STEROIDAL ESTROGEN MINERALIZATION IN LIQUID SWINE MANURE, SEWAGE SLUDGE AND BIOSOLIDS IN THE PRESENCE OF ANTIBIOTICS

By

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ABSTRACT

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Steroidal estrogens and antibiotics used in veterinary and human medicine are detected in livestock manure or sewage sludge and biosolids. Biodegradation is an important process by which estrogenic compounds are removed from organic amendments, but antibiotics have been shown to impede microbial communities. Although both compounds are often present in these media, the fate of estrogens in association with antibiotics has not been previously studied. In this study, both rates of tetracycline (40 and 200 mg kg^{-1}) in liquid swine manure induced a lag phase of 40 to 50 days prior to the onset of a log phase of estrone and 17 β-estradiol mineralization, and tetracycline at 200 mg kg^{-1} significantly reduced maximum mineralization of estrone and 17 β-estradiol in manure. In soils amended with a high rate of manure, penicillin at 200 mg kg^{1} also significantly decreased maximum mineralization of estrone and 17 β-estradiol relative to soils free of antibiotics. Estrogen mineralization almost always significantly decreased in the order of: manure > soil amended with a low rate of manure = soil > soil amended with a high rate of manure.

In order to examine the relationship between physical and chemical parameters of media and estrogen mineralization, sewage sludge and biosolid samples with vastly different characteristics were selected for a study of 17 β-estradiol and 17 α-ethinylestradiol mineralization in the presence of ciprofloxacin, an antibiotic commonly used to treat

urinary and intestinal infections in humans. Ciprofloxacin was persistent in all media, as less than 0.05% mineralization was observed over 133 d. Despite this persistence, no significant effect of ciprofloxacin addition on 17 β-estradiol or 17 α-ethinylestradiol mineralization was observed at 133 days. Consistent with its chemical structure, maximum mineralization of 17 α-ethinylestradiol was always less than that of 17 β-estradiol, indicating resistance to microbial degradation. PCA analysis indicated that total nitrogen, ammonium-nitrogen and total carbon demonstrated a positive association with respiration and maximum mineralization of 17 β-estradiol, but a negative association with 17 α-ethinylestradiol maximum mineralization. Sorption of 17 α-ethinylestradiol was greater than 17 β-estradiol in all media, limiting maximum mineralization of 17 α-ethinylestradiol.

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1. INTRODUCTION

1.1 Steroidal Hormones

Steroidal estrogens are a group of compounds that promote and regulate secondary sexual characteristics in vertebrates. These compounds include estrone and 17 β-estradiol, which are natural estrogens produced by vertebrates, as well as 17 α-ethinylestradiol, the most common component of oral contraceptive pills. Estrogens are C_{18} steroids derived from cholesterol and pregnenolone which are transformed into androstenedione and testosterone, which are the precursors for natural estrogens estrone and 17 β-estradiol (Combalbert and Hernandez-Raquet 2010). Estrone and 17 β-estradiol are differentiated by the functional group on the cyclopentane ring (Fig 1.1). Under many conditions, the inter-conversion between estrone and 17 β-estradiol has been observed in water and sediment samples (Czajka and Londry 2006), sewage sludge samples (Carballa et al. 2007), agricultural soils (Colucci et al. 2001) and soils amended with liquid swine manure (Jacobsen et al. 2005). The synthetic introduction of an ethinyl group to 17 β-estradiol forms 17 α-ethinylestradiol, which is extremely resistant to microbial degradation due to the position of the two quaternary carbon atoms of the ethinyl group (de Mes et al. 2005; Weber et al. 2005; Ying and Kookana 2005).

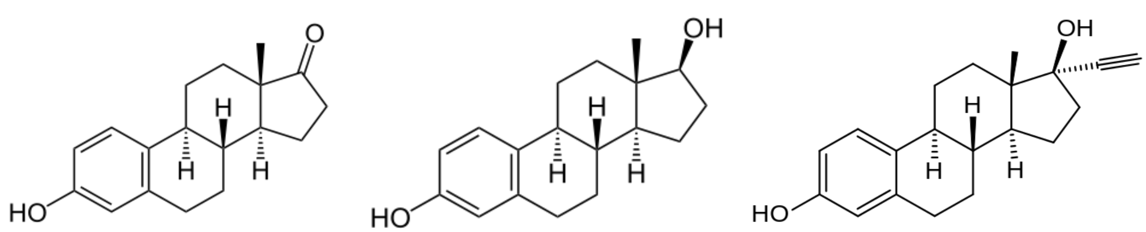

Figure 2.1 Molecular structures of estrone, 17 β-estradiol and 17 α-ethinylestradiol.

Steroidal estrogens are excreted via urine and feces and therefore have been detected in liquid swine manure, sewage sludges and biosolids (Table 1.1). Estrogen discharge into the environment via human excretion alone has been estimated to be 4.4 kg per year for every million inhabitants (Combalbert and Hernandez-Raquet 2010). In addition, it has been estimated that about 720 kg per year of synthetic estrogens are discharged into the environment from some 60 million women using hormonal contraception (Combalbert and Hernandez-Raquet 2010).

Table 1.1 Published concentrations of estrone, 17 β-estradiol and 17 α-ethinylestradiol in select liquid swine manure, sewage sludges and biosolid samples.

Media	Estrone	17 β-estradiol	17 α-ethinylestradiol	Reference
		Solid Media (μg kg^{-1})		
Sewage Sludge	<2-37	5-17	<2-4	Ternes et al. 2002
Sewage Sludge	7	1.7	3	Andersen et al. 2003
Sewage Sludge	12	0.3	0.4	Braga et al. 2005*a*,*b*
Sewage Sludge	2-8	1-5.5	1-18	Muller et al. 2008
Biosolid	<2-16	5-17	<2-4	Ternes et al. 2002
		Liquid Media (ng L^{-1})		
Swine Raw Manure	5300	1250	*not measured*	Furuichi et al. 2006
Swine Primary Lagoon	80	3	*not measured*	Shappell et al. 2007
Swine Farrowing Primary Lagoon	14395	2325	*not measured*	Fine et al. 2003
Swine Farrowing Secondary Lagoon	28	19	*not measured*	Fine et al. 2003
Swine Nursery Primary Lagoon	530	47	*not measured*	(Fine 2002)
Swine Finisher Primary Lagoon	74700	125	*not measured*	(Fine 2002)

Following the application of manure and biosolids to agricultural land, the potential movement of estrogens from agricultural land to surface waters via runoff and erosion is a concern (Nichols et al. 1997; Finlay-Moore et al. 2000; Kjaer et al. 2007). Estrogen hormones found in the environment at very low concentrations have induced a wide range of adverse physiological effects in various species. For example, production of an egg yolk protein precursor has been observed in male fish exposed to concentrations as low as 25 ng L^{-1} estrone or 17 β-estradiol, or 0.1 ng L^{-1} 17 α-ethinylestradiol (Lai et al. 2002).

1.2 Antibiotics in Human and Veterinary Medicine

For over a century, antibiotics have been widely used to control bacterial infections in humans and livestock. In addition to therapeutic applications, antibiotics are commonly administered to livestock at subtherapeutic levels to prevent disease and to promote growth in North America. Penicillin and tetracycline are commonly used antibiotics in both human and veterinary medicine. Penicillin is part of a broad class of antibiotics known as β-lactams, which are used to treat infections caused by Gram-positive bacteria by inhibiting cell wall biosynthesis. Tetracycline is a broad-spectrum antibiotic that inhibits protein synthesis of bacteria. In human medicine, ciprofloxacin, part of the broad class of antibiotics known as fluoroquinolones, is commonly prescribed to treat urinary tract and intestinal infections. Ciprofloxacin is also a broad-spectrum antibiotic that inhibits DNA replication. Ciprofloxacin is also the primary metabolite of enrofloxacin (Mougin et al. 2013), a fluoroquinolone antibiotic that was commonly administered to poultry until approval was withdrawn in 2005 due to the development of fluoroquinolone-resistant bacteria in humans (United States Food and Drug Administration 2005).

Widespread use of antibiotics, particularly at sub-therapeutic levels (Wegener 2003), has been linked to increased prevalence of antibiotic resistant bacteria in humans, animals and the environment (Koike et al. 2007). This may lead to untreatable livestock and human diseases (Kumar et al. 2005). In recent years, over prescription of antibiotics to treat viral infections in humans has become prevalent. For example, recent studies have shown that approximately three quarters of patients with throat inflammation were inappropriately prescribed antibiotics (Gonzales et al. 1997; Linder and Stafford 2001; Ayranci et al. 2005), including tetracycline, penicillin and ciprofloxacin (Palla et al. 2012). This over prescription has contributed to the emergence of resistant strains of microorganisms (Bisno et al. 1997), which has increased morbidity and mortality, as well as the cost of health care (Cohen 1992). In nursery pigs, studies have shown that antibiotic resistance in manure bacteria can develop in a very short period of time, with 70% of fecal bacteria developing resistance to penicillin and tetracycline within three weeks of exposure via feed (Kumar et al. 2005). Antibiotic resistant bacteria have also been documented in terrestrial and aquatic environments. For example, in water samples taken from 16 rivers at 22 sites in the United States over 40% of bacteria exhibited resistance to one or more antibiotics, including tetracycline, penicillin and ciprofloxacin (Ash et al. 2002).

Human and animal wastes are potential sources of both antibiotics and antibiotic resistant bacteria; up to 90% of antibiotics are excreted via feces and urine in an unaltered form (Kumar et al. 2005). Various theories have been put forward to elucidate the development of antibiotic resistant bacteria in the terrestrial environment. Antibiotic resistant bacteria developed in the gut of humans and animals may enter the environment directly via land application of manures and biosolids (Sundin et al. 1995; Kelly et al. 1998); resistance genes from bacteria in manure may transfer to native soil and water microbial populations (Goyal and Hoadley 1979; Gast and Stephens 1988); or the antibiotics present in manures and biosolids applied to soils can induce resistance in native microbial populations (Kumar et al. 2005). Three mechanisms have been proposed for antibiotic resistance in microbes: (1) overproduction of protein pumps to export antibiotics from cells; (2) structural changes in proteins of target cells rendering the organism insusceptible; and (3) modification of pre-existing enzymes to destroy the antibiotic (Walsh 2000).

1.3 Livestock Manures and Biosolids as Organic Amendments

Organic amendments, such as livestock manures or human biosolids, can be applied to agricultural land to improve the physical and chemical qualities of the soil. Manures, biosolids and composted biosolids are a valuable source of macro- and micro- nutrients necessary for plant growth. They are also a source of organic matter, which improves the water holding capacity of soils and reduces soil erosion. Manures are a by-product of livestock production. According to Statistics Canada, Canadian livestock produced approximately 180 million tonnes of manure in 2006, an increase of 16% from 1981

(Hoffman 2008). Increases in Manitoba were primarily due to the increase in the number of localized swine operations (Hoffman 2008). Elevated nutrient loadings in Lake Winnipeg have been directly related to intensive livestock production facilities in the southern portion of the Lake Winnipeg Watershed (Cicek et al. 2006).

Sewage sludges are a residual produced in wastewater treatment. Thoroughly treated sludges that are suitable for beneficial use on agricultural lands are termed “biosolids”; the term “sludge” is used only before beneficial use criteria have been met (Tchobanoglous et al. 2003). Composted biosolids can be produced from a combination of biosolids and municipal waste held at 55˚C for 3days in a static aerated pile or 15 days in a windrow **(**Tchobanoglous et al. 2003). In 2012, the City of Winnipeg produced approximately 13,400 dry-tonnes of anaerobically digested, mechanically dewatered biosolids. These biosolids were deposited at the Brady Landfill rather than being applied to agricultural land (Jones et al. 2012). This is likely due, in part, to eutrophication concerns in Lake Winnipeg, the Winter Spreading of Manure and Biosolids Prohibition Act, as well as emerging concerns about heavy metals, antibiotics, hormones and other endocrine-disrupting substances from pharmaceuticals and personal care products.

