

**Occurrence and Environmental Fate of Chemicals of Emerging Concern in  
Biosolids- and Dairy Manure-Amended Agricultural Soil**

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by

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### **Authorization to Submit Thesis**

This thesis of Danika Noelle Hill, submitted for the degree of Master of Science with a Major in Environmental Science and titled “Occurrence and Environmental Fate of Chemicals of Emerging Concern in Biosolids- and Dairy Manure-Amended Agricultural Soil,” has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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## **Abstract**

Organic fertilizers, such as dairy manure and biosolids, are often applied to agricultural fields as nutrient amendments. In addition to essential nutrients, organic fertilizers regularly contain chemicals of emerging concern (CEC) including hormones, phytoestrogens, antibiotics, pharmaceuticals, and metals. In this thesis, I explore the prevalence of CECs in Idaho's dairy manure and biosolids and their environmental fate from the land-application of organic fertilizers. Two dairy manure samples and one biosolid sample were extracted for 25 CECs, and antibiotics and hormones were detected in all three samples. Undisturbed soil columns were used to determine the environmental mobility of CECs through an agricultural soil. Sulfonamides, flunixin, and ibuprofen leached from the chemical-treated columns, but none of the CECs originally in the dairy manure leached through the soil profile. The continued application of organic fertilizers may pose a threat to future public and environmental health due to the frequent presence of some CECs.

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## **Chapter 1: Literature Review**

### **Environmental Concerns of Land-Applied Organic Fertilizers**

Organic fertilizers provide essential nutrients to the soil as well as sustainably repurposing waste material (Athamenh et al., 2015). Organic fertilizers include composts, animal manure, and biosolids from wastewater treatment facilities. However, organic fertilizers often contain undesired contaminants that threaten environmental health. These chemicals of emerging concern (CECs) are particularly prevalent in biosolids and dairy manure, which can then enter the environment through agricultural runoff (Dutta et al., 2010) or by leaching through the soil profile (Arnon et al., 2008). Evidence continues to show the consistent occurrence and persistence of CECs in the environment as a result of land-applied dairy manure and biosolids (Lorenzen et al., 2004; Zentner et al., 2015; Tucker et al., 2010).

Dairy manure is often applied to agricultural fields as a valuable nutrient amendment. Nationally, the dairy industry ranks as the second largest livestock industry (NASS, 2016). In 2015, an estimated 9.3 million dairy cows produced over 172 billion kg of manure (NASS, 2016). In the state of Idaho, the dairy industry has grown over 500% in milk production in just 20 years (Agricultural Air Quality Task Force). This rapid increase in dairy production has resulted in an increase in the use of dairy manure as a nutrient amendment on agricultural fields in Idaho (Leytem et al., 2011). The long-term environmental effects from annual dairy manure application have not yet been investigated.

Major environmental contaminants in dairy manure include hormones, phytoestrogens, antibiotics, and copper. Hormones and phytoestrogens are endocrine disrupting chemicals (EDCs) that cause developmental effects on many aquatic organisms (Jobling and Tyler, 2003; Milla et al., 2011). An increase in antibiotic presence has been shown to increase

antibiotic resistance, threatening human health (Kemper, 2008). Although governmental regulations exist for manure application on land, they only require that the nutrient loadings of nitrogen and phosphorus do not exceed crop uptake (US EPA, 2012). There are no regulations that monitor the presence or influx of pharmaceuticals or hormones in dairy manure.

Biosolids are also used as organic fertilizers that are regularly applied to agricultural fields (Lu et al., 2012). They frequently contain CECs including hormones, antibiotics, metals, and pharmaceuticals. Biosolids undergo extensive treatment as regulated by the Environmental Protection Agency (EPA) before they can be applied to fields. The EPA has categorized biosolids into three classifications based on the degree of degradation. Class A biosolids are of “exceptional quality” and are deemed safe for direct human handling (EPA/832/R-93/003, 1994). These have no site restrictions and may even be used by the general public to treat their lawns or for public parks (Lu et al., 2012). Class B biosolids may still have some dangerous pathogens, and their use on agricultural fields is more limited. Each state’s Department of Environmental Quality (DEQ) and the federal EPA have limitations on crop harvesting dates for fields receiving Class B biosolids (EPA/832/R-93/003, 1994). Class C biosolids are untreated sludges that cannot be applied to fields (Office of Water Resources; EPA/832/R-93/003, 1994).

The EPA has strict regulations for the land-application of biosolids that focus on bacteria, nutrients, and metals as detailed in 40 CFR Part 503 (EPA/832/R-93/003, 1994). Coliform count determines whether the waste fits into Class A or Class B biosolids (Lu et al., 2012). The nutrient instructions for land application are vague, mandating that biosolids application should not “exceed the agronomic rate . . . of the plants being grown” (EPA/832/R-93/003, 1994). This regulation limits only the loading rates of nitrogen, which often results in

an excess of phosphorus applied to biosolids-amended fields (Lu et al., 2012). However, restrictions of metals include both the maximum concentration and cumulative pollutant loading of 10 specific metals (EPA/832/R-93/003, 1994).

No regulations currently exist for the application of pharmaceuticals or other complex organic contaminants in biosolids or manure. The extensive presence of these compounds in the environment is just becoming clear, as are the detrimental effects that CECs have on the environment. Few methods exist for the extraction of multiple classes of CECs from solid matrices (Białk-Bielińska et al., 2016). Without accurate documentation of CEC presence in organic fertilizers, it is impossible to determine the flux of CECs entering the environment. Furthermore, even less information is known regarding how these chemicals behave in the environment. Do they sorb to organic matter, or do they leach through the soil profile, possibly contaminating groundwater resources? Dairy manure and biosolids have essential macro- and micronutrients necessary for plant growth, but the lack of regulation over CECs so often present in organic fertilizers may cause irreparable environmental damage.

### **Endocrine Disruptors: Hormones and Phytoestrogens**

Dairy cows constantly excrete high concentrations of endogenous hormones in dairy manure due to their pregnancy and lactation cycles (Hanselman et al., 2003).  $17\alpha$ -Estradiol ( $17\alpha$ -E2) is the primary estrogen species excreted by dairy cows (Hanselman et al., 2003) and thus frequently found in high concentrations in dairy manure (Gadd et al., 2010; Zheng et al., 2008; Hutchins et al., 2007). Excretion of hormones increases throughout pregnancy (Tucker, 2000). Approximately 25% of cows on a dairy farm are pregnant at any given time, and consequently these hormones are consistently present in dairy manure.

Biosolids also contain endogenous hormones including estrogens, progestogens, and

testosterones (Lorenzen et al., 2004; Albero et al., 2014b). The major estrogen species excreted by humans is  $17\beta$ -estradiol, which binds to estrogen receptors of vertebrate species stronger than  $17\alpha$ -estradiol (Tyler et al., 1998). Thus,  $17\beta$ -estradiol is a more potent endocrine disruptor. One study found that biosolids produced higher estrogen and androgen responses than multiple animal manures as measured by mammalian and yeast bioassays (Lorenzen et al., 2004). This could be attributed to an increased potency of  $17\beta$ -estradiol in the biosolids.

Aquatic organisms are especially vulnerable to hormonal changes induced by estrogens. Male rainbow trout experience feminization when exposed to  $17\beta$ -estradiol at concentrations of just  $25 \text{ ng L}^{-1}$  (Routledge et al., 1998). The growth rate of fish living downstream from concentrated animal feeding operations (CAFOs) changes from those geographically isolated from such sites (Leet et al., 2012). At one site sampled in 2008 and 2009, all male creek chubs had immature testes due to estrogen exposure (Leet et al., 2012). Two separate sampling sites downstream of wastewater treatment facilities revealed that 100% of male fish had both male and female gonads compared to just 4% of intersex fish in the laboratory population (Jobling et al., 1998). Additionally, researchers found that brown trout exposed to low levels of  $17\beta$ -estradiol ( $3.7 \text{ ng L}^{-1}$ ) for just 2 hours on the day of fertilization had significantly slower hatching and growth rates, demonstrating that any excess exposure to estrogen can cause long-term developmental changes (Schubert et al., 2014).

Terrestrial animals also suffer from estrogenic exposure from manure application. Male lizards captured on agricultural lands amended with animal manure exhibited increased estrogenic activity compared to wild-caught males lizards as determined by the hepatic presence of estrogen-controlled proteins (Verderame et al., 2016). Investigators did not show any contamination from metals (which would indicate industrial or mine exposure) and

concluded that this estrogenic response in male lizards came from manure applied to agricultural fields (Verderame et al., 2016). Thus, hormones directly linked to livestock practices are entering the environment at concentrations high enough to cause significant effects on wildlife.

In addition to natural hormones, biosolids contain synthetic hormones (Clarke and Smith, 2011).  $17\alpha$ -Ethinylestradiol (EE2) is a synthetic estrogen that is the active ingredient in most oral contraceptives. In a comparison of four estrogens on six fish species, EE2 was more toxic than other estrogens in all six fish species (Lange et al., 2012). Research indicates that exposure to concentrations of EE2 as low as  $0.5 \text{ ng L}^{-1}$  during development changed gene expression in a fish species (Nikoleris et al., 2016). Synthetic progestogens used in oral contraceptives and hormone treatments inhibit the production of androgens in aquatic vertebrates (Fernandes et al., 2014). Additionally, EE2 degrades at a slower rate than natural estrogens (Xia et al., 2005), making it a dangerous and potent EDC to aquatic and terrestrial organisms.

Phytoestrogens are plant-made hormones that have endocrine disrupting effects on aquatic species (Milla et al., 2011; Tyler et al., 1998). Just like hormones, they can bind to estrogen receptors of vertebrates (Latonnelle et al., 2003), causing estrogenic effects on that organism (Latonnelle et al., 2002). Flounder fed a diet high in genistein, the primary phytoestrogen in soybeans, experienced significant growth retardation, and the embryos produced a significantly higher proportion of female fish (DiMaggio et al., 2014). These endocrine disrupting effects occur whether the phytoestrogens are consumed (DiMaggio et al., 2014; Latonnelle et al., 2002) or present in the water source (Tyler et al., 1998).

A typical dairy cow's diet contains plants with high concentrations of phytoestrogens

– notably soy, barley, and red clover hay – which are then excreted through their manure (Tucker et al., 2010). Tucker et al. (2010) observed that dairy cows with diets high in phytoestrogen-containing plants excrete higher concentrations of phytoestrogens. Furthermore, manure extracts containing higher concentrations of phytoestrogens are found to increase estrogenic activity in yeast bioassays (Tucker et al., 2010). Dairy cows eating a diet containing soy had statistically higher estradiol equivalents as measured by yeast and human ovarian cell bioassays (Lorenzen et al., 2004). Phytoestrogens excreted by dairy cows contribute to the overall estrogenic activity of their manure.

The effects of organic contamination have long-lasting consequences on organisms. Researchers moved three-spined stickleback fish from a stream receiving wastewater treatment facility (WWTF) effluent into a clean aquarium. After 5 months in the pristine environment, female fish still had significantly higher stress responses that were similar to fish living in contaminated waters (Pottinger and Matthiessen, 2016). This suggests that exposure to anthropogenic CECs can result in extended changes in the non-reproductive endocrine systems of some organisms.

Additional research indicates that EDCs cause epigenetic and transgenerational effects in fish, amphibians, and mammals. Parents can pass their altered endocrine systems onto their offspring, possibly leading to evolutionary effects on that species (Schwindt, 2015). Exposure to endocrine disrupting compounds can thus have not only life-long consequences, but may also alter future generations of species. It is essential that endocrine disrupting compounds entering the environment be closely monitored and regulated.

## **Antibiotics and Antibiotic Resistance**

Dairy manure regularly contains detectable concentrations of antibiotics (Kyselkova et

al., 2015) and antibiotic resistance genes (Wichmann et al., 2014; Kyselkova et al., 2015). As much as 80% of some antibiotics are excreted unmetabolized by dairy cows (Kemper, 2008), meaning they enter the environment in their active form. Although sick cattle are often moved to a separate barn and their milk is discarded, their manure is still combined with the entire operation's waste. The manure is then applied to agricultural fields where antibiotics can enter the environment.

Biosolids also frequently contain antibiotics, as up to 80% of some antibiotics can pass through the human body unchanged (Pamreddy et al., 2013; Du and Liu, 2012; Clarke and Smith, 2011). WWTFs do not include treatment procedures to remove complex organic compounds, allowing most antibiotics to pass through without being broken down (Petrie et al., 2014). Triclosan, the antimicrobial compound commonly used in hand sanitizers, soaps, and personal care products, enters the environment at rates of over 100 million kg annually in the United States alone (Carey and McNamara, 2015). Triclosan partitions to biosolids during wastewater treatment because of its organic structure, resulting in maximum detections of 133 mg kg<sup>-1</sup> (Carey and McNamara, 2015). The antibiotics ciprofloxacin and tetracycline are also frequently found in biosolids (Clarke and Smith, 2011).

The frequent application of antibiotics to soil may spread antibiotic resistance to microbial soil communities (Ross, 2015). Onan and LaPara (2003) found a significant increase in antibiotic-resistant bacterial colonies on a cornfield amended with manure from animals receiving antibiotic treatment compared to fields amended with manure from animals not receiving antibiotics. Antibiotics can persist for long periods of time in the environment, with detectable concentrations measured for as long as 21 months after biosolids application to a field (Clarke and Smith, 2011).

Antibiotic resistance costs the United States over \$50 billion annually and results in two million human antibiotic resistant infections (Carey and McNamara, 2015). Many antibiotic families, such as tetracycline and sulfonamide antibiotics, are used by both the dairy and pharmaceutical industries. The extensive use of these antibiotics in such large quantities on multiple species increases the prevalence of genetic resistance to these antibiotic families, escalating the threat to human health from antibiotic resistant bacteria (Kemper, 2008).

### **Other Pharmaceuticals Originating from WWTFs**

Biosolids contain many of the same CECs found in dairy manure including hormones, antibiotics, and metals, but biosolids contain a greater variety of synthetic pharmaceuticals. Pharmaceuticals frequently found in biosolids include over-the-counter drugs like acetaminophen, ibuprofen, and caffeine, as well as hundreds of antidepressants, antipsychotics, and pain killers (Albero et al., 2014a; Gottschall et al., 2012). A study conducted by New York City Department of Environmental Protection evaluated the city's source waters for 56 CECs in 2009 and 2010, finding that caffeine was the most frequently detected pharmaceutical (Strickland and Rush, 2011). The anti-anxiety drug diazepam (the active ingredient in Valium) appeared in a maximum concentration of  $2.1 \text{ ng L}^{-1}$ ; however the NYC Department of Environmental Protection determined it would take over 3.2 million glasses of drinking water for one person to reach the lowest therapeutic dose for diazepam (Strickland and Rush, 2011).

