 ± 0.281	0.600 ± 0.036	0.081 ± 0.003	0.061 ± 0.004
Beta Blockers	Atenolol	1.442 ± 0.102	0.284 ± 0.010	0.097 ± 0.009	0.099 ± 0.006	0.015 ± 0.001	0.020 ± 0.002
	Nadolol	0.030 ± 0.003	0.030 ± 0.001	0.007 ±0.00 0	0.007 ± 0.001	ND	ND
	Propranolol	ND	ND	ND	ND	ND	ND
	Metoprolol	0.211 ± 0.032	0.025 ± 0.002	0.016 ± 0.001	0.017 ± 0.001	ND	ND
	Sotalol	0.174 ± 0.019	0.148 ± 0.009	0.117 ± 0.005	0.121 ± 0.007	0.031 ± 0.019	0.022 ± 0.003
Sulfonamides	Sulfapyridine	0.068 ± 0.024	0.018 ± 0.008	0.016 ± 0.002	0.016 ± 0.003	ND	ND
	SMX	4.090 ± 0.671	0.918 ± 0.463	0.309 ± 0.055	0.350 ± 0.024	0.328 ± 0.019	0.369 ± 0.124

Standard deviations represent error associated with extraction and analytical precision.
CBZ= Carbamazepine; SMX = Sulfamethoxazole; ND = not detected at concentrations above the LOQ.

2.3.1. Acidic Compounds

Three (acetaminophen, naproxen, Ibuprofen) of the four acidic compound concentrations were reduced below the limits of quantitation within the aeration basins, with gemfibrozil being the exception. The removal of these compounds is similar to concentration reductions observed in previous studies for mechanized wastewater treatment (Ternes, 1998; Heberer et al., 2002; Bendz et al., 2005). The initial gemfibrozil concentration entering the treatment system was 1.65 $\mu g\ L^{-1}$ and a 61% reduction was observed within the aeration basin.

Table 2.3. Removal rates of pharmaceutically active compounds for the wastewater treatment plant at Mandeville, LA, USA and the receiving forested (Bayou Chinchuba) wetland.

Class	Compound	WWTP Discharge (%)	B. Chinchuba Discharge (%)	Total % Removal
Neutrals	Cotinine	>99	0	>99
	Caffeine	>99	0	>99
	Carbamazepine	-53	105	51
	Fluoxetine	ND	ND	ND
Beta Blockers	Atenolol	>99	6	>99
	Nadolol	77	23	>99
	Propranolol	ND	ND	ND
	Metoprolol	92	8	>99
	Sotalol	30	52	82
Sulfonamides	Sulfapyridine	76	24	>99
	Sulfamethoxazole	91	1	92
Acidics	Acetaminophen	100	0	>99
	Naproxen	99	1	>99
	Ibuprofen	>99	0	>99
	Gemfibrozil	64	31	95

ND = not detected at concentrations above the LOQ.

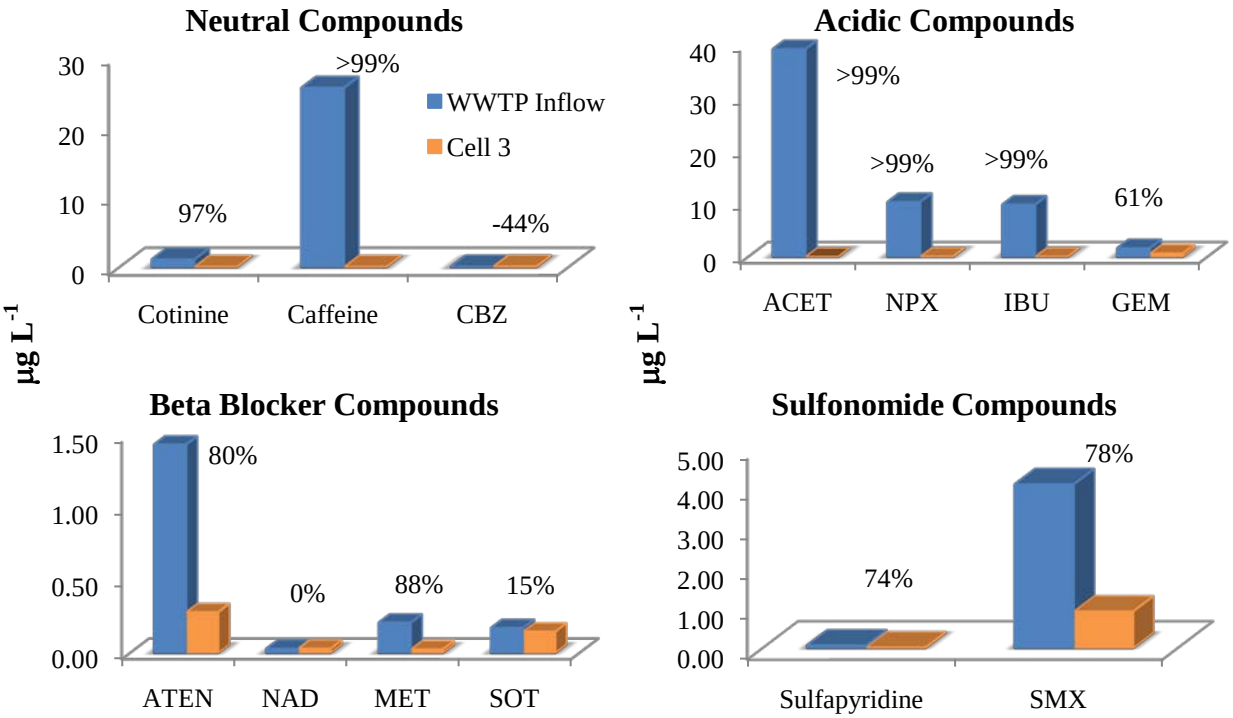
2.3.2. Sulfonamides

Sulfamethoxazole (78%) and sulfapyridine (74%) showed large reductions over initial concentrations of 4.090 $\mu g\ L^{-1}$ and 0.068 $\mu g\ L^{-1}$ within the aeration basins, respectively. The total concentration reduction by the WWTP and forested wetland for each compound was >99%

and 92% for sulfamethoxazole and sulfapyridine, respectively. Both compound concentrations decreased throughout the Mandeville treatment plant, but remained constant after reaching the forested wetland. Previous studies have shown that concentrations of these compounds can increase during the wastewater treatment process (Bendz et al., 2005; Gobel et al., 2007). One study found that sulfapyridine and sulfamethoxazole doubled in concentration during certain aspects of wastewater treatment (Gobel et al., 2007). They hypothesized this increase may be due to the presence of metabolites that were transformed into salfapyridine and sulfamethoxazole during biological treatment. Additionally, these compounds underwent ~96% reductions in concentrations with activated sludge treatment which is more inline with the removal rates observed in this study.

2.3.3. Neutral Compounds

The neutral compounds, cotinine and caffeine, were both nearly reduced to below detection within the aeration basins, while carbamazepine was detected at every step in the treatment process. Fluoxetine was not detected in the treatment system (Table 2.2). There was an increase in carbamazepine concentration in the plant from 0.057 $\mu g\ L^{-1}$ at the inflow to a max of 0.087 $\mu g\ L^{-1}$ at the discharge into Bayou Chinchuba. The carbamazepine concentration increased after the initial measurement at the sewage inflow and remained relatively constant until the measurement of the forested wetland midpoint, where the concentration dropped below the initial level. The concentration of carbamazepine continued to decrease and was only 0.028 $\mu g\ L^{-1}$ when discharged into Lake Pontchartrain, down from 0.058 $\mu g\ L^{-1}$ at the inflow. The results support previous research which suggests carbamazepine is a persistent chemical in the environment.



CBZ= Carbamazepine; ACET=Acetaminophen; NPX=Naproxen; IBU=Ibuprofen; GEM=Gemfibrozil; ATEN=Atenolol; NAD=Nadolol; MET=Metoprolol; SOT=Sotalol; SMX=Sulfamethoxazole

Figure 2.2. The mean concentrations (µg L^{-1}) of the target pharmaceutically active compounds at the inflow into the aeration basins and at Cell 3 of the aeration basins of the Mandeville wastewater treatment plant, where there is a hydraulic retention time of ~13 days.

A possible explanation for this concentration increase within the plant is that there was a greater loading of the compound over a period of a couple of weeks prior to sampling. However, this is unlikely as this drug is administered daily in either one or two doses up to 1600mg to treat chronic symptoms of seizures, ADD, schizophrenia, bipolar disorder and trigeminal neuralgia (USFDA, 2007). An alternate explanation is carbamazepine is retained and persists in the aeration basins. A similar trend was observed in the aeration basins during a November 2006 sampling of the Mandeville WWTP (Unpublished data). During this sampling, the carbamazepine concentration spiked in the aeration basins before dropping below the inflow concentration in the artificial marsh. Miao et al. (2005) suggests that it may be possible that UV

irradiation has the ability to convert hydroxyl metabolites of carbamazepine to the parent compound or even modify the dissolved organic matrix in treated wastewater so that the analytes are released from the dissolved organic materials (Miao et al., 2005). A similar trend was observed with synthetic musks treated with UV irradiation (Yang and Metcalfe, 2006).

The USEPA's Estimation Program Interface (EPI) Suite estimates that carbamazepine removal in WWTP to be 2.96% of the total concentration, with 2.86% by sludge and 0.10% due to biodegradation (USEPA, 2007). Previous research has shown that the removal rates of carbamazepine in WWTPs is greater than the EPI Suite estimate and ranges from less than 10% (Ternes, 1998; Heberer et al., 2002) up to 30% (Bendz et al., 2005; Miao et al., 2005). The aqueous concentration reduction observed for the Mandeville WWTP and forested wetland is at 50%, indicating that this natural wastewater treatment system may be more effective at reducing concentrations of carbamazepine than conventional wastewater treatment.

2.3.4. Beta Blockers

Sotalol has the next lowest removal rate (behind carbamazepine). This drug compound was reduced by 82% compared to removal below the detection limits for the other beta blockers (atenolol, metoprolol). Previous studies have shown that beta blockers are relatively persistent through the wastewater treatment process, with 30-80% removal of concentrations less than 1 ppb in multiple WWTPs studied (Castiglioni et al., 2006; Maurer et al., 2007). The removal rates from this study show that the Mandeville treatment system may be more effective at reducing the aqueous concentration of this class of pharmaceuticals from the aqueous phase. Other studies in conventional WWTPs indicated that atenolol decreased between 30-53% and metoprolol from 10-83% (Castiglioni et al., 2006; Maurer et al., 2007). These values are also lower than what was observed for the Mandeville WWTP system.

2.3.5. Compound Loading

Estimates of the annual loadings of the PhAC compounds were based on a daily wastewater flow of ~7,600 m^3 and on the mean concentrations detected in the samples. The treatment system can potentially remove several hundred kg of PhACs annually, with caffeine (~70 kg) and acetaminophen (107 kg) being the largest contributors (Table 2.5).

There could be competition between compounds for binding sites, where one compound may decrease or prevent sorption due to the presence of another (Li and Werth, 2001; Bonin and Simpson, 2007). Competition could lead to an increase in downstream concentrations of the desorbed compound. However, this may not be significant in vegetated wetland systems that receive nutrient rich, secondarily treated wastewater which can cause an increase in vegetation growth. The extra growth leads to increased accrual of organic matter which replenishes organic matter binding sites as new organic matter is deposited (Rybczyk et al., 2002).

None of the PhACs enter Lake Pontchartrain in kilogram amounts annually, and concentrations are likely further diluted in this large lake (1,630 km^2). However, it has been shown that some of these compounds can have a detrimental effect on aquatic organisms exposed at ppb concentrations (Huggett et al., 2002; Flaherty and Dodson, 2005; Mimeault et al., 2005; Lienert et al., 2007).

2.3.6. Conventional vs Natural Wetlands

Previous studies have shown that conventional wastewater treatment plants reduce the concentrations of PhACs in the aqueous phase, but the efficacy of removal varies widely with the drug and the treatment technology (Table 2.4). However, the present study demonstrated that there was a high potential for reduction of PhACs in the constructed wetland within the Mandeville WWTP. A major reason for the high degree of removal is that the Mandeville system functions with a hydraulic retention time (HRT) of approximately 14 days, whereas

conventional treatment plants have HRTs of 10-60 h, depending on the treatment technology. The extended treatment time allows for greater removal of microcontaminants, including PhACs, through processes of biodegradation, photolysis, etc. There was an additional concentration reduction between 0 and 50% in the forested wetland, although the mechanism (degradation, sorption, dilution) is unknown due to a lack of stream flow data in Bayou Chinchuba.

2.3.7. Seasonal Removal

When comparing the sampling event for this study with a previous sampling of the Mandeville WWTP from 11/20/2006 (Unpublished Data) there appears to be seasonal variation (12 to 300%) with regards to inflow concentrations of several compounds. The most variation between seasons occurs with acidic (acetaminophen, naproxen, ibuprophen, gemfibrozil) and neutral (cotinine, caffeine, carbamazepine) compounds, while beta blockers and sulfonamides generally vary by less than 50%. With the exception of metoprolol and sulfamethoxazole, all compounds exhibited higher concentrations during November compared to May.

While there may be variation with the inflow concentrations for the Mandeville WWTP, the percent removal within the WWTP is very similar for each sampling. Despite the varying inflow concentrations, the November ‘06 and May ‘07 samplings had 6, and 8 compounds, respectively, that exhibited >90% removal, while both had 10 compound with >75% removal. The percent concentration reduction difference between the two samplings was <18% for 10 of the 13 compounds detected, with gemfibrozil, carbamazepine and sotalol being the exceptions.

It is important to note that these data only represent the PhAC compounds in the aqueous phase, since all samples were filtered prior to analysis to remove particulate material. However, previous studies have shown that PhACs adsorbed to suspended particulate material represents a small fraction of the total loads (Miao and Metcalfe, 2007). In addition, this sampling scheme provided only a snapshot of the removal of PhAC compounds in the wastewater stream, and did

Table 2.4. Percent removal and concentrations in treated wastewater reported in the literature for the target pharmaceutically active compounds in conventional wastewater treatment plants, compared to the removal rates of this study.

Drug	% Reduction (this study)	% Reduction (Conventional)	Effluent μg/L	Study	Wastewater Treatment Method
Atenolol	99%	30-53%		Castiglioni et. al (2006)	N/I
		50-80%	0.16	Bendz, D., N. A. Paxeus, et al. (2005)	Clarification, Activated Sludge, P removal
Caffeine	>99%	>99%	0.19	Ternes, T., M. Bonerz, et al. (2001)	Aeration tank, P removal, Clarification
		99%	0.18	Heberer, T., K. Reddersen, et al. (2002)	N/I
		94%	0.22	Bendz, D., N. A. Paxeus, et al. (2005)	Clarification, Activated Sludge, P removal
CBZ	51%	7%	2.10	Ternes, T. A. (1998)	Clarification, Activated Sludge, P removal
		8%	1.63	Heberer, T., K. Reddersen, et al. (2002)	N/I
		30%	1.18	Bendz, D., N. A. Paxeus, et al. (2005)	Clarification, Activated Sludge, P removal
Gemfibrozil	91%	69%	0.40	Ternes, T. A. (1998)	Clarification, Activated Sludge, P removal
		75%	0.18	Bendz, D., N. A. Paxeus, et al. (2005)	Clarification, Activated Sludge, P removal
Ibuprofen	99%	90%	0.37	Ternes, T. A. (1998)	Clarification, Activated Sludge, P removal
		96%	0.15	Bendz, D., N. A. Paxeus, et al. (2005)	Clarification, Activated Sludge, P removal
Metoprolol	>99%	30-65%	0.19	Bendz, D., N. A. Paxeus, et al. (2005)	Clarification, Activated Sludge, P removal
Naproxen	99%	66%	0.30	Ternes, T. A. (1998)	Clarification, Activated Sludge, P removal
		93%	0.25	Bendz, D., N. A. Paxeus, et al. (2005)	Clarification, Activated Sludge, P removal
Sotalol	81.90%	25%	0.25	Maurer et. al (2007)	N/I
SMX	92%	0%	0.07	Bendz, D., N. A. Paxeus, et al. (2005)	Clarification, Activated Sludge, P removal

CBZ= Carbamazepine ; SMX = Sulfamethoxazole; N/I = no info provided

Table 2.5. Loadings (kg year^{-1}) of the target pharmaceutically active compounds to points within the Mandeville, LA, USA wastewater treatment plant and the Bayou Chinchuba receiving wetland.

Drug Class	Compound	Loading at Inflow to WWTP (kg/year)	Loading to Bayou. Chinchuba (kg/year)	Loading to L. Pontchartrain (kg/year)	WWTP Reduction (kg/yr)	Bayou Chinchuba Reduction (kg/yr)	Total Reduction (kg/yr)
Neutral	Cotinine	2.99	0.04	0.03	2.95	0.00	2.95
	Caffeine	69.68	0.09	0.18	69.59	0.00	69.59
	CBZ	0.15	0.24	0.08	-0.09	0.16	0.08
	Fluoxetine	ND	ND	ND	ND	ND	ND
Acidic	Acetaminophen	107.11	0.03	0.00	107.08	0.00	107.11
	Naproxen	28.39	0.25	0.08	28.14	0.17	28.31
	Ibuprofen	27.04	0.11	0.05	26.94	0.06	27.00
	Gemfibrozil	4.50	1.66	0.22	2.85	1.44	4.28
Beta Blockers	Atenolol	3.93	0.27	0.04	3.66	0.23	3.89
	Nadolol	0.08	0.02	0.00	0.06	0.02	0.08
	Propranolol	ND	ND	ND	ND	ND	ND
	Metoprolol	0.57	0.05	0.00	0.53	0.05	0.57
	Sotalol	0.47	0.34	0.09	0.14	0.25	0.39
Sulfonamides	Sulfapyridine	0.19	0.04	0.00	0.14	0.04	0.19
	SMX	11.15	0.97	0.91	10.18	0.06	10.24

Compound loading rates were calculated from the observed concentrations at various points in the treatment process, based on a flow rate of 7600 m^3d^{-1}.

CBZ= Carbamazepine; SMX = Sulfamethoxazole; ND = not detected at concentrations above the LOQ.

not take into account temporal variations in PhAC inputs into the WWTP. As mentioned previously, we believe that daily and weekly concentration variations are normalized due to basin retention times. Also, national prescription patterns (USFDA, 2007) show that for many of the compounds monitored (excluding the sulfonamide antibiotics, ibuprofen, naproxen, and acetaminophen), the remaining drugs are prescribed for long term usage (e.g. carbamazepine, beta-blockers, gemfibrozil, fluoxetine) or otherwise show homogeneous levels of consumption (e.g. caffeine, cotinine). Future work will focus on the temporal and seasonal variability of PhAC concentrations in wastewater and removal within the wetland system.

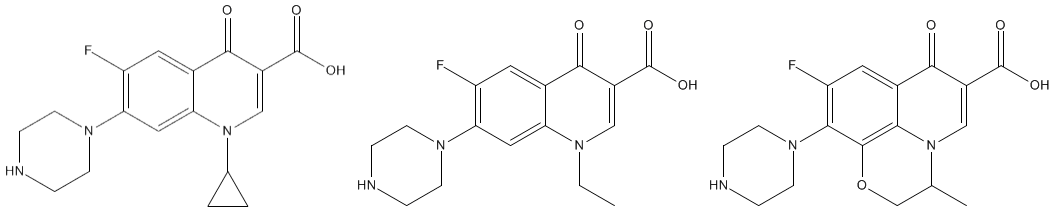
2.4. Conclusions

Results demonstrate that a wastewater treatment system consisting of earthen lagoons and a constructed wetland such as those used in the Mandeville WWTP show a greater reduction in compound concentration than previous studies demonstrated for conventional plants. Further polishing in a natural forested wetland produced removal rates for PhACs that averaged 96% for the entire system. There is variation with inflow concentration in the WWTP, but these concentrations are normalized on a daily to weekly basis by mixing within the aeration basins. Seasonal variability may be more pronounced, with greater concentrations entering the facility during colder months. However, removal rates for both November and May were similar. These removal rates are equal or greater than published removal rates in conventional wastewater treatment plants. In particular, carbamazepine and sotalol appear to be more persistent in conventional WWTPs than in the Mandeville wastewater treatment system. The higher removal rates may be due to a longer HRT in the constructed and natural treatment system. The entire system is capable of removing several kilograms per year of PhACs from wastewater, significantly reducing the annual loadings of these compounds to Lake Pontchartrain.

CHAPTER 3: PHARMACEUTICAL ANALYSIS FOR ENVIRONMENTAL SAMPLES: INDIVIDUAL AND SIMULTANEOUS DETERMINATION OF CIPROFLOXACIN, OFLOXACIN AND NORFLOXACIN USING AN HPLC WITH FLUORESCENCE AND UV DETETECION WITH A WETLAND SOIL MATRIX[2]

3.1. Introduction

Pharmaceuticals, including fluoroquinolone antibiotics, have been detected in surface waters around the world (Golet et al., 2001; Nakata et al., 2005; Batt et al., 2006; Batt et al., 2007; Conkle et al., 2008). The fate of these compounds in the environment needs further investigation, specifically pertaining to sorption, desorption, transport, and biotic and abiotic degradation (White et al., 2006).



Ciprofloxacin Norfloxacin Ofloxacin

Figure 3.1. Structure of the three compounds for which analytical methods were developed.

Methods have been developed for the determination of ofloxacin (OFL), norfloxacin (NOR) and ciprofloxacin (CIP) in sewage using an HPLC (Carlucci, 1998; Golet et al., 2001; Samanidou et al., 2003; Lee et al., 2007a). However, these methods require solid phase extraction (SPE) and have retention times in excess of 10 minutes. Laboratory scale experiments aimed at elucidating removal mechanisms yield large numbers of samples (ranging from 100s to 1000s), which, when combined with long preparation and analysis times makes such approaches impractical for most laboratories. The motivation behind the presented study was to reduce the time required to analyze fluoroquinolone antibiotic as well as developing a method which is

[2] Re-print with permission from Taylor & Francis, Appendix B

effective for complex environmental matrices while, at the same time, having detection limits appropriate for environmental analysis. All three of these compounds have similar structures and properties, which can make separation of each compound more challenging in the presence of the others.

Therefore, the goal of this research was to develop a method for individual and simultaneous analysis of CIP, NOR and OFL (Figure 3.1) using standard HPLC equipment (with UV and fluorescence detection), 1) with short retention times, 2) that eliminates the need for SPE, and 3) provides detection at parts per billion ($\mu g\ L^{-1}$) up to high parts per million ($mg\ L^{-1}$) concentrations.

3.2. Experimental

3.2.1. Materials

CIP, NOR and OFL (HPLC grade) were obtained from Sigma-Aldrich (St. Louis, MO) in powder form. HPLC grad solvents, acetonitrile and water were purchased from Mallinckrodt chemicals. Methanol (HPLC grade), glacial acetic acid (biochemical grade, 99.8%) and sodium azide (99%) were obtained from Acros Organics. Sodium acetate (anhydrous, 99.7%) and calcium chloride (anhydrous, 96.0%) were purchased from Fisher Scientific and Sigma-Aldrich respectively. All solvents used were HPLC grade. Trimethylammonium phosphate buffer solution (pH = 3), as well as citric acid and sodium citrate monobasic (both anhydrous, ultra grade ≥99.5%) were supplied by Fluka Bio Chemika. An 18 mΩ sensitivity water filter with a 0.1 μm filtering device (Modular Water systems, United States Filter Corporation) was used to treat all water used in the stock, standard electrolyte solution and sample solution preparation.