Treatment of sewage in lagoon-based systems is extensive in rural and northern communities throughout Canada. In 1985, there were 127 lagoon-based wastewater treatment systems in Manitoba, representing 85% of total treatment facilities in the province (Smith and Finch 1985). However, lagoons represent a considerably smaller volume of the total sewage treated in Manitoba because of lower population densities in rural and northern communities compared to cities. Facultative lagoons with controlled discharge are the most common type of lagoon (United States EPA). Several treatment processes occur simultaneously in sewage lagoons including sedimentation, bioflocculation, chemical precipitation, biochemical oxidation, fermentation and disinfection (National Guide to Sustainable Municipal Infrastructure 2004). The sludge layer, which is covered in an aerobic effluent, is anaerobic and contains bacteria that decompose organic matter and produce methane and hydrogen sulphide. Biological activity in lagoons is highly dependent on temperature. Therefore, sewage sludge accumulates more rapidly in colder climates and may require more frequent desludging. In a study of 25 respondents who operated lagoons, 32% reported never desludging, 40% reported desludging every 10 to 20 years, and a desludging frequency of five years or less was reported by 20% of respondents (National Guide to Sustainable Municipal Infrastructure 2004). Desludging of a lagoon commonly involves draining the lagoon and removing the sewage sludge mechanically. The microbiological quality of the removed sewage sludge typically exceeds that of biosolids from a wastewater treatment plant (National Guide to Sustainable Municipal Infrastructure 2004) and is generally applied to agricultural land.

1.4 Steroidal Hormone and Antibiotic Fate in Various Media

Soils amended with livestock manures or human biosolid contain steroidal estrogens and antibiotics. In the terrestrial environment, contaminants dissolved in the soil solution or sorbed by soil particles can be transported to surface waters via runoff and erosion, respectively. The fate and transport of a contaminant in soil is strongly influenced by degradation and sorption processes.

The degradation of steroidal hormones and antibiotics is mainly driven by microbial processes (Munnecke et al. 1982; Chen et al. 1997; McGrath et al. 1998; Al-Ahmad et al 1999). Mineralization is a biological degradation process in which microbial populations convert organic molecules, such as estrogens, into CO_2 and other inorganic compounds. The extent of mineralization is determined by the chemical characteristics of the contaminant, the chemical, physical and biological characteristics of the media in which it resides and the environmental characteristics of the system. These characteristics include the solubility and molecular structure of the contaminant; the nutrient content, pH and moisture content of the media; and the temperature of the environment. Mineralization of 17 β-estradiol and estrone is expected to be comparable due to similarities in chemical structure and the inter-conversion between the two molecules. In contrast, due to the inherent stability of the 17 α-ethinylestradiol molecule, previous studies have consistently reported higher mineralization of 17 β-estradiol than 17 α-ethinylestradiol in soils, sewage sludges and biosolids (Ternes et al. 1999; Layton et al. 2000; Stumpe and Marschner 2009).

In agricultural soils amended with livestock manures or human biosolids, a wide range of maximum mineralization values have been reported for steroid estrogens such as estrone and 17 β-estradiol (Lucas and Jones 2006). This variation suggests that physical and chemical parameters of the media likely have a strong influence on estrogen fate. In a study of agricultural soils amended with beef cattle manure, 17 β-estradiol mineralization was significantly positively correlated with soil organic carbon, total carbon, ammonium-nitrogen and total nitrogen (Caron et al. 2012). In contrast, Stumpe and Marschner (2009) found no significant relationship between physical or chemical parameters of soil and the mineralization of 17 β-estradiol or 17 α-ethinylestradiol.

Sorption, which is the binding of a chemical to a solid particle either internally (absorption) or externally (adsorption), can also effect mineralization of a compound. Sorption is determined by chemical characteristics of the sorbate, the sorbent and the environment. These characteristics include the water solubility, polarity and charge distribution of the sorbate; the chemical content, pH and moisture content of the sorbent; and the temperature of the environment (Loffredo and Senesi 2006). Batch equilibrium sorption studies measure sorption at equilibrium in a media slurry. Sorption can be expressed by isotherms, such as the Freundlich isotherm (Freundlich 1909), that represent the relationship between the amount of chemical sorbed by the media and left in the equilibrium solution. Previous studies of estrogen sorption in soils have reported a wide range of Freundlich sorption coefficients for estrone, 17 β-estradiol and 17 α-ethinylestradiol (Stumpe and Marschner 2009). In soils, Stumpe and Marschner (2009) reported greater estrone sorption than sorption of 17 β-estradiol and 17 α-ethinylestradiol, which were comparable. In contrast, Ying and Kookana (2005) reported that sorption decreased in the order of: 17 α-ethinylestradiol > 17 β-estradiol > estrone.

Fewer studies have examined the environmental fate of antibiotics. Bansal (2012) reported that tetracycline degradation was generally greater in manure than soil, but that degradation in soils was influenced by factors such as soil moisture content, nitrogen content, temperature, and the amount of manure applied. Also, other researchers have reported that the additions of livestock manure or sewage sludges increases biodegradation of antibiotics (Ingerslev and Halling-Sørensen 2001; Ingerslev et al. 2001), possibly due to the enrichment of microorganisms that are present in these amendments.

The chemical structure of the antibiotic largely influences its mineralization potential (Thiele Bruhn 2003), for example, penicillin has been identified as easily degradable in soils (Gavalchin and Katz 1994; Midtvedt 2001). In contrast, in manure amended soils, tetracycline concentration did not significantly decline over a period of 5 months following manure application (Aga et al. 2005). Sorption of antibiotics may inhibit biodegradation by reducing bioavailability (Samuelsen et al. 1992; Gavalchin and Katz 1994). Sorption of penicillin in soils is significantly less than that of tetracycline (Subbiah et al. 2011) which is, in turn, less strongly sorbed by soil than ciprofloxacin (Tolls 2001). The sorption of an antibiotic in soil is particularly influenced by soil pH (Yeager and Halley 1990; Holten Lützhøft et al. 2000), soil organic matter (Langhammer 1989; Gruber et al. 1990) and clay minerals (Batchelder 1982). Sorption has been shown to reduce the antimicrobial effect of some antibiotic compounds (Ingerslev and Halling-

Sørensen 2001). For example, it has been suggested that greater sorption causes tetracycline to be neutralized in soils while β-lactam antibiotics, such as penicillin, remain bioactive (Subbiah et al. 2011).

1.5 Research Objectives

The objective of this thesis is to quantify estrogen mineralization in organic amendments in the presence or absence of human and veterinary antibiotics. The first study, described in Chapter 2 and published in the Journal of Environmental Science and Health Part B, examines estrogen mineralization in soil, soil amended with liquid swine manure, and liquid swine manure alone in the presence or absence of penicillin or tetracycline. The practical implication of this study is to add to the discussion of how one can reduce the potential for estrogen contamination in the terrestrial and aquatic environments, for example, by improving on storage methods or the timing and rates of liquid swine manure applications on agricultural land. In the second study, described in Chapter 3 and submitted for publication in the same journal, the impact of ciprofloxacin on estrogen mineralization in sewage sludges and biosolids was quantified, as influenced by estrogen sorption and the characteristics of the media. The practical implication of this study is to improve the understanding of the dissipation of estrogens and antibiotics in a range of media produced during various stages of the wastewater treatment process. Collectively, this Masters of Science research will contribute to the body of knowledge about the interaction between human and veterinary antibiotics and estrogens in the environment.

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2. ESTRONE AND 17 β-ESTRADIOL MINERALIZATION IN LIQUID SWINE MANURE AND SOIL IN THE PRESENCE AND ABSENCE OF PENICILLIN OR TETRACYCLINE

2.1 Abstract

Natural steroid estrogens (e.g., 17 β-estradiol) and antibiotics (e.g., penicillin) are chemicals detected in livestock manure storage facilities and in manure amended agricultural soils. The fate of natural steroid estrogens in these media has been studied for the past two decades but seldom in association with antibiotics. This factorial experiment examined estrone (E1) and 17 β-estradiol (E2) mineralization in liquid swine manure, soil and manure amended soil containing 0, 40 and 200 mg kg^{-1} penicillin or tetracycline. Maximum mineralization (MAX) across treatments ranged from 13.5% to 49.9% for E1 and from 15.4% to 51.2% for E2. Estrogen mineralization almost always significantly decreased in the order of: manure > soil amended with a low rate of manure = soil > soil amended with a high rate of manure, suggesting that periodically agitated manure was a more favorable medium for biotic removal of estrogens than soil. Both rates of tetracycline in manure induces a lag phase of 40 to 50 days prior to the onset of a log phase of mineralization, and tetracycline at 200 mg kg^{-1} significantly decreased E1 and E2 MAX in manure. For soils amended with a high rate of manure, penicillin at 200 mg kg^{-1} significantly decreased E1 and E2 MAX and also the other antibiotic additions resulted in numerically lesser E1 and E2 MAX values, relative to soils free of antibiotics. We conclude that storage of liquid swine manure for 3-4 months with periodic agitation prior to application to agricultural land may reduce the potential for estrogens to become

environmental contaminants, but that the persistence of estrogens in manure storage lagoons is also influenced by farm management practices such as the types and rates of antibiotics administered to livestock. When both estrogens and antibiotics are present in manure applied to soils, estrogens may have slower dissipation rates in soil particularly when manure is applied at high rates or not mechanically incorporated in the surface horizon.

2.2 Introduction

Estrone (E1) and 17 β-estradiol (E2) are natural steroid hormones excreted by vertebrates including livestock such as swine. Potential sources for the estrogenic activity detected in both surface and ground water include the transport of E1 and E2 from agricultural land amended with livestock manure, and from on-farm storage facilities such as swine waste holding lagoons (Matthiessen et al. 2006). The presence of E1 or E2 in water resources is a concern (Matthiessen 2003). For example, increased vitellogenin synthesis has been observed in male rainbow trout exposed to concentrations as low as 1 and 25 ng L^{-1} for E2 and E1, respectively (Routledge et al. 1998).

Antibiotics can be toxic to soil microorganisms (Girardi 2011) while microbial degradation is the most important process for the removal of estrogens in soil. Mineralization rates of E1 and E2 in soils have been reported to be similar, likely due to the rapid conversion of E2 to its first metabolite, E1, which can exceed 95% of the initial E2 within 1-3 h (Stumpe and Marschner 2010). Maximum E2 mineralization in soils ranged from as low as 5.1% over 23 days in soils from Israel irrigated with freshwater (Stumpe and Marschner 2010) to 90% over 100 days in soils from North Wales amended with sheep urine (Lucas and Jones 2006). Previous research results have evaluated the impact of manure characteristics on estrogen mineralization in soil. For example, in a laboratory microcosm study conducted over 21 days, additions of liquid or solid swine manure and liquid cattle manure to soil increased E2 mineralization, but solid cattle manure and poultry manure addition had no significant effect (Stumpe and Marschner 2010).

Antibiotics are commonly used in the livestock industry for therapeutic, prophylactic and sub-therapeutic purposes (Koike et al. 2007). Studies have shown that up to 90% of orally and intravenously administered antibiotics are excreted in an unaltered form (Thiele-Bruhn 2003). Few studies have examined the impact of the presence of antibiotics on estrogen fate in soil (Chun et al. 2005; Shareef et al. 2009). Shareef et al. (2009) concluded that antimicrobials triclosan and triclocarban did not significantly reduce E2 and 17 α-ethinylestradiol biodegradation in a sandy soil, except when triclosan was applied at 1000 mg kg^{-1}. Chun et al. (2005) reported that sulfamethazine, tylosin and chlortetracycline significantly decreased the transformation of E2 to E1 in a loam.

Estrogen concentrations in liquid swine manure range from 0.027 to 74.7 μg L^{-1} and 0.003 to 2.5 μg L^{-1} for E1 and E2, respectively (Combalbert and Hernandez-Raquet 2010). Information about E1 and E2 concentrations in soils is lacking, but Finlay-Moore et al. (2000) measured 0.675 µg kg^{-1} of E2 in soil amended with poultry manure. Following manure applications to land, penicillin and tetracycline, which are commonly used antibiotics in the livestock industry, can be found in soils at concentrations ranging from 199 µg kg^{-1} to 200 mg kg^{-1} (Chun et al. 2005; Shareef et al. 2009). The objective of this study was to quantify the impact of penicillin or tetracycline on E1 and E2 mineralization in liquid swine manure, soil and manure-amended soil.

2.3 Materials and Methods

Urine and solid manure samples were collected in May of 2011 from Berkshire sows at Zinn Farms, located near Springstein, Manitoba. All sows appeared to be in good health and had not received prophylactic antibiotics in their lifetime. Solid manure, urine samples and water were mixed to obtain a liquid swine manure sample with a dry matter content of approximately 7% (Table 2.1). Newdale clay loam soil (0-15 cm) (Table 2.1) was collected from the Ap-horizon in an agricultural field in western Manitoba, Canada. It is the provincial soil of Manitoba and represents a large area of farm land onto which livestock manure is commonly applied. The soil is an Orthic Black Chernozem (nearest equivalent to the U.S. Soil Taxonomy are the Udic Boroll subgroups) (Soil Classification Working Group 1998).