Pharmaceutical residues in water systems pose a minimal threat to human health at current environmental concentrations (Strickland and Rush, 2011), but they do exist in high enough concentrations to affect other organisms. Effects on organisms include retarded growth, lowered survival rates, predominance of one gender, and diminished chloroplasts in



algae (Li, 2014). In a systematic review of over 100 pharmaceuticals, the CEC categories of antidepressants, antibiotics, and antipsychotics are the “most ecotoxic” to aquatic wildlife (Webb, 2004).

Ibuprofen is a non-steroidal, anti-inflammatory drug (NSAID) that is one of the six most common pharmaceuticals found in soil (Li, 2014). Ibuprofen is considered a high concern due to its mass influx, as well as a limited number of ecotoxicity studies (Christensen et al., 2009). In a comparison of 18 pharmaceuticals, ibuprofen was the most toxic to the common earthworm (Pino et al., 2015). This is especially threatening to this species since biosolids are applied to the soil.

Acetaminophen does not have anti-inflammatory effects, but is frequently classified as an NSAID due to its similar metabolic pathway (Parolini et al., 2009). Studies in Korea and the United States have identified acetaminophen as a priority pollutant to the environment because of the mass quantities produced annually (Ji et al., 2016). Treated effluent containing acetaminophen from WWTFs can enter the environment at concentrations as high as  $11.7 \mu\text{g L}^{-1}$ , which was the third highest effluent concentration of 94 common pharmaceuticals in one study (Petrie et al., 2014). Out of three common NSAIDs, acetaminophen caused the greatest cytogenotoxic effects on freshwater zebra mussel hemocytes (Parolini et al., 2009). Although frequently used human pharmaceuticals seem benign, they have toxic consequences to many species of wildlife.

## **Metals**

In addition to organic compounds, dairy manure may also introduce metals into the environment. Copper sulfate or zinc sulfate solutions are regularly used in dairies as hoof baths to prevent disease, entering the environment when excess solution is poured into waste

lagoons (Downing et al., 2010). When this lagoon wastewater is sprayed on agricultural fields, copper and zinc can accumulate to toxic levels in the soil. Neither of these metals is regulated in regards to dairy manure application, making this a viable threat from manure application.

Metal concentration in biosolids is one of the primary limitations for application on agricultural fields (Lu et al., 2012). There is concern that repeated applications will result in metal accumulations in soil or metal leaching into a drinking water source. Especially with toxic metals such as lead, cadmium, and mercury, this could lead to dire public health consequences. However, metals sorb strongly to soils, thus limiting their mobility and bioavailability (Ashworth and Alloway, 2004; Lu et al., 2012). Even after 14 years of annual applications of metal-contaminated biosolids to an agricultural field, over half of the metals could only be extracted with very strong acid (Lu et al., 2012). Furthermore, concentrations of metals are strictly regulated by the EPA, making metal contamination from biosolids application an unlikely concern (EPA/832/R-93/003, 1994).

### **Environmental Fate of Chemicals of Emerging Concern**

Chemicals of emerging concern are frequently documented in the environment, but the transport of these compounds is difficult to predict. Much of the research focus has been on the influx of CECs from WWTF effluent into aqueous environments, and little is understood about CEC movement through soil (Białk-Bielińska et al., 2016). Although many models exist for mapping the fate of metals and pesticides in the environment, such models are inefficient in predicting the movement of pharmaceuticals and hormones due to the physiochemical differences among compounds (Kay et al., 2005a).

Chemical transport through the soil is complicated and variable. The fate of CECs depends not only on the physiochemical characteristics of the compound (including charge,

$K_{OW}$ , and  $K_d$ ), but also on the physical properties of the soil (such as pH, clay content, mineral composition, porosity, and cation exchange capacity) (Thiele-Bruhn, 2003; Li, 2014).

Additionally, the weather following application of manure-introduced CECs will impact environmental movement of the compound. A heavy rainfall will favor surface runoff; whereas a steady, long-lasting rain will result in a deeper penetration of the compound through the soil profile (Jones et al., 2014). Contaminants in organic fertilizers have four potential fates once they are applied to the soil: sorption, degradation, runoff by overland flow, or vertical leaching through the soil profile. Although all four outcomes can occur separately, they generally occur simultaneously in the environment.

Many CECs sorb to the soil, rendering them immobile (Du and Liu, 2012). Antibiotics have significantly longer half-lives in soil than when in aqueous solutions due to sorption and shielding by soil organic matter (Du and Liu, 2012). The strength and type of bonding involved in sorption to soil controls bioavailability and consequently the potential harm a CEC can induce (Kumar et al., 2005). If a compound is sorbed, then it is less bioavailable (Williams et al., 2009). Although an antibiotic's activity is reduced, antibiotics sorbed to the soil still exhibit some antimicrobial activity (Thiele-Bruhn, 2003).

Hormones sorb strongly to soils and sediments (Salvia et al., 2014; Das et al., 2004). There is a positive correlation between organic carbon content and hormone sorption to sediments (Lai et al., 2000). Estrogens with higher  $K_{OW}$  values (such as synthetic 17 $\alpha$ -ethinylestradiol) sorb in higher concentrations than natural estrogens when in the same soil suspension, suggesting that estrogens compete for binding sites (Lai et al., 2000). Hormones are extremely immobile in soil due to these high sorption rates. In a soil column study, 100% of progesterone stayed in the top 6 cm of a soil column after 30 days of watering (Salvia et al.,

2014).

Crop roots can uptake CECs that are not sorbed to the soil. Sulfonamides, a common class of antibiotics found in biosolids and dairy manure, have been detected in corn, wheat, and potato plants (Du and Liu, 2012; Thiele-Bruhn, 2003). Cabbage, corn, and green onions were found to uptake chlortetracycline from soil amended with pig manure containing chlortetracycline (Kumar et al., 2005). In the same study, none of the plants adsorbed the veterinary antibiotic tylosin despite its lower adsorption coefficient to the clay soil compared to chlortetracycline. The researchers concluded that tylosin was too large (molar mass = 916 g mol<sup>-1</sup>) for root uptake (Kumar et al., 2005). Tetracycline was also found to be absorbed by wheat and alfalfa plants (Du and Liu, 2012). Tetracyclines and sulfonamides are antibiotic families used commonly by the dairy industry and are regularly detected in solid dairy manure (FDA 2009; Kyselkova et al., 2015; Snow et al., 2009). Considering that wheat and potato are two of Idaho's major crops, application of dairy manure to these crops may result in the presence of antibiotics in the final produce (Koong et al., 2015).

If CECs are not sorbed by soil or taken up by plants, they are more susceptible to biotic and abiotic degradation (Li, 2014). The degradation rate can vary widely across CEC families. Estrogens and progestogens regularly have half-lives of less than 12 hours in soil, varying slightly with soil type and weather (Salvia et al., 2014; Xuan et al., 2008). Sulfonamides also degrade significantly in less than half a day; whereas tetracyclines experience half-lives of up to 56 days in aerobic conditions (Salvia et al., 2014; Yang et al., 2009; Aga et al., 2005). Half-lives increase for hormones, sulfonamides, and tetracyclines in sterile soils, suggesting that microbial degradation plays a primary role in breakdown of these compounds (Yang et al., 2009; Xuan et al., 2008). Photodegradation also plays a considerable

role for CECs in surface water, in which exposure to UV radiation from the sun cleaves bonds of these compounds (Li, 2014; Qu et al., 2012). Photodegradation is unlikely a contributing factor for any CECs in soil due to limited sunlight exposure.

Although a compound may degrade rapidly, degradation products can cause equal or greater damage than the parent compound. Three months after a manure application containing oxytetracycline (OTC), LC-MS analysis detected less than half of the original OTC concentration in soil. However, bioassays indicated the same intensity of antibiotic activity from tetracyclines 5 months after application (Aga et al., 2005). This suggests that the activity of OTC degradation products causes an equivalent biological response. Additionally, a chlorinated by-product of caffeine degradation was found to be a more toxic mutagen than caffeine itself to a freshwater rotifer, a representative aquatic organism (Zarrelli et al., 2014). Degradation is a necessary step in the removal of CECs from the environment, but some of the degradation products can still cause significant detrimental effects on biota.

Despite high rates of sorption and constant degradation, CECs are consistently detected in groundwater, surface water, aqueous sediments, and soil (Li, 2014; Thiele-Bruhn, 2003; EPA, 2013). Organic fertilizers significantly decrease vertical movement of solutes due to the sealing of soil pores from organic matter (Burkhardt and Stamm, 2007). This encourages surface runoff and overland flow of CECs (Burkhardt and Stamm, 2007; Kay et al., 2005a). Indeed, sulfonamides in manure experienced over a tenfold increase in surface runoff as opposed to sulfonamides added to the soil in an aqueous solution (Burkhardt and Stamm, 2007). Runoff from agricultural fields recently treated with organic fertilizers is a serious threat to the environment. Studies have confirmed that hormones, sulfonamides, and tetracyclines can leave agricultural fields in significant amounts by runoff (Kay et al., 2005b;

Jones et al., 2014).

Sulfonamides are some of the most mobile compounds through soil environments (Salvia et al., 2014; Kay et al., 2005b; Burkhardt and Stamm, 2007). As weak acids, sulfonamides are anions when exposed to the basic pH of manures and biosolids. Their negative charge makes sulfonamides particularly mobile in soil due to anion-anion repulsion from the negatively charged clays and organic matter in most soils (Burkhardt and Stamm, 2007). Furthermore, sulfonamides have been shown to move through preferential pathways, which are densely populated with microorganisms. Increased antibiotic presence in these pores may detrimentally impact the soil microbial community (Burkhardt and Stamm, 2007).

Additionally, repeated application of contaminated manure or biosolids can result in the accumulation of CECs in soil, and if mobile, CECs can threaten groundwater sources (Hamscher et al., 2002). This was demonstrated in an experiment in Israel in which an agricultural field received annual applications of sewage effluent for over 50 years. Nine of the tested 21 pharmaceuticals were found in the vadose zone at a maximum depth of 26 m below the surface. Five compounds (carbamazepine, acridone, venlafaxine, sulfamethoxazole, and caffeine) were in detectable concentrations ( $\text{ng L}^{-1}$ ) in the groundwater (Zentner et al., 2015). In another study, just one application of biosolids to an agricultural field resulted in ibuprofen, triclosan, and triclocarban detections in the groundwater immediately following the first rainfall event (Gottschall et al., 2012).

Finally, manure and biosolids rarely contain just one contaminant. Over 80 CECs have been detected in just one biosolid sample (Gottschall et al., 2012). Accumulated CECs in soils are not only a threat because they build up in high activity zones, such as preferential pathways (Burkhardt and Stamm, 2007), but also because combinations of contaminants may

cause synergistic effects (González-Naranjo and Boltes, 2014). For example, amphibians exposed to a combination of contaminants experienced severer toxic effects than exposure to any one of the compounds individually (Hayes et al., 2006).

CECs do not have to be resistant to degradation to cause environmental damage. They are continually being introduced into the environment, and land-applied organic fertilizers are a major source of that influx (González-Naranjo and Boltes, 2014). The regular occurrence and concentration of these chemicals is not yet thoroughly documented, especially in soil (Białk-Bielińska et al., 2016). Documenting where CECs partition in the environment is essential to understanding their transport and fate, and doing so will allow better monitoring and protection of environmental quality.

## **Research Overview and Objectives**

The presence of contaminants in land-applied dairy manure and biosolids threatens wildlife, environmental health, and public health. In Idaho, use of dairy manure on agricultural fields has increased substantially due to increased availability (Leytem et al., 2011). Over 70% of all dairy cows are located in the Magic Valley region along the Snake River in southern Idaho (United Dairymen of Idaho, 2013). This area is unique due to the concentration of agriculture, aquaculture, dairies, and cities along the Snake River. Runoff or leaching of dangerous chemicals may threaten wildlife, downstream aquaculture, and drinking water. Thus, accurately measuring potential CECs in dairy manure and biosolids is essential to protect human and environmental health.

Our research was focused on developing and testing methods for extracting a wide variety of CECs from complex biosolids and manure matrices. Methods were developed to effectively extract antibiotics, hormones, and pharmaceuticals and to quantify their respective

concentrations by high performance liquid chromatography-mass spectrometer-time of flight (HPLC-MS), a highly sensitive instrument capable of detecting compounds at concentrations as low as  $\text{ng mL}^{-1}$ . Regularly collected, random manure samples from the Magic Valley Compost Facility near Twin Falls, Idaho, were extracted to determine the prevalence of CECs in dairy manure.

In a separate experiment, we monitored the transport and fate of CECs through undisturbed soil columns. Soil columns were treated with dairy manure, biosolids, selected CECs, or urea fertilizer. The leachate was collected weekly for analysis and shared with two other experimental groups who tested for antibiotic resistance and endocrine disrupting capacity. The soil columns were incubated for 3 months to match an average growing season.

We first determined which chemicals of emerging concern frequently occur in dairy manure and biosolids, identifying compounds that are most likely to be a threat. The mobility of these threatening compounds was then evaluated by the soil columns experiment, indicating whether they pose a significant threat of entering the groundwater. In addition to organic contaminants, the soil water effluent was monitored for nutrient and metal contents. Together this data will highlight any potential effects of manure application after one growing season.



## **Chapter 2: Extraction Optimization of a Vast Array of Chemicals from Dairy Manure and Biosolid Matrices**

### **Introduction**

Organic wastes such as dairy manure and biosolids are often applied to agricultural fields as nutrient sources. However, their application to soil also provides direct access for undesired and potentially harmful chemicals to enter the environment. Contaminants can leach through the soil profile threatening groundwater sources (Arnon et al., 2008), or enter surface water through agricultural runoff (Dutta et al., 2010). These chemicals of Emerging Concern (CECs) encompass a vast variety of physiochemical traits. These anthropogenic-introduced chemicals cause detrimental effects on the environment and include hormones, antibiotics, and pharmaceuticals. Accurate quantification of a variety of CECs in land-applied biosolids and manure is necessary to monitor the input of CECs to the environment.