3.2.2. UV and Fluorescence

UV-vis (on a Cary 50Bio, Palo Alto, CA) and fluorescence (on a Fluorolog, Horiba Jobin Yvon, Edison, NJ) characterization was carried out on all three antibiotics. The UV-vis and

fluorescence data yielded absorbance and emission maxima of 272, 273 and 289 nm and 421, 414 and 458 nm for CIP, NOR, and OFL, respectively. CIP and NOR have similar absorbance and emission spectra, while OFL has a higher range.

3.2.3. Chromatography

The liquid chromatographic system used in this study consisted of an Agilent 1100 (Santa Clara, CA). This LC instrument is equipped with the following parts: solvent degasser (G1379A), quaternary pump (G1311A), automatic liquid sampler (G1329A), temperature controlled column compartment (G1316A), DAD detector (G1315B) and fluorescence detector (G1321A). The instrument was fitted with a Zorbax (Santa Clara, CA) eclipse XDB C18 (4.6 mm x 150mm x 5μm) column, and a Phenomenex (Torrance, CA) C18 guard column (4.0 mm x 3.0 mm x 5 μm).

For the analysis of CIP, NOR and OFL as separate components, the mobile phase consisted of sodium acetate (pH 3), acetonitrile (ACN) and triethylamoniumphosphate (TEA) (10 mM). The TEA solution was added to solutions to minimize peak tailing (Snider et al., 1997; Zendelovska and Stafilov, 2005). A 60:40 v:v ratio sodium acetate to ACN was used for CIP and NOR, while a 70:30 ratio was used for OFL. For simultaneous analysis of all three fluoroquinolones, an aqueous citric acid buffer (pH 2.5), ACN, methanol (MeOH) in a 82/8/10 v/v ratio mobile phase was utilized (a modified adaptation from Canada-Canada et al. [9]). A column temperature of 35°C, a flow rate of 1 mL min^{-1} and an injection volume of 20 μL were used. For UV-vis detection, a wavelength of 280 nm and $\lambda_{ex}/\lambda_{em}$, of 280/450nm was found optimal for fluorescence detection. All methods were run in isocratic mode and used a direct injection of aqueous samples without prior sample pretreatment. All samples were injected into the HPLC in triplicate.

3.2.4. Preparation of Standard Solutions

Stock solutions with concentrations of 80 mg L^{-1} were prepared in triplicate. In addition, each stock solution contained 100 mg L^{-1} sodium azide (to remove possible biological components) and 0.01 M $CaCl_2$ (to mimic the ionic strength of environmental samples). Stock solutions were used to create a 9-point standard curve for each fluoroquinolone. The standard solution was subsequently diluted with water to yield concentrations spanning three orders of magnitude from 0.05 to 80 mg L^{-1}. The 80 mg L^{-1} stock solutions were also used to create the 10-point standard curve for the simultaneous method, with standard solution concentrations ranging from 0.04 to 20 mg L^{-1}. All standards were prepared from the triplicate stock solutions prior to its use and each standard was injected into the instrument in triplicate. When standards and stock solution were not in use they were stored at 4 ^{o}C in darkness. Peak area was used for determination of compound concentrations, not height.

Table 3.1. Parameters from the Bayou Castine wetland soil used for environmental method application. All standards used in method development were prepared in triplicate and injected in triplicate. (± values represent standard deviation)

Parameter	Value
Cation Ex Capacity ($cmol_c$ kg^{-1})[‡]	19.8 ± 0.8
Moisture content (%)	65 ± 0.0
pH	6.9 ± 0.2
Organic matter (%) [†€]	18.6 ± 1.0
Total Carbon (g kg^{-1})[¥€]	88.4 ± 3.3
Total Phosphorus (mg kg^{-1})[€]	474.2 ± 15.8
Total Nitrogen (g kg^{-1})[¥€]	6.2 ± 0.2
Clay (%)	31.3

[‡] (Sumner and Miller, 1996)
[†] Loss on ignition
[€] Dry soil basis
[¥] (White and Reddy, 2000)

3.2.5. Method Validation with a Wetland Soil

These methods were tested using an environmental matrix; soil from a local wetland classified as an Arat Silty Clay Loam, which is a fine silty, siliceous, non-acid, thermic Typic Hydraquent (Trahan et al., 1990). This particular soil was chosen because it is the same class as a soil in a nearby treatment wetland that receives treated wastewater containing pharmaceuticals and its parameters are shown in Table 3.1. Woody and root materials were removed and the soil was homogenized and refrigerated at 4 ^{o}C prior to experimental analysis. A 20 mL aliquot of solution containing 5 mg L^{-1} of CIP, NOR, and/or OFL (the remainder of the solution composition was identical to that of the standard solutions) was added to glass vials containing 50 mg of field moist soil. Blank vials containing only the antibiotic solution were prepared to account for sorption to the glass scintillation vials, which is essential for mass balance calculations. Four replicates of each treatment were prepared and shaken for 5 days. At the end of the incubation, samples were centrifuged and 2 mL was extracted for analysis using the methods presented in this study. Blanks containing only the spiked solution demonstrated that there was little if any sorption to the glass vials.

3.3. Results and Discussion

3.3.1. Overall Chromatographic Performance

In recent years the presence of pharmaceuticals in the environment has been a topic of growing concern. While there is a need for data obtained in the field with regards to compound identification, transport and fate, controlled lab studies can provide a baseline for understanding compound interactions in the environment. We developed two methods for the analysis of three fluoroquinolone compounds using an HPLC with UV and fluorescence detection that improve upon previous methods by decreasing analysis times.

For the methods developed in this work, the retention times for analysis of individual compounds (1.5-1.7 min) are significantly shorter than those obtained with the multiple compound detection method (6.5-8.0 min) (Table 3.2). A short retention time for individual compound detection is important when performing laboratory studies that require hundreds of samples, such as sorption and desorption experiments. However, when analyzing compounds simultaneously there is a significant increase in retention time. The method recently developed by Canada-Canada et al. (2007) demonstrated retention times of 7.8-9.7 min for the same compounds when analyzed simultaneously along with twelve other fluoroquinolone compounds. Therefore, when only analyzing these three compounds, the two methods presented herein provide at a minimum 1) 4.5-6.4 times shorter retention times for individual compounds and 2) 14-17 % shorter retention times for simultaneous compound detection over previously published methods (Golet et al., 2001; Canada-Canada et al., 2007; Lee et al., 2007a).

All UV standard curves achieved >0.995 R^2 value for concentrations ranging from 0.055 (CIP, NOR) or 0.11 (OFL) to 80 mg L^{-1} for individual compound detection. UV detection was effective over the entire range tested for individual compounds. However fluorescence detection of individual compound was only linear at the lower end of the range tested (~0.06 to 1.5 mg L^{-1}). This indicates that UV analysis is best when either unsure of compound concentration or the known range varies from μg L^{-1} to mg L^{-1}.

For the simultaneous method, the standard curve was linear up to the concentration of 20 mg L^{-1} for both UV and fluorescence. The R^2 values was near 1.0 for UV analysis during simultaneous detection and >0.995 for CIP and OFL fluorescence. However, the NOR R^2 was only 0.989 (Table 3.2) indicating that fluorescence detection of NOR may be inferior to UV detection.

Table 3.2. Method development calibration curves of CIP, NOR & OFL using UV and fluorescence. (± values represent standard deviation)

UV							
Method		Retention Time (min)	Slope	Intercept	R^2	Range Tested (mg L^{-1})	LOQ (mg L^{-1})†
	CIP	1.520 ± 0.000	125.63 ± 1.93	28.55 ± 20.59	0.999 ± 0.000	0.0570 ± 0.008 - 80.833 ± 0.684	0.024
	NOR	1.511 ± 0.000	124.61 ± 0.67	19.4 ± 9.68	0.999 ± 0.000	0.0597 ± 0.004 - 79.200 ± 1.266	0.033
Single	OFL	1.704 ± 0.000	52.86 ± 0.42	9.53 ± 2.65	0.999 ± 0.000	0.1093 ± 0.025 - 79.933 ± 0.133	0.017
	CIP	8.041 ± 0.077	91.55 ± 0.49	-7.29 ± 0.45	1.000 ± 0.000	0.1637 ± 0.123 - 20.342 ± 0.158	0.037
	NOR	7.000 ± 0.063	88.41 ± 1.34	-7.32 ± 1.63	1.000 ± 0.000	0.1567 ± 0.112 - 19.742 ± 0.283	0.035
Triple	OFL	6.509 ± 0.057	35.59 ± 0.16	-2.9 ± 0.19	1.000 ± 0.000	0.0533 ± 0.003 - 19.950 ± 0.000	0.078

Fluorescence							
Method		Retention Time (min)	Slope	Intercept	R^2	Range Tested (mg L^{-1})	LOQ (mg L^{-1})†
	CIP	1.552 ± 0.001	590.99 ± 22.27	-6.92 ± 3.91	0.999 ± 0.000	0.0653 ± 0.001 - 1.362 ± 0.280	0.065
	NOR	1.542 ± 0.000	613.8 ± 16.41	5.81 ± 1.76	0.997 ± 0.001	0.0597 ± 0.004 - 0.931 ± 0.357	0.030
Single	OFL	1.737 ± 0.000	432.09 ± 15.24	9.83 ± 2.28	0.997 ± 0.001	0.0827 ± 0.003 - 1.599 ± 0.003	0.024
	CIP	8.088 ± 0.075	350.97 ± 3.35	19.8 ± 4.87	0.999 ± 0.000	0.1637 ± 0.123 - 20.342 ± 0.158	0.011
	NOR	7.026 ± 0.063	356.53 ± 4.6	81.68 ± 4.62	0.989 ± 0.000	0.1533 ± 0.113 - 19.742 ± 0.283	0.019
Triple	OFL	6.533 ± 0.057	61.47 ± 0.30	-3.65 ± 0.40	1.000 ± 0.000	0.0533 ± 0.003 - 19.950 ± 0.000	0.061

‡ limit of quantitation (S/N=10), LOQs were calculated from the standard solutions, not sample analysis.

There did not appear to be any degradation of the compounds, as indicated by the absence of significant peaks other than the target compounds during both sample and standard analysis (Figures 3.2 & 3.3). It should be noted that both methods are isocratic in nature, and hence, can be run on an HPLC instrument with a single channel pump. In comparison, the method developed

by Canada-Canada et al. (2009) requires, at a minimum, a three channel pump. In addition, it was found that the mobile phase is required to be at a pH below 3 in order to resolve NOR and OFL satisfactorily. All standard samples were prepared in triplicate and injected into the HPLC in triplicate to account for variation in standard preparation and the detector. There is a small signal between 1.1-1.6 minutes that was attributed to contamination of the HPLC water that was detected by UV analysis (Figure 3.2). This signal appeared during fluorescence analysis as well, but with a much lower response. This HPLC water signal was most noticeable during UV analysis when compound concentrations were low and was observed during environmental application (Figure 3.3).

3.3.2. Buffer Solutions

The use of sodium acetate buffer (pH = 3) in the individual analysis of antibiotics or citrate buffer (pH = 2.5) in the simultaneous separation has a two-fold purpose. The mobile phase pH is below the pKa's of the fluoroquinolones and prevents ionization of the molecules. For example, CIP has the following reported pKa's: carboxylic group (5.85 – 6.35), amino (8.24 – 8.95) and the other two N groups (5.05, 3.64) (De Witte et al., 2007). In addition the use of low pH ($2.0 < pH < 2.5$) minimizes the presence of free unprotonated silanol groups of silica-based columns. Previous methods employed phosphoric acid (Zendelovska and Stafilov, 2005), citric acid (Canada-Canada et al., 2007), formic acid and trifluoracetic acid (De Witte et al., 2007; Lee

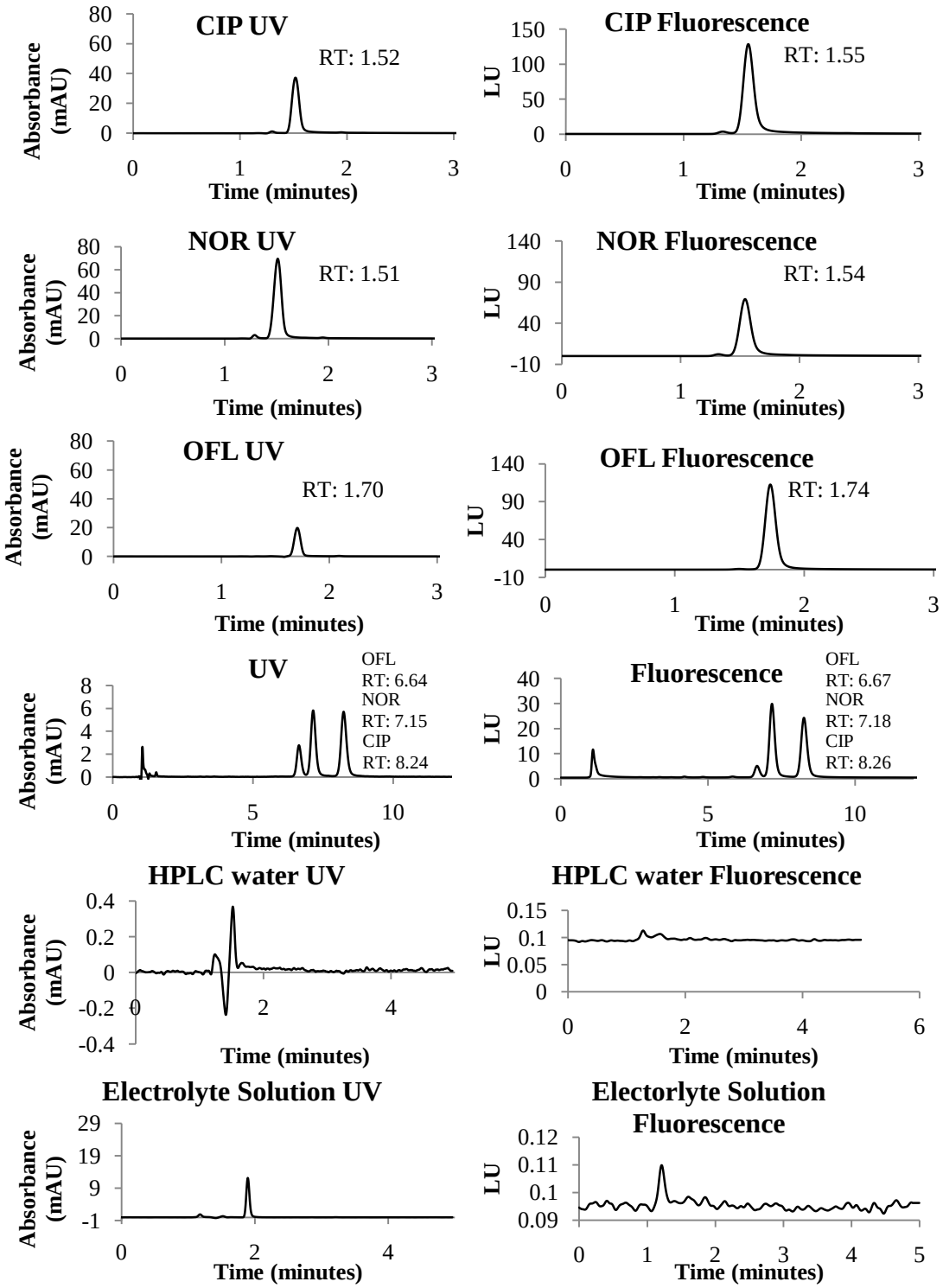


Figure 3.2. UV and Fluorescence signals of individual and simultaneous compound detection at 1 mg L^{-1}. (RT = Retention Time)

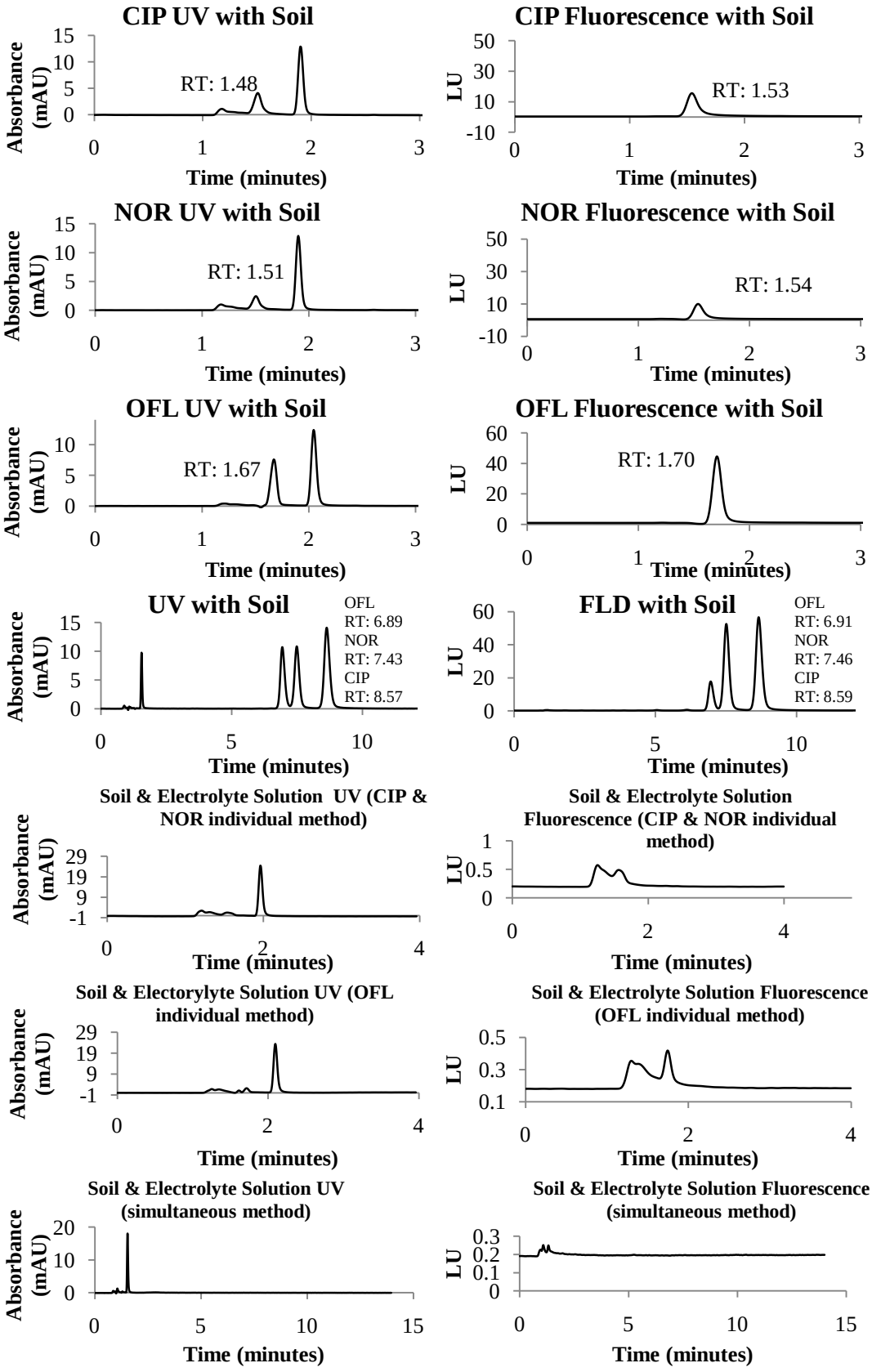


Figure 3.3. UV and fluorescence signals of individual compound detection with a wetland soil at 5 mg L^{-1} loading.

et al., 2007a). Moreover, triethylammonium from TEAP exchanges with less strongly retained ions such as sodium cations, thereby reducing the amount of free ionized silanol groups and suppressing the access of fluoroquinolones to residual silanols (Snider et al., 1997; Zendelovska and Stafilov, 2005). All of the above reasons result in peak shape improvement and decrease in retention times for the fluoroquinolones tested.

3.3.3. Mobile Phase Ratios

For individual compound analysis a 60:40 mobile phase consisting of sodium acetate and ACN was used for CIP and NOR while a 70:30 ratio was used for OFL. When all three compounds are run with the same amount of ACN in the mobile phase, the first fluoroquinolone to elute is OFL. If a 60:40 (A/B) mobile phase for ofloxacin is used, a potential problem arises especially at very low ppb level detection, where ofloxacin may completely overlap with early eluters from the matrix solution. Therefore, the 70:30 ratio is needed for proper OFL separation.

3.3.4. Simultaneous Method

The ability to simultaneously detect fluoroquinolone compounds allows us to analyze samples that are representative of environmental conditions, where multiple compounds may be present in a water sample. The single methods developed for individual compounds would not allow for simultaneous detection of all three compounds because CIP and NOR have similar retention times. The method for simultaneous detection was developed to ensure that there was separation between CIP and NOR.

This simultaneous method provides a closer examination of pharmaceutical and soil interaction in lab microcosm experiments. The method performed well when used to analyze the three compounds in the presence of a wetland soil. There was no interference observed during simultaneous method sample analysis (Figure 3.3).

Table 3.3. UV and Fluorescence results obtained from method application on Bayou Castine soils that validate the application of these methods on environmental matrices. Each compound demonstrated high sorption to the soil, with low concentrations remaining in the aqueous phase. Values obtained for both UV and Fluorescence were similar. (± values represent standard deviation).

UV

	Initial Mass (μg)	Mass on soil (μg)	Mass in solution (μg)	Conc. In soil (g kg^{-1})	Conc. In solution (mg L^{-1})	% in Solution	% on Soil	UV Retention Time
CIP	97.99 ± 0.08	93.07 ± 2.55	4.92 ± 2.53	4.06 ± 0.08	0.25 ± 0.13	5.02 ± 2.58	94.98 ± 2.58	1.50 ± 0.00
NOR	98.39 ± 0.08	91.97 ± 1.21	6.42 ± 1.21	4.01 ± 0.20	0.33 ± 0.06	6.53 ± 1.23	93.47 ± 1.23	1.50 ± 0.00
OFL	98.34 ± 0.08	87.3 ± 2.61	11.04 ± 2.55	3.81 ± 0.11	0.56 ± 0.13	11.23 ± 2.59	88.77 ± 2.59	1.67 ± 0.00

Fluorescence

	Initial Mass (μg)	Mass on soil (μg)	Mass in solution (μg)	Conc. In soil (g kg^{-1})	Conc. In solution (mg L^{-1})	% in Solution	% on Soil	Fluorescence Retention time
CIP	97.99 ± 0.08	92.34 ± 2.85	5.65 ± 2.83	4.03 ± 0.07	0.29 ± 0.14	5.77 ± 2.89	94.23 ± 2.89	1.54 ± 0
NOR	98.39 ± 0.08	89.47 ± 1.42	8.92 ± 1.34	3.90 ± 0.15	0.45 ± 0.07	9.07 ± 1.37	90.93 ± 1.37	1.54 ± 0
OFL	98.34 ± 0.08	88.36 ± 2.47	9.98 ± 2.41	3.85 ± 0.11	0.51 ± 0.12	10.15 ± 2.45	89.85 ± 2.45	1.70 ± 0

3.3.5. Analysis of Soil Samples

Neither interference nor degradation was observed during the soil sample analysis, as indicated by the absence of significant peaks other than the target compounds, a sodium azide peak at ~ 2 minutes and the low background signal (Figure 3.3). Blanks with soil and electrolyte solution showed no additional peaks associated with soil for either method during both UV and fluorescence detection (Figure 3.3). A mass balance was calculated, taking into account any compound sorbed to the surface of the vial, to determine the sorption rates of each compound onto the soil. Peak retention times for the environmental matrix samples were within 2.5% of those observed for the standards (Table 3.3 and Figure 3.3). Both methods produced corresponding results that showed high sorption to the wetland soil. For both UV and Fluorescence at 5 mg L^{-1}, the three compounds exhibited high sorption to the wetland soil, with both CIP and NOR sorbing >90% and OFL ~88% (Table 3.3).