Table 2.1 Analysis of Newdale clay loam soil sample and liquid swine manure sample.

Parameter	Newdale Clay Loam	Liquid Swine Manure (wet wt.)	Liquid Swine Manure (dry wt.)
pH[1]	7.5	8.7	8.7
SOC (%)[2]	3.2	3.5	46.7
N (ppm)	13.5[3]	6800[4]	90667[4]
P (ppm)	11.7[5]	1500[6]	20000[6]
K (ppm)	315[7]	1800[6]	24000[6]
Ca (ppm)	3564[7]	1200[6]	16000[6]
Mg (ppm)	614[7]	700[6]	9333[6]

[1]1:1 soil-water solution. [2]Loss on ignition method. [3]Cadmium reduction method followed by modified Griess-Ilosvay method. [4]LECO model CHN 600 Carbon-Hydrogen-Nitrogen Determinator. . [5]Olsen phosphorous test. [6]Aqua regia digestion followed by Inductively Coupled Plasma Optical Emission Spectroscopy. [7]Extracted with sodium acetate solution at pH 7.

A factorial experiment (2 estrogens x 4 media x 5 antibiotic treatments) was carried out using microcosms incubated under a constant 20˚C temperature in the dark. 2.23 mL manure was placed in 15 mL glass test tubes to provide for the manure only treatment. For the other media, 25 g of soil or soil amended with manure was brought to 80% of field capacity (including manure, antibiotic and estrogen additions) and placed in 90 mL glass beakers. Manure was added to soil at rates of 30 or 90 mL kg^{-1}. The low rate of manure amendment represented a typical application rate of 5.6 L m^2 incorporated to 15 cm depth, while the high rate of manure amendment represented the same application rate incorporated to 5 cm depth. Microcosms consisted of a 500 mL Mason jar containing the glass beaker or test tube, a 20 mL scintillation vial with 5 mL of 0.5 M NaOH to trap evolved CO_2 and a 15 mL test tube with 3 mL of acidified water (pH≤3) to maintain moisture without trapping CO_2. Manure in tubes was periodically agitated to prevent anaerobic conditions. Agitation was done when NaOH traps were replaced (see below) and involved five seconds of rapid lateral movement to produce a visibly homogenous sample. Mechanical agitation is one of the basic methods of aeration in liquid swine manure storage facilities, the other being to introduce air with submerged diffusers or other aeration devices (Tchobanoglous et al. 2003). The experiment also included microcosm controls in which autoclaved and non-autoclaved silica sand was used instead of soil.

Microcosms were pre-incubated at 20˚C for a total of 96 h prior to estrogen additions. Antibiotics were added at 48 h. Antibiotic stock solutions were prepared at concentrations of 1 or 5 g L^{-1} penicillin G sodium salt (≥98% pure, Sigma-Aldrich Chemical Company, Saint Louis, MO) and 1 or 5 g L^{-1} tetracycline (≥88% pure, Sigma-Aldrich Chemical Company, Saint Louis, MO). Antibiotics (1 mL) were added at concentrations of 40 and 200 mg antibiotic per kg $media^{-1}$. The range of antibiotic concentrations used in the study represents the concentrations of antibiotics typically found in swine manure (Kumar et al. 2005). One mL of deionized water was added to treatments not receiving antibiotics. Estrone or 17 β-estradiol stock solutions were prepared at concentrations of 1250 µg L^{-1} by combining analytical grade 17 β-estradiol (≥98% pure, Sigma-Aldrich Chemical Company, Saint Louis, MO) and 17 β-estradiol [4-^{14}C] (99% radiochemical purity, specific activity 45-60 mCi $mmol^{-1}$, American Radiolabeled Chemicals Inc., Saint Louis, MO), or analytical grade estrone (≥99% pure, Sigma-Aldrich Chemical Company, Saint Louis, MO) and estrone [4-^{14}C] (99% radiochemical purity, specific activity 45-60 mCi $mmol^{-1}$, American Radiolabeled Chemicals Inc., Saint Louis, MO). Estrogens (1 mL) were added at a rate of 50 μg estrogen per kg media^{-1}(with 66,680 Bq ^{14}C-estrogens per kg media^{-1}). Although this concentration is higher than what is typically found in manure or soil, this estrogen concentration is that used by Caron et al. (2012) and is within the mid-range of the concentrations used by Casey et al. (2003), who both studied estrogen fate in soil as influenced by manure additions. After the spiking with an estrogen and antibiotic, the dry matter content of the liquid swine manure sample was approximately 4% which is typical for liquid swine manure applications to agricultural land (Manitoba Agriculture, Food and Rural Initiatives 2007).

NaOH traps were removed and replaced every 2 to 4 days for the first two weeks, then every 4 to 7 days until week 13, and finally every 10 to 15 days until the completion of the experiment at 133 days. Scintillation cocktail (8 mL, 30% Scintisafe scintillation cocktail; Fisher Scientific, Fairlawn, NJ) was added to the traps and radioactivity was measured by Liquid Scintillation Counting (LSC) with automated quench correction (#C method) and a maximum counting time of 10 min (LS 7500 Beckman Instruments, Fullerton, CA). The experiment was terminated at 133 days at which time the mineralization had slowed to ≤0.1% per day for all treatments. The cumulative $^{14}CO_2$ evolved was fitted to the rise to max equation using Sigma Plot 2000 for Windows version 11.0 (SPSS Inc., 1986-2000) to test the goodness of fit to first order kinetics.

In order to assist in the explanation of the experimental results obtained, a smaller factorial experiment (2 estrogens x 4 agitation frequencies) was conducted after completion of the antibiotic experiment. This second experiment involved the use of manure only which was again collected from Zinn Farms. All experimental protocols were as described above except that no antibiotics were applied and manure was agitated every 3-4 d, every 14 d, every 28 d, or never.

The effect of liquid swine manure amendment and antibiotic addition on cumulative E1 or E2 mineralization at 133 days was determined using a three-way analysis of variance (ANOVA $P<0.05$) followed by the Tukey-Kramer HSD multiple comparison test ($P<0.05$) applied on transformed data (x^{-1}) that respected normality (Shapiro-Wilks statistic ≥ 0.90) and equality of variance (2002-2010, Windows version 6.1.6701, SAS Institute). The effect of agitation in liquid swine manure samples on cumulative E1 and E2 mineralization at 133 days was determined using a two-way analysis of variance without data transformation since data respected normality and equality of variance.

2.4 Results and Discussion

Maximum mineralization (MAX) was relatively small in silica sand (3.06% ± 0.44% for E1 and 4.60% ± 0.33% for E2) and autoclaved silica sand (1.74% ± 1.33% for E1 and 3.54% ± 0.28% for E2). MAX was larger in soil, manure and manure-amended soil suggesting that degradation of E1 and E2 was biologically mediated by microorganisms in manure and soil, as concluded by others (Ying and Kookana 2005; Caron et al. 2012). Across all treatments, MAX ranged from 13.5% to 49.9% for E1 and from 15.4% to 51.2% for E2. A wide range of E1 and E2 mineralization values have been reported for different media. In a study by Lucas and Jones (2006), E1 mineralization ranged from 15-20% in soils, 15-80% in urine-amended soils, 40-90% in cattle manure amended soils and

40-70% in sheep manure amended soils, while E2 mineralization ranged from 10-35% in soils, 15-90% in urine amended soils, 40-80% in cattle manure amended soils and 40-70% in sheep manure amended soils.

The rise to max equation provided a good fit to most of the mineralization data indicating that E1 and E2 mineralization in the media typically followed first-order kinetics with the goodness of fit (R^2) ranging from 0.73 to 1.0. A clear exception was the manure treatment containing 200 mg kg^{-1} tetracycline for which there was a lag phase of about 40 to 50 days prior to the onset of a log phase of E1 and E2 mineralization (Figure 2.1). Other studies have shown that degradation of E1 and E2 is controlled by first-order kinetics (Colucci et al. 2001; Caron et al. 2010), but zero-order and second-order kinetics have also been reported for E2 (Stumpe and Marschner 2007).

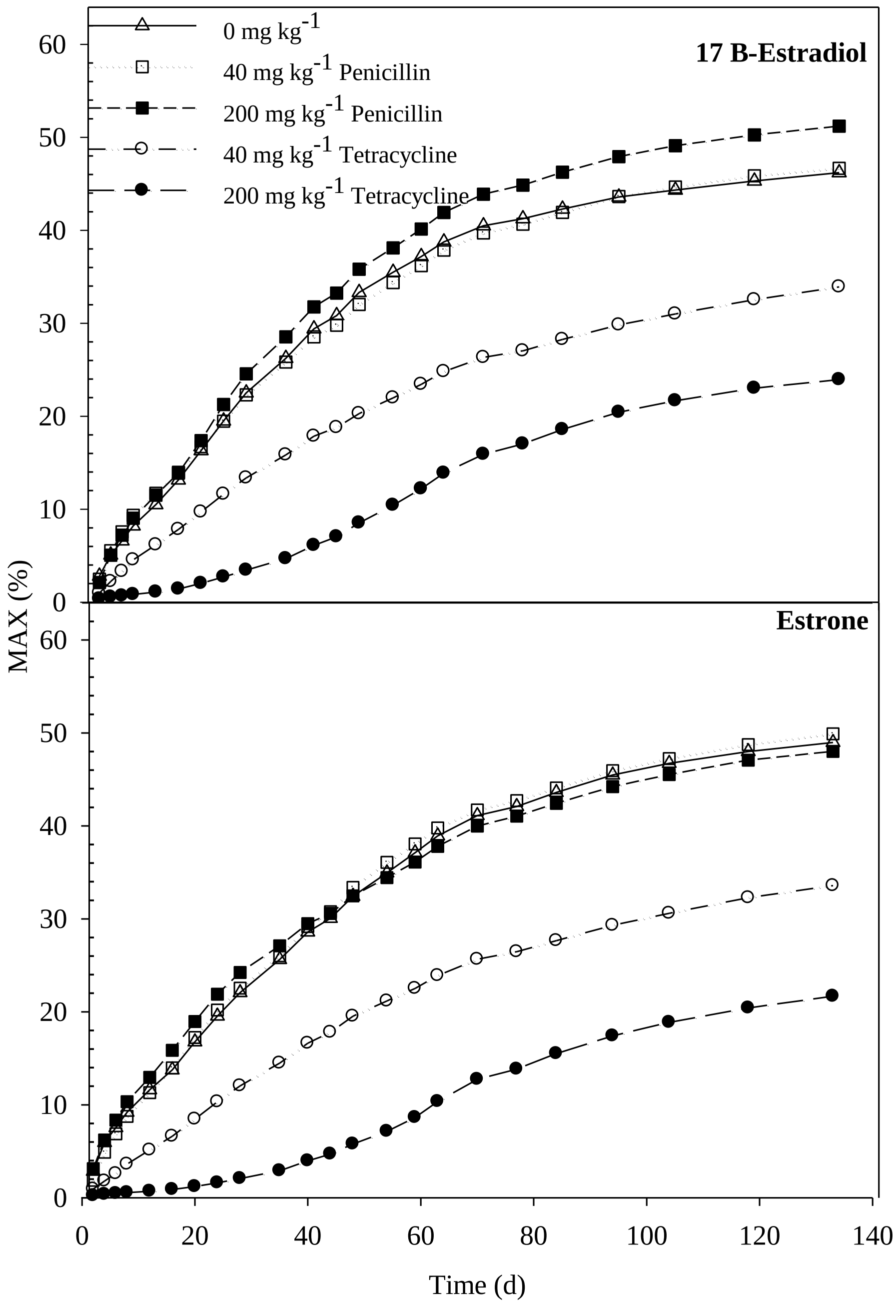

Figure 2.1 Estrone and 17 β-estradiol mineralization in liquid swine manure in the presence of penicillin or tetracycline.

The two-way interactions between media and antibiotic and media and estrogen were significant (Table 2.2). E1 and E2 MAX were statistically similar except in soils amended with a high rate of manure in which E1 MAX < E2 MAX (Figure 2.2). For all antibiotic treatments except for the 200 mg kg^{-1} tetracycline, estrogen mineralization decreased in the following order: manure > soil amended with a low rate of manure = soil > soil amended with a high rate of manure (Figure 2.3), suggesting that the periodically agitated manure was a more favorable medium for biotic removal of estrogens than soil. In the follow up experiment using manure collected from the same sows, the frequency of agitation (every 3-4 d, every 14 d, every 28 d) had no significant impact on E1 or E2 MAX in manure, but E1 and E2 MAX were significantly greater when manure was agitated every 3-4 d, relative to E1 and E2 MAX in manure not agitated. It is likely that agitation increased the breakdown of solids as well as improved contact between microbes, extra-cellular enzymes and solids, thus increasing bioavailability of estrogens and biological activity in the liquid swine manure sample (Pinho et al. 2004).