Dairy manure is a particular concern because of its high concentration of estrogens. Pregnant and lactating cattle excrete large quantities of estrogens and progestogens in their manure, which increase throughout the duration of the pregnancy (Hanselman et al., 2003; Tucker, 2000). Aquatic organisms are especially vulnerable to hormonal changes induced by estrogens and progestogens. Fish downstream from concentrated animal feeding operations (CAFOs) experience different growth rates than undisturbed fish. At one site sampled in 2008 and 2009, all male creek chubs had immature testes (Leet et al., 2012). Estrogens at concentrations as low as 25 ng L<sup>-1</sup> are found to cause feminization of male rainbow trout (Routledge et al., 1998).

In addition to hormones, dairy manure is a potential source of antibiotics. The frequent application of the antibiotics to soil increases antibiotic resistance in microbial soil communities (Ross, 2015). Onan and LaPara (2003) found a significant increase in antibiotic-

resistant bacterial colonies on a cornfield amended with manure from animals receiving antibiotic treatment compared to fields amended with manure from animals not receiving antibiotics. Many of the antibiotics used by dairies are identical or in the same family as those used as human pharmaceuticals, especially the tetracycline and sulfonamide families. This escalates the threat to human health from antibiotic resistant bacteria (Kemper, 2008).

Wastewater treatment facilities (WWTF) treat solid biosolids for harmful pathogens, but do not have the technology to remove pharmaceutical residues like antibiotics and hormones (Lorenzen et al., 2004; Ross, 2015). In an analysis of 110 biosolids samples, tetracycline was measured in all of them with a maximum concentration of  $2.7 \text{ mg kg}^{-1}$  (McClellan and Halden, 2010). Biosolids also contain pharmaceuticals like acetaminophen, ibuprofen, and caffeine (Albero et al., 2014a; Gottschall et al., 2012). Although these seem like benign drugs, common pharmaceuticals can be toxic to other organisms. Out of 18 studied pharmaceuticals, ibuprofen was found to be the most toxic to earthworms (Pino et al., 2015). Some pharmaceuticals are very mobile in the environment. Compounds from land-applied treated sewage effluents were found to travel 28 m through the vadose zone before entering the ground water (Zentner et al., 2015).

Antibiotics, hormones, phytoestrogens, and pharmaceuticals have all been well documented in dairy manure and biosolids. Most of these compounds are slightly water-soluble and sorb strongly to organic matter, making extraction processes difficult and time-intensive (Białk-Bielińska et al., 2016). Most research studies focus on extraction of just one family of chemicals such as estrogens (Gadd et al., 2010; Raman et al., 2004), progestogens (Liu et al., 2014), or tetracyclines (Aga et al., 2005). Occasionally, research includes combinations of similar chemicals such as steroid hormones (Zheng et al., 2008; Hansen et al.,

2011) or two classes of antibiotics (Pamreddy et al., 2013; Zhao et al., 2009; An et al., 2015). In one study, both antibiotics and hormones were extracted from manure, but the focus was on only one hormone (progesterone) (Ho et al., 2012). In another investigation, four antibiotics and two estrogens were extracted from biosolids, but recovery rates of only 11 – 59% were achieved (Shafrir and Avisar, 2012). There are methods for removing a wide variety of compounds with different physiochemical properties from surface water (Hernandez et al., 2015; Campanha et al., 2014); however no methods exist for the extraction of multiple classes of hormones, antibiotics, and pharmaceuticals from the complex solid matrices of manure or biosolids.

Many techniques exist for measuring chemical concentrations, but gas chromatography (GC) or liquid chromatography (LC) coupled to a mass spectrometer (MS) offers sensitive quantification of individual compounds from complex environmental matrices with detection limits as low as nanogram per liter. However, GC-MS analysis requires that the compounds are first derivitized; whereas LC-MS analysis offers the benefit of analyzing compounds in a liquid phase. This saves both time and derivitizing materials (Białk-Bielińska et al., 2016).

In this research, previously published extraction methods were altered to develop a more robust procedure to better accommodate a wider range of potential contaminants from both dairy manure and biosolids. Additionally, methods were tested for efficiency in extracting chemical families not previously tested. For example, concentrations of  $\alpha$ -hydroxyprogesterone have never been measured in either matrix, and phytoestrogen concentrations have never been reported for biosolids. The objectives of this study were to 1) develop a method for extraction of 25 CECs from dairy manure and biosolids and 2) compare

the findings to current knowledge on the concentrations of these compounds in the environment.

## **Materials and Methods**

### **Chemicals and Materials**

All chemicals purchased were from Sigma Aldrich (St. Louis, MO, USA). This consisted of seven hormones (17 $\beta$ -estradiol, 17 $\alpha$ -estradiol, estrone, estriol, ethinyl estradiol, progesterone, and  $\alpha$ -hydroxyprogesterone), five phytoestrogens (coumestrol, enterodiol, formononetin, biochanin A, and equol), nine antibiotics (sulfamethazine, sulfadimethoxine, sulfamethoxazole, sulfathiazole, tetracycline, oxytetracycline, chlortetracycline, trichlorocarbide, and penicillin G), one veterinary drug (flunixin), and three human pharmaceuticals (caffeine, acetaminophen, and ibuprofen). Two internal, isotopic standards were used for method confirmation: 2,3,4-<sup>13</sup>C<sub>3</sub>-17 $\beta$ -estradiol and <sup>13</sup>C<sub>6</sub>-phenyl-sulfadimethoxine. Stock solutions (1.00 g L<sup>-1</sup>) of each chemical were prepared in methanol and stored at -20 °C. New stock solutions were prepared every 6 months. Dilutions for standard calibration curves were prepared in methanol.

Methanol, water, acetonitrile, acetone, and ethyl ether were all HPLC-grade solvents (Fisher Scientific or Sigma Aldrich). Ultrapure deionized water was filtered in-lab with an Ion X Charger (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) and then a Bion Exchanger (Pierce Chemical Company, Rockford, IL, USA). Formic acid (98%) was purchased from Fluka Analytical, and sodium hydroxide (NaOH) was purchased from Macron Fine Chemicals (Pennsylvania, USA). Oasis HLB SPE cartridges (30  $\mu$ m, 3 mL, 60 mg sorbent) were obtained from Waters Inc. (Massachusetts, USA).

Glass centrifuge tubes (50 mL) and glass vials (15 mL) were silanized prior to manure

extractions, as described by Seed (1994). Briefly, glassware was fumigated with dichlorodimethylsilane (3 mL) under vacuum for 24 h. After fumigation, glassware was rinsed with DDI water and air-dried.

### Dairy Manure and Biosolids Collection

Manure was collected from the University of Idaho Dairy (Moscow, ID) for the optimization of extraction techniques. This dairy does not use hormonal supplements or sub-therapeutic doses of antibiotics on the cows (personal communication with Josh Peak). Sick cows given antibiotics are isolated in an off-site facility. Manure was collected from lactating cattle that had given birth within the previous 6 months. Manure samples were scraped from the concrete floor and were fewer than 6 h old, as the pen was cleaned every morning. Two samples were taken directly from two cows at different stages of pregnancy: 130 d pregnant and 283 d pregnant. All manure samples were extracted within 24 h, and excess manure was stored at 4 °C.

Once methods had been determined, two samples of dairy manure from southern Idaho were analyzed for CEC concentration. Manure 1 was collected in June 2015, and Manure 2 was taken in August 2015. In both samples, non-composted dairy manure samples were shipped on ice from Magic Valley Compost (MVC; Jerome, ID). This facility collects manure from multiple dairy farms and may include manure from pregnant and antibiotic-treated cows. Manure was extracted within 24 h of arrival. Additionally, one biosolid sample from Boise's WWTF was received and extracted in July 2015. This was categorized as Class B biosolids, meaning the solids had undergone some treatment but may still contain harmful pathogens. Subsamples of biosolids and manure were sent to Soiltest Farm Consultants, Inc. (Moses Lake, WA, USA) for total elemental analysis of some nutrients and metals (Table 2.1).

## Solid-liquid Extraction of CECs from Dairy Manure

Manure and biosolid samples were extracted twice: a basic extraction optimized for hormones, and an acidic extraction optimized for antibiotics and pharmaceuticals. Every extraction had three additional samples spiked with 100  $\mu\text{L}$  of 1  $\text{mg L}^{-1}$  of 2,3,4- $^{13}\text{C}_3$ -17 $\beta$ -estradiol and  $^{13}\text{C}_6$ -phenyl-sulfadimethoxine isotopic standards before extraction to determine recovery rates. Recovery rates of all compounds were determined by spiking water and solid matrices at 1  $\mu\text{g mL}^{-1}$  or 1  $\mu\text{g g}^{-1}$  prior to extraction. The estrogen and sulfonamide families were normalized to relative recovery rates of the isotopic standards to account for matrix effects. All extractions were run in triplicate.

The basic extraction follows methods from Zheng et al. (2008). Briefly, 2.5 g of field-moist manure ( $\sim 0.5$  g dry weight) was weighed into a silanized glass centrifuge tube. Ten milliliters of ethyl ether and 5.0 mL of 1 M NaOH were added to the manure. This was shaken horizontally for 1 h and centrifuged at 1500 rpm for 15 min. The organic layer was decanted into a glass vial. This extraction was repeated twice more, and the organic layers were combined. The organic solvent was evaporated under a gentle stream of nitrogen gas until near dryness. The dried extract was reconstituted to 1 mL in methanol, filtered through 0.2  $\mu\text{m}$  syringe filter, and analyzed by HPLC-MS-ToF.

The acidic extraction for removal of antibiotics and pharmaceuticals followed an adaptation of Aga et al. (2005) methods. Field-moist manure (2.5 g) was weighed into a 50-mL glass centrifuge tube. Acetone acidified with formic acid (10 mL, pH = 4) was added to each sample. Samples were shaken for 30 min and centrifuged at 1500 rpm for 15 min. The organic layer was decanted into a glass vial. This process was repeated a second time, and acetone extracts were combined. The manure was then extracted twice with 10 mL of 1 M

citric acid, and each was added to the acetone extract. Combined extractions were concentrated under nitrogen gas to approximately 50% of the original volume (~20 mL), reducing organic solvent concentration to less than 5%. The combined extract was then diluted to 100 mL with DDI water and passed through an Oasis HLB SPE cartridge, previously conditioned with 3 mL methanol and 3 mL of water. The cartridge was allowed to dry under vacuum for 30 min. Cartridges were stored at -20 °C and eluted within 12 weeks. SPE cartridges were eluted with 3 mL of methanol and 3 mL of 50:50 methanol:acetone. SPE extracts were evaporated to 1 mL under nitrogen gas and analyzed by HPLC-MS-ToF.

Multiple extraction techniques were tested to develop an optimal method for meeting our objectives. Various trials tested the difference between extracting freeze-dried and field moist manure. For these trials, the manure was spiked with 100  $\mu\text{L}$  of 10  $\text{mg L}^{-1}$  estrogen standards (17 $\beta$ -estradiol, 17 $\alpha$ -estradiol, estrone, and estriol), placed either in the freezer or the freeze dryer overnight, and extracted the next day using the basic extraction. The basic extraction was tested for recovery rates of antibiotics but was found to be insufficient, ranging from 1 – 16% (data not shown). The acidic extraction was therefore incorporated into the final method to extract a wider range of compounds.

#### HPLC-MS-TOF Analysis of CECs in Manure and Biosolid Extracts

Chemical analysis was performed with an Agilent 1200 Series HPLC system containing a diode array detection (DAD) system. The HPLC was coupled to an Agilent G1969A TOF-MS system with an electrospray ion (ESI) source (Agilent Technologies, Santa Clara, CA, USA).

Studied compounds were separated on a Kinetex 5 $\mu$  EVO C18 (50  $\mu\text{m}$  x 4.6 mm) column equipped with an ULTRA guard cartridge for a Kinetex column (Phenomenex,

Torrance, CA, USA) maintained at 30 °C. The needle was washed in isopropyl alcohol between samples and all injections were 10 µL. Two methods were created: Method 1 optimized separation of hormones in negative ionization and Method 2 separated antibiotics and pharmaceuticals under positive ionization.

Method 1 used the mobile phases 0.02% NH<sub>4</sub>OH in water (Solvent A) and 0.02% NH<sub>4</sub>OH in methanol (Solvent B). The solvent gradient began with 60% A and 40% B. For the first 2.5 min, solvent B was brought up to 50%. B was then slowly ramped up to 65% over 8.5 min. Solvent B was then brought to 100% over the next 2 min and held at 100% for 50 s. The solvent was then brought back to 40% B and 60% A over 10 s. This was held steady for the last 4 min for a total run time of 18 min. The flow rate was at 0.5 mL min<sup>-1</sup>.

Method 2 used the mobile phases 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The solvent gradient began with 95% solvent A and 5% solvent B. This was held for 2 min, followed by a linear gradient to 100% B over 8 min. Solvent B was held at 100% for 3 min and then decreased to 5% B over the course of 1 min. This was maintained for an additional 2 min, creating a total runtime of 18 min. The flow rate was set at 0.4 mL min<sup>-1</sup>.

The nebulizing gas was nitrogen. Both methods had the following optimized parameters: nebulizer pressure (25 psig), drying gas flow rate (10 L min<sup>-1</sup>), drying gas temperature (350 °C), capillary potential (3500 V), and transients per scan (8000). The analyses were conducted with an *m/z* range from 90 to 1000 amu. Each compound was analyzed with different fragmentor voltages (125, 175, 250, 375) and two ESI modes (positive or negative) to determine peak ionization. The retention time, ionization, limits of detection (LOD), and recovery rates from spiked deionized water of each compound were determined.



## Data Analysis

Chromatographic data processing was done with the software Analyst QS 1.1.

Compounds were identified by their retention times and the specific product masses resulting from fragmentation. Method limit of detection was defined as a signal three times background noise from the chromatogram.

LOD for HPLC-MS-ToF were based on a least square regression of  $3.3(s_y b^{-1})$  where  $s_y$  equals the residual standard deviation of the calibration curve and  $b$  is the slope of the line. Limits of quantification (LOQ) were determined by  $10(s_y b^{-1})$  of the calibration curve. Each calibration curve had an  $r^2$  value of 0.98 or greater, except for ibuprofen ( $r^2 = 0.96$ ). Significant differences among treatments were evaluated by one-way analysis of variance (ANOVA), with the level of significance  $p < 0.05$ .