3.4. Conclusions

In comparison to previously published methods, the methods developed in this work 1) allow for faster analytical throughput , with retention times that are 4.5-6.4 times shorter for individual compounds and 14-17% shorter for simultaneous detection of three antimicrobial pharmaceuticals (CIP, NOR and OFL), 2) remove the necessity of time consuming solid phase extraction if detection down to the low part per billion is desired, and 3) require minimal HPLC hardware, especially in the isocratic mode.

CHAPTER 4: SORPTION, DESORPTION AND COMPETITION BETWEEN THREE FLUOROQUINOLONE ANTIBIOTICS IN A SOUTHEAST LOUISIANA FRESHWATER WETLAND SOIL

4.1. Introduction

Fluoroquinolones (FQs) are used in human, veterinary and agricultural applications with CIP, OFL and NOR being three of the most widely used (Pico and Andreu, 2007). Fluoroquinolones have been detected at levels up to 0.12 and 0.33 μg L^{-1} in surface water and wastewater effluent, respectively (Kolpin et al., 2002). This raises concerns about the potential ecotoxicity of these compounds, individually or as a mixture (Isidori et al., 2005; Robinson et al., 2005; Lee et al., 2007b) and the evolution of resistance to frontline antibiotics bacterial strains (Waters and Davies, 1997; Kern, 2007; Yamane et al., 2007). Fluoroquinolones enter the environment by passing through wastewater treatment plants (WWTP) and the application of WWTP sludges to soil (Golet et al., 2003; Pico and Andreu, 2007). Once in the environment, FQs may sorb to solid matrices, such as soils and sediments, and there may be competition among multiple pharmaceutical compounds for preferred soil binding sites (Li and Werth, 2001; Bonin and Simpson, 2007).

Wastewater treatment facilities discharge into adjacent wetlands for tertiary treatment of the wastewater to reduce nutrient concentrations to background levels before they enter larger surface water bodies and alter ecosystem dynamics (Verhoeven and Meuleman, 1999; Day et al., 2006). Pharmaceutically active compounds (PhAC) are consistently found in WWTP effluent and, in some areas, WWTP effluent is discharged into wetlands for additional treatment (Metcalfe et al., 2003; Watkinson et al., 2007; Conkle et al., 2008). Recent studies have shown that surface and subsurface flow wetlands are effective at reducing PhAC concentrations

(Matamoros and Bayona, 2006; Waltman et al., 2006; White et al., 2006; Conkle et al., 2008). More research is required to address the chemical interactions, fluxes and system mitigation within these wetlands, with fluoroquinolone antibiotics being the focus of this study.

The goal of this study was to examine the sorption and desorption behavior of three related and highly used compounds, CIP, NOR, and OFL to a wetland soil and specifically we sought to 1) determine the sorption and 2) desorption of compounds within a wetland soil, and 3) assess competition among these similar antibiotics for binding sites on the soil.

4.2. Methods

4.2.1. Stock and Spike Solutions

Bayou Castine (15R 784949 E, 3361530 N) wetland soils were chosen for this study as 1) the Mandeville, LA wastewater treatment plants continuously discharges trace amount of numerous PhACs into the adjacent Bayou Chinchuba and Bayou Castine serves as the control wetland for Bayou Chinchuba research (Conkle et al., 2008), 2) Bayous Chinchuba and Castine are classified as Arat Silty Clay Loam, which is a fine silty, siliceous, non-acid, thermic Typic Hydraquent (Trahan et al., 1990). The surface soil (0-10) was collected by pushcore, homogenized and stored at 4 °C. Soil characterization included total and extractable metals (DeLaune et al., 2008) and total carbon, total nitrogen and organic matter (White and Reddy, 2000) and the data are reported in Tables 4.1 and 4.2. CIP, NOR and OFL were obtained from Sigma-Aldrich (St. Louis, MO) and used to create an aqueous stock solution of 100 mg L^{-1}, from which a 6-point standard curve and spike concentrations were created. All solutions contained 0.01 M $CaCl_2$ and 100 ppm sodium azide (NaN_3). Sodium azide is added to samples, as a biocide to prevent microbial degradation of compounds. Antibiotic compound spike levels are above environmentally relevant concentrations. However, we set out to simulate longer-term loading where a wastewater treatment plant discharges for example, 7.5 million liters daily with 2 μg L^{-1}

of a compound, which roughly equals 15 g into the environment. Therefore, these soils may be loaded with much larger amounts of the compound than just a few μg and the sorption incubation concentrations were increased to mimic longer-term soil exposure amounts.

Table 4.1. Bayou Castine soil metal concentrations

Metal	Exchangeable	Total
	mg kg^{-1}	
Al	11.6 ± 8.2	29284.3 ± 478.6
Ca	1765.4 ± 20.6	1830.9 ± 19.5
Fe	170.9 ± 78.4	11072.9 ± 158.2
K	202.6 ± 20.1	1527.5 ± 53.4
Mg	1117.1 ± 18	2155.2 ± 31.2
Na	499.6 ± 17.9	502.8 ± 8.8

Metal analysis was performed using an ICP in accordance with DeLaune et al. 2008

Table 4.2 Bayou Castine soil chemical properties

Parameter	Value
Cation Ex Capacity ($cmol_c$ kg^{-1})[‡]	19.8 ± 0.8
Moisture content (%)	65 ± 0.0
pH	6.8 ± 0.2
Organic matter (%) [†€]	18.5 ± 1.0
Total Carbon (g kg^{-1})[¥€]	88.3 ± 3.3
Total Phosphorus (mg kg^{-1})[€]	474.1 ± 15.8
Total Nitrogen (g kg^{-1})[¥€]	6.1 ± 0.2
Clay (%)	31.3

[‡] (Sumner and Miller, 1996)
[†] Loss on ignition
[€] Dry soil basis
[¥] (White and Reddy, 2000)

4.2.2. Sorption and Desorption

The Organization for Economic Cooperation and Development (OECD) method 106, adsorption – desorption batch equilibrium method was followed (European Union, 2000). However, samples were incubated longer than the suggested 24 hrs in the method to allow for pseudo-equilibrium to be reached. Sodium azide was also added to soils to prevent microbial

degradation of test compounds. The soil was homogenized and all woody debris and plant materials were removed. Samples were prepared with 100 mg (dry basis) field moist soil in glass scintillation vials in quadruplicate and incubated at 24 °C while being orbitally shaken at 150 rpm for 3-5 days in the dark. Immediately after incubation, samples were centrifuged at 3000 rpm (604 x g) for 15 minutes and the supernatant was extracted. Desorption incubations were run using the same sorption samples. For desorption, most of the remaining solution in the vials was removed and the amount of solution remaining was determined using mass balance. Then, the original volume of matrix solution was then added to each vial. From the concentration of the solution at pseudo-equilibrium, and knowing the volume remaining in the vial prior to matrix solution addition, the concentration of the antibiotic concentration at desorption time 0 (zero) was determined. The samples were then incubated, extracted and analyzed the same manner as the sorption experiment.

Table 4.3. Instrument analytical parameters for measuring the three analytes of interest both individually and simultaneously.

Fluoroquinolone	Retention time (min)	Slope	Intercept	R^2	LOQ[1] ($\mu g\ L^{-1}$)
Ofloxacin[a]	7.027	32.264	-7.0027	0.9997	97.8
Norfloxacin[a]	7.552	61.163	-9.5596	0.9998	55.7
Ciprofloxacin[a]	8.714	80.803	-13.623	0.9998	49.3
Ofloxacin[b]	1.672	52.302	3.8022	0.9999	84.3
Norfloxacin[b]	1.514	131.21	-0.403	0.9999	58.8
Ciprofloxacin[b]	1.513	167.49	-0.403	0.9999	65.3

[a] simultaneous detection of fluoroquinolones using DAD 280 nm
[b] individual detection of fluoroquinolone using DAD 280 nm
[1]LOQ- limit of quantitation (S/N=10)

All samples were analyzed following Conkle et al (2009) using an Agilent 1100 (Santa Clara, CA) series HPLC with UV and fluorescence detectors fitted with a Zorbax eclipse XDB C18 (4.6 x 150 mm x 5 μm) column. Only UV detection was used during this experiment. For NOR and CIP analysis, a 60:40 mobile phase consisting of acetate buffer (pH=3) with

triethylammoniumphosphate buffer (TEA); acetonitrile (ACN) was used. For OFL analysis, the mobile phase remained the same but the ratio was adjusted to 70:30. The simultaneous detection method for all 3 FQs used a mobile phase solvent ratio of 82:8:10 with citric acid buffer (pH 2.5): ACN: methanol. Analytical parameters are provided in Table 4.3 (Conkle et al., 2009).

4.2.3. Data Analysis

Percent and total amount sorbed were determined using the OECD guidelines. The amount sorbed (C_s^{ads}) (Equation 4.1) was calculated using mass balance between the compound concentration initially in solution (C_0) and the amount remaining in solution at equilibrium (C_{aq}^{ads}), which accounts for sorption to the glass vials. The calculations also account for the initial solution volume (V_0) and dry soil mass (m_{soil}).

Equation 4.1: $C_s^{ads}(eq) = \frac{[C_0 - C_{aq}^{ads}(eq)] \times V_0}{m_{soil}}$ $(\mu g\ g^{-1})$

The sorption distribution coefficient (K_d) (Equation 4.2) for a particular concentration was calculated by dividing the C_s^{ads} at equilibrium by the concentration in solution (C_{aq}^{ads}) at equilibrium.

Equation 4.2: $K_d = \frac{C_s^{ads}(eq)}{C_{aq}^{ads}(eq)}$ $(cm^3\ g^{-1})$

Freundlich Isotherms were determined by graphing the log of the compound concentration in soil on the Y-axis and the log of the aqueous compound concentration on the X-axis. The equation of the best fit line was determined and the y-intercept is the Freundlich (K_F) coefficient, while the 1/slope is the regression coefficient. It should be noted that although $N \neq 1$, the use of K_D to compare the sorption affinity of these individual FQs to its mixture may be allowed due to similarity in N, or by the use of the equation $K_D = K_F C_e^{(N-1)}$ (Carmo et al., 2000).

A one-way ANOVA was run using SPSS 15 (SPSS Inc.) to determine the difference between the K_D means for sorption and desorption of 60 ppm, 20 ppm and competition (20 ppm each of CIP, NOR, OFL in solution). If the homogeneity of variance (HOV) was >0.05, a Latin Square Design (LSD) was used to determine differences between the K_D means. If the HOV was <0.05, the Dunnett's T3 test was used.

Electrostatic potential surface models were generated using Sybyl 8.0 (Tripos International, St. Louis, MO). These models were used to examine the charged surface of each of the fluoroquinolone antibiotics to aid in understanding differences in sorption and desorption for each compound.

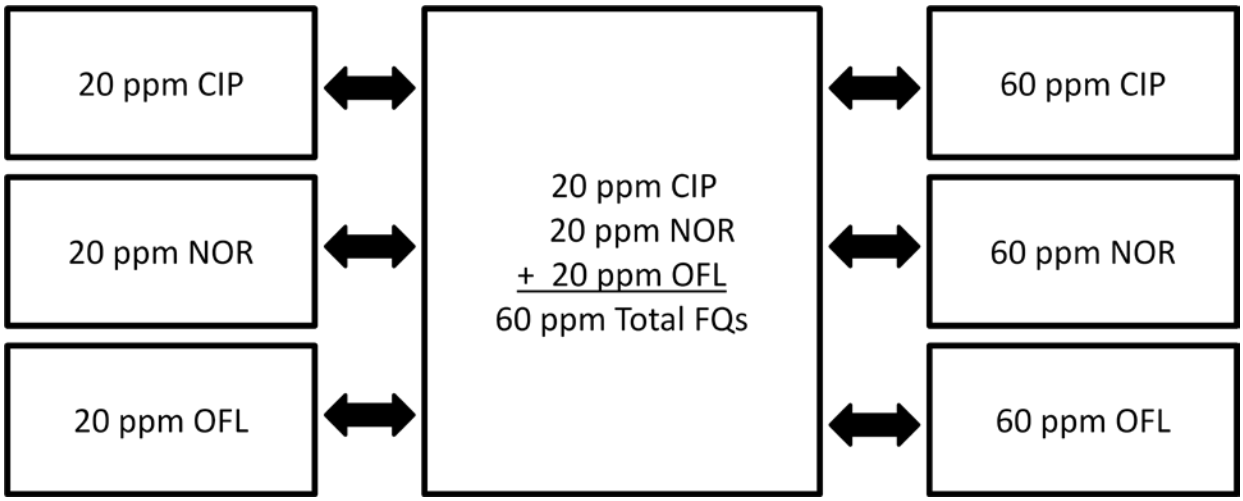


Figure 4.1. Competition analysis diagram demonstrating a conceptual framework for the competition comparisons.

4.2.4. Simultaneous Compound Analysis

Sorption and desorption of soil samples spiked with 20 and 60 ppm FQs were examined along with an additional set of samples spiked with a solution containing 20 ppm of each compound, for a total of 60 ppm of antibiotics in the mixture. The 60 ppm multiple compound samples were used to determine effects on sorption or desorption due to competition between compounds for binding sites. Sorption and desorption K_D values at 20 and 60 ppm for CIP,

NOR and OFL were compared to determine any difference in sorption potential at these two concentration levels. The individual 20 and 60 ppm K_D values were then compared to the K_D values determined for multiple compound analysis, and a comparative matrix is provided in Figure 4.1.

Table 4.4. Sorption kinetics data for 20 ppm solutions with mass and percent sorbed.

Kinetics at 20 ppm

Hours	g kg^{-1} Dry Soil			% Sorbed		
	CIP	NOR	OFL	CIP	NOR	OFL
8	4.49 ± 0.57	3.67 ± 0.92	3.71 ± 0.16	34 ± 4	34 ± 9	32 ± 2
12	5.83 ± 1.07	5.29 ± 0.97	4.99 ± 0.73	44 ± 7	47 ± 9	43 ± 7
16	6.31 ± 1.44	7.33 ± 0.54	5.65 ± 0.53	49 ± 11	63 ± 4	49 ± 5
20	8.21 ± 0.90	7.11 ± 0.38	7.75 ± 0.68	62 ± 7	62 ± 2	65 ± 6
24	7.39 ± 0.54	6.33 ± 2.50	7.17 ± 0.91	58 ± 4	56 ± 22	62 ± 8
48	9.24 ± 0.20	8.83 ± 0.66	8.87 ± 0.56	71 ± 1	78 ± 5	78 ± 7
72	9.63 ± 0.87	10.21 ± 0.31	9.17 ± 0.49	73 ± 6	89 ± 4	81 ± 5
120	9.41 ± 1.01	8.88 ± 0.38	8.58 ± 0.40	73 ± 6	76 ± 3	75 ± 2

4.3. Results and Discussion

4.3.1. Single Compound Sorption and Desorption

Compound sorption reached steady state within 2-3 days at 20 ppm of each compound, with 50-60% being sorbed within the first 16 hours of incubation (Table 4.4). While the *total mass* sorbed of each compound *increased* from ~10 g in a 20 ppm solution to 28-35 g per 1 kg of dry soil in an 80 ppm solution (Figure 4.2), the *percent* of the sorbed FQs *decreased* from ~90% for all three FQs at 20 ppm to 76.7 ± 5.1% for NOR, to 72.1 ± .06% for OFL, to 62.3 ± 3.2% for CIP at 80 ppm.

The data in Table 4.5 show that K_D also decreased as a function of sorbate concentration. In regards to desorption, the K_D values for all concentrations yielded the following trend: NOR > CIP > OFL. For OFL, CIP, and NOR the percentage desorbed (20 ppm : 80 ppm) were 4.07 ± 0.76 : 11.83 ± 0.97; 3.76 ± 0.75 : 6.09 ± 1.18 and 2.04 ± 0.25 : 5.31 ± 1.44, respectively (Figures

4.3a-c). The overall trend is that FQs bind less tightly at higher concentration, with OFL being most loosely bound to the studied soil. These results lead to three questions: 1) why does the studied soil have its sorption capacity, where does a soil sorption capacity come from, 2) what is the mechanism of sorption and 3) are there different sorption sites? In order to answer the first question one must study the sorbent. The studied soil contained 1830 mg kg^{-1} Ca (1756 mg kg^{-1} exchangeable), 29,284 mg kg^{-1} Al (11 mg kg^{-1} exchangeable) and 11,072 mg kg^{-1} Fe (170 mg kg^{-1} exchangeable), an overall cation exchange capacity (CEC) of 19.8 $cmol_c$ kg^{-1} with a clay content of 31.3%, and organic matter content of 18.5 ± 1.0% (Tables 4.1 and 4.2). Ca, Al, and

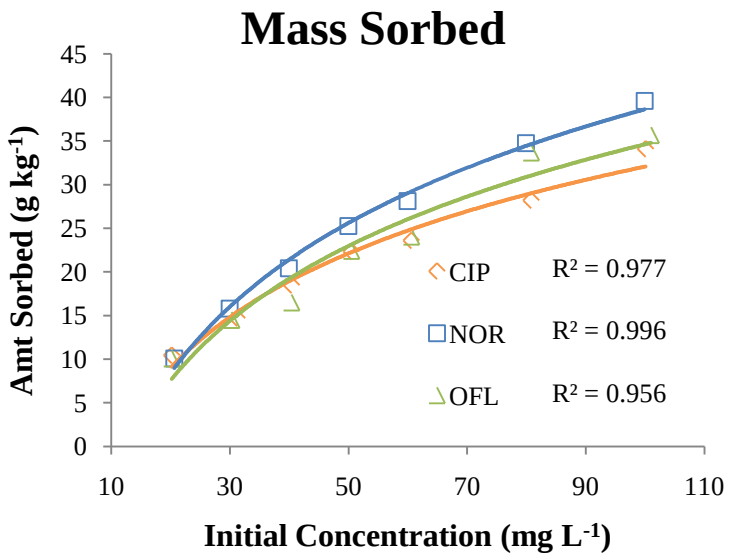


Figure 4.2. Soil sorption capacity for an initial concentration range from 20 to 100 mg L^{-1}

Fe are known to complex with fluoroquinolones and have been found to play an important role in a soil's FQs sorption capacity (Gu and Karthikeyan, 2005; Zhang and Huang, 2007; Zhang and Dong, 2008). In addition, cation exchange is also an important mechanism for the sorption of FQs (Lee et al., 2007b). The soil has a high percentage (18%) of organic matter which has also been shown to have a high sorption affinity for fluoroquinolones (Golet et al., 2003). This means

Table 4.5. Sorption and desorption data for mass of soil and percent of each compound sorbed along with the K_D values at each loading concentration.

Initial Conc. (ppm)	g kg^{-1} Dry Soil			% Sorbed			K_D (cm^3 g^{-1})		
	CIP	NOR	OFL	CIP	NOR	OFL	CIP	NOR	OFL
Sorption									
20	10.5 ± 0.4	10.1 ± 0.13	10.1 ± 0.3	89.2 ± 1.3	91.0 ± 1.9	88.3 ± 1.3	4844 ± 628	5791 ± 1280	4325 ± 634
30	14.7 ± 0.8	15.8 ± 0.64	14.5 ± 0.8	85.2 ± 4.7	91.5 ± 0.8	85.0 ± 5.5	3587 ± 1730	6234 ± 727	3541 ± 1291
40	18.5 ± 3.2	20.5 ± 0.4	16.5 ± 1.5	80.5 ± 9.5	90.0 ± 2.8	72.8 ± 5.9	2798 ± 1405	5382 ± 1422	1576 ± 530
50	22.3 ± 0.6	25.2 ± 0.2	22.4 ± 1.0	75.6 ± 2.6	89.4 ± 2.5	80.7 ± 3.9	1835 ± 267	4984 ± 1177	2387 ± 587
60	23.6 ± 2.6	28.1 ± 1.6	24.0 ± 3.0	69.0 ± 6.7	83.6 ± 4.4	70.5 ± 8.0	1338 ± 520	3073 ± 1035	1471 ± 630
80	28.2 ± 1.3	34.8 ± 2.4	33.7 ± 1.1	62.3 ± 3.3	76.7 ± 5.2	72.1 ± 0.6	934 ± 127	1942 ± 450	1496 ± 82
Desorption									
20	10.1 ± 0.3	9. 9 ± 0.1	9.7 ± 0.3	3.8 ± 0.8	2.0 ± 0.3	4.1 ± 0.76	5431 ± 1029	10005 ± 1308	5042 ± 1044
30	14.0 ± 0.7	15.3 ± 0.5	13.6 ± 0.7	4.8 ± 0.7	3.1 ± 0.6	5.9 ± 1.2	4021 ± 659	6664 ± 1530	3376 ± 762
40	17.7 ± 2.7	19.6 ± 0.4	15.1 ± 1.4	6.3 ± 1.3	3.3 ± 0.2	7.6 ± 2.0	3168 ± 765	6281 ± 375	2519 ± 644
50	21.1 ± 0.6	24.3 ± 0.3	20.8 ± 1.1	5.4 ± 1.4	3.7 ± 0.7	7.2 ± 0.4	4071 ± 1390	5407 ± 972	2569 ± 166
60	22.0 ± 2.6	27.1 ± 1.4	21.8 ± 2.9	6.9 ± 1.3	3.6 ± 0.9	10.4 ± 2.1	2788 ± 621	5733 ± 1257	1796 ± 395
80	26.5 ± 1.0	32.9 ± 1.8	29.7 ± 0.9	6.1 ± 1.2	5.3 ± 1.4	11.8 ± 1.0	3224 ± 717	4017 ± 1449	1573 ± 135

that a number of mechanisms can be envisioned to be at play in the sorption of FQs to this soil, and hence, within a wetland WWTP. From an applied point of view, the results above do show that: (i) the wetland soil under study has a high sorption capacity for the FQs, (ii) FQs are rapidly sorbed, and (iii) other removal mechanisms (e.g. photodegradation) probably play a minor role reducing the remaining concentration in the aqueous phase.

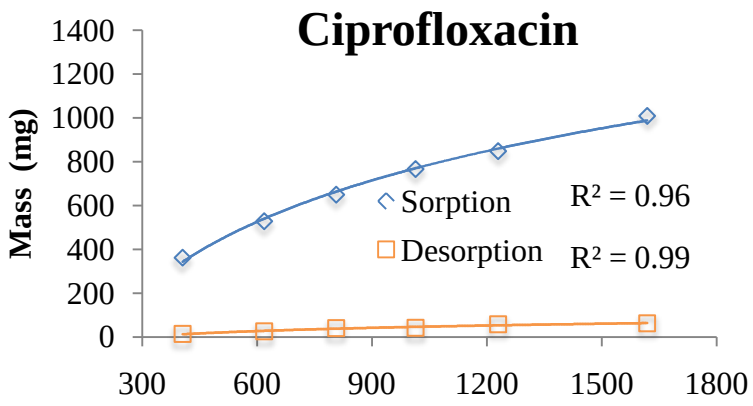
Insight into the sorption mechanisms comes from batch sorption data fitted with the Freundlich isotherm. Log transformed values of Freundlich sorption coefficients (K_F) of FQs give the trend NOR (4.09) > CIP (4.01) > OFL (3.9). Desorption K_F values are higher but show a similar behavior: NOR (4.24) > CIP (4.12) > OFL (4.05). In addition, sorption-desorption isotherms are hysteretic and highly non-linear (Table 4.6; Fig 4.3). In another study, OFL had Log K_F of 3.75 in a soil with pH = 4.3, 7.1% OC, 57.52% sand, 26.64% silt and 15.84% clay, which is slightly lower, but the same order of magnitude as our value (Drillia et al., 2005). In contrast, our K_F values are lower than those published (Zhang and Dong, 2008) for two of the

Table 4.6. Freundlich Isotherm values for sorption and desorption of each compound. Information on Freundlich calculations are located in supplemental information.