Table 2.2 Three-way ANOVA, P < 0.05 with factors: medium, estrogen, antibiotic.

Effect	Num DF	Den DF	F Value	Pr > F
Medium	3	89.4	318.84	<.0001
Estrogen	1	30.4	4.3	0.0468
Antibiotic	4	30.4	9.51	<.0001
Medium x Estrogen	3	89.4	5.71	0.0013
Medium x Antibiotic	12	89.3	11.61	<.0001
Estrogen x Antibiotic	4	30.4	0.24	0.9113
Medium x Estrogen x Antibiotic	12	89.3	0.90	0.5522

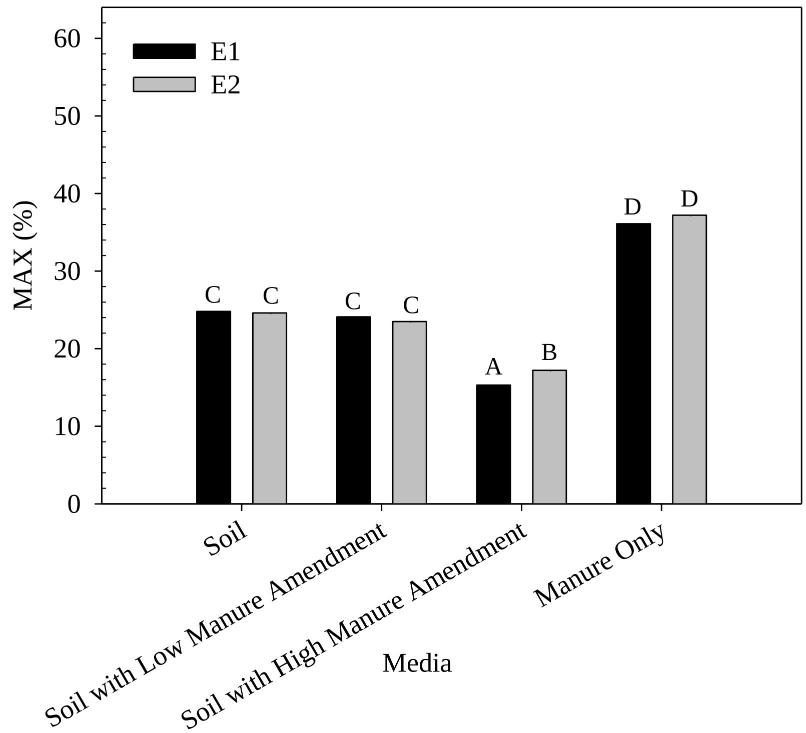

Figure 2.2 Maximum mineralization of estrone (E1) and 17 β-estradiol (E2) in soil, soil with low manure amendment, soil with high manure amendment and manure only.

The addition of 40 and 200 mg kg^{-1} tetracycline to manure decreased E1 and E2 mineralization in manure throughout the duration of the experiment (Figure 2.1). The impact of tetracycline in reducing estrogen mineralization was most pronounced for manure containing 200 mg kg^{-1} tetracycline since the treatment showed significantly lesser estrogen mineralization at 133 d, relative to manure without antibiotics (Figure 2.3). Relative to manure free of antibiotics, manure with 40 and 200 mg kg^{-1} tetracycline had 1.4 and 2.1 times lesser estrogen mineralization at 133 d.

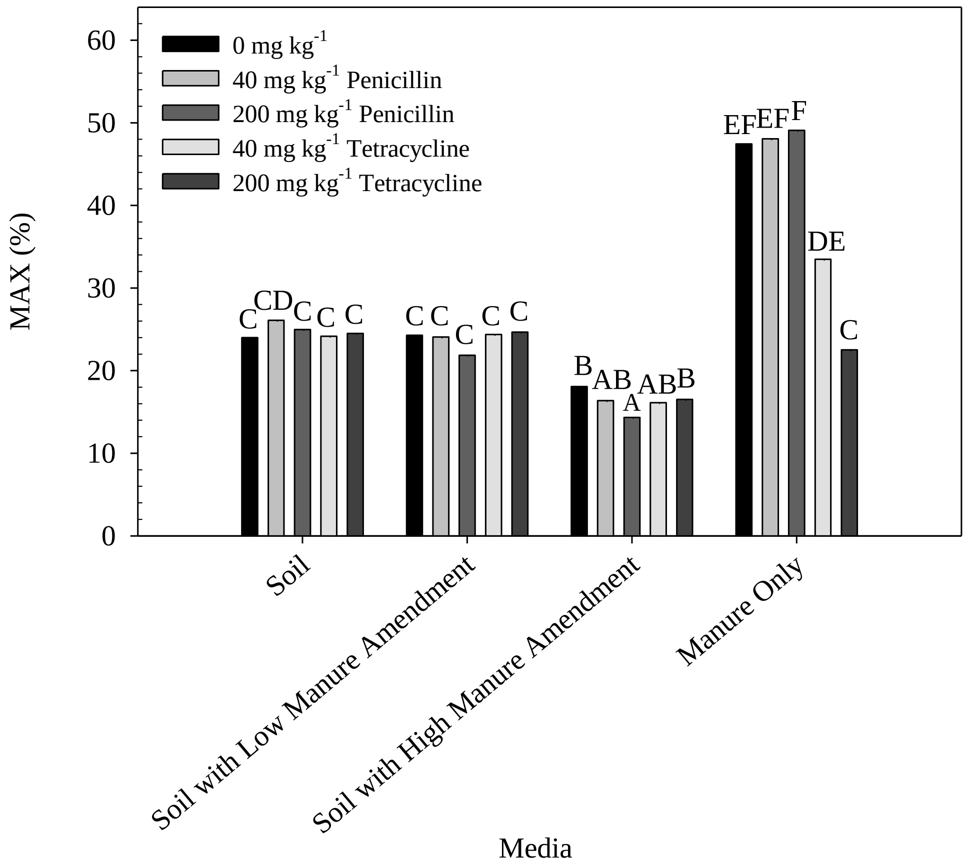

Figure 2.3: Maximum estrogen mineralization in soil, soil with low manure amendment, soil with high manure amendment and manure only in the presence of absence of penicillin or tetracycline.

Relative to manure free of antibiotics, neither rate of penicillin additions to manure had a significant effect on E1 and E2 MAX. The greater impact of tetracycline than penicillin on estrogen degrading microorganisms is likely because tetracycline is relatively persistent while the β-lactam ring of penicillin is readily cleaved (Thiele-Bruhn 2003). It is also possible that the estrogen degrading microorganisms in manure are more susceptible to tetracycline than to penicillin, or that a greater portion of the microorganisms were resistant to penicillin than tetracycline. Resistance to antibiotics has been shown to develop rapidly in swine, for example, a study demonstrated that more than 70% of fecal bacteria of nursery pigs were resistant to a range of antibiotics within three weeks of feeding (Kumar et al. 2005). The Berkshire sows at Zinn Farms had not been exposed to prophylactic antibiotics, but some animals have been administered therapeutic antibiotics, while naturally occurring antibiotics may also contribute to low levels of resistance in animals not treated with synthetic antibiotics (Docic and Bilkei 2003). In addition, resistance can be passed from generation to generation. For example, in a swine herd that had not been exposed to sub-therapeutic, prophylactic, or therapeutic antibiotics for more than 10 years, Langlois et al. (1988) found that between < 1% to 64% of fecal coliforms tested positive to be resistant for a wide range of antibiotic agents.

Unlike the striking impact of tetracycline on estrogen mineralization in manure, tetracycline additions had no significant impact on E1 and E2 MAX values in soil (Figure 3). It was recently reported that the bioactivity of some antibiotics in soil is reduced because of sorption (Thiele-Bruhn 2003; Subbiah et al. 2011). For example tetracycline bioactivity was minimal in a silt-loam and sandy-loam soil, but not in the sandy soil which had lesser sorption (Subbiah et al. 2011). The Newdale soil was a clay loam with a soil organic carbon content of 3.2% and hence tetracycline sorption is expected to be relatively strong (Subbiah et al. 2011). Sorption of β-lactam antibiotics to soil is generally weaker than tetracycline and bioactivity is greater (Subbiah et al. 2011).

Regardless of whether antibiotics were applied or not, soils amended with a high rate of manure always demonstrated significantly lesser estrogen mineralization at 133 d than other media (Figure 2.3). Caron et al. (2012) found that addition of fresh beef cattle manure in the laboratory also reduced E2 mineralization rates. Liquid swine manure samples used in this study had approximately 100 and 500 times more phosphorous and nitrogen, respectively, than the Newdale soil (Table 2.1) potentially impacting the activity, size and composition of the soil microbial community for a period of time (Lucas and Jones 2006; Saison et al. 2006). Soils amended with a high manure rate and incubated with 40 and 200 mg kg^{-1} penicillin or tetracycline demonstrated throughout the experiment numerically smaller E1 and E2 mineralization rates than the same soil free of antibiotics (Figure 2.4). Since this was not observed in soil free of manure, these results suggest that the ability of antibiotics to decrease the functionality of estrogen degrading soil microorganisms may be increased at greater nutrient levels. Penicillin in manure-amended soils had a greater impact on estrogen degrading microorganisms than tetracycline (Figure 2.4). In soils with a high rate of manure, the 200 mg kg^{-1} penicillin treatment significantly reduced MAX by a factor of 1.3 relative to soils free of antibiotics (Figure 2.3). In addition, in soils with a low rate of manure, the 200 mg kg^{-1} penicillin treatment demonstrated throughout the experiment numerically lesser E1 and E2 mineralization rates than any other antibiotic treatment (Figure 2.4).

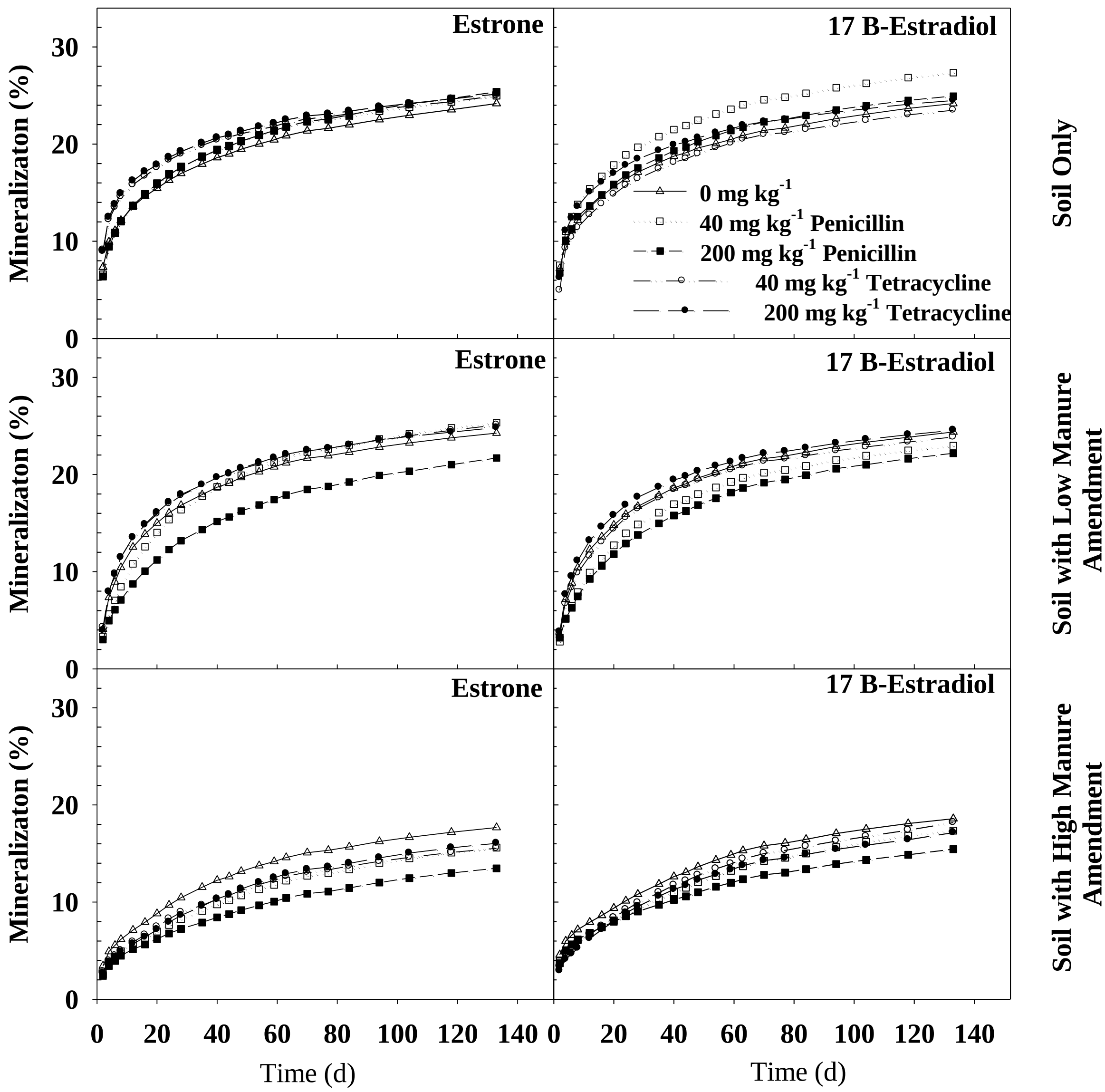

Figure 2.4: Estrone and 17 β-estradiol mineralization in soil, soil with low manure amendment, and soil with high manure amendment in the presence of penicillin or tetracycline.