## Results and Discussion

### Extraction Optimization

There was no significant difference in recovery rates between freezer-stored and freeze-dried manure samples (Table 2.2;  $p = 0.37$ ). Estrone recovery rates were the least variable between the treatments, ranging from 30.8 – 60.0% and 48.2 – 61.0% from freeze-dried and field-moist manure, respectively. Manure was spiked 24 h before extraction, and both samples remained frozen during the incubation period. Estrogens sorb irreversibly to manure in this time frame (Colucci and Topp, 2002), explaining the low recovery rates.

Freeze drying the manure before extraction may prove most useful when using a solvent that is miscible in water, such as acetonitrile or methanol. In this case, the organic solvent would be diluted by water present in the matrix, increasing the drying time during concentration, the number and variety of compounds removed from the manure, and possibly

increasing interferences observed in HPLC-MS chromatography. Diethyl ether, the solvent used in the basic extraction, is not miscible with water. Thus, salts and other water-soluble compounds that possibly cause chromatographic interference were not extracted. The acidic extraction used a water-based solvent (citric acid) that was then diluted in water, effectively eliminating the benefits of using freeze-dried manure. Thus, field-moist manure was used for both extractions.

The acidic extraction successfully removed a wide variety of contaminants from soil (Table 2.3). These recovery rates were similar to those reported in the literature from studies extracting organic contaminants from soil matrices. Recovery rates for the estrogens were considerably higher than recoveries of the same five estrogen species in a similar extraction by Salvia *et al.* (2012), which used acidified acetonitrile to extract soil and a Strata-X SPE cartridge (Phenomenex) for purification and concentration. Their procedure yielded 41 to 48% recoveries for the estrogens when spiked at levels of  $1.5 \text{ ng g}^{-1}$  (Salvia *et al.*, 2012).

A previous study found that sulfonamide extraction depends more upon the specific sulfonamide species than the extraction technique (Raich-Montiu *et al.*, 2010). In a comparison of four extraction techniques on six sulfonamides, sulfadimethoxine consistently yielded the highest recovery rates, whereas sulfathiazole produced the lowest rates. This may partially explain the lower recovery rate of sulfathiazole (16%) in this study, compared to the higher recovery rate of sulfadimethoxine (67%). Another study, using acidified acetonitrile buffer ( $\text{pH} = 4$ ) and HLB SPE cartridges yielded sulfonamide recoveries of 55 to 65% (Bian *et al.*, 2015), which is similar to this study's recoveries of sulfadimethoxine and sulfamethoxazole.

Unfortunately, the acidic extraction technique was not best suited for human

pharmaceuticals (caffeine, acetaminophen, or trichlorocarbide). This may be due their hydrophilicity, resulting in their passing through the HLB SPE cartridge without sorption. Alternatively, they may have volatilized during the evaporation process.

The basic extraction produced estrogen recovery rates of 18 – 136%, which is comparable to previous extraction efficiencies (Salvia et al., 2012). The acidic extraction yielded higher recovery rates for the estrogens (ranging 92 – 112%), suggesting that basic extraction is not necessary for future extractions. Using only the acidic extraction will save time and procedural costs in the future.

### HPLC-MS-ToF Optimization

Hormones and phytoestrogens were best ionized with negative ionization, whereas antibiotics, pharmaceuticals, and progestogens were optimized with positive ionization (Table 2.4). Positive ionization required two fragmenters for best optimization: 125 and 175. Only one fragmenter (175) was necessary during negative ionization.

The limits of detection were low with most compounds below  $0.1 \mu\text{g mL}^{-1}$  (Table 2.4). The exceptions were  $17\alpha$ -estradiol,  $17\beta$ -estradiol, equol, sulfathiazole, tetracycline, chlortetracycline, flunixin, and ibuprofen. All compounds were below  $1 \mu\text{g mL}^{-1}$ , except for ibuprofen.

The separation between peaks was adequate for identification and quantification (Figures 2.1 – 2.4). The most overlap was among the estrogens (Fig. 2.1), but the calibration curves remained highly linear ( $r^2 = 0.99$ ). Although the three tetracycline peaks are not ideal (Fig. 2.4), the data still gave linear calibration curves ( $r^2 = 0.99$  or more) and adequate limits of detection ( $0.16 \mu\text{g mL}^{-1}$  or lower). Techniques that would have increased the resolution of the tetracycline peaks would have consequences. For example, changing the solvent would

decrease the resolution of other compounds. Increasing the run time would have increased time and instrumentation costs. It was determined that the methods were sufficient for tetracycline detection because of 1) the statistical evidence listed above and 2) the spiked peaks could still be identified in complex matrices.

### Quantification of CECs in Dairy Manure and Biosolids

No estrogens were measured in the four manure samples taken from lactating cows at the University of Idaho dairy (data not shown). In contrast,  $17\beta$ -estradiol,  $17\alpha$ -estradiol, estrone, and estriol were found in pregnant dairy cattle manure from the University of Idaho dairy (Fig 2.5). The manure excreted later in pregnancy contained higher concentrations of  $17\beta$ -estradiol,  $17\alpha$ -estradiol, and estrone, which is consistent with the literature (Hanselman et al., 2003).  $17\alpha$ -Estradiol had the highest concentration of all estrogen species found in both stages of pregnancy at  $0.08$  and  $1.24 \mu\text{g g}^{-1}$  for 130 and 283 d pregnant cows, respectively. Previous research has also shown that dairy cows excrete  $17\alpha$ -estradiol in the highest concentrations (Hanselman et al., 2003; Zheng et al., 2008).

Estrone and estriol were the only estrogen species found in the two manure samples from MVCF (Table 2.5). Neither  $17\alpha$ -estradiol nor  $17\beta$ -estradiol was detected in MVCF manure samples. Although  $17\alpha$ -estradiol is found in the highest concentrations in fresh manure (Zheng et al., 2008; Gadd et al., 2010), estrone is the most frequently detected estrogen species in dairy waste kept outside for extended periods (Leet et al., 2012; Kolodziej et al., 2004). Time-dependent studies have shown that as estradiol (both enantiomers) concentrations decrease, the concentration of estrone increases (Colucci and Topp, 2002). Likely, this manure had experienced estradiol degradation in the June and August heat. The measured estrone concentrations are in the same range of other stacked dairy solids (Raman et

al., 2004).

Progesterone and  $17\alpha$ -hydroxyprogesterone were also detected in both MVC manure samples. Progesterone has been found previously in piled manure (less than 2 weeks old), but not fresh manure (less than 2 h old) (Zheng et al., 2008). Progesterone has also been detected in swine waste, with concentrations of 1000 times our current findings (Liu et al., 2014). We are the first to quantify  $17\alpha$ -hydroxyprogesterone from solid dairy manure samples, however solid swine waste was found to contain  $17\alpha$ -hydroxyprogesterone concentrations similar to our findings (Liu et al., 2014).

Multiple antibiotics were found in the two manure samples, although there was some variation between samples (Table 2.5) The antibiotics sulfathiazole and tetracycline were detected only in the second manure sample. However, the antibiotic sulfadimethoxine was detected in both manure samples at 206 and 128  $\mu\text{g kg}^{-1}$  dry weight manure. These are all antibiotics approved by the FDA for use in dairies (FDA), justifying their presence in the manure.

$17\beta$ -Estradiol,  $17\alpha$ -estradiol, estrone, and estriol were all detected in the biosolid sample (Table 2.5). The concentrations of progesterone and  $17\alpha$ -hydroxyprogesterone were higher than reported values from dewatered sludge in China, but within the same order of magnitude (Liu et al., 2014). Additionally, tetracycline and sulfathiazole were detected in the biosolids sample. A study of five WWTFs in Spain detected sulfathiazole in the highest concentrations of all sulfonamides (Pamreddy et al., 2013), consistent with our study. Tetracycline was detected at 59.5  $\mu\text{g kg}^{-1}$  in Boise's biosolids. Tetracycline is a very common antibiotic frequently found in biosolids (McClellan and Halden, 2010). This is the first published study documenting pharmaceuticals in Boise's WWTF.

The biosolids sample had higher concentrations of all estrogens compared to either manure sample. This may be because the biosolids were fresher than the manure, as it is unknown how long the manure samples sat before being transferred to MVC. Estrogens decompose rapidly, with estrogenic activity diminishing by over 50% in less than 24 h (Colucci and Topp, 2002). Dairy manure contained larger concentrations of progesterone and sulfadimethoxine. This may be explained by the elevated production of progesterone during pregnancy (Kirsch et al.). Additionally, sulfadimethoxine is one of the few veterinary drugs approved for lactating cattle (FDA), making its detection in dairy manure feasible.

## **Conclusion**

The developed extraction and chromatographic methods successfully quantified many environmental contaminants present in organic fertilizers. Multiple extraction techniques were tested, but the acidic extraction on fresh, field-moist manure was determined to adequately remove a large variety of organic compounds. New methods were created for optimal quantification of potential contaminants on an HPLC-MS-ToF. The methods were tested on manure from the University of Idaho Dairy, and estrogens were detected only in the manure from pregnant cattle. This pattern suggests that application of manure from pregnant cows may cause greater endocrine-disrupting effects in the environment. Hormones and antibiotics were detected in two manure samples from southern Idaho and in one Boise WWTF Class B biosolids, suggesting that land application of organic fertilizers may be a route of CEC contamination in Idaho. The monitoring of CECs in land-applied biosolids and dairy manure is essential for preventing undesired environmental effects.

Table 2.1 Selected Elemental Analyses in Biosolids and Manure, concentration per dry weight

	Manure 1	Manure 2	Biosolids
Date Received	6/12/15	8/18/15	7/9/15
Water Weight (%)	69.7	51.1	88.6
Total N ( $\text{g kg}^{-1}$ )	10.1	24.1	47.8
Available $\text{NH}_4^+$ -N ( $\text{g kg}^{-1}$ )	2.5	1.6	14.5
Available $\text{NO}_3^-$ -N ( $\text{mg kg}^{-1}$ )	49.5	10.0	30.5
P ( $\text{g kg}^{-1}$ ) <sup>+</sup>	2.4	9.0	30.7
K ( $\text{g kg}^{-1}$ )	10.1	40.4	3.4
Cu ( $\text{mg kg}^{-1}$ )	74	127	1229
Zn ( $\text{mg kg}^{-1}$ )	116	348	1040
pH	7.6	8.6	8.3

+ P, K, Cu, and Zn all represent total elemental analysis

Table 2.2 Ranges of Averaged Recovery Rates from Freeze Dried and Field Moist University of Idaho Dairy Manure

	17 $\alpha$ -Estradiol	17 $\beta$ -Estradiol	Estrone	Estriol
Freeze Dried <sup>+</sup>	49.5 - 96.4%	24.3 - 51.2%	30.8 - 60.0%	4.8 - 58.6%
Field Moist <sup>+</sup>	44.6 - 55.2%	32.2 - 50.3%	48.2 - 61.0%	0.0 - 2.7%
Post-Hoc <i>p-value</i>				
(Fisher LSD) <sup>++</sup>	0.29	0.87	0.67	0.17

+ All samples were extracted 24 hours after spiking.

++ Fisher LSD post-hoc test compares each individual compound to each other. Overall Two-Way ANOVA for Freeze Dried vs Field Moist resulted in a non-significance of  $p = 0.36$ .



Table 2.3 Recovery Rates of Acidic and Basic Extractions from Manure or Biosolids

Compounds	Recovery Rate from Basic Extraction (%) <sup>+</sup>	Recovery Rates from Acidic Extraction (%)
<b>Hormonal Steroids</b>		
17 $\alpha$ -Estradiol	117.2	107.5
17 $\beta$ -Estradiol	78.4	92.2
2,3,4- <sup>13</sup> C <sub>3</sub> -17 $\beta$ -Estradiol	58.2	96.3
Estrone	120.5	112.2
Estriol	18.2	111.3
17 $\alpha$ -Ethinylestradiol	135.8	114.8
Progesterone	14.5	4.2
17 $\alpha$ -Hydroxyprogesterone	23.1	6.4
<b>Phytoestrogens</b>		
Coumestrol		86.6
Enterodiol		130.8
Formononetin		22.4
Biochanin A		13.5
Equol		115.9
<b>Antibiotics</b>		
Sulfamethazine		13.2
Sulfadimethoxine		67.7
<sup>13</sup> C <sub>6</sub> -phenyl-sulfadimethoxine		61.2
Sulfamethoxazole		56.3
Sulfathiazole		16.7
Tetracycline		25.6
Oxytetracycline		29.3
Chlorotetracycline		41.2
Penicillin G		33.1
Flunixin		22.2
<b>Human Pharmaceuticals</b>		
Acetaminophen		0.5
Caffeine		0.0
Ibuprofen		12.0
3,4,4'-Trichlorocarbanilide		2.0

+ Basic extraction was not evaluated for the recovery of phytoestrogens, antibiotics, or pharmaceuticals

Table 2.4 Optimized HPLC-MS-ToF Parameters and Recovery Rates from Water

	tR (min)	Fragmenter (V)	LOD <sup>+</sup> ( $\mu\text{g mL}^{-1}$ )	Recovery Rate (%)
<b>Hormonal Steroids</b>				
Estrogens (ESI-)				
17 $\alpha$ -Estradiol	10.34	175	0.41	101.5
17 $\beta$ -Estradiol	9.90	175	0.39	95.9
2,3,4- <sup>13</sup> C <sub>3</sub> -17 $\beta$ -Estradiol	9.90	175	0.12	100.0
Estrone	9.62	175	0.08	101.4
Estriol	4.65	175	0.07	100.6
17 $\alpha$ -Ethinyl estradiol	10.30	175	0.15	116.7
Progestagens (ESI+)				
Progesterone	12.37	175	0.02	35.1
17 $\alpha$ -Hydroxyprogesterone	11.88	175	0.04	53.3
<b>Phytoestrogens</b>				
Coumestrol (ESI -)	1.37	175	0.12	18.7
Enterodiol (ESI -)	4.62	175	0.04	90.7
Formononetin (ESI +)	9.51	175	0.04	102.6
Biochanin A (ESI +)	9.76	175	0.04	60.6
Equol (ESI -)	5.22	175	0.78	0.0
<b>Veterinary Drugs</b>				
Sulfonamides (ESI+)				
Sulfamethazine	8.04	125	0.03	48.1
Sulfadimethoxine	9.59	125	0.04	94.1
<sup>13</sup> C <sub>6</sub> -phenyl-sulfadimethoxine	9.59	125	0.06	100.0
Sulfamethoxazole	8.61	125	0.02	83.7
Sulfathiazole	7.03	125	0.19	0.8
Tetracyclines (ESI+)				
Tetracycline	8.23	175	0.16	8.0
Oxytetracycline	6.95	175	0.04	50.0
Chlorotetracycline	8.53	175	0.12	103.7
$\beta$ -Lactam (ESI+)				
Penicillin G	7.43	125	0.07	28.8
NSAID				
Flunixin (ESI+)	12.34	175	0.17	72.3
<b>Human Drugs (ESI +)</b>				
Acetaminophen	5.05	125	0.08	3.7
Caffeine	8.16	125	0.10	6.6
Ibuprofen	9.11	125	1.54	18.2
3,4,4'-Trichlorocarbanilide	12.70	125	0.15	37.9