Fluoroquinolone	Sorption			Desorption		
	N	K_F	R^2	N	K_F	R^2
Ofloxacin	0.40 ± 0.05	3.90 ± 0.05	0.74	0.46 ± 0.03	4.05 ± 0.02	0.92
Ciprofloxacin	0.28 ± 0.04	4.01 ± 0.04	0.74	0.44 ± 0.03	4.12 ± 0.01	0.92
Norfloxacin	0.38 ± 0.04	4.09 ± 0.03	0.81	0.49 ± 0.03	4.24 ± 0.01	0.91

three mineral soils for NOR, 4.32, 4.45. This wetland soil has a pH of 6.87, ~10% OC, 66.40% sand, 31.31% clay and 19 cmol kg^{-1} CEC. Thus, it is likely that sorption of these FQs is not only due to its associations with the minerals present in soil, but also to the soil organic matter (SOM) content. In addition, pH and ionic strength also influence sorption of charged organic

a)



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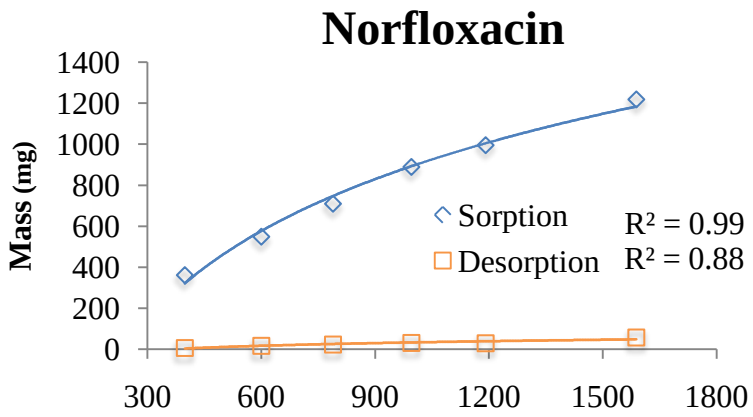

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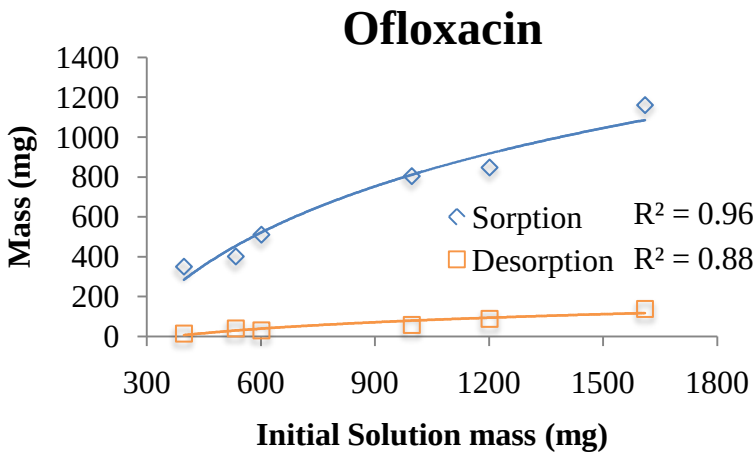


Figure 4.3. Sorption and desorption of each compound. The x-axis represents the initial compound mass in solution. The y-axis represents either the compound mass on the soil (sorption) or the compound mass desorbed (desorption) at pseudo-equilibrium after three days for a) CIP, b) NOR, and c) OFL.

compounds. Also, Zhang and Dong (2008) found that the presence of low molecular weight organic acids decreased the sorption capacity of NOR in a mineral soil. Therefore, the presence of organic materials in the Bayou Castine soil may have contributed to a decrease in the fluoroquinolone sorption capacity.

The studied fluoroquinolones have two different pK_a values: carboxylic (pK_{a1} = 5.90 - 6.23) and amino (pK_{a2} = 8.28 - 8.89) (Tolls, 2001; Pico and Andreu, 2007). Since the pH of Bayou Castine soil is 6.87, during the sorption process a greater fraction of FQs (~80%) exist in zwitterionic or neutral form, whereas the remaining portion is cationic. The presence of charged groups in an organic molecule enables ion-ion as well as ion-dipole interactions, which in turn greatly influence sorption to mineral/clay surfaces as well as to SOM. Neutral molecules can also be sorbed by various retention mechanisms, such as hydrophobic forces, hydrogen-bonding, ion-dipole, and aromatic electron donor-acceptor processes depending on their chemical structures. For this reason, high potential for sorption to both the mineral phase and SOM within this soil is expected.

The highly nonlinear nature of Freundlich isotherms of FQs in this study suggests that site specific interactions of varying energies largely contribute to FQ sorption, aside from nonspecific hydrophobic forces. Nonlinearity in sorption of organic compounds with charged groups, and even of unionized polar organic compounds, is expected (Chiou et al., 2000; Schwarzenbach et al., 2003). Sorption isotherm nonlinearity of NOR and OFL (N=0.76 and 0.61, respectively) to highly mineral soils with OC<0.5% have been observed (Drillia et al., 2005; Zhang and Dong, 2008). The hysteresis is further enforced by desorption K_F > sorption K_F. The experimental design makes artificial hysteresis very unlikely. Thus, the observed hysteresis can be considered as real and attributable to mineral hydrophobic nanosites, sorbate-induced rearrangement of the SOM matrix during sorption, and the creation of rigid and dilated pores,

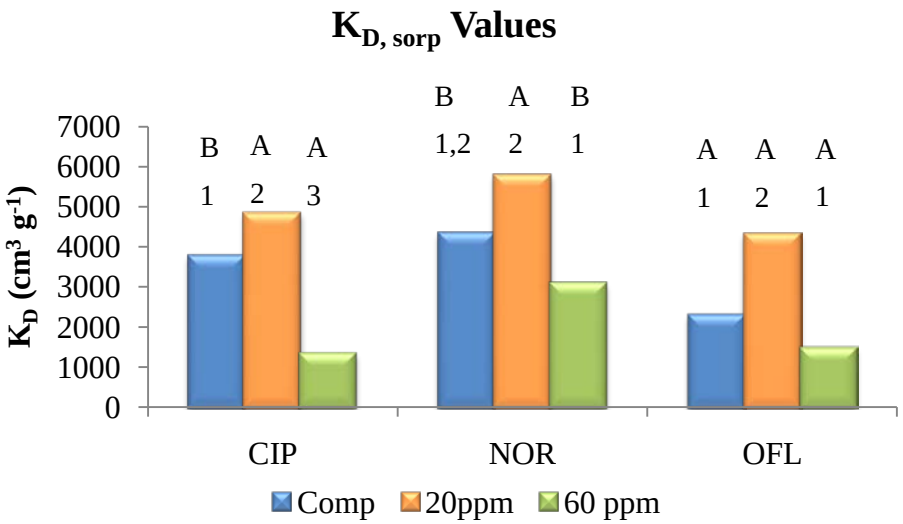
which may not fully relax back during the desorption process (Xia and Pignatello, 2001; Sander et al., 2006). The organic matter within the Bayou Castine soil may actually compete with the FQs for available binding sites, as indicated by the decreased the sorption capacity of NOR in a mineral soil in China in the presence of low molecular weight organic acids (Zhang and Dong, 2008).

The data above indicate that there is a distribution of sites capable of sorbing FQs within the investigated wetland soil. This conclusion comes from 1) the difference observed in binding constants at different concentrations, 2) the change in the order of binding constants among FQs as a function of concentration and 3) the observed hysteresis. These points also lead to the possibility of competition between FQs for sorption sites within the wetland soil under study.

4.3.2. Multi-compound (Competitive) Sorption and Desorption

In order to address the possibility of competition between the studied FQs for the sorption sites within the soil, three different scenarios were explored, as illustrated in Figure 4.1. Each individual FQ was sorbed at a 20 ppm concentration. Additionally, all three compounds, each at 20 ppm, were simultaneously sorbed for a cumulative 60 ppm FQ concentration. Finally, each FQ was studied individually at a concentration of 60 ppm. The results of this analysis are presented graphically in Figure 4.4. From the data it can be seen that the individual compound sorption K_D values at 20 ppm showed no significant difference (CIP & NOR $p = 0.523$, CIP & OFL $p = 0.601$, NOR & OFL $p = 0.243$), while at 60 ppm, NOR had a statically greater K_D value compared to CIP ($p = 0.01$) and OFL ($p = 0.016$), with a general trend of NOR>OFL=CIP. The K_F from the Freundlich fits also show preferential sorption for NOR, followed by CIP. These K_D results are not unexpected and are in fact consistent with what would be predicted based on the isotherm data.

a)



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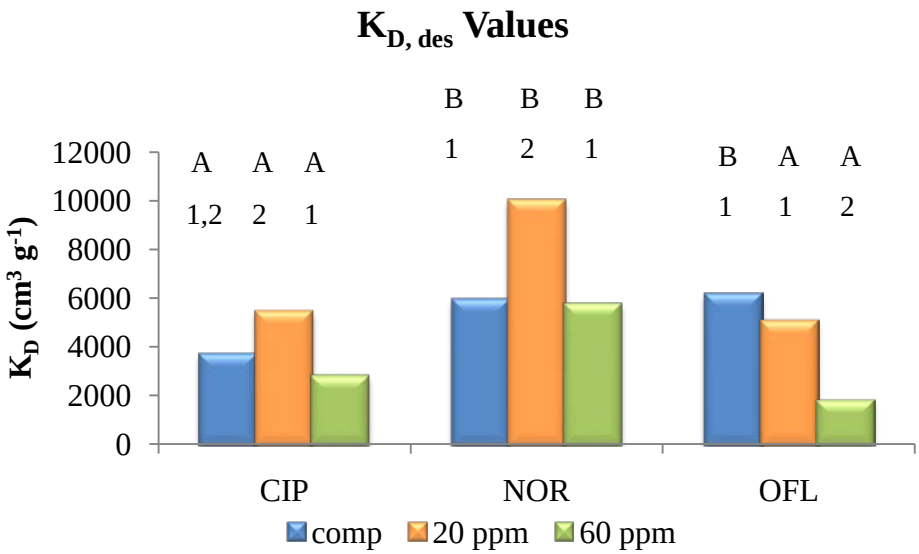
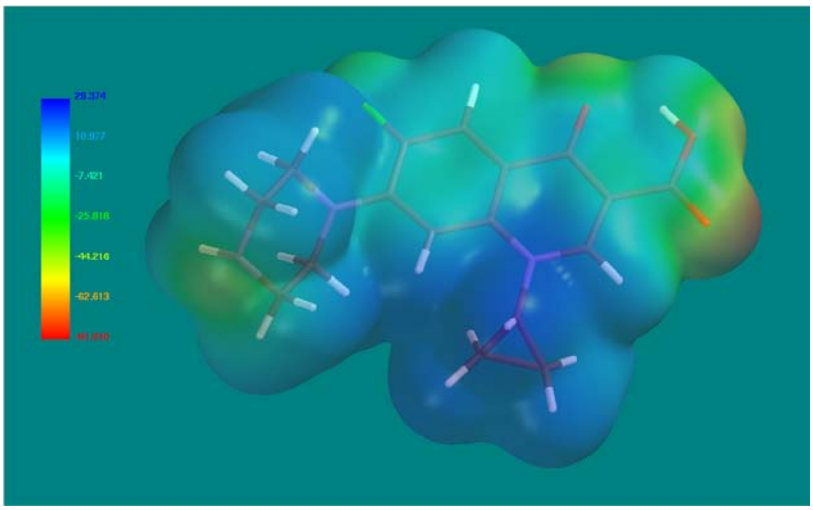


Figure 4.4. Sorption and desorption K_D values showing competition compared to individual values at 20 and 60 ppm. The 20 and 60 ppm values represent 1 single compound in solution at that concentration. Competition data represents all 3 compounds (CIP, NOR, OFL) in solution at 20 ppm each, for a cumulative concentration of 60 ppm. Letters represent subsets of comparisons between treatment levels (ie. 60 ppm CIP vs. 60 ppm NOR vs. 60 ppm OFL, etc.). Numbers represent subsets of comparisons between treatments for each compound (20 ppm CIP vs. Competition CIP vs. 60 ppm CIP, etc.).

If one now focuses on the multi-compound sorption data, it can be seen that the 20 ppm individual K_D values are significantly higher than the 60 ppm mixture K_D values for CIP ($p = 0.048$) and OFL ($p = 0.001$). NOR K_D values for 20 ppm individual analysis are not significantly different ($p = 0.093$) than the mixture K_D (Figure 4.4a). The 60 ppm individual K_D for NOR is not statistically different from the K_D for NOR in the 60 ppm mixture. Both of these findings reinforce the hypothesis that NOR outcompetes the other two compounds for binding sites. In fact, carrying out the same type of analysis reveals that the preferential sorption of NOR occurs when OFL is also present. The same analysis shows that CIP lies between NOR and OFL. These findings suggest that: 1) NOR competes for OFL sites only and CIP has unique sites and/or 2) NOR and CIP compete with OFL for sorption sites.

In terms of desorption, the K_D values are as expected from the adsorption isotherm analysis at both 20 and 60 ppm. No significant difference was observed between the mixture and 20 ppm results for CIP and OFL ($p = 0.095$ and 0.267, respectively) (Figure 4.4b). For NOR, the 20 ppm individual K_D value was significantly greater than that in the mixture K_D ($p = 0.01$). Only OFL ($p < 0.01$) had a mixture K_D that was significantly greater than that in the 60 ppm individual solution. In fact, the K_D for OFL in the mixture was the same as for NOR mixture, which was unexpected, given the sorption data discussed above. The data indicate that, while OFL is "outcompeted" in terms of total sorption site occupation, the site "quality", as determined by sorption strength or irreversibility, partially reverses that effect. Results demonstrate that the competition and 20 ppm values of OFL are significantly higher than 60 ppm OFL $K_{D,des}$ values, but that the 20 ppm and competition are not significantly different (Figure 4.4b). The lack of a difference between these two values demonstrates that while OFL sorbs less than NOR and CIP, it sorbs to higher quality sites in the presence of the other two compounds. While each of the three antibiotic compounds are similar, there are some structural differences which lead to OFL

a)



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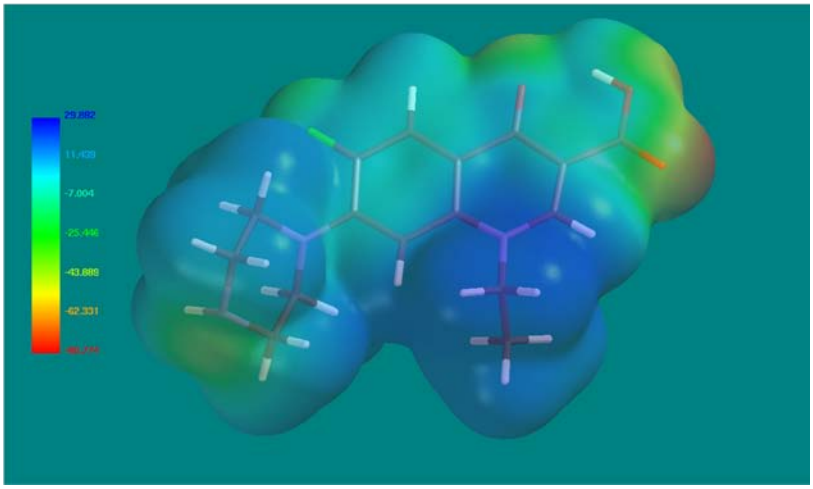

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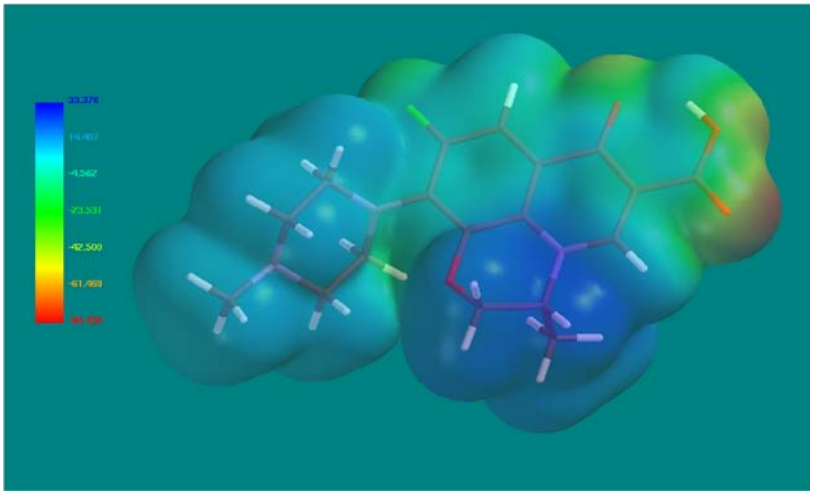


Figure 4.5. Electrostatic potential surface models for a) CIP, b) NOR and c) OFL. Models, courtesy of Dr. Charisma Lattao, were generated using Sybyl 8.0 (Tripos International, St. Louis, MO).

sorbing to higher quality binding sites than for the other two compounds. The reason for this phenomenon is understood when examining the electrostatic surface potential models of each compound (Figures 4.5a-c). A highly negative surface potential exists on the carboxylic group while on the other end of the molecule, a positive electrostatic surface potential is situated on the amino group, depicting the zwitterionic or neutral form of FQs as having localization of charges. OFL however, has a more positive surface potential than CIP and NOR on the opposite end of the carboxylic group. Moreover, the aromatic moieties in FQ display neutral to minimal negative charges on its surface, whereas the different substituents attached to the nitroaromatic moiety exhibit the greatest positive charge concentration along the surface of the molecule.

This is consistent with a previous suggestion by Carrasquillo et al. (2008) that the distribution of charges on the FQ molecule permits an orientation that allows optimal interactions with sorption sites. Hence, the carboxylate groups in FQs are capable of electrostatic interaction with the multivalent cations on the mineral phase and/or hydrogen bonding with natural organic matter, which is enhanced by a greater charge separation on the molecule. The net dipole of FQs is in the order OFL (8.63) > CIP (8.38) > NOR (8.09), which suggests that the neutral OFL molecule is the most polarized hydrophobic compound among the three FQs. Aside from other interactions, hydrophobic forces are also higher for OFL because of its larger hydrophobic surface, which also contributes to the observed trend. In addition, Zhu and Pignatello (2005) found that polarizability contributes between ~15-40% of sorption free energy. Therefore, compounds that are more polarizable are more likely to bind. In this case, charge-dipole interactions are probable between the carboxylate and phenolate groups of the natural organic matter and the positively charged surface of the FQs. The combined sorption and desorption data (Figures 4.4a ad 4.4b) collectively provide the following evidence: 1) overall

NOR and, to a lesser extent, CIP outcompete OFL for sorption sites, 2) OFL sorbs to its share of "quality" sorption sites, and 3) competition only occurs for lesser "quality" binding sites.

4.4. Environmental Implications

Treatment wetlands continuously receive wastewater containing a suite of pharmaceutical compounds. The soils of these wetlands are responsible for a significant amount of aqueous concentration reduction of pharmaceutical compounds through sorption. The treatment wetland soil used in this study sorbed 60-90% of each compound within 2-3 days. The rapid sorption to the soil indicates that treatment wetlands do not need extended retention times in order to be effective at reducing aqueous concentrations of these compounds. If loading of each compound were halted, which is unlikely in a wetland receiving treated wastewater, the wetland soil would release a small percentage of the amount sorbed in the short term. Freundlich isotherm values were ~4 L kg^{-1}, which also points to sorption as is a major removal pathway. These data indicate that this soil has a significant ability to sorb and immobilize these FQs, mitigating any downstream releases during wastewater treatment. However, studies need to establish whether the release of low amounts of these FQs would continue over the long term. The pH of freshwater wetlands, which are used to treat wastewater, have a pH between 6-7, therefore the small pH range will not significantly alter sorption when comparing different treatment wetlands.

Sorption of compounds in wastewater to a wetland soil is complicated by the presence of a suite of pharmaceutical compounds and other wastewater contaminants. There is competition between the compounds studied for preferred binding sites. Therefore, one compound may decrease the sorption capacity of another compound, which may cause greater migration of compounds downstream. The competition effect is of particular concern in a treatment wetland wastewater where 10s to 100s of compounds have been detected (White et al., 2006). White et al. 2006 also determined that some treatment wetlands may receive a significant mass of these

compounds annually (27 kg). Therefore, if sorption is the major removal pathway of these compounds from the aqueous phase, soils would need to be able to sorb large amounts of compounds without reaching saturation. This soil is capable of sorbing a significant amount of the pharmaceuticals studied, well above a daily loading rate.

These points, as well as viability of treatment wetlands over the course of decades, require further studies due to soils becoming increasingly more loaded with a range of pharmaceutical compounds, including antibiotics. The presence of competition between these three compounds demonstrates the need for more studies that examine the sorption of pharmaceuticals and personal care products in the aqueous environment in the context of waters containing multiple compounds.

CHAPTER 5: ANTIBIOTIC EFFECTS ON MICROBIAL RESPIRATION IN TWO WETLAND SOILS

5.1. Introduction

Since Alexander Fleming's discovery of Penicillin in 1928, antibiotics have become a cornerstone for the health of our society and are used to maintain high levels of livestock and aquaculture production, along with human health (Kummerer, 2009a). These compounds are valued for their ability to interrupt the proliferation of specific bacteria. While antibiotics can occur naturally in the environment, a wide range of synthetic pharmaceuticals are now regularly detected in soil and water impacted from various sources (Kolpin et al., 2002; Batt et al., 2006; Karthikeyan and Meyer, 2006; White et al., 2006; Conkle et al., 2008; Barber et al., 2009).

In wetlands, microbes facilitate many biogeochemical transformations, such as organic matter decomposition and a wide variety of nutrient transformations (Wright and Reddy, 2007; Ye et al., 2009). This important ecosystem function relies on microbes that break down organic matter in wetlands, thereby contributing to nutrient availability. Wetland microbes produce carbon dioxide (CO_2), and methane (CH_4) during oxidative respiration. Additionally, denitrifiers facilitate the gaseous removal of nitrogen from wetlands by utilizing nitrate (NO_3^-) as their terminal electron acceptor, converting it to nitrogen gas (N_2) through denitrification and in some systems N_2O is also produced.

Nitrogen is a limiting nutrient in many aquatic ecosystems. Therefore, it is important to reduce N loading to prevent eutrophication (Broussard and Turner, 2009). In some Louisiana communities, and many other places around the world, wetlands are used to treat wastewater (Chapman, 2003; Gray and Sedlak, 2005; Conkle et al., 2008). Wastewater can contain a range of pharmaceuticals, including antibiotics (Conkle et al., 2008). It is therefore important to determine if the loading of antibiotics to treatment wetlands has an adverse impact on the

function of the natural microbial communities, especially denitrifiers responsible for removing nitrate from wastewater.