2.5 Conclusion

This is the first study to quantify E1 and E2 mineralization in media as influenced by the presence of penicillin or tetracycline in liquid swine manure, soil and manure-amended soil. While manure that is periodically agitated was a more favorable media for estrogen mineralization than soil, soil amended with the highest rates of manure additions showed lesser total estrogen mineralization than soils amended with the low rate of manure and non-amended soils. While both rates of tetracycline numerically decreased MAX, only the high rate had a significant effect relative to manure free of antibiotics. Tetracycline additions had no significant effect on MAX in the silt loam soil, possibly due to greater sorption. Throughout the experiment, soils amended with a high rate of manure and incubated with 40 and 200 mg kg^{-1} penicillin or tetracycline demonstrated numerically smaller E1 and E2 mineralization rates than the same soil free of antibiotics, suggesting a positive correlation between bioactivity of antibiotics and nutrient levels in the media. This study concludes that storage of liquid swine manure with periodic agitation for 3 to 4 months prior to application to agricultural land may reduce the potential for estrogens to enter the aquatic environment via runoff and leaching of soils, but that biological removal of estrogens through mineralization may be inhibited by the presence of some veterinary antibiotics particularly when manure applied at a high rate or not incorporated into the soil.

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3. 17 β-ESTRADIOL AND 17 α-ETHINYLESTRADIOL MINERALIZATION IN SEWAGE SLUDGE AND BIOSOLIDS

3.1 Abstract

Natural steroid estrogens (e.g., 17 β-estradiol, E2), synthetic steroid estrogens (e.g., 17 α-ethinylestradiol, EE2) and pharmaceutical antibiotics (e.g., ciprofloxacin) are chemicals detected in biosolids and sewage sludges because they partition to the solids fraction during the wastewater treatment process. This research utilized a three-way factorial design (6 media x 2 estrogens x 3 antibiotic treatments) to quantify cumulative E2 and EE2 mineralization (MAX) in a range of sewage sludge and biosolid samples in the presence (4 and 40 mg kg^{-1}) and absence of ciprofloxacin. The same three-way factorial design was utilized to quantify the impact of the six media, E2 or EE2, and ciprofloxacin on cumulative soil respiration over time (RESP). MAX at 133 d ranged from 38.38% to 48.44% for E2 but from only 0.72% to 24.27% for EE2 even though RESP at 133 d was relatively similar, ranging from 101.00 to 866.54 mg CO_2 in E2 and from 69.55 to 893.95 mg CO_2 in EE2. Minimal ciprofloxacin mineralization was observed (<0.05% over 133 d), but despite its persistence, no significant effect of ciprofloxacin addition on E2 or EE2 MAX was observed. Ciprofloxacin had no significant effect on RESP either, except that composted biosolids demonstrated increased RESP upon the addition of 4 or 40 mg kg^{-1} in some cases. Sorption limited EE2 bioavailability and biodegradation because EE2 MAX and EE2 Freundlich sorption constants (K_f) were negatively associated. EE2 MAX was significantly greater in secondary sludges than primary sludges, while EE2 K_f values

were significantly greater in primary sludges than secondary sludges. EE2 K_f values were largest in composted biosolids in which EE2 was particularly resistant to microbial degradation with EE2 MAX (<3%) being near the cumulative mineralization observed in sterile silica sand (<1%). In contrast, E2 MAX showed a positive association with E2 K_f because some steps in the E2 transformation process have been found to occur in the sorbed phase, and E2 MAX was significantly larger in biosolids than sewage sludges. Considering the impact of estrogen sorption as well as the physical and chemical parameters of the media, the results of this study suggest that sewage sludges in municipal lagoons and pre-treatment holding lagoons are a more favourable media for mineralization of EE2, while biosolids in post-treatment storage lagoons are a more favourable media for mineralization of E2. The presence of ciprofloxacin will have no impact on the potential E2 or EE2 mineralization rates in these cases.

3.2 Introduction

On a global scale, humans excrete annually an estimated 720 kg of synthetic steroid estrogens (e.g., 17 α-ethinylestradiol) and 29,500 kg of natural steroid estrogens (e.g., 17 β-estradiol) (Combalbert and Hernandez-Raquet 2010). During the wastewater treatment process, sorption to solids is an important pathway by which estrogens are removed from wastewater (Johnson and Sumpter 2001; Andersen et al. 2003), particularly for 17 α-ethinylestradiol (EE2) and 17 β-estradiol (E2) (Holbrook et al. 2002). Consequently, EE2 and E2 have been detected in sewage sludge and biosolids at concentrations ranging from 0.4 to 18 μg kg^{-1} for EE2 and from 0.2 to 49 μg kg^{-1} for E2 (Ternes et al. 2002; Braga et al. 2005*a*; Braga et al. 2005*b;* Muller et al. 2008; Andaluri et al. 2012). Approximately 30% of biosolids produced in Canada are applied to agricultural land as an organic amendment (Weber et al. 2006). The potential movement of estrogens from agricultural land to surface water by runoff has raised concerns (Nichols et al. 1997; Finlay-Moore et al. 2000; Kjaer et al. 2007). For example, increased vitellogenin synthesis has been observed in male rainbow trout exposed to environmentally-relevant concentration as low as 0.1 ng L^{-1} and 1.0 ng L^{-1} of EE2 and E2, respectively (Routledge et al. 1998).

Antibiotics have also been detected in sewage sludge and biosolids (Kumar 2005; Lindberg 2005). Ciprofloxacin is a fluoroquinolone antibiotic that is commonly administered to humans in treating urinary tract and intestinal infections. A human excretes about 90% of the administered ciprofloxacin in an unaltered form (Girardi et al. 2011). Ciprofloxacin is strongly sorbed to solids during the wastewater treatment process (Picó and Andreu 2007) and has been detected in sewage sludge at 102 to 405 ng L^1 and in biosolids at 0.5 to 4.8 mg kg^{-1} (Lindberg et al. 2005). Ciprofloxacin impacts both gram-positive and gram-negative bacteria and when added to soil at 20 mg kg^{-1}, ciprofloxacin has been shown to inhibit soil respiration (release of CO_2) by 70% after 2 d, and by 35% after 77 d (Girardi et al. 2011). Ciprofloxacin in soil is known to be transformed by abiotic reactions resulting in three degradation products (Wetzstein et al. 1999; Girardi et al. 2011). Ciprofloxacin and its degradation products displayed a similar antimicrobial activity in soil interstitial water and activated sludge water (Halling-Sorensen et al. 2003). Ciprofloxacin mineralization (release of $^{14}CO_2$) has been shown to be negligible when ^{14}C-ciprofloxacin was added to soil incubated for 84 or 93 d (Girardi et al. 2011, Mougin et al. 2013). No ciprofloxacin mineralization was observed in biosolid samples incubated for 77 d (Chenxi et al. 2008).

The antibiotics triclosan, tetracycline and penicillin have been shown to reduce E1 and E2 mineralization in liquid swine manure and soils (Shareef et al. 2009; Rose and Farenhorst 2014). E2 and EE2 mineralization has been examined in biosolids (Layton et al. 2000), but not in combination with antibiotics. In recent publications, the need for additional experimental work on estrogen fate in sewage sludge and biosolids was highlighted (Combalbert and Hernandez-Raquet 2010; Andaluri et al. 2012). The objective of this study was to quantify E2 and EE2 mineralization in a range of sewage sludge and biosolid samples in the presence or absence of ciprofloxacin.

3.3 Materials and Methods

3.3.1 Chemicals

The experiments used analytical grade 17 β-estradiol (98% pure), 17 α-ethinylestradiol (98% pure) and ciprofloxacin (≥98% pure) from Sigma-Aldrich Chemical Company, Saint Louis, MO; 17 β-estradiol [4-^{14}C] (99% radiochemical purity, specific activity 45-60 mCi mmol^{-1}) and 17 α-ethinylestradiol [4-^{14}C] (99% radiochemical purity, specific activity 50-60 mCi mmol^{-1}) from American Radiolabeled Chemicals Inc., Saint Louis, MO; and ciprofloxacin [2-^{14}C] (97% radiochemical purity, specific activity 10-15 mCi mmol^{-1}) from Moravek Biochemicals and Radiochemicals, Brea, CA.

3.3.2 Sewage Sludge and Biosolid Samples

Six media (Table 3.1) were collected in September 2012. The biosolid and composted biosolid media were obtained from a wastewater treatment plant in central Alberta that treats more than 100,000 million litres of wastewater annually. Treatment processes, to transform untreated sewage sludge into biosolids that can be applied to agricultural land, include pre-treatment screening, enhanced primary treatment, primary treatment clarifying, solids settling, fermentation, and anaerobic digestion. Following treatment, it is often necessary to store biosolids in storage lagoons before they are disposed of such as through application on agricultural land. In the case of composted biosolids, residential solid waste is combined with biosolids and composted indoors for 14 to 21 days and cured outdoors for 4 to 6 months. Composting is used to further degrade organic contaminants in biosolids before application to agricultural soils (Xia et al. 2005), although the effectiveness of this process is unclear (Girardi et al. 2011).

Table 3.1 Select physical and chemical parameters of sewage sludge and biosolid samples.

Parameter	Primary 1	Primary 2	Secondary 1	Secondary 2	Biosolid	Compost
% Moisture[1]	62.9	55.7	39.0	43.6	77.0	39.4
pH[2]	8.02	7.47	8.37	8.30	7.40	6.11
Total N[3]	8.80	7.68	2.75	3.55	23.3	25.5
Total C[4]	92	121	48	31	164	286
NH_4-N[5]	1.19	1.03	0.35	0.71	6.50	5.67

[1]Determined gravimetrically. [2]1:1 solution. [3]LECO model CHN 600 Carbon-Hydrogen-Nitrogen Determinator. [4]Loss on ignition method. [5]Steam distillation into boric acid followed by titration with standard acid.

The four sewage sludge media were obtained from two different facultative lagoon-based sewage treatment facilities in southern Manitoba that service approximately 2000 and

8000 people each. Treatment processes, including sedimentation, bioflocculation, chemical precipitation, biochemical oxidation, fermentation and disinfection, occur simultaneously within these lagoon-based sewage treatment facilities. At both facilities, grab samples were taken from a primary stabilization cell that receives raw sewage and from a secondary stabilization cell that receives the discharge from the primary cell. Samples were taken from the outer edge of each lagoon and mixed thoroughly. Facultative lagoons are typically up to 1.5 m deep and consist of an anaerobic layer of sewage sludge below an aerobic layer of liquid (National Guide to Sustainable Municipal Infrastructure 2004). Due to the accumulation of sludge in primary and secondary cells, desludging may be required every 5 to 20 years (National Guide to Sustainable Municipal Infrastructure 2004). If the series of stabilization cells has not received raw sewage in the past three months, the quality of the sludge is generally equal to or exceeding that of a biosolid from a wastewater treatment plant and can potentially be applied to agricultural land as an organic amendment (National Guide to Sustainable Municipal Infrastructure 2004). However, all sewage sludge samples used in this study were taken from series of stabilization cells that were currently receiving raw sewage.