+ LOD = Limits of Detection

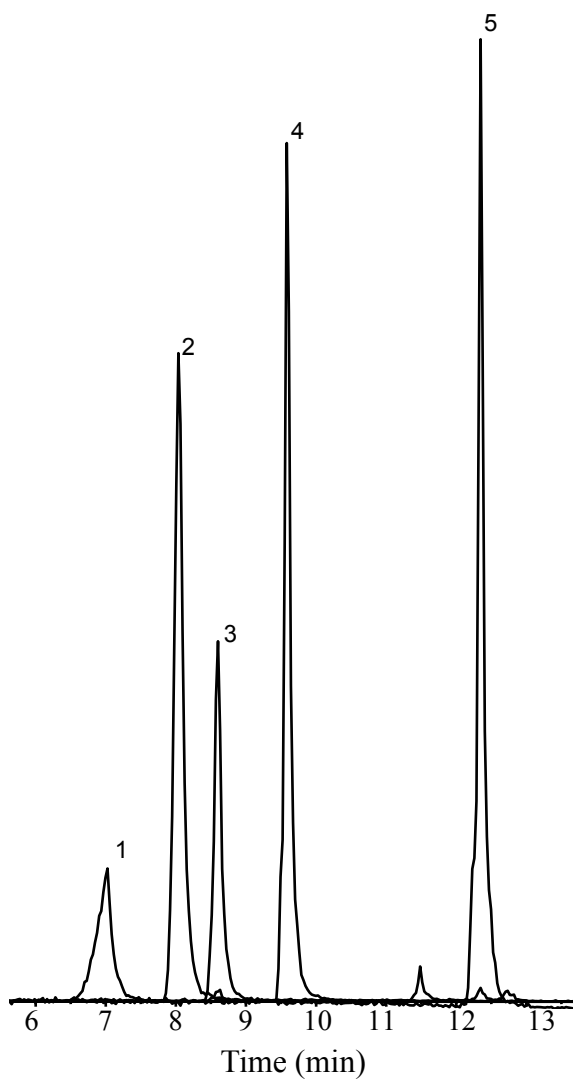


Figure 2.1 Chromatographic separation of drugs in positive ionization, fragmenter 175: 1) sulfathiazole; 2) sulfamethazine; 3) sulfamethoxazole; 4) sulfadimethoxine and  $^{13}\text{C}$ -sulfadimethoxine; 5) flunixin.

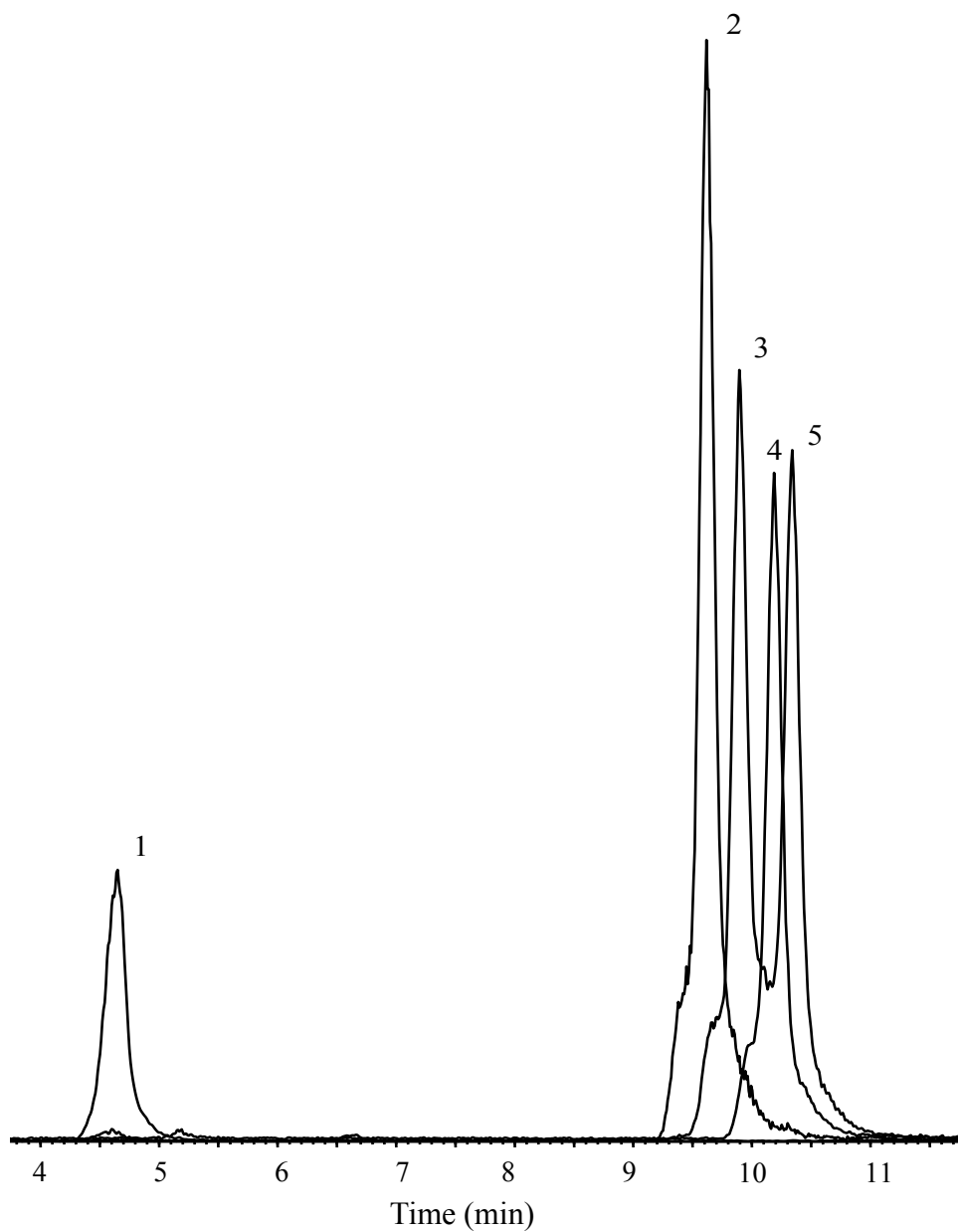


Figure 2.2 Chromatographic separation of hormones in negative ionization: 1) estriol; 2) estrone; 3) 17 $\beta$ -estradiol; 4) 17 $\alpha$ -ethinyl estradiol; 5) 17 $\alpha$ -estradiol

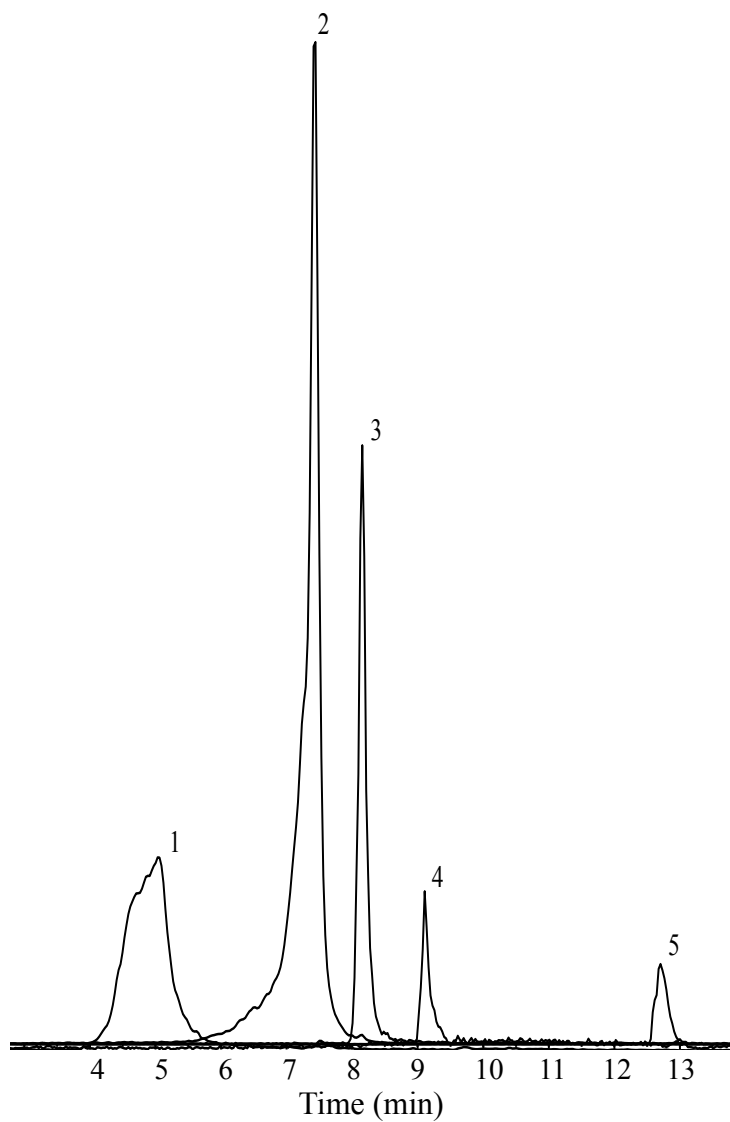


Figure 2.3 Chromatographic separation of human pharmaceuticals: 1) acetaminophen; 2) penicillin G; 3) caffeine; 4) ibuprofen; 5) trichlorocarbide

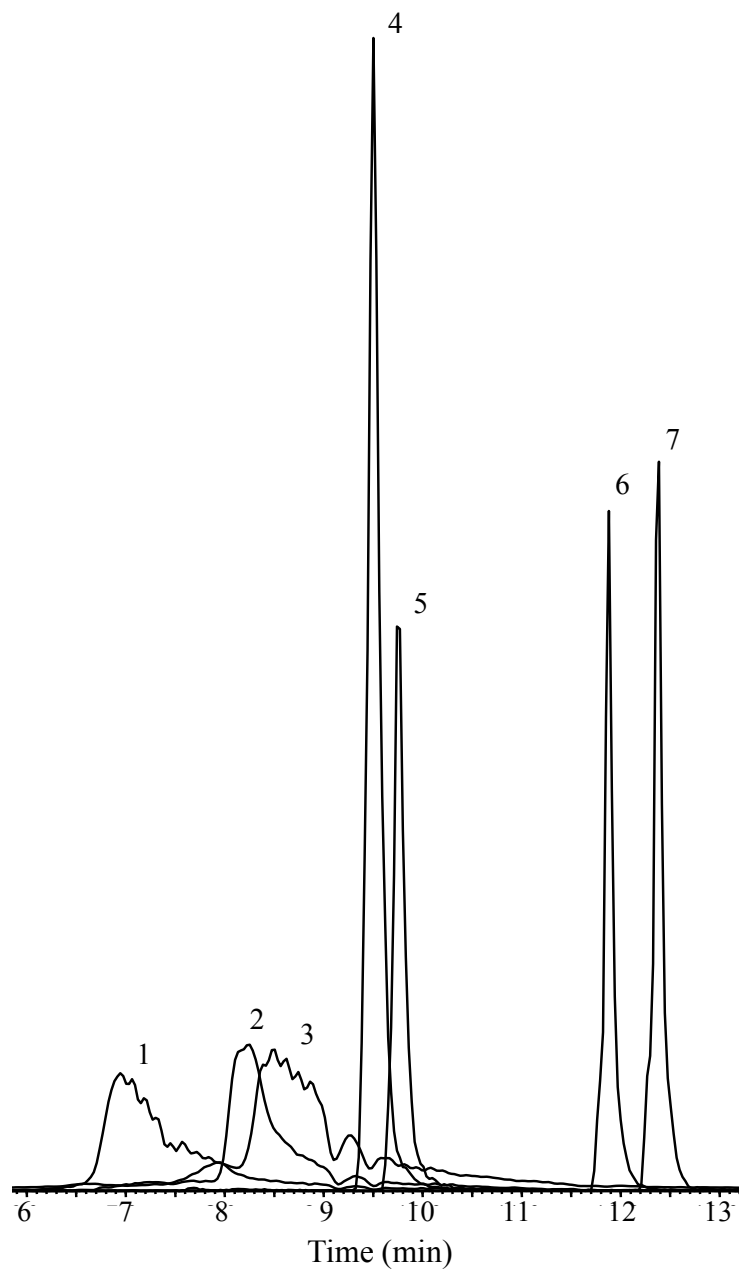


Figure 2.4 CECs in positive ESI, fragmenter 175: 1) oxytetracycline; 2) tetracycline; 3) chlortetracycline; 4) formonentin; 5) biochanin A; 6)  $17\alpha$ -hydroxyprogesterone; 7) progesterone

Table 2.5 CEC Concentration in Two MVC Manure and One Boise WWTF Biosolids Samples ( $\mu\text{g kg}^{-1}$  dried weight)<sup>+</sup>

Date Received	Manure 1 6/12/2015	Manure 2 8/18/2015	Biosolids 7/9/2015
17 $\alpha$ -Estradiol	0.00	0.00	11.29
17 $\beta$ -Estradiol	0.00	0.00	9.34
Estrone	102.87	35.94	82.94
Estriol	27.01	17.86	30.24
Equol	675.44	0.00	0.00
Progesterone	28.87	7.04	20.41
17 $\alpha$ -Hydroxyprogesterone	66.01	49.75	92.01
Sulfadimethoxine	206.12	127.92	0.00
Sulfathiazole	0.00	133.46	63.22
Tetracycline	0.00	364.62	59.54

+ Unlisted compounds were not detected.