Only two studies, to the author's knowledge, have examined microbial respiration in the presence of pharmaceuticals. Costanzo et al (2005) found that some antibiotics reduced the rates of denitrification (erythromycin, clarithromycin, amoxicillin), while amoxicillin/clavaulini showed no effect when soils were loaded with 1000 ppb. Ciprofloxacin was also tested over a concentration gradient from 0.1 to 1000 ppb and no effects were noticed (Costanzo et al., 2005). Fountoulakis et al (2004) examined the influence of pharmaceuticals on methanogenesis and determined that propranolol hydrochloride, diclofenac (sodium), carbamazepine and ofloxacin all inhibited rates, while sulfamethoxazole and clofibric acid showed no significant effects. However, in the study by Fountoulakis et al (2004), samples were tested over a concentration gradient of 10 to 400 ppm, which is 2 to 6 orders of magnitude higher than environmentally relevant concentrations (Fountoulakis, 2004).

This study compliments their research by examining the effect of three antibiotic compounds, ciprofloxacin (CIP), tetracycline (TET) and sulfamethoxazole (SULF) on N_2O, CH_4 and CO_2 production in two wetland soils, with contrasting organic matter contents; peat and mineral (Table 5.1). Substrate induced respiration (SIR) rates were determined for CH_4, N_2O and CO_2 production, while basal respiration rates were determined for CH_4 and CO_2.

5.2. Materials and Methods

5.2.1. Test Solutions

Tetracycline, ciprofloxacin and sulfamethoxazole (all > 98% purity) were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of 20 ppm were prepared for each compound, which were then further diluted to a 5 ppm spike solution. The 5 ppm spike solution of each

compound was then injected into the sample bottles or tubes containing soil and solution at specific volumes to achieve the pre-determined treatment concentration.

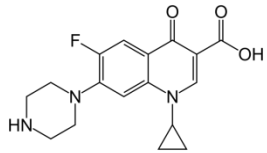
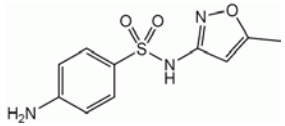
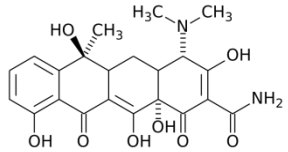
5.2.2. Site Description and Soil

Mandeville, LA utilizes a system of lagoons and wetlands to treat wastewater, and is effective at reducing the concentration of pharmaceuticals discharged in its effluent (Conkle et al., 2008). However, several of the detected compounds are still released, but at much lower concentrations into the adjacent forested wetland (Bayou Chinchuba) and ultimately Lake Pontchartrain. A nearby wetland soil from Bayou Castine (BC, 15R 784949 E, 3361530 N) was chosen for this study due to its proximity to Bayou Chinchuba and similar soil classification as an arat silty clay loam, which is a fine silty, siliceous, non-acid, thermic typic hydraquent (Trahan et al., 1990). A peat soil was also chosen for analysis to compare the effects of antibiotics on soils with a significantly higher organic matter contents. The peat soil was taken from the Davis Pond (DP) freshwater diversion wetland (15R 0765814, 3307688) (Gardner and White, IN PRESS). This wetland receives water diverted from the Mississippi River, which has been determined to contain a wide range of pharmaceutical compounds (Zhang et al., 2007). Soils from the top 10 cm were collected, woody debris removed, homogenized and stored at 4 °C. Soil characterization included total and extractable metals (DeLaune et al., 2008) and total carbon and nitrogen and organic matter (White and Reddy, 2000). All analysis was performed on field moist soil.

5.2.3. Methane and Carbon Dioxide Respiration Methods

For determination of CH_4, and CO_2 production, 0.75 g dry weight of each soil was added to 27 mL anaerobic tubes, capped with a gas impermeable butyl rubber stopper and sealed with an aluminum crimp. Each sample tube was evacuated to a pressure of < -88 Kpa and then flushed

Table 5.1. Compounds used to examine antibiotic effects on wetland soil microbial respiration and various properties of each drug.

Compound		Antibiotic Effect	Targets	Human Excretion	Treats	Log K_{ow}	Log K_F
CIP		Bactericidal	Gram Negative Gram Positive	35% in 24 hrs	Urinary Tract Infections	0.4[a]	4.01[c]
SULF		Bacteriostatic	Gram Negative Gram Positive	30% in 72 hrs	Urinary Tract Infections Chronic Bronchitis Pneumonia	0.89[b]	0.04 – 1.27[b]
TET		Bacteriostatic	Gram Negative Gram Positive	---	Respiratory Tract Infections Skin and Tissue Infections	-1.19[a]	1.66 – 3.63[d]

[a] Tolls 2001
[b] Yu et al. 2009
[c] Conkle et al. IN REVIEW
[d] Jia et al. 2008, Gu et al. 2007

with 99.9% O_2 free N_2 for five minutes. Each vial was incubated at 25^{o}C in an incubated orbital shaker at 100 rpm for 72 hrs and the headspace was again flushed for five minutes, prior to solution, substrate and antibiotic additions. Each vial received DI water to bring the total liquid volume of the vial to 15 mL, which includes the additions of one of five concentrations (1, 50, 100, 500, 1000 ppb) of CIP, TET or SULF. Substrate induced incubations for CH_4, and CO_2 were identical to basal incubations, except sodium acetate (23 g C kg^{-1} dry soil) was added to CH_4 vials while glucose (30 g C kg^{-1} dry soil) was also added to CO_2.

Headspace gas samples were collected and analyzed once during the first 48 hours and then a week later, followed by bi-weekly sampling for up to two months for basal respiration. Substrate induced respiration samples were sampled daily for one week. Gas samples were analyzed for CO_2 using a Shimadzu (Koyoto, Japan) GC-2014 fitted with a thermo conductivity detector operated at 160^{o}C, utilizing a packed Poropak N (6 ft; 80/100 mesh) column, supplied by Sigma-Aldrich (St. Louis, MO), with an oven temperature of 80^{o}C.

Gas samples for methane basal respiration were analyzed bi-weekly for up to 2.5 months. Substrate induced CH_4 samples were incubated and analyzed for 3 months. Samples were analyzed using a Shimadzu GC-2014 fitted with a flame ionization detector operated at 160^{o}C, utilizing a packed Carboxyn 1000 (6 ft; 40/60 mesh) column, supplied by Sigma-Aldrich (St. Louis, MO), with an oven temperature of 110^{o}C.

Five point standard curves were run weekly and continuing calibrations within 5% were performed prior to each analysis. Standard gas consisting of 1.0% CO_2, 1.0% CH_4, 1.0% O_2 and a balance of N_2 was used for the calibration of both CO_2 and CH_4.

5.2.4. Denitrification Methods

The acetylene block method was used to determine the effect of antibiotics on denitrification (Yoshinari et al., 1977; Sorensen, 1978). Therefore, N_2O evolution is used as a

proxy for the denitrification rate, where acetylene blocks the final reduction transformation of N_2O to N_2. Approximately 0.75 g dry weight of the two soils were added to 70 mL serum bottles, capped with a gas impermeable butyl rubber stopper and sealed with an aluminum crimp. Only substrate induced respiration (added carbon and nitrate) was measured for denitrification. Serum bottles were evacuated to < -88 Kpa and then flushed with 99.9% O_2 free N_2 gas for five minutes to assure anaerobic conditions. Each bottle was incubated at 25^{o}C in an orbital shaker at 100 rpm for 72 hrs and the headspace was again flushed with N_2 gas for 5 minutes. Each bottle then received one of five concentrations (1, 50, 100, 500, 1000 ppb) of CIP, TET or SULF and DI water before being incubated for an additional 72 hours prior to additions of acetylene (15% of headspace) and substrate (glucose: 597 g C kg^{-1} dry soil , potassium nitrate: 73.8 g N kg^{-1} dry soil). Headspace gas samples were collected within the first five hours after substrate addition and then daily for 4-7 days. Gas samples were analyzed using a Shimadzu GC-8A fitted with an electron capture detector operated at 150^{o}C, utilizing a packed Poropak Q (6 ft; 80/100 mesh) column, supplied by Sigma-Aldrich (St. Louis, MO), with an oven temperature of 50^{o}C. All samples were incubated in triplicate, with blanks consisting only of soil, DI water and substrate (if SIR).

Standard curves were run as needed and continuing calibrations (within 5%) were performed prior to each analysis. Standard gas consisting of 10 or 100 ppm and a balance of N_2 was used for the calibration of the N_2O standard curve.

5.2.5. Data Analysis and Statistics

Substrate induced respiration rates were calculated for the initial rate, prior to an exponential increase in gas concentration, and for the maximum (potential) rate. Basal respiration rates were determined to be the maximum rate observed during the incubation. If there was a visible lag with one or more of the treatments, an intermediate rate was also

calculated, and is referred to as the delay rate. The potential rates represent the maximum capacity of the microbes produce each gas. Due to the addition of substrates to create non-limiting conditions, potential rates are significantly higher than what would be expected in normal environmental conditions. All rates are the average of the maximum rates of each respiration phase for each individual sample vessel within that treatment. Outliers were removed from each analysis, and respiration rate differences were determined using a one-way ANOVA. A Latin Square Design (LSD; $p < 0.05$) model was used when equal variance was met, and a Dunnett's T3 test when equal variances were not met. Determination of outliers and ANOVA analysis was performed using SPSS 17 (SPSS Inc., Chicago, IL), while correlations were determined using Excel 2007 (Microsoft Corp., Redmond, Wash). All drug compounds were obtained from Sigma-Aldrich (St. Louis, MO).

5.3. Results

The total carbon content varied between the two soils, with the Davis Pond peat soil containing 232 g C Kg^{-1} and the Bayou Castine containing 60 g C Kg^{-1}. This carbon difference let to significant differences between the respiration rates of blanks (no antibiotic) for each of the soil types. The respiration rates were higher in the peats soils for CH_4 and CO_2 basal respiration (5.8, 2.5 times larger, respectively) and SIR initial (65.4, 5.1 times larger, respectively) than the mineral soil. However, there is no significant difference between the mineral and peat soils when measuring the potential SIR rate for CO_2 or CH_4 and N_2O. Therefore, under non carbon limiting conditions, both soils are potentially capable of reaching similar microbial activities.

5.3.1. Basal Respiration

Carbon dioxide production in wetlands is an indicator of overall microbial activity, while methane production is linked to a specific group of organisms called methanogens. Both gases are a byproduct of microbial breakdown of organic matter. These two gases are produced by

different bacteria that are predominant at either highly reduced (CH_4) or moderately reduced to aerobic (CO_2) conditions in wetland soils. Microbial function in wetlands is essential to organic matter decomposition and nutrient availability.

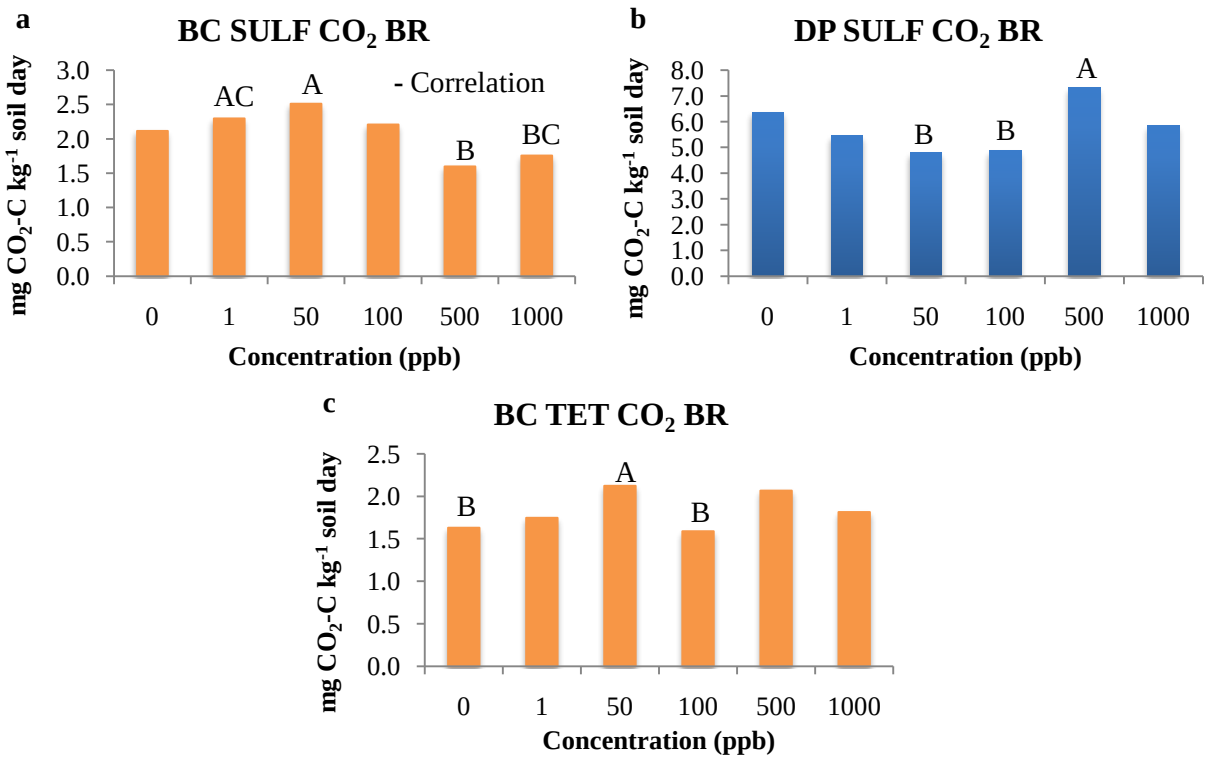


Figure 5.1. Basal respiration rates of carbon dioxide in the peat and mineral soils. Letters represent significant differences in treatment respiration rates. Respiration was negatively correlated with treatment concentrations for 5.1a.

CIP-CO_2: There was no significant correlation for CO_2 respiration rate with concentration or differences between treatment concentrations for the peat and mineral soils.

SULF-CO_2: The mineral soils exhibited lower respiration rates at the higher concentrations (500 < 1, 50; p = 0.036, 0.010 and 1000 < 50; p = 0.026) (Figure 5.1a). There was a significant negative correlation with respiration rate and treatment concentration for the mineral soil under basal respiration for CO_2 (ρ = -0.569). In the peat soil there were lower respiration rates at the low treatment concentrations (500 > 50, 100; p = 0.038, 0.042) (Figure 5.1b).

TET-CO_2: There were no significant relationships in the peat soil. The mineral soils amended with TET produced a significantly higher respiration rate for the 50 ppb treatment compared to 100 ($p = 0.048$), and blank ($p = 0.044$) (Figure 5.1c).

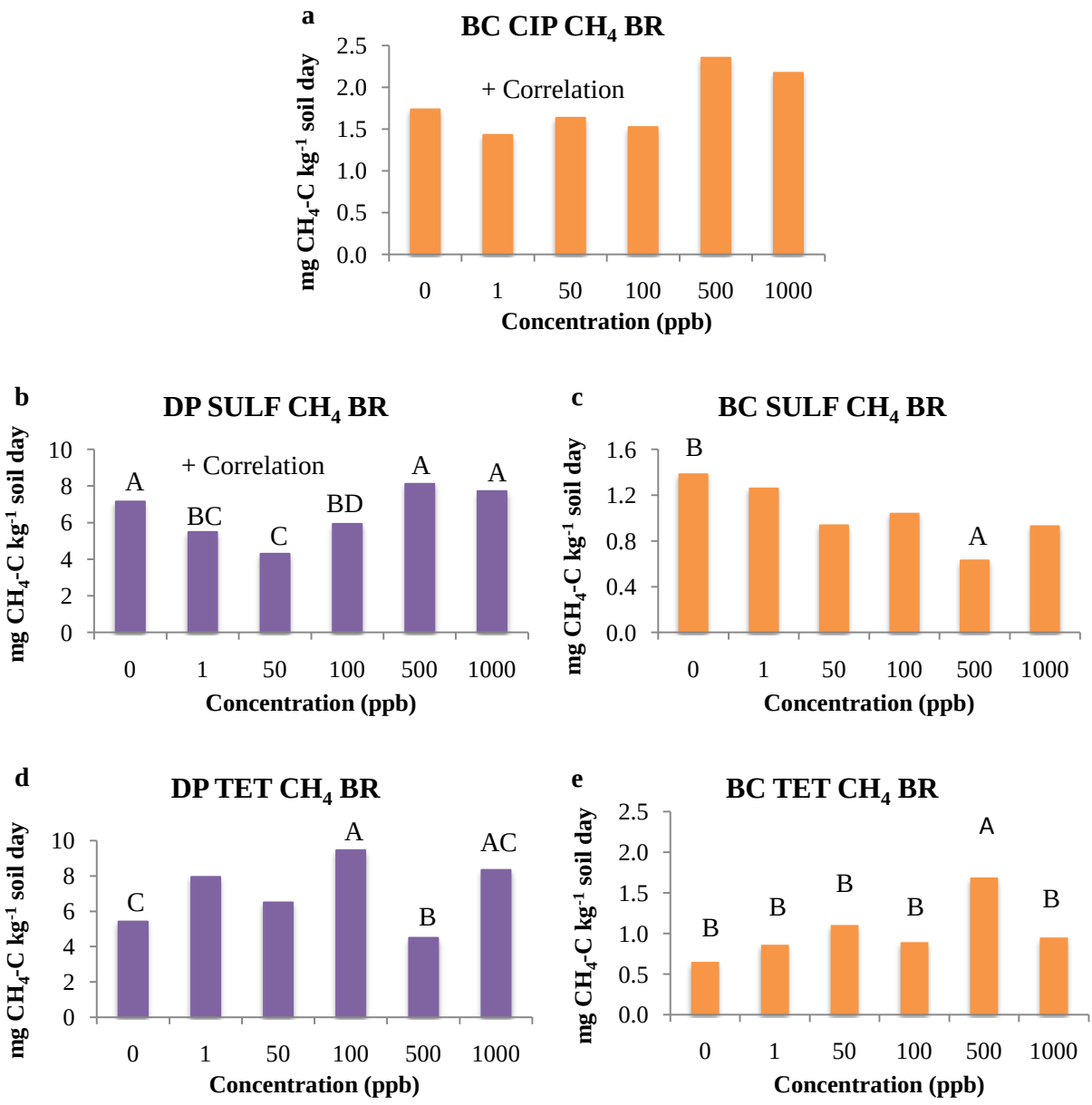


Figure 5.2. Basal respiration rates of methane in both the mineral and peat soils. Letters represent significant differences in treatment respiration rates. Respiration was positively correlated with treatment concentrations for 5.2a and b.

CIP-CH_4: There was only one significant effect between treatments for CH_4 basal respiration in either the peat or mineral soils. Higher CIP treatment concentrations in the mineral soil were significantly correlated with higher CH_4 basal respiration rates ($\rho = 0.489$) (Figure 5.2a).

SULF-CH_4: There was significant basal respiration suppression at lower treatment concentrations in the peat soil, where the blank, 500 and 1000 ppb were significantly higher than 1 ($p = 0.022, 0.003, <0.00$) and 50 ($p = 0.001, <0.00, <0.00$) ppb, and 500 and 1000 were higher than 100 ($p = 0.09, 0.015$ respectively) ppb (Figure 5.2b). Also 100 ppb was significantly higher than 50 ($p = 0.025$) ppb, indicating that there is less of an effect at 100 ppb, even though it is still measurable. There was also a significant positive correlation for basal respiration rates and treatment concentration in the peat soil ($\rho = 0.590$).

The mineral soil CH_4 basal respiration rates exhibited the opposite trend from the peat soil in the presence of SULF, with suppression of respiration at higher concentrations. Respiration in the 500 ppb treatment was significantly lower than respiration in the blank ($p = 0.024$) ppb (Figure 5.2c).

TET-CH_4: There were a couple significant relationships associated with basal respiration in the peat soil for CH_4, where 100 is greater than 500 ($p = 0.023$) ppb and the blank ($p = 0.037$), while the 1000 ppb treatment is also greater than 500 ($p = 0.045$) ppb (Figure 5.2d). In the mineral soil the 500 ppb treatment respiration rate was significantly higher than the other treatments, including the blank (Figure 5.2e).

5.3.2. Substrate Induced Respiration

CIP-CO_2: There were no significant differences between SIR treatments or correlations with concentration for CO_2 in the mineral soil. Only the 50 ppb treatment was significantly greater ($p = 0.016$) than the 100 ppb treatment for the peat soil (Figure 5.3a).

SULF-CO_2: Respiration rates were significantly different between several treatments in the peat soil. The initial CO_2 respiration rates of the blank and 500 ppb were higher than 1 (p = 0.011, 0.010 respectively), 50 (p = 0.044, 0.043 respectively) and 100 (p = 0.031, 0.030 respectively) ppb treatments. This was evidence of respiration supressesion at lower concentrations for the initial rate, since the blank was higher than the 1, 50, and 100 ppb treatments (Figure 5.4a).

Higher concentration treatments for the peat soil CO_2 potential respiration produced significantly higher respiration rates, with 500 ppb being higher than blank (p = 0.004), and 100 (p = 0.045). An increase in respiration rate was significanly correlated with SULF treatment concentration (ρ = 0.4908) (Figure 5.3b).

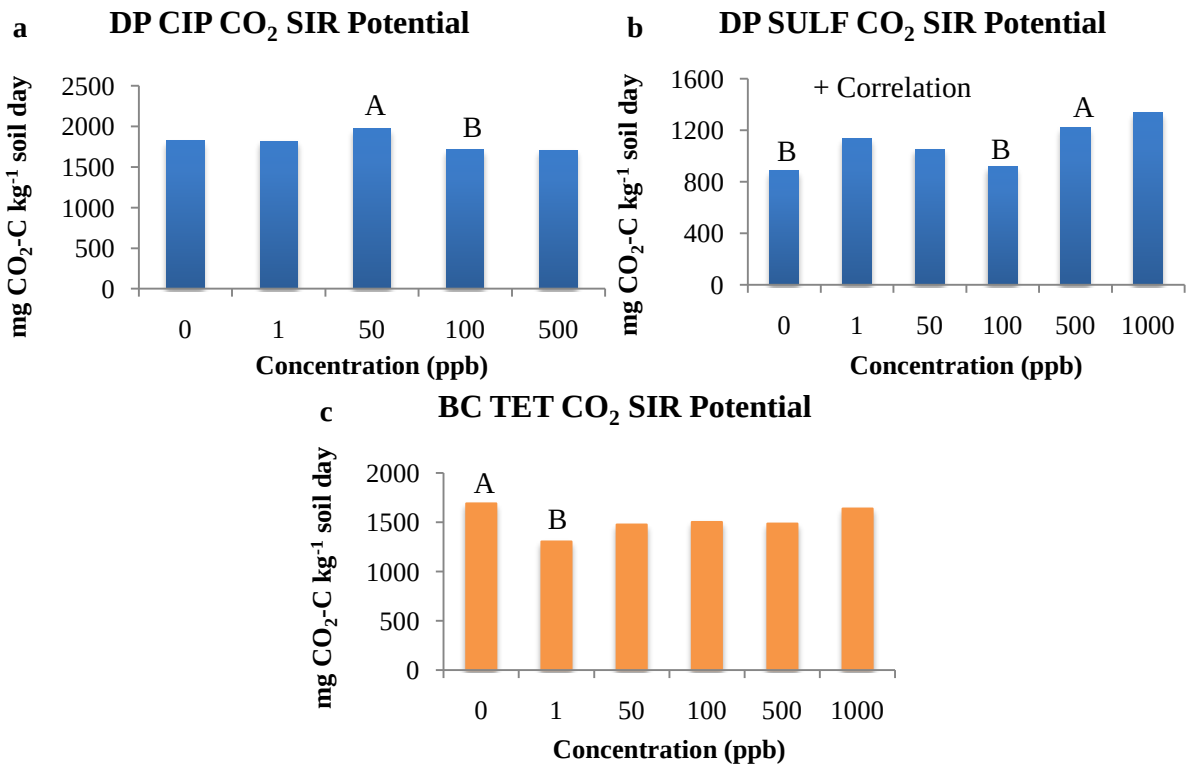


Figure 5.3. Potential substrate induced carbon dioxide respiration rates for the peat and mineral soils. Letters represent significant differences in treatment respiration rates. Respiration was positively correlated with treatment concentrations for 5.3b.