3.3.3 Properties of Samples

Media were commercially analyzed (Alberta Innovates Technology Futures, Edmonton, AB) for background levels of hormones and pharmaceuticals. Hormones analyzed for were E2, EE2, estriol, estrone, progesterone, and testosterone. Pharmaceuticals were acetaminophen, benzoylecgonine, chloramphenicol, ciprofloxacin, clindamycin, cocaine, codeine, cotinine, enrofloxacin, erythromycin, fluoxetine, lincomycin, methamphetamine, norfloxacin, norfluoxetine, ofloxacin, oxolinic acid, pentoxifylline, pipemidic acid, sulfabenzamide, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfapyridine, sulfaquinoxaline, sulfathiazole and trimethoprim. Detection limits ranged from 0.00 to 0.10 ng g^{-1} for hormones and from 0.2 to 5.0 ng g^{-1} for pharmaceuticals.

Media was also analyzed for a range of physical and chemical parameters (Table 3.1) as well as for the sorption of E2 and EE2 by batch equilibrium experiments. For the sorption determinations, initial concentrations of the E2 or EE2 solutions (prepared in 0.01 M $CaCl_2$) were 1000, 2000, 4000, 8000 and 16000 µg L^{-1}. The solutions also contained 17 β-estradiol [4-^{14}C] or 17 α-ethinylestradiol [4-^{14}C] in the amount of 8000, 16000, 32000, 64000 and 132000 Bq L^{-1}, respectively. All equipment and media were autoclaved at 121° C for 30 minutes before use. Solutions (10 mL 0.01 M $CaCl_2$) were added to media (5 g) in glass tubes in triplicates which were rotated for 24 h at 5 °C in the dark and then centrifuged for 60 min at 7000 rpm. Supernatant (1 mL) was added to scintillation vials (duplicates) with 5 mL of scintillation cocktail (30% Scintisafe scintillation cocktail; Fisher Scientific, Fairlawn, NJ) to determine radioactivity remaining in the equilibrium solution by Liquid Scintillation Counting (LS 7500 Beckman Instruments, Fullerton, CA) with automated quench correction (#C Method) and a maximum counting time of 10 min. The Freundlich sorption constant, K_f (μg^{1-N} g^{-1} mL^N), was calculated by Cs/Ce^N where Cs= the amount of estrogen sorbed by the media (µg kg^{-1}), Ce= the estrogen concentration in the equilibrium solution (µg mL^{-1}) and N= the Freundlich slope (1/n).

3.3.4 Experimental Design

Microcosm experiments were conducted under a constant 20˚C temperature in the dark as previously described (Rose and Farenhorst 2014). The impact of ciprofloxacin on [4-^{14}C] E2 and [4-^{14}C] EE2 mineralization was quantified using a three-way factorial design (6 media x 2 estrogens x 3 antibiotic treatments). The experiment was conducted for 133 days at which time mineralization had slowed to <0.20% per day for al treatments and the experiment was terminated. In a parallel experiment, only analytical grade chemicals were applied, but the same factorial design was utilized to quantify cumulative soil respiration over 133 days. In addition, a two-way factorial experiment (6 media x 3 antibiotic concentrations) was used to determine the impact of media on [2-^{14}C] ciprofloxacin mineralization over 133 days. No estrogens were used in the two-way factorial experiment. In both experiments, there were four replicates per treatment.

Microcosms consisted of a 500 mL Mason jar containing a 90 mL glass beaker with 25 g sewage sludge or biosolid samples (wet weight), a 20 mL scintillation vial with 5 mL of 0.5 M NaOH and a 15 mL test tube with 3 mL of acidified water (pH≤3) to preserve a humid environment without trapping CO_2. Each experiment also included microcosm controls consisting of autoclaved and non-autoclaved silica sand. In addition, a microcosm containing only the NaOH trap and no media was included in the respiration study to quantify background levels of CO_2.

Microcosms were pre-incubated at 20˚C for a total of 9 days. Ciprofloxacin stock solutions consisted of 0.1 g and 1.0 g L^{-1} ciprofloxacin dissolved in deionized water acidified with 0.5 M HCl (pH 4.1). Flasks were wrapped in tin foil to minimize photolysis and solutions were used within 2 hours after preparation. Ciprofloxacin solutions (1 mL) were added after 168 h to achieve a concentration within the range reported for biosolids (4 mg kg^{-1}) (Lindberg et al. 2005), and at a ten times greater concentration. Due to the addition of antibiotic solution (pH 4.1), the pH decreased by 0.2 in the composted biosolid and primary-1 sewage sludge sample, by 0.3 in the biosolid, by 0.4 in the secondary-1 sewage sludge sample, and by 0.5 in the primary-2 and secondary-2 sewage sludge samples. For the treatments not receiving antibiotics, 1 mL acidified water (pH 4.1) was added to the media to ensure similar decreases in pH. Estrogen stock solutions were prepared at concentrations of 2500 µg L^{-1} E2 or EE2 and contained 66,680 Bq ^{14}C-estrogen per kg media. Estrogens (0.5 mL) were applied to microcosms to achieve a concentration of 50 µg kg^{-1}. This estrogen concentration is that used in soil microcosm experiments by Caron et al. (Caron et al. 2012) and is within the mid-range of the concentrations used by Casey et al. (2003). [4-^{14}C] ciprofloxacin solutions were prepared at concentrations of 0.1 mg kg^{-1} ciprofloxacin and 66,680 Bq ^{14}C-ciprofloxacin per kg media and applied to microcosms to achieve a concentration of 4 mg kg^{-1}, the concentrations found in biosolids (Lindberg et al. 2005).

NaOH traps were removed and replaced every 2 to 4 days for the first two weeks, every 4 to 7 days until week 13, and every 10 to 15 days until completion of the experiment at 133 days. $^{14}CO_2$ in traps was quantified by adding scintillation cocktail (8 mL) to the removed vials and measuring radioactivity by LSC as described for the sorption experiment above. CO_2 in traps was quantified by titrating with 0.5 M HCl to two final endpoints (pH=8.3 and 3.7) using the T50 Titrator (Mettler Toledo, Mississauga, ON). Mineralization data typically followed first-order kinetics with the goodness of fit ranging from 0.96 to 1.0 for EE2 MAX but from 0.68 to 1.0 for E2 MAX. Respiration data followed first-order kinetics with the goodness of fit ranging from 0.96 to 1.0 for both estrogens.

3.3.5 Statistical Analysis

MAX, RESP and transformed K_f ($x^{-0.3}$) respected normality (Shapiro-Wilks statistic ≥ 0.90) and equality of variance (2002-2010, Windows version 9.3, SAS Institute, Cary, NC). Analysis of variance were conducted on these parameters in SAS (2002-2010, Windows version 9.3, SAS Institute, Cary, NC) with the Tukey-Kramer HSD multiple comparison test ($P<0.05$) applied for multiple comparison. Principal component analysis (PCA) was used to identify the most discriminatory effects of moisture content, pH, total nitrogen (TN), ammonium nitrogen (NH_4-N), total carbon (TC), K_f (Table 3.1) and RESP on E2 and EE2 MAX. These parameters were selected because they have been previously included in studies quantifying estrogen degradation in a range of media (Vader et al. 2000; Shi et al. 2004; Stumpe and Marschner 2009; Caron et al. 2012).

3.4 Results and Discussion

Hormones were below detection limits in all samples. Pharmaceuticals detected were fluoxetine (8.0 ng g^{-1}) and norfluoxetine (4.7 ng g^{-1}) in the composted biosolid, and cotinine in biosolid (26.8 ng g^{-1}) and primary-1 sewage sludge (7.3 ng g^{-1}). Although the detections of pharmaceuticals were most pronounced in the composted biosolid and biosolid, these media demonstrated significantly larger RESP than the primary and secondary sewage sludge samples (Figure 3.1). Fluoxetine is an antidepressant and cotinine is a metabolite of nicotine. Fluoxetine and its metabolite norfluoxetine are likely to enter the terrestrial environment through application of biosolids (Oakes et al. 2010) but have been reported to have low toxicity in bacteria (Richards et al. 2004). In a study of river sediments, nicotine inhibited microbial respiration (Bunch and Bernot 2010) but the impact of cotinine on microbial communities is unknown.

E2 MAX was greater than EE2 MAX in all media (Figure 3.2). MAX ranged from 38.38% to 48.44% for E2 and from only 0.72% to 24.27% for EE2 (Figure 3.2) even though RESP was relatively similar, ranging from 101.00 to 866.54 mg CO_2 in E2 and from 69.55 to 893.95 mg CO_2 in EE2 (Figure 3.1). EE2 and E2 are structurally comparable, except that EE2 has an ethinyl group which makes the synthetic steroid hormone relatively resistant to microbial degradation (de Mes et al. 2005; Ying and Kookana 2005; Weber et al. 2005). It is for this reason that EE2 is more slowly mineralized than E2 in soils, sewage sludges and biosolids (Layton et al. 2000, Stumpe and Marschner 2009, Ternes et al. 1999).

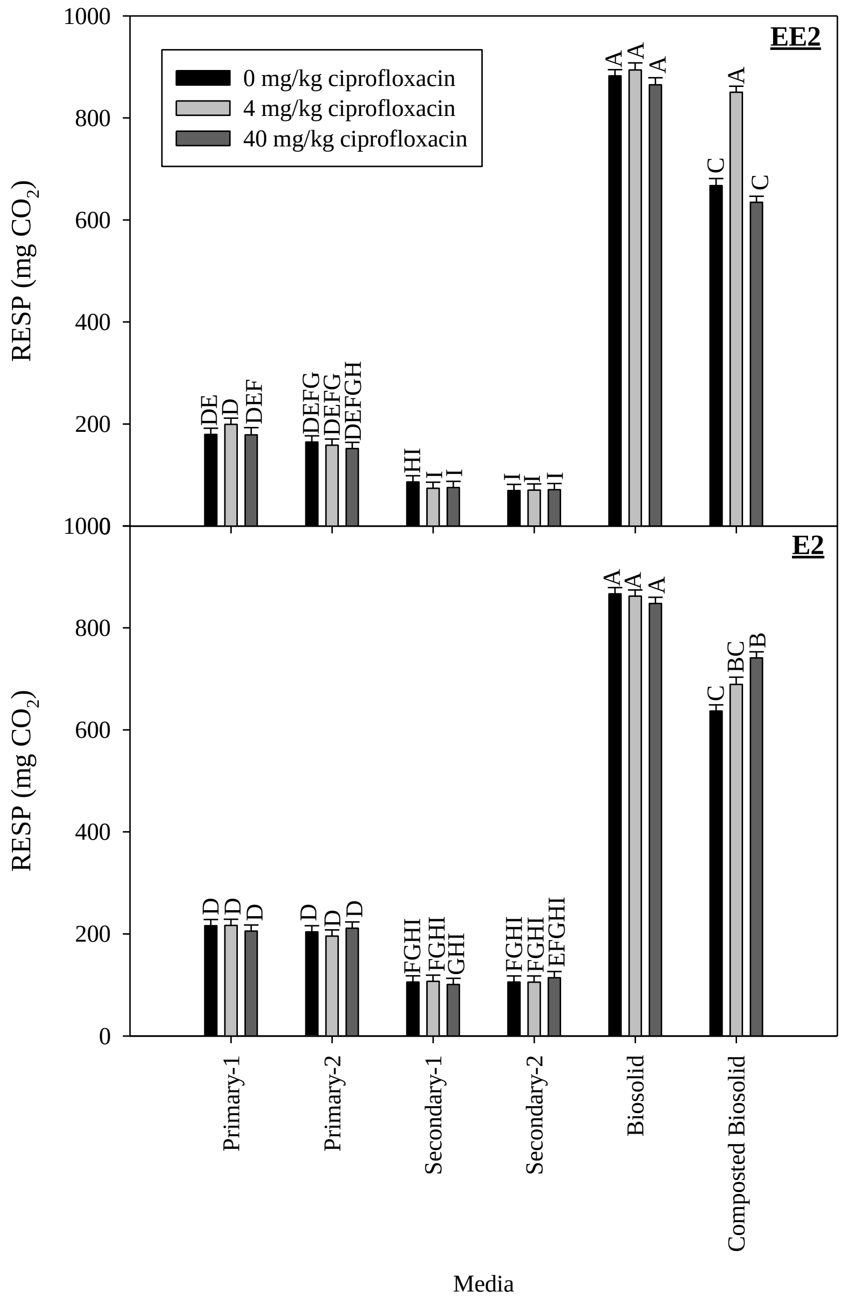

Figure 3.1 Maximum respiration in sewage sludges and biosolids in the presence of 17 β-estradiol (E2) or 17 α-ethinylestradiol (EE2), with three rates of ciprofloxacin addition.