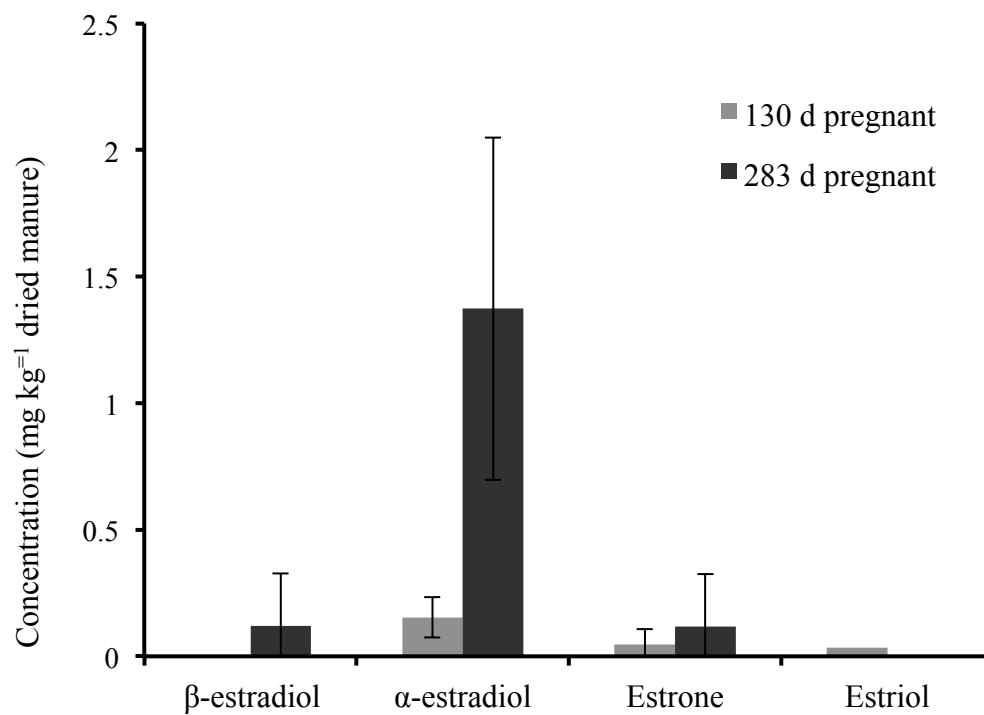


Figure 2.5 Concentrations ( $\text{mg kg}^{-1}$  dry weight) from two different cows at different states of pregnancy from the University of Idaho dairy; Error bars represent standard deviation.



## **Chapter 3: Transport of Chemicals of Emerging Concern through Undisturbed Soil Columns from Southern Idaho**

### **Introduction**

Idaho's dairy industry has grown rapidly over the past decade, making Idaho the 3<sup>rd</sup> largest dairy producing state in the country (USDA NASS, 2011). Consequently, dairy manure has become widely and cheaply available as a form of nutrient amendment, and there has been a marked increase in the number of Idaho producers applying dairy manure to their fields (Leytem et al., 2011). Biosolids, treated solid waste from municipal wastewater treatment facilities (WWTF), are also frequently applied to fields for their nutrient content. Although these organic fertilizers contain essential nutrients, they consistently contain environmentally harmful chemicals of emerging concern (CECs) including endocrine-disrupting chemicals, pharmaceuticals, nutrients, and metals. Application of organic fertilizers to agricultural lands creates a direct route for CECs to enter the environment by leaching through the soil profile, potentially impacting groundwater resources.

CECs encompass a wide array of biologically active compounds including hormones, human and veterinary pharmaceuticals, and inorganic contaminants, all of which are frequently detected in organic fertilizers. Hormones are endocrine disrupting compounds (EDCs) that cause detrimental effects on wildlife by mimicking the role of endogenous hormones, and aquatic organisms are especially vulnerable to these effects (Jobling and Tyler, 2003; Leet et al., 2012). Hormones are detected in both biosolids and dairy manure, but hormone concentrations are a particular concern in dairy manure. Pregnant cattle excrete increasing concentrations of estrogens and progestogens throughout the duration of pregnancy (Hanselman et al., 2003; Tucker, 2000). When aquatic organisms are exposed to these

hormones, they can experience drastic biological effects. Estrogens at concentrations as low as 25 ng L<sup>-1</sup> are found to cause feminization of male rainbow trout (Routledge et al., 1998). Hormones also interrupt the immune systems of fish, possibly making fish in contaminated waters more susceptible to illnesses (Milla et al., 2011).

Phytoestrogens are estrogen-like compounds produced by plants. Like animal hormones, phytoestrogens cause endocrine-disrupting effects on aquatic life. Phytoestrogens can bind to the estrogen receptors of multiple fish species (Latonnelle et al., 2003), creating an estrogenic response *in vivo* (Latonnelle et al., 2002). Many plants in the average dairy cows diet such as soy, barley, and red clover contain high concentrations of phytoestrogens (Tucker et al., 2010). Dairy cows fed diets higher in phytoestrogens have increased estrogenic activity in their manure (Tucker et al., 2010; Lorenzen et al., 2004).

Antibiotics are another class of CECs frequently detected in both dairy manure and biosolids (Kyselkova et al., 2015; Wichmann et al., 2014; Pamreddy et al., 2013). Introduction of antibiotics into the environment is associated with an increase in antibiotic resistance, which is a major public health concern (Kemper, 2008; Ross, 2015). Similar classes of antibiotics are used by both the veterinary and human pharmaceutical industries. Notably, both parties frequently use the tetracycline and sulfonamide classes of antibiotics, increasing the risk of antibiotic resistance to human health (Kemper, 2008). The Environmental Protection Agency (EPA) found antibiotics to be the most frequently detected class of compounds in 110 biosolid samples (McClellan and Halden, 2010). Dairy manure contaminated with antibiotic resistance genes spreads those genes to soil bacteria after manure application (Kyselkova et al., 2015; Ross, 2015).

Several pharmaceuticals used by humans and frequently detected in biosolids are toxic

to aquatic and terrestrial wildlife. For example, ibuprofen is the most toxic of 18 pharmaceuticals to the common earthworm (Pino et al., 2015). Acetaminophen caused the greatest cytogenotoxic effects on freshwater zebra mussel haemocytes, out of three common pain killers (Parolini et al., 2009). Furthermore, human pharmaceuticals are continuously emitted from WWTFs, making them a constant environmental threat.

Inorganic contaminants include metals, nutrients, and salts in organic fertilizers. High metal concentrations in biosolids or manure can accumulate to toxic levels in the soil (Brandt et al., 2008; Lu et al., 2012). Biosolids are strictly regulated for metal application to prevent soil toxicity, but manure does not currently have such limitations (Lu et al., 2012). Some bacteria carry antibiotic and metal resistance genes on the same plasmid, and exposure to either antibiotics and high metal concentrations can accelerate the transfer of antibiotic resistance by co-selection (Seiler and Berendonk, 2012; Snow et al., 2013). The dairy industry uses copper or zinc salts as disinfectants in footbaths, often disposing the used footbath with the manure (Downing et al., 2010). Thus, the combination of metals and antibiotics in manure and biosolids can exacerbate the public health concern over the spread of antibiotic resistant genes.

Excessive nutrient concentrations (nitrogen and phosphorus) can detrimentally impact the environment. Nutrients that leach or runoff from fields receiving biosolids or dairy manure applications cause eutrophication to nearby bodies of surface water (Lu et al., 2012).

These environmental concerns are magnified in southern Idaho where nearly all agriculture, aquaculture, and dairy farms are concentrated along the Snake River (Fig. 3.1). The rainbow trout industry provides significant economic stability within the Magic Valley region, and Idaho is the largest producer of the nation's domestic trout supply (USDA and

NASS, 2015). Endocrine-disrupting chemicals present in dairy manure, including endogenous hormones or phytoestrogens, may enter the groundwater supply by leaching through the soil profile of irrigated fields. Such transport may threaten the drinking water supply of cities further downstream. There is little data on the movement of CECs through the soil profile, and none relevant to the specific conditions in southern Idaho. As the application of dairy manure to agricultural fields increases, it is necessary to understand how repeated application of organic fertilizers can impact the environment (Leytem et al., 2011).

This project focused on understanding the potential threat of land-applied manure and biosolids to agricultural fields using undisturbed soil columns. An interdisciplinary approach was used to address this concern, using soil chemistry, microbiology, and fish biology to thoroughly analyze the environmental impacts. The project used undisturbed soil columns to 1) evaluate the vertical transport of chemicals through a soil profile; 2) determine the spread of antibiotic resistance from organic fertilizer application; and 3) test the endocrine-disrupting potential of soil leachate. My specific objective was to determine the fate of CECs originating in dairy manure and biosolids when applied to the soil, and this chapter will focus on CEC identification and quantification in the column leachate.

## **Materials and Methods**

### **Field Site Description and Column Extraction**

The field site was located at the USDA's Agricultural Research Service facility in Kimberly, Idaho (Fig. 3.1). The studied soil was a Portneuf silt loam (coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcid), which is representative of many cropped fields in southern Idaho. The field site was in a crop rotation of wheat-potatoes-barley-sugar beets beginning with the field season of 2013. Undisturbed soil columns were sampled from a

neighboring field, which had never received manure amendments. A total of 24 soil columns were collected in June 2014. Undisturbed soil columns (10.2 cm diameter, 0.6 m length) were sampled by a tractor-mounted hydraulic soil probe with PVC tubing liners. Columns were stored at 4° C until the start of the experiment (August 2015). A subsample from the top 20 cm of the soil column was analyzed by the Analytical Services Laboratory at the University of Idaho (Moscow, ID). The soil had the following characteristics: pH of 7.7, organic matter content of 1.5%, available potassium of 94  $\mu\text{g g}^{-1}$ , available phosphorus of 38  $\mu\text{g g}^{-1}$ , and nitrogen as nitrate and nitrite at 32  $\mu\text{g g}^{-1}$ . The pH below 30 cm averaged 8.3.

## Chemicals and Materials

All chemicals purchased were from Sigma Aldrich (St. Louis, MO, USA). This consisted of seven hormones (17 $\beta$ -estradiol, 17 $\alpha$ -estradiol, estrone, estriol, ethinyl estradiol, progesterone, and  $\alpha$ -hydroxyprogesterone), five phytoestrogens (coumestrol, enterodiol, formononetin, biochanin A, and equol), nine antibiotics (sulfamethazine, sulfadimethoxine, sulfamethoxazole, sulfathiazole, tetracycline, oxytetracycline, chlortetracycline, trichlorocarbide and penicillin G), one veterinary drugs (flunixin), and three human pharmaceuticals (caffeine, acetaminophen, and ibuprofen). Two internal, isotopic standards were used for method confirmation as internal standards: 2,3,4- $^{13}\text{C}_3$ -17 $\beta$ -estradiol ( $^{13}\text{C}$ -E2) and  $^{13}\text{C}_6$ -phenyl-sulfadimethoxine ( $^{13}\text{C}$ -SDX). Stock solutions (1.00 g L $^{-1}$ ) of each chemical were prepared in methanol and stored at -20 °C. New stock solutions were prepared every 6 months. Dilutions for standard calibration curves were prepared in methanol.

Methanol, water, acetonitrile, acetone, and ethyl ether were all HPLC-grade solvents (Fisher Scientific or Macron Fine Chemicals, Pennsylvania, USA). Ultrapure deionized water was filtered in-lab with an Ion X Charger (Cole-Parmer Instrument Company, Vernon Hills,

IL, USA) and then a Bion Exchanger (Pierce Chemical Company, Rockford, IL, USA).

Formic acid (98%) was purchased from Fluka Analytical, and sodium hydroxide (NaOH) from Macron (Center Valley, PA, USA). Oasis HLB SPE cartridges (30  $\mu$ m, 3 mL, 60 mg sorbent) were bought from Waters Inc. (Milford, MA, USA).

Glass centrifuge tubes (50 mL) and glass vials (15 mL) were silanized prior to use in manure extractions as described by Seed (1994). Briefly, glassware was fumigated with dichlorodimethylsilane (3 mL) under vacuum for 24 h. After fumigation, glassware was rinsed with deionized water and air-dried.

Soil columns were designed as passive capillary samplers to prevent saturated conditions (Boll et al., 1992). A fiberglass wick (9.5 mm, Pepperell Braiding Co., Pepperell, MA) was attached to the bottom of each column. Before installation, wicks were combusted at 400 °C for 3 h to remove carbon compounds. Wicks were then soaked in DDI water, changed weekly, for 120 d to remove impurities that may impact the study (Jabro et al., 2008).

Untreated dairy manure was obtained from Magic Valley Compost Facility (Jerome, ID). Manure was extracted within 24 h of arrival to determine concentration of CECs. Class B biosolids were donated by Boise Wastewater Treatment Facility (Boise, ID). Biosolids were stored at 4° C and analyzed within a week.

### Determination of CECs in Initial Manure and Biosolids

Subsamples of biosolids and manure were sent to Soiltest Farm Consultants, Inc. (Moses Lake, WA, USA) for analysis of nutrients and metals (Table 3.1). For determination of CECs, manure and biosolids were extracted using two different methods. The basic extraction optimized for hormone extraction, and the acidic extraction optimized removal of antimicrobials and pharmaceutical drugs. Extracts were then analyzed on HPLC-MS-ToF to

determine CEC concentrations.

The basic extraction followed methods from Zheng (2008). Briefly, 2.5 g of field-moist manure (~0.5 g dry weight) was weighed into a silanized glass centrifuge tube, and 10.0 mL of ethyl ether and 5.0 mL of 1 M NaOH were added to the manure. The tube was shaken horizontally for 1 h, centrifuged at 1500 rpm for 15 min, and the organic layer was decanted into a glass vial. This extraction was repeated a total of three times, and the organic layers were combined. The organic solvent was evaporated under nitrogen gas until near dryness. The dried extract was reconstituted to 1 mL in methanol and filtered through 0.2  $\mu$ M polyethersulfone syringe filter (Corning Scientific, Corning, NY).