The application of SULF to the mineral soil presented the clearest example of antibiotic suppression of microbial respiration. There was a time lag (Figure 5.5) where CO_2 production in 500 and 1000 ppb treatment lagged behind all other treatments during the first 60 hours of the incubation. However, the total amount of gas evolved in these two treatments eventually reached comparable rates and concentrations to the other treatments. This result suggests that either the microbial community consortia changed or the microbial consortia present adapted to the antibiotics.

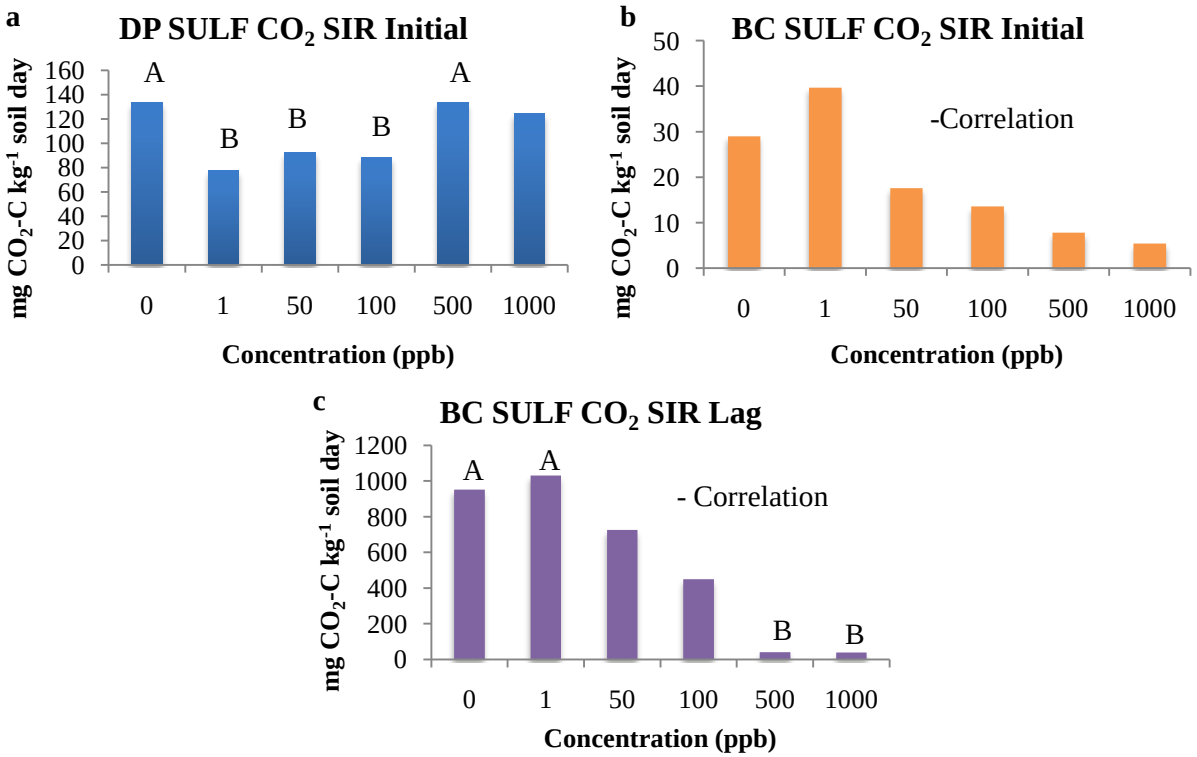


Figure 5.4. Initial substrate induced carbon dioxide respiration rates for the mineral and peat soils for Sulfamethoxazole, along with the lag rates in the mineral soil. Letters represent significant differences in treatment respiration rates. Respiration was negatively correlated with treatment concentrations for 5.4b and c.

There was a significant decrease in respiration rate correlated ($\rho = -0.696$) with treatment concentration and CO_2 respiration rates for the initial SIR rate in the mineral soil (Figure 5.4b). Additional rates were calculated during the lag period on the graph between 20 - 60 hours. As

expected, the lag in respiration rates were negatively correlated with increasing concentration (□ = -0.823) (Figure 5.4c). The blank and 1 ppb concentration were significantly higher than 500 (p = 0.017, 0.027 respectively) and 1000 (p = 0.016, 0.021 respectively) ppb treatments for the delay rates.

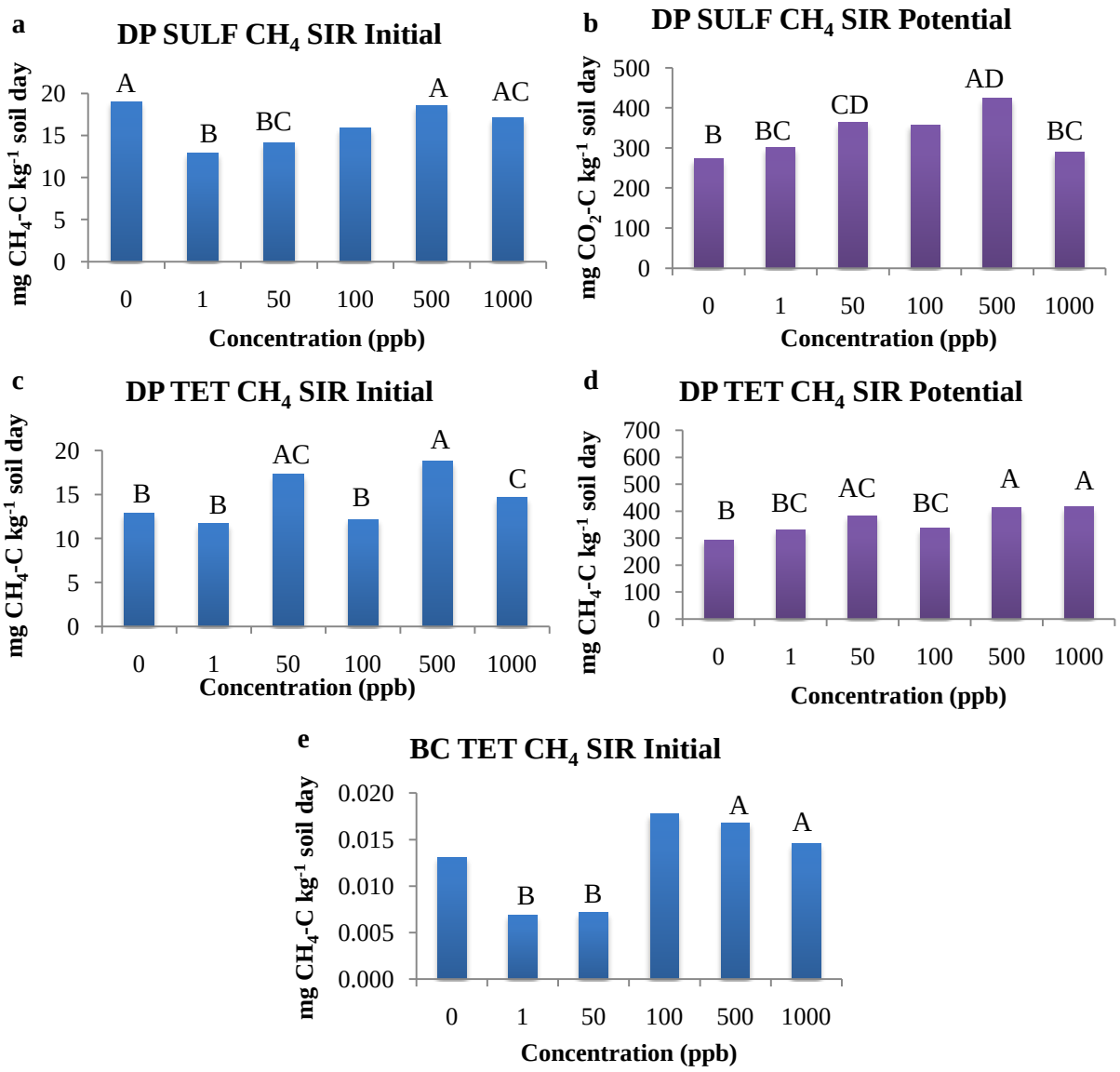


Figure 5.5. Initial and potential substrate induced methane respiration rates for the peat soil. Letters represent significant differences in treatment respiration rates.

TET-CO_2: The only significant relationship observed in the mineral soil was for respiration in the blank, which was significantly higher than respiration in the 1 ppb treatment ($p = 0.036$) (Figure 5.3c).

CIP-CH_4: For CH_4, there were no significant differences between treatments or correlations for the peat and mineral soils.

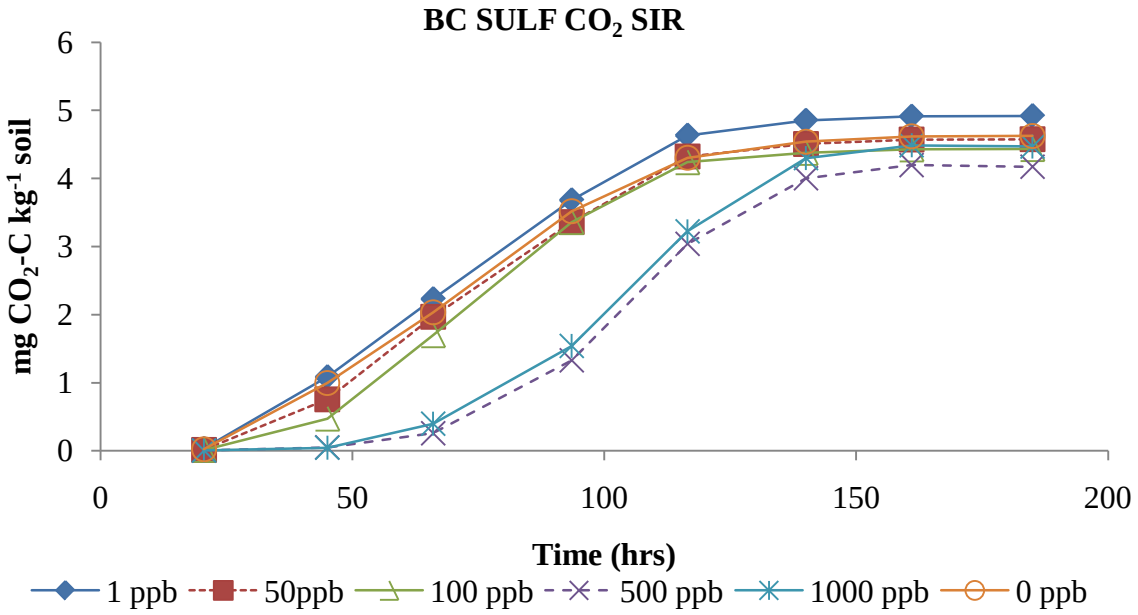


Figure 5.6. Carbon dioxide concentration increase with time for sulfamethoxazole in the mineral soil. A lag in respiration is observed for both the 500 and 1000 ppb treatment levels between 20 and 60 hours.

SULF-CH_4: There were several significantly different respiration rates with the initial SIR for the peat soil (Figure 5.6a). There was suppression at lower concentrations where respiration in the 1 treatment ppb was significantly less than respiration in the blank ($p = 0.005$), 500 ($p = 0.008$) and 1000 ($p = 0.035$) ppb treatments. The respiration for the 50 ppb treatment was also significantly less than the blank ($p = 0.016$) and 500 ($p = 0.026$) ppb treatments. The data for potential methane SIR rate in the peat soil showed that methane production in the 500 ppb treatment was significantly higher than production in the blank ($p = 0.002$), 1 ($p = 0.009$)

and 1000 ($p = 0.005$) ppb treatments. Respiration in the 50 ppb treatment was also significantly higher than respiration in the blank ($p = 0.041$) (Figure 5.6b). There were no significant differences between treatments or correlations for the mineral soil associated with substrate induced methane respiration

TET-CH_4: The initial SIR methane respiration rate for the peat soil produced inconclusive results, with 50 ppb treatment respiration rates being significantly higher than respiration in 1 ($p = 0.008$), 100 ($p = 0.012$) ppb treatments and the blank ($p = 0.026$), while respiration in the 500 ppb treatment was also higher than respiration in the 1 ($p = 0.002$), 100 ($p = 0.003$), 1000 ($p = 0.035$) ppb and the blank ($p = 0.005$) (Figure 5.6c). The peat potential SIR rates were significantly higher treatments for the 1000 and 500 ppb treatments compared to the blank ($p = 0.003, 0.002$), 1 ($p = 0.027, 0.022$) and 100 ($p = 0.034, 0.028$) ppb treatments (Figure 5.6d) The 50 ppb treatment was also higher than the blank ($p = 0.016$). There was also a significant positive correlation with increased concentration ($\rho = 0.635$).

The only significant substrate induced relationship observed for methane in the mineral soil was for the initial rate, where the rates in the 1,000 and 500 ppb treatments were significantly higher than the respiration rates in the 1 ($p = 0.026, 0.024$) and 50 ($p = 0.030, 0.027$) ppb treatments (Figure 5.6f).

5.3.3. Denitrification

CIP-Denitrification: The initial denitrification rate for the peat soil revealed that at lower concentrations there was a significant increase in N_2O rates compared to rates in the blank and in treatments at higher concentrations (Figure 5.7a). Denitrification in the 1 ppb treatment was significantly higher than rates in the 50 ($p = 0.012$), 500 ($p = 0.002$) and 1000 ($p = 0.008$) ppb treatments and the blank ($p = 0.050$), while denitrification at 50 ppb was higher than the rates in the 500 ($p = 0.019$) and 1000 ($p = 0.010$) ppb treatments and the rates in the 100 ppb treatment

was higher than the rates for the blank (p = 0.027). There were no significant relationships between treatments for the potential rate in the peat soil.

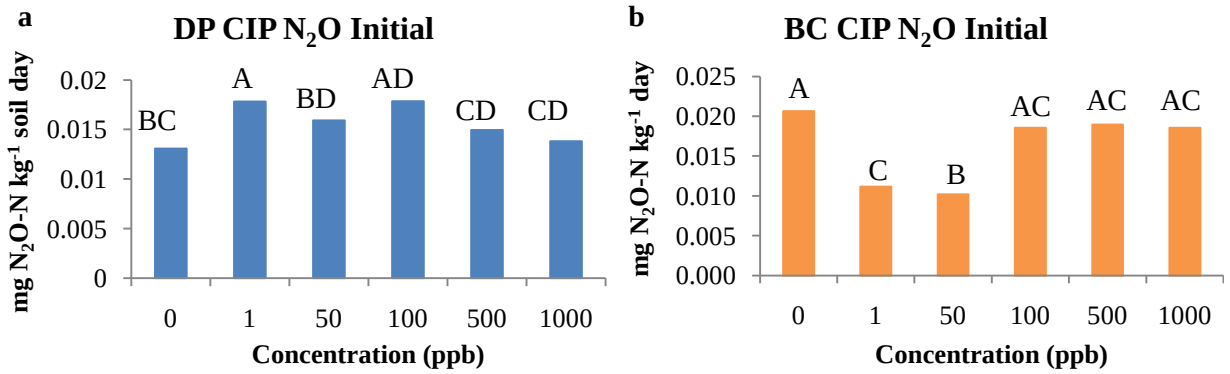


Figure 5.7. Potential and initial denitrification rates for CIP in both the mineral and peat soils. Letters represent significant differences in treatment respiration rates.

There was a significant difference for the initial denitrification rate for mineral soil, where the rate in the 50 ppb treatment was significantly lower than the rate in the 100 (p = 0.045), 500 (p = 0.037), 1000 (p = 0.045) ppb treatments and the blank (p = 0.016) (Figure 5.7b). The rates for the 1 ppb treatment was also significantly lower than the rates in the blank (p = 0.025). There were no significant differences between treatments for the mineral soil with the denitrification potential rates.

SULF-Denitrification: There were no significant differences in denitrification rates between treatments or correlations observed in experiments with the peat soil. Three denitrification rates (initial, delay, potential) were calculated for mineral soil due to an apparent lag in respiration by the two highest concentration treatments (Figure 5.8a,b). There were no significant differences or correlations for the initial respiration rates. The lag and potential respiration rates both showed a significant negative correlation with increased concentration (ρ = -0.626, -0.846). However, only one significant relationship was observed, where 1000 ppb treatment respiration rate was significantly lower than respiration rate for the 1 (p = 0.042) ppb treatment and the blank (p = >0.000) for the potential rate.

TET-Denitrification: No significant relationships for denitrification rates were observed in the peat soil. Only one significant difference was observed for denitrification rates between treatments in the mineral soil, where the respiration rate for the 1 ppb treatment was significantly higher than the respiration rates of the blank ($p = 0.023$) and 50 ($p = 0.0$) and 1000 ($p = 0.035$) ppb treatments for the initial rate (Figure 5.8c). Any increase in respiration due to 1 ppb of TET was overcome when measuring the potential respiration rates, where there were no significant relationships between treatments.

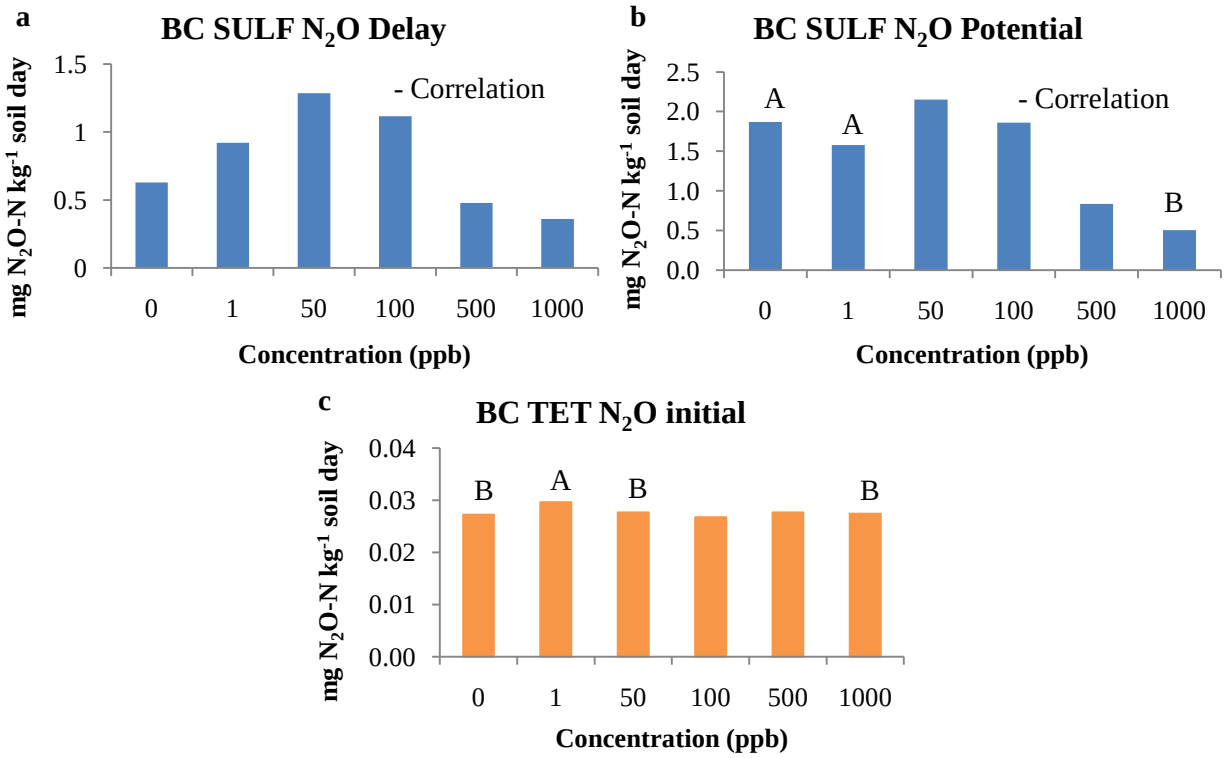


Figure 5.8. Delay and potential denitrification rates for sulfamethoxazole and the initial denitrification rates for tetracycline. Letters represent significant differences in treatment respiration rates. Respiration was negatively correlated with treatment concentrations for 5.8a and b.

5.4. Discussion

Antibiotics are used to either kill bacteria or prevent their proliferation. Consequently, it was expected that the exposures to antibiotics in high (500, 1000 ppb) concentrations would stop, decrease or delay microbial respiration. No treatments resulted in complete cessation of

respiration and only in the mineral soil exposed to SULF, did we observe a decrease in respiration and a delay, which was relatively short-lived. The remaining samples produced mixed results, with some treatments increasing respiration rates, while others only decreasing respiration with the low concentration treatments.

The respiration suppression due to SULF was observed for basal rate of CH_4 and the initial and delayed SIR rate for CO_2. The suppression of CO_2 rates for the initial and delay were short lived (~40 hours). The CO_2 rates for SIR potential recovered over the span of a couple days to exceed the mean potential rates for the other treatments, while also reaching similar total respiration concentrations of CO_2. Therefore, there was equal activity after the microbial community recovered from the initial exposure to SULF when in the presence of a substrate. In the mineral soil, the short term effects of SULF were overcome in a non-carbon limiting environment. We offer two possible explanations for the rapid recovery are: (1) there was a shift in the composition of the microbial community at the higher drug concentration treatments, or (2) the higher concentrations drug created a new readily available carbon pool of dead microbes, which fed the remaining microbes sparking a surge in respiration activity. Soil contains a wide range of consortia of microbial organisms. Antibiotics could only affect certain microbes in this consortia, which may have lead to the mixed results we observed.

Several treatments revealed that there was suppression of respiration at lower concentration (1, 50 and sometimes 100 ppb) in relation to the blank, 500 and 1000 ppb treatments. This was observed for three instances with SULF and once with CIP. All instances occurred during basal respiration or with the initial rate for substrate induced respiration. The relationship was observed for CH_4 and CO_2 respiration with SULF treatments and for denitrification with CIP treatments. These results demonstrate that the concentrations, which are "environmentally relevant", have the potential to affect the microbial population. The reason for

impacts at lower concentration and not higher concentrations is not fully understood. However, as previously mentioned, there may have been a shift in the microbial community composition at high concentrations to make up for the impacts of the antibiotics. There may be some antibiotic influence on the microbial community at higher concentrations, but it was not observed with regards to the respiration rates. Experiments examining the microbial community composition are needed to understand the effects observed at higher treatment concentrations.

Respiration increased at the lower concentration treatments in the peat soil with CIP for the initial denitrification rate. The increase in respiration was reversed with the potential rate, where the mean rates of respiration in the 1 and 50 ppb treatments were suppressed compared to respiration in the blank and high concentrations after 140 hours (Figure 5.9).