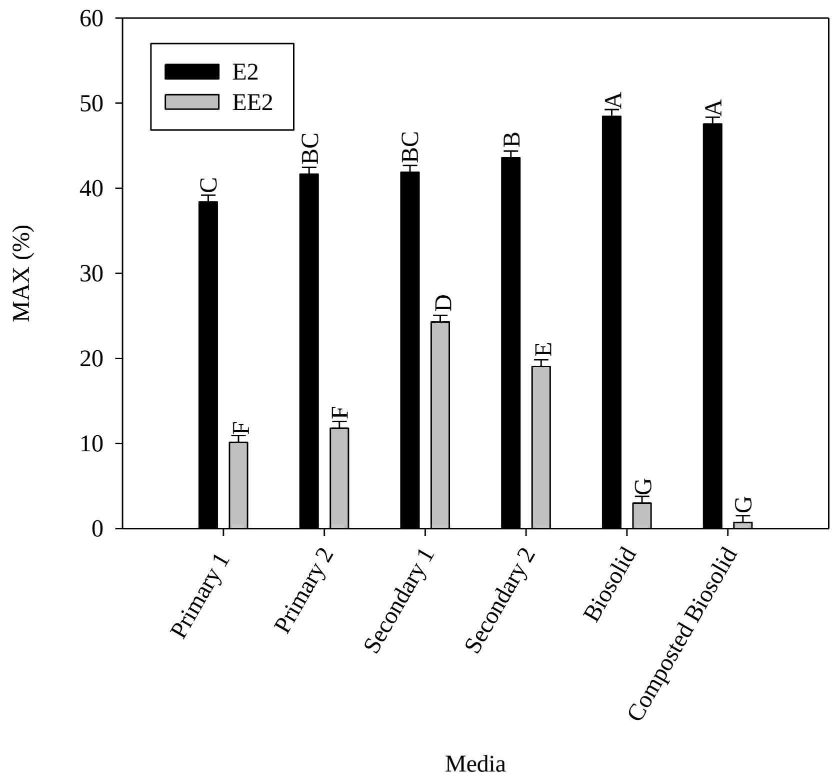

Figure 3.2 Maximum mineralization of 17 β-estradiol (E2) and 17 α-ethinylestradiol (EE2) in sewage sludges and biosolids.

The three way interaction (media x estrogen x antibiotic) was highly significant ($P<0.0001$) for RESP (Table 3.2). RESP significantly increased in the order of secondary sewage sludge < primary sewage sludge < composted biosolids < biosolids, except that for the EE2 treatment with 4 mg kg^{-1} ciprofloxacin, there were no significant differences in RESP between composted biosolids and biosolids. RESP was most strongly positively associated with TN, NH_4-N and Total C (Figure 3.3). For soils that had received various rates of beef cattle manure application for 35 years, Caron et al. (2012) also observed a positive association among microbial activity (measured by fluorescein diacetate hydrolysis assay), TN, NH_4-N and TC.

Table 3.2 Three-way ANOVA for maximum respiration (RESP), P < 0.01 with factors: medium, estrogen, antibiotic.

Effect	Num DF	Den DF	F Value	Pr > F
Medium	5	86.7	4718.99	<0.0001
Estrogen	1	18.6	9.67	0.0059
Antibiotic	5	86.7	9.88	0.0032
Medium x Estrogen	2	18.6	7.95	<0.0001
Medium x Antibiotic	10	86.7	7.92	<0.0001
Estrogen x Antibiotic	2	18.6	10.62	0.0008
Medium x Estrogen x Antibiotic	10	86.7	8.28	<0.0001

The interaction media x estrogen was highly significant (P<0.0001) for MAX (Table 3.3). E2 MAX was significantly greater in biosolid = composted biosolid than in sewage sludges (Figure 3.2). In contrast, EE2 MAX significantly decreased in the order of secondary-1 > secondary-2 > primary-2 = primary-1 > biosolid = composted biosolid (Figure 3.2). E2 MAX was two to four times larger than EE2 MAX in sewage sludge samples, but the differences between E2 and EE2 MAX were up to 66 times in biosolids (Figure 3.2). EE2 MAX was <3% in biosolids, close to the total mineralization observed in silica sand (<1.4%) and autoclaved silica sand (<1%), indicating that EE2 was particularly resistant to microbial degradation in composted biosolids and biosolids. EE2 K_f values were significantly (P <0.01) greater than E2 K_f values (Table 3.4), which is in agreement with that observed in soils (Ying and Kookana 2005). There was a negative association between EE2 MAX and EE2 sorption (Figure 3.3, EE2), suggesting that sorption may have limited EE2 bioavailability and biodegradation. EE2 K_f values were largest for composted biosolids (Figure 3.4) that had the smallest EE2 MAX (Figure 3.2) despite the relatively large RESP in this treatment (Figure 3.1). For soils (Lee et al. 2003), dissolved organic matter surrogates (Yamamoto et al. 2003) and activated sludge (Weber et al. 2005), other studies also observed greater persistence of EE2 than E2 and attributed this to reduced bioavailability of EE2 due to sorption to solid particles. E2 MAX showed a positive association with RESP, and as well as with E2 K_f (Figure 3.3, E2). The positive association between E2 MAX and E2 Kf values has been previously observed in soils (Casey et al. 2003; Caron et al. 2012) and biosolids (Layton et al. 2000) because some steps in the E2 transformation process occur in the sorbed phase.

Table 3.3 Three-way ANOVA of maximum mineralization (MAX), P < 0.01 with factors: medium, estrogen, antibiotic.

Effect	Num DF	Den DF	F Value	Pr > F
Medium	5	107	44.22	<0.0001
Estrogen	1	107	4876.36	<0.0001
Antibiotic	2	107	108.92	0.9206
Medium x Estrogen	5	107	0.08	<0.0001
Medium x Antibiotic	10	107	2.41	0.0127
Estrogen x Antibiotic	2	107	1.05	0.3527
Medium x Estrogen x Antibiotic	10	107	2.40	0.0129

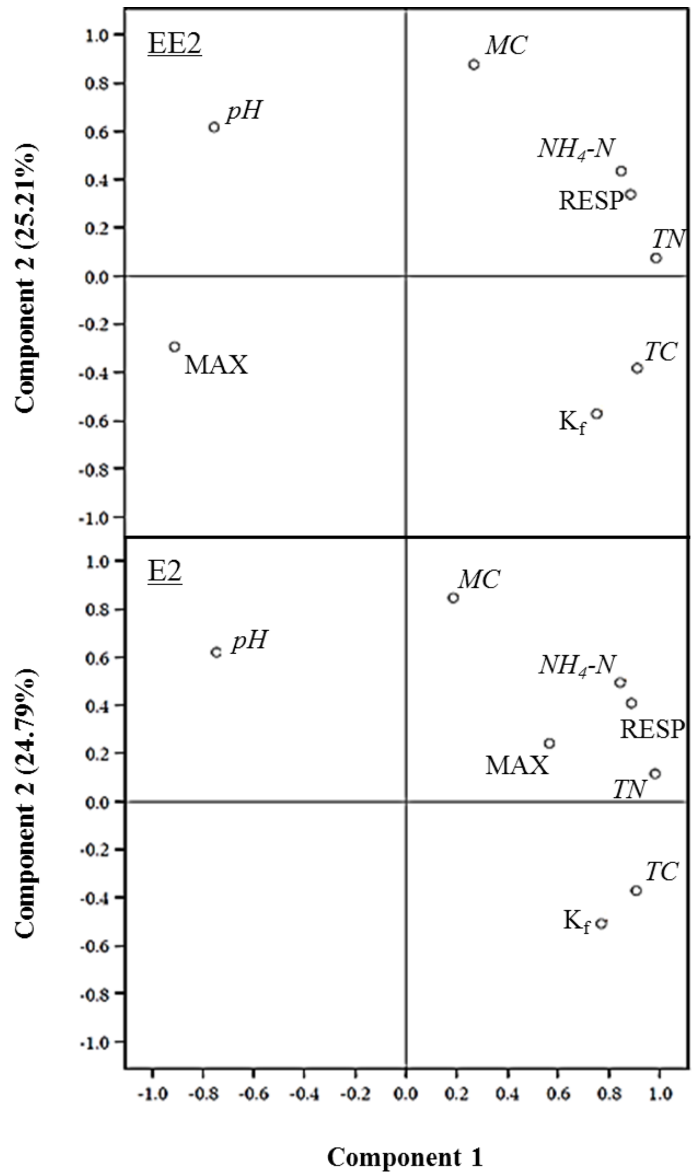

Figure 3.3 Principal component analysis of 17 β-estradiol (E2) and 17 α-ethinylestradiol (EE2) mineralization parameters constrained by select physical, chemical and biological parameters of the media. Independent variables (indicated by italics) include moisture content (MC), ammonium-nitrogen (NH_4-N), total nitrogen (TN) and total carbon (TC). For E2, 84.57% of the model was explained by Component 1 (59.78%) and Component 2. For EE2, 91.89% of the model was explained by Component 1 (66.68%) and Component 2.

Table 3.4 Two-way ANOVA for Freundlich sorption coefficient (K_f), P < 0.01 with factors: medium, estrogen.

Effect	Num DF	Den DF	F Value	Pr > F
Medium	5	24	12.52	<0.0001
Estrogen	1	24	8.28	0.0083
Medium x Estrogen	5	24	1.69	0.1745

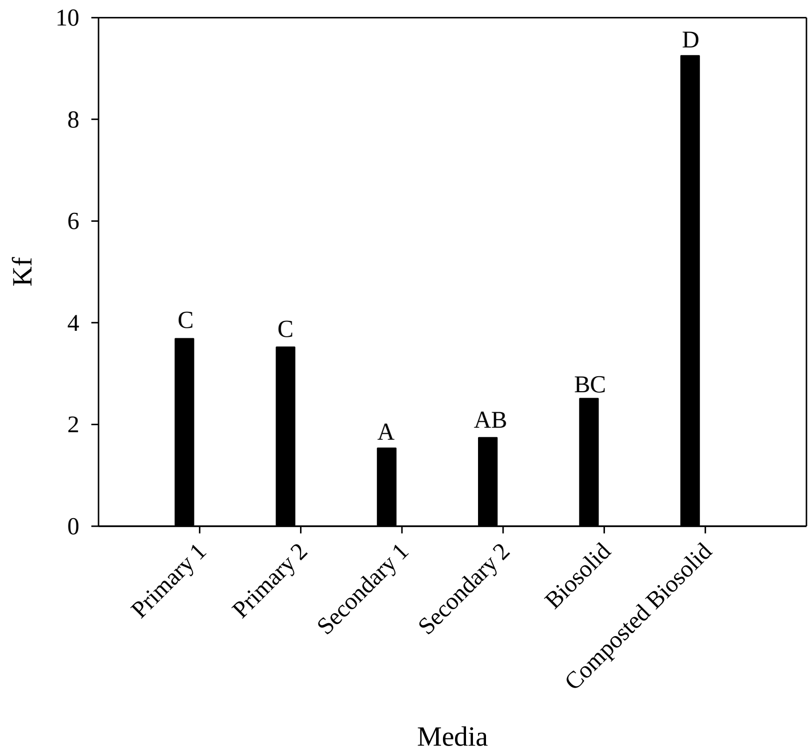

Figure 3.4 Back-transformed least squares means of Freundlich sorption coefficients (K_f) for sewage sludges and biosolid samples.

Ciprofloxacin MAX was negligible (<0.03%) in all media which is in agreement with other studies (Chenxi et al. 2008; Girardi et al. 2011; Mougin et al. 2013). Despite being persistent in each media as the parent molecule or transformation products, the ciprofloxacin addition had no significant effect on E2 or EE2 MAX (Table 3.3). Ciprofloxacin additions had also no significant effect on RESP in any media, except in the composted biosolid in which, relative to treatments free of ciprofloxacin, RESP significantly increased with the addition ciprofloxacin at 4 mg kg^{-1} (Figure 3.1, EE2) or 40 mg kg^{-1} (Figure 3.1, E2). Previous studies have reported inhibition of various soil microbes at ciprofloxacin concentrations as low as 2 mg kg^{-1} (Zhou et al. 2008) and of RESP at ciprofloxacin concentrations as low as 20 mg kg^{-1} (Girardi et al. 2011). Ciprofloxacin concentrations below 0.2 mg kg^{-1} have been shown to increase bacterial biomass in manure amended soils, suggesting that some soil microbes have the ability to use ciprofloxacin as a substrate at low concentrations (Zhou et al. 2008; Igel-Egalon et al. 2012). The reason for the increase in RESP observed in our study upon the addition of 4 and 40 mg kg^{-1} ciprofloxacin to composted biosolids is not known, but these observations were consistent among replicates.