An adaptation of Aga et al.'s (2005) method was used for extraction of antibiotics and hormones. Field-moist manure (2.5 g) was weighed into a 50-mL glass centrifuge tube, and 10 mL of acetone acidified with formic acid (pH = 4) was added to each sample. Samples were shaken for 30 minutes, centrifuged at 1500 rpm for 15 minutes, and the organic layer was decanted into a glass vial. This process was repeated a second time, and acetone extractions were combined. The manure was then extracted with 10 mL of 1 M citric acid, shaken, and centrifuged. The citric acid solution was added to the acetone extractant layers, and the citric acid extraction was repeated a second time. Combined extracts were concentrated under nitrogen gas to approximately 50% of the original volume to remove most of the organic solvent from solution. The concentrated extract was then diluted to 100 mL with DDI water. The diluted sample was further concentrated under vacuum on an Oasis 3cc HLB SPE cartridge, previously prepared with 3 mL methanol and 3 mL of water. The cartridge was allowed to dry under vacuum for 30 min. Cartridges were stored at -20 °C for up to 3 mo before elution. SPE cartridges were eluted with 3 mL of methanol and 3 mL of 1:1

methanol:acetone. The solvents were evaporated to 1 mL under nitrogen gas and then analyzed using HPLC-MS-ToF.

### Preparation of Column Experiment

To determine the hydraulic conductivity of the soil, one column was cut into six 10.2-cm length sections. Hydraulic conductivity was measured in each section using a mini disk infiltrometer (Decagon Devices, Pullman, WA). The lowest hydraulic conductivity ( $0.04 \text{ cm h}^{-1}$ ) set the flow rate of the entire column to ensure steady-state unsaturated flow conditions during the experiment. Multichannel peristaltic pumps (Models 205S and 205U, Watson Marlow, Wilmington, MA, USA) were optimized to drip 100 mL (+/- 3 mL) of 0.005 M  $\text{CaCl}_2$  solution per day on each column. The  $\text{CaCl}_2$  solution was autoclaved to prevent microbial contamination.

Soil columns were equilibrated at room temperature for 2 weeks to allow soil microorganisms to adjust to ambient temperature ( $30^\circ \text{C}$ ). The bottom cap of the column had a 2.5 cm diameter hole drilled in its center to allow insertion of Tygon 2375 Ultra Chemical Resistant tubing ( $\frac{1}{2}$ " ID,  $\frac{3}{4}$ " OD, US Plastic Corporation, Lima, OH). A fiberglass wick was pulled through the Tygon tubing. Individual fibers of the wick (5 cm length) were separated and laid radially across the inside of the cap to provide adequate contact with the soil, which produced a negative soil water potential and prevented the bottom of the soil column from becoming saturated. Autoclaved sand (2 cm deep) was spread evenly on top of the wick fibers, also serving to prevent saturated conditions in the soil. The bottom 2 cm of the soil was removed to make room for the sand, and the bottom cap was reconnected to the column. The columns were flushed with 0.005 M  $\text{CaCl}_2$  for 4 days before the start of the experiment. The leachate from the columns was collected in an autoclaved amber bottle stored at  $4^\circ \text{C}$  (Fig.



3.2). This effluent was prepared and analyzed to determine the  $t_0$  concentrations of CECs.

Columns were randomly assigned into four treatments by unsorted, blind selection: chemical, manure, biosolid, and fertilizer. For each treatment, 4 cm of soil was removed from the top of the column and homogenized. The treatment was then mixed with 300 g of soil and reapplied to the top of the soil column. Manure-treated columns had field-moist dairy manure ( $98 \text{ g column}^{-1}$ ) applied at a similar rate as the field experiment ( $47.2 \text{ ton acre}^{-1}$ ). Biosolid-treated columns had wet biosolids (210 g) applied at a rate to match the manure's total nitrogen content. Urea fertilizer (0.226 g; NPK: 46-0-0) was added to the fertilizer-treated columns at a rate to match manure's available N content. It was determined that phosphorus and potassium additions were not needed because of the available concentrations of these nutrients already in the soil. The chemical treatment was spiked with 10 mg of each compound listed under "Chemicals and Materials," except for the two isotopes and the five phytoestrogens due to cost. The mixture of compounds was dissolved in MeOH and mixed into the soil. The soil was dried under  $\text{N}_2$  to remove most of the organic solvent, and the soil was added back on the column.

Bromide was used as a conservative tracer. Potassium bromide (0.1 M) was added in 5.0-mL increments to each column at the start of the study. Filter paper (11.0 cm d., Q8 Course Porosity, Fisher Scientific, Pennsylvania, USA) was set on the top of each column to ensure the equal spread of the water but was removed after 1 week because of mold growth.

$\text{CaCl}_2$  solution (0.005 M) was dripped onto the top of each soil column at  $100 \text{ mL day}^{-1}$  by use of a peristaltic pump and collected in sterile amber bottles kept inside a refrigerator ( $4^\circ \text{C}$ ). The column leachate was weighed and emptied twice a week into a 1-L amber bottle, producing a composited 1-week sample for each column. The leachate was split evenly: half

of the volume was used for microbiological evaluation and the other half for chemical analyses.

### Column Leachate Treatment

Column leachate was stored at 4 °C until analysis. Each of the 5 replicates of the chemical treatment leachate was spiked with 100 µL of 1 mg L<sup>-1</sup> of 2,3,4-<sup>13</sup>C<sub>3</sub>-17β-estradiol and <sup>13</sup>C<sub>6</sub>-phenyl-sulfadimethoxine isotopic standards before concentration on SPE cartridge to determine recovery rates.

SPE cartridges were conditioned with 3 mL of MeOH followed by 3 mL of DDI H<sub>2</sub>O (Hernandez et al., 2015). Conditioned cartridges were attached to 500-mL vacuum flasks, and column leachate was loaded onto the cartridges under vacuum at approximately 100 mL h<sup>-1</sup>. Cartridges were rinsed with 3 mL of DDI H<sub>2</sub>O, dried under vacuum for 30 min, and stored at -20 °C until analysis.

SPE cartridges were allowed to adjust to room temperature for 30 min before elution. Elution of the cartridge was performed with 3 mL MeOH and 3 mL 1:1 MeOH:acetone. The collected solvent was concentrated to 1 mL under N<sub>2</sub> and analyzed by HPLC-MS-ToF to determine the concentration of CECs. Excess sample was then stored at -20 °C until used for determination of endocrine disruption potential in a rainbow trout hepatocyte bioassay.

Soil column leachate that passed through the SPE cartridge was saved in the 500 mL vacuum flasks for additional analyses. Subsamples were taken for elemental analysis (10 mL) by ICP-AES and for determination of nutrient ions (5 mL) by IC. Each ICP-AES subsample had 1 drop of concentrated HCl added to keep metals suspended in solution. The IC subsample was left untreated. Both subsamples were stored at 5 °C until analysis.

### ICP-AES Analysis of Column Leachate

Acidified subsamples from column effluent as obtained above were tested for total P, Zn, and Cu by ICP-AES (iCAP 6000 series, Thermo Scientific, Waltham, MA). Standards were prepared with the same acidification procedure as samples, and a quality control sample was tested after every 12 samples to ensure validation of the calibration curve.

### Ion Chromatographic Analysis of Anions in Column Leachate

Subsamples of the column leachate were tested for bromide, nitrate, and sulfate on a Dionex Ion Analyzer (Dionex Corporation, California, USA) equipped with a GP40 gradient pump, ED40 electrochemical detector, and an AS40 autosampler. A Dionex IonPac AS18 Analytical column (4  $\mu\text{m}$  x 250 mm) separated anions using 16 mM NaOH as the mobile phase. The flow rate was kept constant at 1 mL min<sup>-1</sup>. The anion suppressor was set to 300 mA, and the detector stabilizer temperature was controlled at 30 °C with temperature compensation of 1.7% per °C. The injection volume was 20  $\mu\text{L}$ .

### HPLC-MS-TOF Analysis of CECs in Column Leachate

Compound analysis was performed with an Agilent 1200 Series HPLC system containing a diode array detection (DAD) system. The HPLC was coupled to an Agilent G1969A TOF-MS system with an electrospray ion (ESI) source (Agilent Technologies, Santa Clara, CA, USA).

Studied compounds were separated at 30 °C on a Kinetex 5 $\mu$  EVO C18 (50  $\mu\text{m}$  x 4.6 mm) column paired with the appropriate ULTRA guard cartridge designated for the column (Phenomenex, Torrance, CA, USA). The needle was washed in isopropyl alcohol between samples, and all injections were 5  $\mu\text{L}$ . Three methods were created: Method 1 optimized separation of hormones in negative ionization; Method 2 optimized separation of

sulfonamides in positive ionization; and Method 3 optimized ionization and separation of pharmaceuticals and tetracyclines in positive ionization. All three methods relied on linear gradients during solvent proportion changes.

The method for negative ESI (Method 1) used the mobile phases 0.02%  $\text{NH}_4\text{OH}$  in water (Solvent A) and 0.02%  $\text{NH}_4\text{OH}$  in methanol (Solvent B). The program began with 60% A and 40% B. For the first 2.5 min, solvent B was brought up to 50%, after which it was then slowly ramped up to 65% over 8.5 minutes. Solvent B was then brought to 100% over the next 2 min and held at 100% for 50 s. The solvent was then brought back to 40% B and 60% A over 10 sec. This was held steady for the last 4 min with a total run time of 18 min. The flow rate was at  $0.5 \text{ mL min}^{-1}$ .

Method 2, with positive ESI, used the mobile phases 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The program began with 95% solvent A and 5% solvent B. This was held for 2 min followed by a linear gradient to 100% B over 8 min. Solvent B was held at 100% for 3 min, then decreased to 5% B over the course of 1 min. This was maintained for an additional 2 min, creating a total runtime of 18 min. The flow rate was set at  $0.4 \text{ mL min}^{-1}$ .

Method 3 also used positive ESI, but the mobile phases were 0.02%  $\text{NH}_4\text{OH}$  in water (A) and 0.02%  $\text{NH}_4\text{OH}$  in methanol (B). The program began with 95% solvent A and 5% solvent B. This was held for 2 min followed by a linear gradient to 100% B over 8 min. Solvent B was held at 100% for 3 min, then decreased to 5% B over the course of 1 min. This was maintained for an additional 2 min, creating a total run time of 18 min. The flow rate was set at  $0.4 \text{ mL min}^{-1}$ .

The nebulizing gas was nitrogen. All methods had the following optimized

parameters: nebulizer pressure (25 psig), drying gas flow rate (10 L min<sup>-1</sup>), drying gas temperature (350 °C), capillary potential (3500 V), and transients per scan (8000). The analyses were conducted with an  $m/z$  range from 90 to 1000 amu. Method 1 used only one fragmentor strength (175 V); whereas Methods 2 and 3 analyzed compounds at fragmentor strengths of 125, 175, and 250 V.

## Statistical Analysis

Data processing for the HPLC-MS was performed with the software Analyst QS 1.1. Compounds were identified by retention time and the specific product masses resulting from fragmentation. The method limit of detection was determined by a signal three times the background noise from the chromatogram. Limits of detection (LOD) for HPLC-MS and ICP-AES were based on a least square regression of  $3.3*(s_y b^{-1})$  where  $s_y$  equals the residual standard deviation of the calibration curve and  $b$  is the slope of the line. Compounds that exceeded three times the signal-to-noise ratio, but were calculated below the method LOD, were given the value of  $\frac{1}{2}$  LOD. This was found to be an effective and appropriate method when less than 15% of the results are censored (Manly, 2008; EPA, 2000).

Statistical analysis of the data was performed using SAS 9.4 software in two steps (SAS Institute Inc., 2016). First, the data was fit to a model and assessed for good fit individually. Then, the data was fit to a full dummy variable model for each treatment\*compound interaction in the nonlinear mixed-model procedure. The model was:

$$\mu = \frac{(m + e)}{(1 + \exp^{(-B * \text{LOG}(\text{Week}) - \text{LOG}(L)))}}$$

$$\text{Model } y \sim \text{NORMAL}(\mu, s^2)$$

$$\text{Random } e \sim \text{NORMAL}(0, S m^2)$$

where the parameters  $m$ ,  $B$ , and  $L$  represented the maximum value, slope, and median lag time (time =  $\frac{1}{2} m$ ), respectively. The  $s$  and  $Sm$  variables accounted for randomness in the model. The parameter  $y$  represented the compound being studied. This model predicted the cumulative values by week. Table 3.2 lists the parameters for each treatment's model cumulative leaching of bromide. Figure 3.3 depicts the model against a scatter plot of the data for cumulative bromide leaching as an example for how all models were evaluated. The remainder of the model parameters are found in Appendix A.

For each compound,  $m$  was compared to determine significant difference across the four treatments using the *contrast* command in the *nlmixed* procedure. The  $L$  parameter was also compared in the bromide models across treatments to determine if the treatment affected transport time of the tracer. The random parameters  $s$  and  $Sm$  were averaged for the compared models, except for the phosphorus model in which each treatment had its individual random parameters.

In the chemical treatment columns, the compound's  $L$  factor was compared to the control compound bromide to determine a difference in lag time between compounds.

## Results and Discussion

### Column Observations

The data from the biosolids-treated columns revealed limitations that prevent the treatment from being compared quantitatively to the other treatments. Although 100 mL day<sup>-1</sup> was dripped on the top of each biosolids column, the collected effluent averaged 41 mL day<sup>-1</sup> by week 5. It is assumed that organic matter in the biosolids plugged soil pores, which inhibited the flow of water. Consequently, the columns began to leak from the lids daily. Due to leaking, the calcium chloride solution flow rate was decreased for only the biosolids

treatment to just  $20 \pm 3 \text{ mL day}^{-1}$  on week 7. The biosolids effluent was analyzed using the same procedure as the other treatments, but the pattern of bromide leaching made it clear that water flow in the biosolids-treated columns was impeded (Fig. 3.3). The biosolids data was not statistically compared to the other treatments, but a summary of the leachate is included in Appendix B.

Despite the difference in water movement, there was no significant difference in bulk density among treatments. This suggests that the limited water mobility in the biosolids columns was not the result of soil compaction. However, the bulk densities of the top sections of the soil columns were significantly lower than the middle or bottom sections for all treatments ( $p < 0.001$ ; Table 3.3).