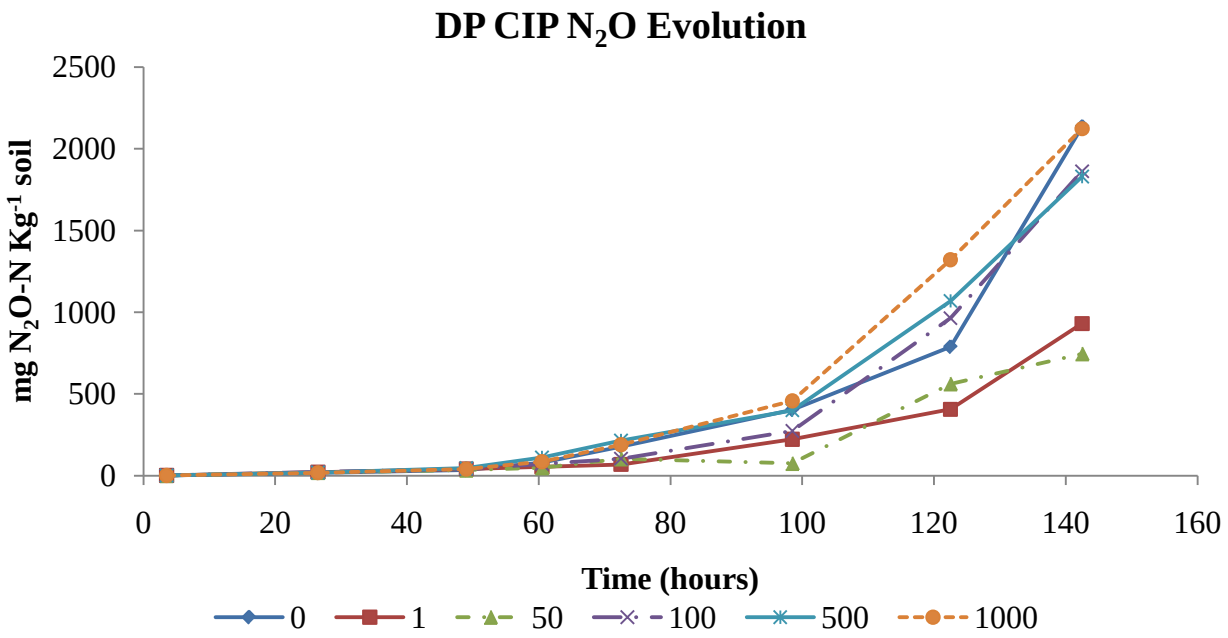


Figure 5.9. N_2O increase with time for CIP in the peat soil. A decrease in respiration is observed for both the 1 and 50 ppb treatment levels between 80 hours and the end of the incubation at 140 hours.

Increased respiration rates for CIP basal methane respiration were significantly correlated with higher gas evolution in the mineral soil. The incubation period for these samples was 2.5 months, in which time the tubes became highly reduced. This example demonstrates that in the

mineral soils under highly reducing conditions, the antibiotics may lose some of their antibiotic properties and act as a substrate for the initial background microbe community in this soil. A previous study found that CIP increased microbial biomass when tested under anaerobic conditions (Cordova-Kreylos and Scow, 2007). This result was explained as a decrease or complete loss of antibiotic activity in an anaerobic environment (Lewin et al., 1991; Zabinski et al., 1995; Cordova-Kreylos and Scow, 2007) or even bacterial resistance. Bacteria have also been found to subsist on antibiotics (Dantas et al., 2008), but this has not been studied in wetland or submerged soils.

The potential CO_2 respiration rate exhibited a positive increase with concentration for the peat soil. The positive correlation occurred after suppression at the low antibiotic concentration in the peat for the initial rate measurements. In the mineral soil, there were negative rate correlations with antibiotic concentrations for both the initial and delay respiration rates. The decreasing rate trend was overcome for the potential rates, and the high concentrations exhibited slightly higher rates than the lower concentration treatments. This may be due to some selective pressure exerted on the microbial community by SULF. The positive correlation in the mineral soil appeared to be a result of a rebound effect after the high concentration treatments respiration was suppressed and delayed compared to the blank and low concentration amendments.

All of the negatively correlated respiration rates with drug concentration occurred with the SULF treatments. They were seen in the mineral soil CO_2 basal, initial and delay SIR, and the mineral N_2O delay and potential SIR. With the exception of the N_2O potential rate, all other negative correlations occur prior to respiration rates peaking due to the substrate addition. The mineral N_2O incubation was ended after only 96 hours. Therefore, it is not known if the rate or total concentration respired would have reached that of the other treatments for SIR CO_2 in the mineral soil

Cordova-Kreylos and Scow (2007) determined that redox potential may play a role in the function of antibiotic compounds in the environment. The antibiotic properties of antibiotic compounds may be reduced or negated under highly reducing conditons (Cordova-Kreylos and Scow, 2007). This may be what is occuring for basal methane respiration at high CIP (mineral) and TET (mineral and peat) concentrations. Under highly reducing (CH_4) and basal conditions the respiration rates were positivley correlated with treatment concentration or the high concentration treatments were significantly higher than the blanks for CIP and TET, indicating that the antibiotic may act as carbon source (Cordova-Kreylos and Scow, 2007). Under less reducing (N_2O) conditions, there appears to be a mixed effect on microbes, where the lower concentrations affected respiration the most. The background (initial rate) microbe population in the peat soils produced more N_2O when low concentrations were introduced, but as the incubation continued and the respiration rates increased the lower concentrations respired less than the higher concentration treatments. In addition, soil respiration under nitrate reducing conditions (denitrification) is mediated by a wide range of microbial groups (Salles et al., 2009) and antibiotics may only affect a small portion of these organisms, which explains the mixed results.

Sorption of antibiotic compounds to soil (organic or mineral fraction) can be a pathway for PhAC removal from the water column (Hildebrand et al., 2006; Pan et al., 2009; Conkle et al., IN REVIEW). Cordova-Kreylos and Scow (2007) found that the magnitude of the effect of CIP on microbial communities was inversely related to compound sorption to the soil. This resulted in a reduced bioavailability and antibiotic potency of the compound (Cordova-Kreylos and Scow, 2007). CIP sorption to the mineral soil from Bayou Castine produced a Log K_F value of 4.01 (Conkle et al., IN REVIEW). Log K_F values from the literature for TET and SULF range between 1.66 – 3.63 and 0.04 – 1.27, respectively (Gu et al., 2007; Jia et al., 2008; Yu et al.,

2009). Based on published values K_F values, the general sorption potential follows this order: CIP > TET > SULF (Gu et al., 2007; Jia et al., 2008; Yu et al., 2009; Conkle et al., IN REVIEW). Reduction of antibiotic activity due to sorption explains why SULF exerts more of a negative influence on the soil microbes.

TET and SULF are broad spectrum antibiotics which target a wide range of bacteria, while CIP is designed to target specific bacteria (Halling-Sørensen, 2001). Since CIP only targets a specific suite of bacteria, it could cause a shift in the microbial community dominance or alter their activity (Halling-Sørensen, 2001). TET is known to easily degrade in the environment due to light, pH and chelating metals (Halling-Sørensen et al., 2002). This coupled with sorption may explain why there are less pronounced effects on respiration rates than what is found with CIP and SULF.

5.5. Conclusions

Overall, SULF exhibited the greatest and TET the least influence on all microbial respiration parameters. Samples amended with TET demonstrated mixed interaction with regard to methane production in both soils. No effects of TET addition were seen with regards to CO_2 or N_2O production. The only significant relationships observed for CIP were seen in the mineral soil, where respiration was suppressed at low antibiotic concentrations during the initial phase and basal respiration for CH_4 and N_2O. A non-significant trend was also observed with the potential respiration rate in the peat soil for N_2O production, where the low concentrations were suppressed compared to the high antibiotic concentrations and the blank.

In general SULF impacted both soils and the production of all three gasses (CH_4, CO_2 and N_2O). Suppression at high concentrations was observed in the mineral soils for the basal and initial rates. The suppression was overcome for both the CH_4 and CO_2 respiration rates when determining the potential rates, but N_2O showed signs of suppression. The opposite trend was

observed in the peat soil, where there was suppression at lower concentrations for both CO_2 and CH_4. Any initial suppression of gas production rates were overcome when measuring the potential rate.

The general effect of each compound on microbial respiration appears to be tied to the sorption potential of each compound and the compounds overall stability. The general sorption potential is CIP > TET > SULF. TET is also known to be very unstable in the environment. Therefore, SULF is relatively more stable than TET and has a lower sorption potential, which may have resulted in the greater impact observed on microbial respiration.

At environmentally relevant concentrations, antibiotics, specifically sulfamethoxazole, may negatively impact microbial respiration in these wetland soils. Release of CH_4, CO_2 and N_2O is a byproduct of microbial respiration/activity in wetland soils, and a decrease in respiration indicates that the microbial pool is less active. This finding has significant implications for wetland systems used to improve water quality, a common practice around the world. The efficiency of wetlands to treat and polish wastewater may be diminished due to the presence of antibiotics.

Microbes are essential to the overall function of wetlands, through the transformation of nutrient to bioavailable forms. The bioavailable forms of nutrients are essential to wetland plants and higher trophic levels. A reduction in bioavailable nutrients due to antibiotic impacts on the microbial pool may decrease the overall productivity of the wetland. In order to further understand the antibiotics effects on wetland soil microbial processes, studies addressing microbial community structure need to be undertaken.

CHAPTER 6: CONCLUSIONS

The environmental health of coastal Louisiana is directly tied to the maintenance of wetlands and "good" water quality. Wastewater containing excess nutrients and pharmaceutically active compounds is routinely discharged into surface waters, and has been shown to impair ecosystem health. Certain pharmaceutical compounds have been shown to negatively affect some fish species in rivers and streams (Chambers and Leiker, 2006). In a several Louisiana towns, wetlands are used to "polish" wastewater by removing excess nutrients prior to release into surface waters downstream. These treatment plants are not designed (nor are they required) to remove pharmaceuticals during the treatment process. The interactions and fate of many pharmaceutical compounds in the environment remains unknown. Research into these processes must be conducted prior to implementing any policies requiring pharmaceutical removal during wastewater treatment. We investigated whether wetlands are effective at removing certain pharmaceutical compounds.

This research is among the first studies to examine the fate, transport and microbial effects of PhAC compounds in wetlands. Studies were conducted that focused on identifying compounds entering wetlands and surface waters in Southeast Louisiana and their removal by wetland processes, understanding the fate of these compounds through sorption and desorption and examining the effects of antibiotics on wetland soil microbial respiration (CO_2, CH_4 and N_2 production).

Research on compound loading from the Mandeville wastewater treatment plant, found that 13 of 15 target compounds were detected within the plant. Treated wastewater leaving the treatment plant contained only 9 of the 13 detected compounds above detection limits (low parts per-trillion). The concentrations of many analytes were reduced by greater than 90% within the

wastewater treatment plant (WWTP). Several pharmaceuticals were not completely removed before discharge into Lake Pontchartrain, although their collective concentration was reduced by 96% and lake loading was reduced to less than 1 kg yr^{-1}. The Mandeville WWTP and treatment wetland effectively reduced concentrations of various pharmaceuticals by several kilograms annually, decreasing potential contamination of Lake Pontchartrain and its fragile fisheries.

In order to investigate sorption and desorption of antibiotics to a wetland soil, analytical methods utilizing direct analysis of aqueous samples (i.e. without extraction) and short retention times that allow for analysis of 100s to 1000s of samples in a reasonable time frame were required. Previously published methods were time consuming and not feasible for the proposed experiment. Two HPLC methods were developed for individual and simultaneous determination of ciprofloxacin, norfloxacin and ofloxacin. Chromatography of individual compounds produced retention times between 1.5 and 2 minutes, and for simultaneous detection, retention times between 6.5 to 8 min. These methods are compatible with complex geomatrices, such as wetland soil. The methods provide 1) detection limits in the low parts per-billion range, 2) decreases in retention times up to 6x times for single compounds, and up to 2x for simultaneous detection over published methods, and 3) requires no solid phase extraction.

Sorption and desorption of three antibiotic compounds (ciprofloxacin, norfloxacin and ofloxacin) was determined on a mineral wetland soil from Bayou Castine, the control wetland for the Mandeville WWTP. Roughly 50% each compound sorbed within the first 20 hours of incubation and pseudo-equilibrium (60-90% sorption) was reached between 2 and 3 days. Only 5-12% of the compound that sorbed was released from the soil over a 3 day period, indicating high loading onto the soil and low release. Therefore, sorption to soil can be a major removal pathway for these compounds from the water column. We also examined competition between individual compounds for sorption sites. When multiple compounds were introduced to the soil,

the sorption potential of each compound decreased. Sorption and desorption results (single component and mixture) provided the following conclusions: 1) overall NOR and, to a lesser extent, CIP outcompete OFL for sorption sites, 2) OFL sorbes to its share of prefered sorption sites, and 3) competition only occurs for lesser "quality" binding sites. Multiple compounds in surface waters could lead to a decrease in sorption of certain compounds and a greater migration downstream. Therefore, future work that examines sorption of PhACs in wastewater need to investigate sorption in the presence of multiple compounds, since we have shown that there in competition between pharmaceuticals for sorption sites within this soil.

The impact of antibiotics on soil microbial respiration was investigated in two wetland soils of contrasting carbon content. Tetracycline exhibited the least influence on microbial respiration, with only mixed effects on CH_4 respiration in both the peat and mineral soil. There were, however, no noticeable trends or correlations between the respiration rates observed for TET and the concentration added to the soil. Ciprofloxacin only affected the respiration rates in the mineral soil, where at low antibiotic concentrations there was respiration suppression during the initial and basal rates for CH_4 and N_2O.

Sulfamethoxazole impacted both soils and the respiration rates of all three analytes (CH_4, CO_2 and N_2O). Suppression at high concentrations was observed in the mineral soil for the basal and initial rates. The suppression was overcome for both the CH_4 and CO_2 respiration rates when determining the potential rates, but N_2O respiration potential still showed signs of suppression throughout the incubation period. The opposite trend was observed in the peat soil, where there was suppression at lower concentrations for both CO_2 and CH_4. This initial suppression of gas production rates was overcome when measuring the potential rate.

Antibiotics, specifically sulfamethoxazole, may negatively impact microbial respiration in these wetland soils. A reduction in respiration would indicate that the antibiotic is altering the

vital normal function of microbially mediated nutrient transformation in the wetland soil. This finding has significant implications when using wetlands to treat and polish wastewater, which is known to contain multiple pharmaceutical compounds including antibiotics. The efficiency of wetlands to treat and polish wastewater may be diminished due to the presence of antibiotics. In order to further understand the antibiotics effects on wetland soil microbial processes, studies must be conducted in the field and address the microbial community structure.

Our original hypotheses was that natural wetland treatment systems will remove pharmaceuticals from domestic wastewater in amounts that are equal to or greater than conventional wastewater treatment systems, and the major mechanism for removal (for most compounds) is sorption to particulates and sediments. The presence of multiple antibiotic compounds in a wetland will also result in competition for preferred binding sites between the compounds studied. Antibiotics will reduce the microbial activity in treatment wetlands. However, sorption may decrease the impact of antibiotics on wetland soil microbial communities. In addition, wetland soils that have previously sorbed pharmaceuticals may act as a source of pharmaceuticals if loading concentrations are decreased or stopped.

We found that these natural systems are effective at treating and removing PhACs from wastewater and that sorption can be a major removal pathway for particular compounds. However, when multiple compounds are present, which is normally the case in wastewater, there can be a decrease in the compound sorption potential due to competition between compounds for binding sites. Sorption to the soil appears to be strong enough to prevent significant desorption in the short-term (several days). Although, there is some minor desorption (4 to 12% at 20 to 80 ppm loading respectively) when the concentration gradient was reversed. We found that antibiotics, at environmentally relevant concentration do possess the ability to affect the microbial communities in soils.

The conclusions of this research have answered several questions but also shed light on areas that require more attention. Future research that examines sorption and desorption in the context of a treatment wetland or wastewater in general should focus in greater detail on the effects of competition between the compounds for sorption sites. Studies that detect PhACs in the environment rarely find a single compound. Therefore, to more accurately understand removal mechanisms, sorption competition must be investigated. The microbial activity was determined based on gas evolution. However, an analysis of microbial communities, using molecular techniques also needs to be studied to shed more light on the results presented herein. Microbial activity was studied in a lab scale setting, which allows for determination of potential environmental effect. This study could be scaled up to the field level to determine impacts on microbial activity at a larger scale. Using enclosed soil cores with a flow-through system would be one way to incorporate both sorption and respiration into an experiment to gain a broader, collective understanding of antibiotic behavior in the wetland environment.

The unique landscape of Louisiana allows small municipalities to use the natural environment to treat and "polish" wastewater using lagoons and wetlands. Most cities and towns discharge treated wastewater directly into surface waters such as the Great Lakes, Mississippi River or Atlantic Ocean. This research shows that using wetlands to "polish" waste water, rather than directly discharging treated effluent into surface waters, significantly reduces or prevents pharmaceutical migration downstream by reducing pharmaceutical compound concentrations in the water column. Antibiotics were found to exert a negative influence on microbial respiration, but the longer-term effects require further investigation. Overall, wetlands appear to be a useful tool in decreasing or preventing the release of potentially harmful pharmaceutical compounds into surface and coastal waters.

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APPENDIX A: PERMISSION REQUEST TO RE-PRINT

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APPENDIX C: SORPTION AND DESORPTION DATA

Table A-C.1. Average data for the sorption and desorption of CIP (a), NOR (b), OFL (c) and competition (d).

a)

Ciprofloxacin									
	Sorption					Desorption			
Initial Conc. (ppm)	C_s^{ads} (eq) μg g^{-1}	C_{aq}^{ads} (eq) μg cm^{-3}	$K_{D,\ ads}$ (cm^3 g^{-1})	% in Solution	% on Soil	C_s^{des} (eq) μg g^{-1}	C_{aq}^{des} (eq) μg cm^{-3}	$K_{D,\ des}$ (cm^3 g^{-1})	% Desorbed
20.20	10455.34	2.18	4844.02	10.80	89.20	10060.31	0.72	5431.06	3.76
31.23	14703.83	4.61	3587.42	14.77	85.23	14012.77	1.36	4023.83	4.77
40.40	18484.48	7.89	2797.78	19.53	80.47	17737.01	2.21	3116.54	6.41
50.50	22341.69	12.32	1834.63	24.39	75.61	21140.64	2.69	4070.81	5.37
60.60	23593.78	18.82	1337.56	31.05	68.95	21978.00	3.56	2787.91	6.90
80.80	28189.97	30.43	934.41	37.66	62.34	26464.54	4.35	3223.96	6.09

b)

Norfloxacin									
Sorption						Desorption			
Initial Conc. (ppm)	C_s^{ads} (eq) $\mu g\ g^{-1}$	C_{aq}^{ads} (eq) $\mu g\ cm^{-3}$	$K_{D,\ ads}$ ($cm^3\ g^{-1}$)	% in Solution	% on Soil	C_s^{des} (eq) $\mu g\ g^{-1}$	C_{aq}^{des} (eq) $\mu g\ cm^{-3}$	$K_{D,\ des}$ ($cm^3\ g^{-1}$)	% Desorbed
20.64	10092.50	1.81	5790.66	9.04	90.96	9887.01	0.46	10004.69	2.04
29.97	15788.16	2.55	6233.86	8.51	91.49	15327.13	0.95	6663.62	3.12
39.96	20427.70	3.99	5382.29	9.98	90.02	19604.16	1.30	6281.23	3.28
49.95	25240.80	5.29	4984.17	10.58	89.42	24299.44	1.86	5407.29	3.73
59.94	28133.33	9.81	3073.48	16.36	83.64	27111.10	2.25	5732.66	3.61
79.92	34753.61	18.61	1942.41	23.28	76.72	32883.34	4.09	4016.51	5.31

c)

Ofloxacin									
Sorption						Desorption			
Initial Conc. (ppm)	C_s^{ads} (eq) $\mu g\ g^{-1}$	C_{aq}^{ads} (eq) $\mu g\ cm^{-3}$	$K_{D,\ ads}$ ($cm^3\ g^{-1}$)	% in Solution	% on Soil	C_s^{des} (eq) $\mu g\ g^{-1}$	C_{aq}^{des} (eq) $\mu g\ cm^{-3}$	$K_{D,\ des}$ ($cm^3\ g^{-1}$)	% Desorbed
20.20	10070.64	2.36	4324.84	11.66	88.34	9660.37	0.81	5042.36	4.07
30.30	14488.53	4.53	3541.12	14.96	85.04	13638.55	1.61	3376.11	5.86
40.40	16491.32	11.00	1575.61	27.23	72.77	15105.77	2.36	2518.52	7.61
50.50	22408.69	9.75	2386.90	19.30	80.70	20801.25	3.16	2569.32	7.19
60.60	24042.74	17.86	1471.08	29.47	70.53	21847.30	4.72	1796.23	10.36
80.80	33685.20	22.54	1495.70	27.90	72.10	29695.76	7.70	1573.45	11.83

(table cont.)

d)

Competition									
Sorption						Desorption			
Initial Conc. (ppm)	C_s^{ads} (eq) $\mu g\ g^{-1}$	C_{aq}^{ads} (eq) $\mu g\ cm^{-3}$	$k_{d,\ ads}$ ($cm^3\ g^{-1}$)	% in Solution	% on Soil	C_s^{des} (eq) $\mu g\ g^{-1}$	C_{aq}^{des} (eq) $\mu g\ cm^{-3}$	$k_{d,\ des}$ ($cm^3\ g^{-1}$)	% Desorbed
20.20	9971.40	2.75	3768.27	13.61	86.39	3275.97	0.95	3681.71	5.21
20.64	10372.69	2.49	4336.11	12.05	87.95	3479.99	0.64	5939.13	3.30
20.20	9190.23	4.12	2304.19	20.38	79.62	3073.90	0.59	6157.42	3.18

(table cont.)

APPENDIX D: MICROBIAL RESPIRATION AVERAGE RAW DATA

Table A-D.1. Average concentrations of BR methane evolved at each time interval for CIP (a), TET (b) and SULF (c) in the mineral soil

a)

Tmt (ppb)	BC CIP CH_4 BR				
	Time (hours)				
	529.5	864.5	1178	1532.5	1822.5
	mg CH_4-C Kg^{-1} dry soil Day				
1	0.003	0.023	0.036	0.038	0.036
50	0.004	0.026	0.039	0.041	0.040
100	0.003	0.024	0.037	0.039	0.037
500	0.012	0.045	0.055	0.058	0.051
1000	0.011	0.041	0.061	0.060	0.051
BLK	0.016	0.040	0.054	0.055	0.050

b)

Tmt (ppb)	BC TET CH_4 BR				
	Time (hours)				
	529	864	1174.5	1534.5	1851
	mg CH_4-C Kg^{-1} dry soil Day				
1	0.001	0.011	0.024	0.027	0.025
50	0.001	0.016	0.031	0.031	0.031
100	0.009	0.033	0.048	0.045	0.040
500	0.005	0.032	0.051	0.050	0.049
1000	0.001	0.014	0.027	0.030	0.029
BLK	0.001	0.008	0.018	0.023	0.022

c)

Tmt (ppb)	BC SULF CH_4 BR				
	Time (hours)				
	527.5	863	1171.5	1531.5	1846
	mg CH_4-C Kg^{-1} dry soil Day				
1	0.002	0.018	0.029	0.030	0.025
50	0.001	0.011	0.023	0.025	0.027
100	0.001	0.013	0.022	0.034	0.035
500	0.001	0.009	0.017	0.018	0.016
1000	0.001	0.012	0.019	0.018	0.016
BLK	0.001	0.015	0.018	0.030	0.029

(table cont.)