3.5 Conclusion

Despite persistence in sewage sludge and biosolids throughout the duration of the experiment, no significant effect of ciprofloxacin (or its potential transformation products) on estrogen mineralization was observed. Regardless of media, E2 MAX was always greater than EE2 MAX, with up to 50% of E2 as compared to only 25% of EE2 being mineralized in the treatments examined. Differences between E2 MAX and EE2 MAX varied greatly with media type, with E2 MAX in biosolid samples being up to 66 times greater than that of EE2, as compared to only 2 to 4 times greater in sewage sludge samples. Biosolids showed significantly greater RESP than sewage sludge samples and hence the differences in the potential for E2 and EE2 mineralization are likely due to the inherent resistance to microbial degradation associated with the chemical structure of EE2. In addition, EE2 MAX was smallest in the composted biosolids that demonstrated the strongest sorption of EE2 and hence reduced bioavailability for mineralization, while E2 MAX was enhanced in this media with greater sorption and RESP because some steps in the E2 transformation process would have occurred in the sorbed phase. Considering the vast differences observed in estrogen mineralization between media types, it is valuable to recognize the stage of treatment at which estrogen removal can be maximized in the wastewater treatment process prior to the utilization of waste materials in agricultural land applications. Considering the impact of estrogen sorption as well as the physical and chemical parameters of the media, the results of this study suggest that there is a greater potential for EE2 mineralization in sewage sludges than biosolids, while the potential for E2 mineralization is greater in biosolids than sewage sludges.

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4. OVERALL SYNTHESIS

4.1 Summary of Research

Steroidal estrogens and antibiotics are introduced into the terrestrial environment through land application of organic amendments such as manure and biosolids. They have the potential to enter aquatic environments via runoff and erosion and become contaminants of concern. Steroid hormone contamination in rivers has been shown to have adverse effects on the secondary sexual characteristics of fish, including vitellogenin production in male fish (Lai et al. 2002). There is the potential for steroidal estrogens to be removed from organic amendments or soil by degradation processes such as estrogen mineralization. While degradation of estrogen is primarily related to microbial processes, antibiotics have been shown to decrease microbial activity in organic amendments and soils (Shareef et al. 2009). The impact of antibiotics on steroidal estrogen mineralization in organic amendments and soils has been quantified for a limited number of compounds and media.

Chapter 2 of this thesis focused on mineralization of estrone and 17 β-estradiol in liquid swine manure and soils in the presence and absence of penicillin or tetracycline. This research successfully identified liquid swine manure as a more favorable media for removal of estrogens than soil, particularly when manure was frequently agitated (every 3 to 4 days). Relative to treatments without antibiotics, maximum estrogen mineralization was decreased when tetracycline was present in liquid swine manure, and when penicillin was present in soils amended with a high rate of manure. These results suggest that both tetracycline and penicillin have the potential to reduce estrogen mineralization but that their actual impact is determined by the characteristics of the media.

In Chapter 3, the relationship between estrogen mineralization and the characteristics of the media was examined by quantifying maximum mineralization of 17 β-estradiol and 17 α-ethinylestradiol in sewage sludge and biosolid samples with vastly different properties. Total nitrogen, ammonium-nitrogen, and total carbon were identified as exhibiting a positive association with respiration and maximum mineralization of 17 β-estradiol but a negative association with maximum mineralization of 17 α-ethinylestradiol. As a result, biosolids were identified as a more favourable media for removal of 17 β-estradiol while raw sewage sludges were more favourable for 17 α-ethinylestradiol. Although ciprofloxacin was persistent throughout the duration of the study, additions had no significant inhibitory effect on the mineralization of either estrogens, suggesting that its bioavailability or bioactivity was minimal.

4.2 Practical Implications

Through identifying the environmental conditions and physical-chemical parameters of media that are most favourable for estrogen removal through mineralization, measures can be taken to potentially reduce the amount of steroidal hormones entering the environment via land application of organic amendments such as liquid swine manure and biosolids. Results of this thesis research indicate that steroidal estrogens can be removed or reduced by natural processes when organic amendments are stored or applied to soils.

In Chapter 2, the potential for estrogen removal through mineralization was greater in liquid swine manure with periodic agitation than in soils. However, the amendment of soils with relatively high rates of liquid swine manure reduced estrogen mineralization as compared to soils that had not received manure amendments. This suggests that steroidal estrogens should be removed during on-farm livestock manure storage prior to land application. Estrogen mineralization approached maximum limits between 90 and 133 days after being added to liquid swine manure, indicating that liquid swine manure should be stored for a minimum of 3 to 4 months prior to land application to maximize removal of estrogens through mineralization. In Manitoba, liquid swine manure storage facilities are generally emptied once or twice per year. As a result of Manitoba's Bill 217, which bans application of manures or biosolids from November 10 to April 10, a minimum storage period of 5 months during the winter is mandatory. Therefore, swine producers in Manitoba may already be implementing optimum practices with respect to storage periods for estrogen removal in on-farm storage facilities.

Agitation is a type of diffused-air aeration system commonly used in wastewater treatment process (Tchobanoglous et al. 2003). While aeration systems are commonplace in wastewater treatment systems, liquid swine manure is generally agitated prior to land application only. Aeration of liquid swine manure results in a reduction of odorous compounds due to oxidation (Van der Hoek 1977). Storage aeration also reduces the nitrogen concentration of liquid swine manures (Van der Hoek 1977). Although nitrogen is a beneficial plant nutrient, reductions in nitrogen content may beneficial in situations where the land base for manure application is limited. As this study indicates, aeration systems can also be used to optimize estrogen mineralization in on-farm manure handling systems.

Associations between physical-chemical parameters of the media and mineralization of 17 β-estradiol and 17 α-ethinylestradiol were successfully identified in Chapter 3. While maximum mineralization of 17 β-estradiol was significantly greater than 17 α-ethinylestradiol in all media, the difference was most pronounced in composted biosolids and biosolids. Since the estrogenicity of 17 α-ethinylestradiol is ten times greater than that of 17 β-estradiol, these results emphasize the importance of determining optimum conditions for bio-removal prior to applying biosolids on land. Based on the research, 17 α-ethinylestradiol has a greater mineralization potential in secondary sludges than in primary sludges, biosolids and composted biosolids.

However, the results of this thesis indicate that the presence of certain antibiotics may limit the potential for estrogen mineralization in media. The impact of antibiotics on estrogen degrading microbes is dependent on the chemical availability of the antibiotic as well as the susceptibility of the microorganism. Considering the observed reduction in estrogen mineralization of penicillin in soils and tetracycline in liquid swine manure, as well as the observed persistence of ciprofloxacin in sewage sludges and biosolids, there is significant cause for concern with regards to reduced microbial activity and developing resistance in the terrestrial and aquatic environments.

4.3 Recommendations for Further Studies

Organic wastes and amendments may contain a wide range of steroidal estrogens (Combalbert and Hernandez-Raquet 2010) as well as a wide range of veterinary and pharmaceutical antibiotics (Kumar et al. 2005; Lindberg 2005). Previous studies have investigated estrogen (Caron et al. 2012; Lucas and Jones 2006) or antibiotic fate (Aga et al. 2005; Al-Rajab et al. 2009) in a range of organic amendments, but not the interaction between estrogens and antibiotics in these amendments. This research indicates that the impact of antibiotics on estrogen mineralization depends on the type of organic amendment as well as the amount of antibiotic present. This research was limited in that it only examined the impact of a single antibiotic on estrogen mineralization and not the presence of multiple antibiotics which is more realistic in manure, sewage sludge and biosolid samples (Kumar et al. 2005; Lindberg 2005). There are a wide range of possible antibiotics to include in such studies, but future studies are best to include antibiotics that are (a) highly relevant to both veterinary and human medicine, and (b) commonly detected in organic amendments. Examples of these antibiotics include those that were studied in this research, as well as sulfathiazole in livestock manure, and erythromycin in biosolids (Kumar et al. 2005).

This and previous research on estrogen fate in organic amendments and soil (Chun et al. 2005; Lucas and Jones 2006; Shareef et al. 2009; Caron et al. 2012) was conducted under aerobic conditions in the laboratory on a microcosm scale. However, organic amendments are typically stored in large tanks or lagoons and the environmental conditions at such scales are difficult to simulate at the microcosm scale. For example, facultative lagoons typically consist of an anaerobic layer of solids below an aerobic layer of liquid (National Guide to Sustainable Municipal Infrastructure 2004) while estrogen degradation is primarily an aerobic process (Ying and Kookana 2005; Czajka and Londry 2006). Ideally, the effect of environmental conditions on estrogen degradation in lagoons could be examined by directly quantifying estrogen concentrations in the media at various depths over time, but difficulties associated with analyzing estrogens in solid matrices currently limit this approach (Cicek et al. 2006). However, different anaerobic conditions could be simulated in the laboratory, as described by Czajka and Londry (2006), and applied to laboratory microcosm studies of estrogen mineralization in various organic amendments to provide some insight on this subject.

The research presented in this thesis was conducted at a constant temperature of 20˚ C. Similarly, other laboratory mineralization studies are typically performed at a constant temperature between 10 and 30˚ C (Colucci et al. 2001; Lucas and Jones 2006; Caron et al. 2010; Stumpe and Marschner 2009). However, storage facilities for organic amendments are typically outdoors and therefore subject to temperature fluctuations.

Temperature fluctuations affect microbial populations, which may subsequently impact estrogen mineralization (Howell et al. 1971; Biederbeck 1973). In order to elucidate the effect of temperature fluctuations on estrogen mineralization in organic amendments, future laboratory experiments should be conducted with temperature fluctuations that mimic diurnal and seasonal variations in temperature.

Liquid swine manure, sewage sludge and biosolid samples used in this study were frozen immediately after collection and then thawed within 1 to 3 months, prior to the commencement of the experiments. The effect of freezing on estrogen mineralization is not well understood; several studies have found no significant effect of freezing on chemical mineralization in soils, while a recent study demonstrated that long term freezing of soil samples significantly reduced mineralization of 17 β-estradiol, possibly due to a reduction in soil microbial communities over time (Andronak 2013). In colder climates, such as the Canadian prairies, storage facilities such as lagoons are also subject to seasonal freeze-thaw cycles. While microbial populations are likely replenished in lagoons receiving raw sewage, freezing could potentially decrease microbial populations in lagoons not receiving raw sewage. Before biosolids can be applied to agricultural land, municipal wastewater lagoons must not receive raw sewage for a minimum period of time, usually three months (National Guide to Sustainable Municipal Infrastructure 2004). Similarly, decommissioned municipal lagoons or post-treatment holding lagoons at a wastewater treatment plant do not receive raw sewage. In these situations, estrogen removal through mineralization may be hindered by seasonal freezing. Laboratory microcosm studies, such as the ones described in this thesis, could be implemented to determine the impact of freezing on estrogen-degrading microbes by quantifying estrogen mineralization in organic amendments that have never been frozen and those that have been frozen for different lengths of time.

In Chapter 3 of this thesis, microbial respiration was successfully quantified in sewage sludge and biosolid samples. Although respiration was negatively associated with mineralization of 17 α-ethinylestradiol, degradation of natural estrogens is mainly driven by microbial processes (Munnecke et al. 1982; Chen et al. 1997; McGrath et al. 1998; Al-Ahmad 1999). Therefore, microbial activity should be considered in future estrogen fate studies. Various estrogen degrading microorganisms have been identified in manure, sewage sludge, compost and soil samples (Combalbert and Hernandez-Raquet 2010). However, the microbial communities in organic amendments vary greatly with the nature of the amendment (Pascual et al. 1998; Garcia-Gil 2000) and the response of the soil microbial community to organic amendments is dependent on the rate of amendment (Albiach et al. 2000; Garcia-Gil 2000). Rather than attempting to characterize the microbial community present in an organic amendment, future studies should focus on the response of the microbial community to changes in the physical and chemical parameters, environmental conditions, or antibiotics and then relate this information to estrogen mineralization. This can be done successfully through parallel laboratory microcosm studies quantifying media respiration and estrogen mineralization, as described in this thesis.

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