Table A-D.2. Average concentrations of SIR methane evolved at each time interval for CIP (a), TET (b) and SULF (c) in the mineral soil

a)

Tmt (ppb)	BC CIP CH_4 SIR																				
	Time (hours)																				
	240	313.5	409.5	527.5	621	742.4	870.5	983.5	1087	1176	1272	1370	1463	1582	1680.5	1777.5	1917.5	2038.5	2185	2353.5	2502.5	2641
	mg CH_4-C Kg^{-1} dry soil Day																				
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.004	0.008	0.031	0.090	0.244	0.871	1.761	4.322	5.088	5.285	5.575
50	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.002	0.004	0.008	0.017	0.054	0.128	0.326	1.164	2.616	7.409	7.955	8.079	8.107
100	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.003	0.008	0.042	0.248	0.727	1.235	1.373	1.987	3.530	3.843	4.274	4.821	5.201	5.506
500	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.003	0.006	0.012	0.043	0.279	1.257	2.163	2.977	4.450	4.936	5.959	6.423	6.207	6.242
1000	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.002	0.002	0.006	0.026	0.193	0.947	3.111	3.980	4.285	4.901	5.377	7.000	7.104	6.997	6.993
BLK	0.000	0.000	0.000	0.000	0.000	0.001	0.002	0.002	0.004	0.007	0.017	0.035	0.087	0.405	0.900	1.793	3.253	4.471	7.060	7.293	7.363	7.315

b)

Tmt (ppb)	BC TET CH_4 SIR																				
	Time (hours)																				
	240	313.5	408.5	527	621.5	743	870.5	983	1086	1174.5	1271.5	1369	1462.5	1581.5	1679	1777	1918.5	2039	2186	2353	2497	2637
	mg CH_4-C Kg^{-1} dry soil Day																				
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.002	0.009	0.081	0.195	1.190	1.936	2.507	3.079	3.993	4.746	6.669	6.744	6.774	6.780
50	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.002	0.008	0.078	0.676	1.586	1.913	2.084	2.552	2.852	2.583	1.012	1.050	1.103	1.281
100	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.009	0.012	0.022	0.042	0.084	0.209	0.604	1.696	2.463	3.088	3.417	4.556	4.875	4.990	5.129
500	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.003	0.008	0.024	0.102	0.467	1.610	3.138	3.054	3.381	4.710	4.808	4.842	4.836	4.821
1000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.003	0.011	0.110	0.750	1.737	3.027	4.410	5.130	5.500	5.724	5.682	5.620	5.542	5.478
BLK	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.002	0.005	0.013	0.024	0.078	0.263	0.704	1.382	2.039	3.462	3.776	3.923	3.862	3.803

c)

BC SULF CH_4 SIR																			
Tmt (ppb)	Time (hours)																		
	241	527.5	622	750	876	989.5	1091.5	1182.5	1278	1376	1470	1588.5	1687.5	1784	1925.5	2045	2190	2358	2502	2643
	mg CH_4-C Kg^{-1} dry soil Day																			
1	0.000	0.000	0.000	0.001	0.001	0.002	0.003	0.012	0.077	0.503	1.180	1.496	1.709	1.956	3.307	4.621	4.800	5.018	5.256	5.098
50	0.000	0.000	0.000	0.001	0.002	0.002	0.005	0.012	0.028	0.072	0.252	0.879	1.868	2.860	4.979	6.392	6.300	6.586	6.548	6.353
100	0.000	0.000	0.000	0.001	0.001	0.002	0.003	0.008	0.016	0.037	0.091	0.274	0.786	1.815	4.443	4.953	5.237	5.390	5.435	5.394
500	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.002	0.004	0.008	0.022	0.067	0.229	0.432	1.478	3.055	4.425	5.199	6.139	6.305
1000	0.000	0.000	0.001	0.001	0.002	0.002	0.003	0.007	0.015	0.038	0.383	0.284	0.948	1.799	3.732	4.646	5.181	5.562	5.666	5.722
BLK	0.000	0.000	0.000	0.001	0.003	0.008	0.036	0.358	1.114	1.643	1.421	1.989	1.995	2.139	2.920	3.407	3.572	3.619	3.607	3.574

(table cont.)

Table A-D.3. Average concentrations of BR methane evolved at each time interval for CIP (a), TET (b) and SULF (c) in the peat soil

a)

Tmt (ppb)	DP CIP CH_4 BR									
	Time (hours)									
	48	73	95.5	168	241.5	430.5	598.5	764.5	1100.5	1436
	mg CH_4-C Kg^{-1} dry soil Day									
1	0.028	0.027	0.024	0.029	0.039	0.078	0.100	0.081	0.107	0.121
50	0.024	0.031	0.027	0.032	0.049	0.094	0.116	0.142	0.169	0.182
100	0.024	0.024	0.025	0.035	0.044	0.084	0.105	0.109	0.162	0.158
500	0.020	0.022	0.023	0.033	0.044	0.078	0.099	0.122	0.145	0.160
100	0.017	0.018	0.020	0.024	0.035	0.067	0.096	0.114	0.145	0.142
BLK	0.016	0.019	0.018	0.027	0.045	0.086	0.104	0.119	0.145	0.154

b)

Tmt (ppb)	DP TET CH_4 BR			
	Time (hours)			
	204.5	539.5	871.5	1189.5
	mg CH_4-C Kg^{-1} dry soil Day			
1	0.121	0.232	0.260	0.288
50	0.235	0.394	0.451	0.465
100	0.163	0.278	0.364	0.374
500	0.169	0.329	0.392	0.415
1000	0.118	0.234	0.247	0.257
BLK	0.104	0.180	0.232	0.256

c)

Tmt (ppb)	DP SULF CH_4 BR			
	Time (hours)			
	210	546.5	860	1194
	mg CH_4-C Kg^{-1} dry soil Day			
1	0.107	0.184	0.215	0.207
50	0.141	0.201	0.208	0.233
100	0.122	0.205	0.217	0.222
500	0.193	0.323	0.331	0.378
1000	0.163	0.272	0.306	0.336
BLK	0.148	0.248	0.278	0.305

(table cont.)

Table A-D.4. Average concentrations of SIR methane evolved at each time interval for CIP (a), TET (b) and SULF (c) in the peat soil

a)

DP CIP CH_4 SIR																						
Tmt (ppb)	Time (hours)																					
	23	29	79.5	93.5	143	168	199	217.5	238.5	262.5	287	318	334	358	388	409	433	454.5	504.5	553	599	650.5
	mg CH_4-C Kg^{-1} dry soil Day																					
1	0.004	0.003	0.014	0.020	0.068	0.157	0.315	0.404	0.568	0.801	1.070	1.455	1.694	1.873	2.257	2.365	2.234	2.353	3.173	4.078	4.370	4.452
50	0.007	0.005	0.022	0.029	0.080	0.137	0.292	0.405	0.542	0.823	1.067	1.535	1.705	1.939	2.284	2.380	2.644	2.806	3.656	4.668	4.705	4.380
100	0.007	0.005	0.023	0.029	0.091	0.169	0.389	0.501	0.668	0.997	1.120	1.641	1.762	2.233	2.498	2.632	2.858	3.065	3.985	4.915	5.357	5.051
500	0.009	0.006	0.020	0.030	0.097	0.212	0.442	0.583	0.838	1.222	1.665	2.335	2.406	2.768	3.287	3.261	3.440	3.531	4.342	4.871	4.827	4.542
100	0.006	0.004	0.019	0.021	0.077	0.145	0.299	0.403	0.563	0.805	1.058	1.337	1.381	1.642	2.000	2.197	2.185	2.500	3.255	3.974	4.114	4.032
BLK	0.007	0.004	0.021	0.028	0.081	0.159	0.306	0.460	0.611	0.883	1.111	1.374	1.461	1.813	2.132	2.392	2.652	2.654	3.573	4.356	4.506	4.510

b)

DP TET CH_4 SIR													
Tmt (ppb)	Time (hours)												
	30	72.5	121.5	167.5	217	266	313	363	409.5	458	531	601	647.5	719
	mg CH_4-C Kg^{-1} dry soil Day													
1	0.013	0.029	0.058	0.144	0.401	1.078	1.614	2.216	2.127	2.544	3.189	3.383	3.754	4.245
50	0.008	0.024	0.074	0.175	0.484	1.265	1.801	2.328	2.249	2.791	3.217	3.426	3.853	4.450
100	0.009	0.022	0.055	0.133	0.389	1.075	1.536	2.106	2.175	2.656	3.260	3.769	4.095	4.891
500	0.018	0.045	0.089	0.211	0.590	1.432	1.976	2.539	2.663	3.110	3.921	4.322	4.740	5.570
1000	0.018	0.039	0.074	0.169	0.440	1.288	1.821	2.521	2.635	3.153	3.827	4.159	4.467	5.036
BLK	0.014	0.032	0.063	0.143	0.347	0.947	1.445	1.945	2.059	2.424	2.916	2.994	3.152	3.522

c)

Tmt (ppb)	DP SULF CH_4 SIR													
	Time (hours)													
	30.5	72.5	120.5	166	216	266	312	362	410	458	531	601	647.5	719
	mg CH_4-C Kg^{-1} dry soil Day													
1	0.008	0.024	0.056	0.120	0.332	0.976	1.540	2.120	1.814	2.325	2.965	3.284	3.717	4.416
50	0.011	0.031	0.063	0.147	0.376	1.054	1.662	2.418	2.593	3.151	3.703	4.099	4.454	4.648
100	0.015	0.048	0.092	0.233	0.506	1.251	1.827	2.366	2.540	3.198	3.656	4.297	4.632	5.209
500	0.020	0.040	0.090	0.204	0.578	1.460	2.184	2.892	3.054	3.688	4.325	5.146	5.399	6.043
1000	0.021	0.043	0.085	0.191	0.468	1.113	1.588	1.949	2.244	2.588	2.759	3.310	3.799	4.216
BLK	0.021	0.049	0.093	0.200	0.488	1.167	1.689	2.199	2.318	2.841	3.131	3.330	3.278	3.429

(table cont.)

Table A-D.5. Average concentrations of BR carbon dioxide evolved at each time interval for CIP (a), TET (b) and SULF (c) in the mineral soil

a)

Tmt (ppb)	BC CIP CO_2 BR				
	Time (hours)				
	24.5	189.5	528	862	1175.5
	mg CO_2-C Kg^{-1} dry soil Day				
1	0.005	0.014	0.024	0.048	0.042
50	0.013	0.023	0.032	0.053	0.054
100	0.014	0.026	0.035	0.059	0.052
500	0.008	0.021	0.034	0.066	0.052
100	0.007	0.022	0.031	0.060	0.054
BLK	0.007	0.017	0.033	0.060	0.058

b)

Tmt (ppb)	BC TET CO_2 BR				
	Time (hours)				
	26	193.5	525.5	860	1172.5
	mg CO_2-C Kg^{-1} dry soil Day				
1	0.005	0.016	0.021	0.043	0.042
50	0.006	0.021	0.030	0.049	0.047
100	0.006	0.017	0.026	0.054	0.056
500	0.009	0.023	0.034	0.061	0.063
1000	0.007	0.017	0.023	0.046	0.043
BLK	0.006	0.017	0.026	0.044	0.038

c)

Tmt (ppb)	BC SULF CO_2 BR				
	Time (hours)				
	25.5	192.5	542.5	860	1169.5
	mg CO_2-C Kg^{-1} dry soil Day				
1	0.006	0.022	0.029	0.051	0.044
50	0.007	0.024	0.027	0.048	0.043
100	0.006	0.019	0.020	0.045	0.041
500	0.005	0.013	0.015	0.032	0.031
1000	0.004	0.010	0.013	0.036	0.032
BLK	0.004	0.013	0.018	0.044	0.041

(table cont.)

Table A-D.6. Average concentrations of SIR carbon dioxide evolved at each time interval for CIP (a), TET (b) and SULF (c) in the mineral soil

a)

Tmt (ppb)	BC CIP CO_2 SIR					
	Time (hours)					
	18	43.5	64.5	92	114	137.5
	mg CO_2-C Kg^{-1} dry soil Day					
1	0.012	0.963	2.218	3.518	3.898	3.994
50	0.013	1.008	2.071	3.477	3.944	4.048
100	0.012	1.123	2.149	3.693	4.049	4.128
500	0.009	1.171	2.419	3.837	4.218	4.303
1000	0.009	0.894	2.023	3.485	4.115	4.220
BLK	0.010	0.822	1.948	3.338	3.892	3.983

b)

Tmt (ppb)	BC TET CO_2 SIR					
	Time (hours)					
	19	44	65.5	95	116.5	139
	mg CO_2-C Kg^{-1} dry soil Day					
1	0.020	1.190	2.235	3.735	4.347	4.453
50	0.023	1.364	2.595	4.123	4.523	4.627
100	0.020	1.410	2.705	4.064	4.551	4.640
500	0.019	1.057	2.300	4.071	4.413	4.488
1000	0.021	1.232	2.589	4.505	4.783	4.850
BLK	0.018	0.913	2.263	4.095	4.539	4.613

c)

Tmt (ppb)	BC SULF CO_2 SIR							
	Time (hours)							
	20.5	45	66	93.5	116.5	140	161	185
	mg CO_2-C Kg^{-1} dry soil Day							
1	0.034	1.086	2.231	3.682	4.632	4.850	4.911	4.919
50	0.015	0.756	1.969	3.357	4.321	4.509	4.571	4.576
100	0.012	0.471	1.697	3.359	4.239	4.377	4.428	4.433
500	0.007	0.049	0.258	1.328	3.039	4.003	4.198	4.172
1000	0.005	0.044	0.398	1.539	3.224	4.299	4.486	4.470
BLK	0.025	0.997	2.032	3.519	4.303	4.541	4.618	4.625

Table A-D.7. Average concentrations of BR carbon dioxide evolved at each time interval for CIP (a), TET (b) and SULF (c) in the peat soil

a)

Tmt (ppb)	DP CIP CO_2 BR													
	Time (hours)													
	2.5	8	21	27	48	71.5	95	166	242	434	602	765	1103.5	1438.5
	mg CO_2-C Kg^{-1} dry soil Day													
1	0.010	0.006	0.010	0.008	0.012	0.014	0.012	0.012	0.021	0.043	0.058	0.095	0.079	0.100
50	0.012	0.012	0.011	0.010	0.012	0.009	0.009	0.014	0.016	0.036	0.046	0.087	0.071	0.091
100	0.010	0.011	0.011	0.012	0.015	0.010	0.010	0.017	0.019	0.043	0.046	0.089	0.077	0.108
500	0.012	0.009	0.009	0.007	0.009	0.030	0.034	0.042	0.049	0.071	0.091	0.169	0.113	0.153
100	0.011	0.012	0.012	0.010	0.013	0.009	0.011	0.016	0.021	0.040	0.056	0.110	0.071	0.120
BLK	0.009	0.009	0.011	0.011	0.012	0.010	0.010	0.015	0.021	0.045	0.055	0.102	0.083	0.103

b)

Tmt (ppb)	DP TET CO_2 BR				
	Time (hours)				
	42.5	204.5	543.5	876	1191.5
	mg CO_2-C Kg^{-1} dry soil Day				
1	0.029	0.065	0.122	0.160	0.168
50	0.044	0.126	0.202	0.277	0.258
100	0.035	0.104	0.186	0.246	0.240
500	0.031	0.121	0.210	0.275	0.273
1000	0.027	0.065	0.132	0.146	0.150
BLK	0.023	0.058	0.108	0.139	0.153

c)

Tmt (ppb)	DP SULF CO_2 BR				
	Time (hours)				
	46	209	545	852.5	1187
	mg CO_2-C Kg^{-1} dry soil Day				
1	0.03446	0.06569	0.11512	0.13033	0.11901
50	0.04001	0.07261	0.11127	0.13345	0.12152
100	0.032	0.06505	0.10342	0.12519	0.12264
500	0.03981	0.09872	0.16031	0.19714	0.20262
1000	0.0373	0.08598	0.14148	0.17815	0.1694
BLK	0.0321	0.07537	0.12994	0.16723	0.16004

(table cont.)

Table A-D.8. Average concentrations of SIR carbon dioxide evolved at each time interval for CIP (a), TET (b) and SULF (c) in the peat soil

a)

Tmt (ppb)	DP CIP CO_2 SIR								
	Time (hours)								
	3.5	9	21.5	27.5	49	72.5	94	127	151
	mg CO_2-C Kg^{-1} dry soil Day								
1	0.016	0.053	0.118	0.181	1.161	2.940	4.127	5.180	5.729
50	0.015	0.055	0.115	0.160	0.936	2.864	4.170	5.248	5.674
100	0.018	0.067	0.136	0.214	1.430	2.883	3.714	4.630	4.992
500	0.010	0.049	0.129	0.225	1.326	2.982	3.933	4.321	4.388
100	0.009	0.043	0.096	0.126	1.370	3.182	4.312	5.354	5.787
BLK	0.037	0.066	0.155	0.210	1.500	3.290	4.489	5.550	5.816

b)

Tmt (ppb)	DP TET CO_2 SIR							
	Time (hours)							
	9	26.5	49.5	74.5	96	121	144	171.5
	mg CO_2-C Kg^{-1} dry soil Day							
1	0.041	0.083	0.646	1.890	2.885	3.517	3.840	3.800
50	0.029	0.120	0.781	1.745	2.327	2.892	3.247	3.333
100	0.023	0.096	0.683	1.880	2.787	3.461	3.767	3.792
500	0.036	0.106	0.805	1.885	2.770	3.549	3.976	3.981
1000	0.027	0.100	0.695	1.956	2.928	3.546	3.839	3.793
BLK	0.024	0.086	0.707	1.877	2.803	3.445	3.802	3.701

c)

Tmt (ppb)	DP SULF CO_2 SIR							
	Time (hours)							
	5	25	51	73.5	97	117.5	144.5	167
	mg CO_2-C Kg^{-1} dry soil Day							
1	0.008	0.072	0.538	1.207	2.029	2.998	3.831	3.888
50	0.011	0.088	0.661	1.478	2.337	3.166	3.796	3.902
100	0.006	0.080	0.626	1.371	2.182	2.966	3.633	3.701
500	0.009	0.120	0.611	1.576	2.611	3.649	4.187	4.197
1000	0.010	0.113	0.538	1.444	2.498	3.570	4.120	4.071
BLK	0.015	0.126	0.691	1.408	2.238	2.982	3.590	3.673

(table cont.)

Table A-D.9. Average concentrations of SIR nitrous oxide evolved at each time interval for CIP (a), TET (b) and SULF (c) in the mineral soil

a)

Tmt (ppb)	BC CIP N_2O SIR						
	Time (hours)						
	2	25	47.5	59.5	71	96.5	120
	mg N_2O-N Kg^{-1} dry soil Day						
1	0.000	0.011	0.028	0.065	0.115	0.281	0.386
50	0.000	0.010	0.022	0.035	0.074	0.235	0.423
100	0.001	0.019	0.036	0.057	0.093	0.298	0.374
500	0.001	0.019	0.034	0.055	0.092	0.299	0.368
1000	0.001	0.019	0.034	0.053	0.084	0.205	0.457
BLK	0.001	0.021	0.036	0.067	0.138	0.384	0.512

b)

Tmt (ppb)	BC TET N_2O SIR						
	Time (hours)						
	4	27	47.5	57.5	77	99	119
	mg N_2O-N Kg^{-1} dry soil Day						
1	0.002	0.030	0.149	0.276	0.949	2.770	4.546
50	0.002	0.028	0.106	0.325	0.911	2.821	4.102
100	0.002	0.028	0.121	0.273	0.770	2.860	4.053
500	0.002	0.028	0.121	0.198	0.911	3.151	4.939
1000	0.002	0.028	0.127	0.127	1.061	2.849	4.289
BLK	0.002	0.028	0.142	0.114	0.879	2.624	3.592

c)

Tmt (ppb)	BC SULF N_2O SIR						
	Time (hours)						
	2	26.5	47.5	57	73.5	95.5	117
	mg N_2O-N Kg^{-1} dry soil Day						
1	0.001	0.036	0.211	0.460	0.834	2.270	3.562
50	0.001	0.032	0.093	0.253	0.577	2.315	4.129
100	0.001	0.037	0.113	0.290	0.631	2.080	3.382
500	0.002	0.031	0.070	0.121	0.300	0.908	1.656
1000	0.001	0.030	0.071	0.089	0.227	0.683	1.097
BLK	0.001	0.028	0.161	0.350	0.893	2.407	3.677

(table cont.)

Table A-D.10. Average concentrations of SIR nitrous oxide evolved at each time interval for CIP (a), TET (b) and SULF (c) in the peat soil

a)

Tmt (ppb)	DP CIP N_2O SIR							
	Time (hours)							
	3.5	26.5	49	60.5	72.5	98.5	122.5	142.5
	mg N_2O-N Kg^{-1} dry soil Day							
1	0.001	0.018	0.034	0.045	0.057	0.184	0.335	0.767
50	0.001	0.016	0.028	0.040	0.085	0.459	0.464	0.616
100	0.001	0.018	0.034	0.059	0.086	0.225	0.793	1.532
500	0.002	0.016	0.038	0.091	0.177	0.329	0.880	1.507
1000	0.001	0.015	0.033	0.070	0.156	0.376	1.086	1.744
BLK	0.001	0.014	0.029	0.065	0.147	0.333	0.650	1.762

b)

Tmt (ppb)	DP TET N_2O SIR						
	Time (hours)						
	2	24	48	60	72	96	120
	mg N_2O-N Kg^{-1} dry soil Day						
1	0.000	0.029	0.049	0.085	0.324	1.401	1.881
50	0.000	0.029	0.059	0.128	0.512	1.628	3.219
100	0.000	0.030	0.100	0.236	0.800	1.822	2.648
500	0.000	0.027	0.068	0.140	0.695	1.939	2.379
1000	0.000	0.026	0.064	0.135	0.610	1.685	2.202
BLK	0.001	0.034	0.158	0.248	0.506	1.431	2.031

c)

Tmt (ppb)	DP SULF N_2O SIR				
	Time (hours)				
	2	24	48	72	96
	mg N_2O-N Kg^{-1} dry soil Day				
1	0.000	0.036	0.160	0.588	1.254
50	0.000	0.032	0.051	0.530	1.447
100	0.000	0.032	0.052	0.453	1.476
500	0.001	0.034	0.064	0.446	1.348
1000	0.000	0.032	0.053	0.437	1.389
BLK	0.000	0.036	0.198	0.522	1.464

(table cont.)

VITA

Jeremy Landon Conkle was born to Sheryl Lynn Conkle and Joseph Patrick Conkle on the tip of the Northern Neck in Kilmarnock, Lancaster County, Virginia, in March, 1981. He comes from humble beginnings, where his parents were hard working, loving and nurturing individuals. The first 18 years of his life were spent in Lancaster County, but he frequently traveled between Pennsylvania and North Carolina visiting relatives.

Jeremy earned bachelor's degrees in biology and chemistry from Longwood University in Farmville, Virginia in 2003. At Longwood, he was a member of the varsity soccer team and Sigma Phi Epsilon, while also working part time in the work study program with the university. Upon graduation, Jeremy worked for Earth Tech as a field chemist at hazardous waste sites, emergency response sites and natural disasters.

He began work on a master's degree in environmental studies in the fall of 2004 at The College of Charleston (CofC), in Charleston, South Carolina and completed it in the spring of 2006. While at the CofC, Jeremy became interested in the unique properties and values of wetlands. This led him to seek doctoral programs where he could tie two of his interests together: wetlands and environmental contaminants. Jeremy began work on his doctorate at Louisiana State University (LSU), under the guidance of Dr. John White, immediately after graduating from CofC. His research at LSU focused on pharmaceuticals in surface waters and their interactions in wetlands.