The isotopic standards yielded an adequate recovery throughout the duration of the experiment. Over the course of the experiment,  $^{13}\text{C}$ -E2 and  $^{13}\text{C}$ -SDX averaged recovery rates of 110% and 89%, with 95%  $t$ -based confidence intervals of (100.1, 131.8) and (68.4, 109.7), respectively. Matrix effects pose a challenge to analytical analysis of environmental samples, especially in those with high organic matter (Białk-Bielińska et al., 2016). Estrogens have been shown to undergo positive interference in some complex matrices, explaining the slightly over 100% recovery rates (Nguyen et al., 2011; Kumar et al., 2009). In contrast, sulfonamides have frequently shown matrix suppression of up to 80% decreased response (Bian et al., 2015; Salvia et al., 2012). Although many investigators adjust their reported values according to the retention rates of their isotopic standards, this thesis did not make such assumptions. The values reported in this thesis reflect the exact measurement by the mass spectrometer.

## Bromide Elution

Bromide was used as a conservative tracer to measure the time it took for water to move from the top to the bottom of the soil columns. Bromide moved similarly through the chemical, fertilizer, and manure treatments, eluting between weeks 2 and 4 (Fig. 3.4). High recovery rates were found for the chemical, fertilizer, and manure treatments at 85%, 85%, and 87%, respectively. The model revealed lag values ( $L$ ) of 2.3, 2.1, and 2.8 weeks for the chemical, fertilizer, and manure treatments, respectively (Table 3.2). The high recovery rates and quick elution time (over the course of ~2 weeks) suggest that bromide served as a good indication of the time it took for water to reach the bottom of the column.

In contrast, bromide eluted throughout the 13-week duration of the experiment from the biosolids-treated columns and yielded an average recovery rate of 62%. The significant reduction in recovery rate and the extended leaching time verify that the biosolids amendment caused a dramatic difference in the movement of water in those soil cores.

## Nutrient and Metal Leaching

Total phosphorus (P) leached a cumulative of 0.64, 0.75, and 1.21 mg from the fertilizer, chemical, and manure treatments, respectively. P leached primarily during weeks 2 through 5 for all treatments (Fig. 3.5). This matches the bromide elution period, suggesting that the P originated near the soil surface and traveled the length of the soil column.

Phosphorus has traditionally been considered nearly immobile in the soil due to its low solubility and its high affinity to soil particles, but recent studies have noted the importance and prevalence of P movement through the soil (King et al., 2015). Another column study using soil from southern Idaho determined a strong positive correlation with leaching total organic carbon and total phosphorus loss (Tarkalson and Leytem, 2009). The additional



organic matter in the manure-amended treatment may have increased phosphorus movement in those columns.

Nitrate exhibited a very different pattern than phosphorus (Fig. 3.6). Columns leached their highest concentrations during the first 2 weeks, which would be outside the influence of the treatment amendments. Nitrate production began when the columns were wetted one week before the start of the study, stimulating microbial activity. This flush of nitrate after wetting a dried soil is well documented in the literature (Sposito, 2008; Harmel and Haney, 2013; Franzluebbers et al., 2000). The chemical treated columns eluted nitrate at a constant rate, due to the absence of a nitrate source. The linearity of the nitrate leaching for the chemical treatment did not fit the model well (Fig. 3.7), and thus the respective model for nitrate was not included in the statistical comparison of nitrate across treatments. The chemical treatment eluted a maximum of 12.7 mmol, which was just half of what the manure and fertilizer treatments eluted.

The manure and fertilizers treatments did receive nitrate inputs, and the fertilizer was applied at a rate to match the available nitrate of manure. However, the manure treatment leached slightly more nitrate than the fertilizer treatment ( $m = 27.7$  and  $24.1$  mmol, respectively;  $p < 0.05$ ). Nitrate poses a significant public health threat in groundwater. It is known to cause methaemoglobinemia, which is commonly referred to “blue baby syndrome” for the blue-tinge infants develop from the disease. Nitrite binds to hemoglobin in the blood which prevents oxygen from being carried through the blood stream, and in large enough concentrations, nitrite can suffocate the consumer. Nitrate can be transformed to nitrite in the body, and the EPA has a limit of  $10 \text{ mg L}^{-1}$  ( $0.16 \text{ mmols L}^{-1}$ ) in drinking water (Knobeloch et al., 2000). Small family wells are not checked with the same frequency as drinking water

facilities, and those downstream from agricultural fields risk containing toxic concentrations of nitrate and nitrite in the water. The manure-amended soil leached significantly more nitrate than non-treated soil, and the continued leaching of nitrate into the groundwater could threaten the drinking water source of people downstream.

Sulfate leached in the highest concentrations of any of the nutrients from the manure-amended columns (Fig. 3.8). Manure treatments eluted significantly more sulfate ( $m=33.19$  mmol) compared to chemical (4.16 mmol) and fertilizer (6.51 mmol) treatments ( $p<0.001$  check). There was no significant difference in  $m$  between the fertilizer and chemical treatments. The abundance of sulfate in dairy manure may be partially due to the use of copper sulfate by the dairy industries (Downing et al., 2010). When sulfate leaches through the soil, it brings a positive ion (likely  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ ) with it. This decreases the concentration of these essential plant micronutrients in the soil solution, threatening plant productivity (Hao and Chang, 2002). Salt accumulation is another critical concern from repeated manure applications (Hao and Chang, 2002), and some fields have had electrical conductivity measurements double after just two annual applications of dairy manure (Martin et al., 2011). Salinity in soil can decrease water availability to plants and microbial communities, and eventually, lower crop yields (Hao and Chang, 2002). Salinity is especially a threat in dry climates, such as south-central Idaho (Zglobicki et al., 2016).

The metals copper and zinc leached from all treatments (Figs. 3.9 and 3.10). This was also true of the chemical- and fertilizer-treated columns, which did not receive any metal additions in their treatments (Fig. 3.9). Thus, the leached copper and zinc must have originated in the soil. During the procedure of setting up the soil columns, the top few inches of the soil was disturbed and thoroughly hand-mixed. Physical disturbance of soil has been

shown to break apart aggregates, which spatially protect a variety of compounds inside the aggregates by preventing enzymatic or microbial access (McCarthy et al., 2008; Darrouzet-Nardi and Weintraub, 2014). For example, carbohydrates have been protected for decades inside some soil aggregates (Darrouzet-Nardi and Weintraub, 2014). Physical disturbance during the set-up may have released CECs that were spatially protected inside soil aggregates.

Copper and zinc exhibited clear peaks around weeks 2 – 3 and 4 – 6, respectively. The timing of the peak suggests that the metals originated at the surface of the columns. It is important to note that both copper and zinc content was measured after the effluent was run through the SPE cartridges, so any organically-bound metals may have been removed. There was no significant difference among treatments in the cumulative amount of zinc leaching.

Copper leached in greater concentrations than zinc from the manure treatment. Copper elution from the manure treatment peaked during Weeks 4 to 5, which was a bit later than the other treatments that peaked in Week 2 (Fig. 3.10). This may have been due to organic matter in the manure treatment, which delayed the transport of copper (Mohamed et al., 2010). The manure treatment leached slightly more total copper, but not significantly more than the other two treatments.

Copper accumulation in the soil from land-applied dairy manure is an emerging concern (Leytem et al., 2011). Many dairies use copper or zinc sulfate solutions as hoof baths to prevent disease on their cows, which are then disposed with the dairy manure (Downing et al., 2010). If the leached copper and zinc from the manure columns originated in the manure, then only an average of 0.6% and 1.5% leached through the soil column, respectively. Likely, the majority of leached metals were mechanically released during the set-up of the soil columns. If over 99% of the copper in dairy manure is held by soils, then copper will

accumulate rapidly in the soil. After just one application of dairy manure, our soil columns had accumulated  $7.38 \text{ kg ha}^{-1}$  copper. The cumulative loading limit for copper in biosolids is  $1503 \text{ kg ha}^{-1}$  (Lu et al., 2012). At this rate, it would take 203 years to pass the EPA threshold of copper accumulation. However, increased total copper concentration in the soil causes an increase of bioavailable copper in soil solution (Brandt et al., 2008), which can then cause plant and soil organism toxicity. Copper that is sorbed to dissolved organic matter (DOM) represented the majority of bioavailable copper and mobile copper in manure-amended soils (Brandt et al., 2008; Ashworth and Alloway, 2004). Thus, organic fertilizers with more DOM may cause a greater threat because a large proportion of metals are bioavailable due to DOM-metal associations. Studies that have measured the respiration rates of soil microbial communities have determined that copper sulfate is more toxic than organically-bound copper (Bolan et al., 2003). Although it would take generations for one field plot to exceed federal regulations for copper accumulation from manure applications, copper toxicity may begin affect plant and soil organisms before that threshold.

### Leaching of Spiked CECs from the Chemical-Treated Columns

Six compounds exhibited a distinct pulse through the chemical treatment columns: sulfamethazine, sulfadimethoxine, sulfamethoxazole, sulfathiazole, ibuprofen, and flunixin. They eluted from all five replicates of the chemical treatment. In contrast, none of these compounds leached with a clear peak from the fertilizer-treated control columns. The absence of a clear peak of the eluted compounds from the control soil columns suggests that those six compounds originated from chemical additions made to the treatment.

All four of the applied sulfonamides were detected in the leachate of the chemical treatment (Figures 3.11 – 3.14), eluting about the same time ( $L = 5.12 - 5.96$ ) (Table 3.4). The

$L$  values for the sulfonamide models were later than the  $L$  for bromide ( $L = 2.30$ ), meaning that it took longer for the sulfonamide antibiotics to move through the soil profile. This is consistent with another study that found sulfonamides to be delayed by 2 – 5 pore volumes compared to the conservative tracer (Wehrhan et al., 2007).

The recovery rates for the sulfonamides added in the chemical treatment ranged from less than 0.01% (sulfathiazole) to 4.7% (sulfadimethoxine) (Table 3.5). Sulfadimethoxine eluted in the greatest concentrations of the four sulfonamides with an average total amount of 472  $\mu\text{g}$  collected (Fig. 3.12). The variability in elution concentration among sulfonamides may be partially explained by the half-lives ( $t_{1/2}$ ) of each compound. In one degradation study, sulfathiazole was found to have a half-life of 6.1 days in a sandy soil with a pH of 8.2 and clay content of 9.2% (Salvia et al., 2014). In that same soil, sulfadimethoxine had a half-life of 20.4 days, and sulfamethoxazole measured a half-life of 11.8 days (Salvia et al., 2014). Although the exact half-life of each sulfonamide is unknown in this specific soil, sulfadimethoxine experienced the least degradation of the studied sulfonamides in Salvia's study (2014). Those relative degradation rates correspond with the increasing recovery rates found in this study. That is, the sulfonamide yielding the highest recovery rate (sulfadimethoxine at 4.7%) was found to have the longest half-life compared to other sulfonamides (Salvia et al., 2014). Sulfamethoxazole (0.45%) and sulfathiazole (<0.01%) followed this pattern. Thus, it is likely that the very low recovery rates for sulfathiazole are in part due to its high degradation in the soil environment.

Sulfonamides are consistently found to be one of the most mobile chemical families in the environment. Our research agrees with many other studies that found that sulfonamide antibiotics moved in the greatest concentrations and the greatest distances over hormones,

tetracyclines, macrolides, or human pharmaceuticals in the soil (Kay et al., 2005b; Wehrhan et al., 2007; Salvia et al., 2014; Zentner et al., 2015). Sulfonamides have two  $pK_a$  values: the first is the deprotonation of the aromatic amine around pH of 3 (making the compound neutral) and the second is the deprotonation of the sulfonamide group at a pH of 5 – 7 (giving the compound a negative charge) (Thiele-Bruhn and Aust, 2004; Burkhardt and Stamm, 2007; Dmitrienko et al., 2014). The high pH of southern Idaho's calcareous soils favors the anionic form of these compounds (Burkhardt and Stamm, 2007), which results in anion-anion repulsion between the negatively charged soil particles and the compound. Additionally, sulfonamides have lower sorption ( $k_d$ ) to the soil than other CECs in alkaline soils (Kim et al., 2012; Doretto et al., 2014). Thus, the mobility of the sulfonamides in this experiment is explained by their negative charge and the alkalinity of the soil.

Ibuprofen leached from the chemical soil columns from weeks 2 – 4 (Fig. 3.15), the same weeks as bromide ( $L = 2.23 \pm 0.05$ ). Another column study found ibuprofen to leach at the same rate as a conservative tracer (Borgman and Chefetz, 2013). Ibuprofen is highly mobile in the soil, partly due to its high water solubility (Borgman and Chefetz, 2013). Additionally, ibuprofen has a carboxylic functional group with a  $pK_a$  of 4.4, making it an anion with this soil's pH. Thus, anion-anion repulsion would further decrease ibuprofen's affinity to sorb to clay particles in soil and increase its mobility.

No ibuprofen was detected after week 4, and the average recovery rate was only 0.2% (Table 3.5). This is likely due to degradation of the compound. Much variance exists in reporting the half-life of ibuprofen. Experiments in soil and water matrices found the half-life of ibuprofen to be 12 and 15 days, respectively (Petrie et al., 2014; Gottschall et al., 2012). Alternatively, another study found that ibuprofen had completely degraded in soil after 14

days (Pino et al., 2015). Thus, it was very possible for ibuprofen to be largely degraded after 28 days.

Although ibuprofen degrades quickly, it is a constant output from WWTF. As a result, it is continuously entering the environment from WWTF effluent or from the land-application of biosolids. Ibuprofen is toxic to organisms in soil (Pino et al., 2015) and aqueous environments (Ortiz de Garcia et al., 2014). The combination of ibuprofen's constant presence, high water solubility, and ecotoxicity make it a significant threat to the environment.

Flunixin eluted with the highest recovery rates of any CEC studied, with an average of 12.76% (Table 3.5). Like the sulfonamides and ibuprofen, flunixin is a weak acid with a pKa of 5.8 (SDS, 2016). In this alkaline soil, the carboxylic functional group would be fully deprotonated, making flunixin a mobile anion in the soil environment.

Flunixin did not begin to elute from the columns until week 7, which is considerably later than the other studied compounds (Fig. 3.16). The  $L$  for flunixin is  $10.67 \pm 0.28$  compared to  $2.30 \pm 0.05$  weeks for bromide. Flunixin has a log  $K_{OW}$  of 3.83, which is much higher than the sulfonamides ( $K_{OW} \sim 1.5$ ) or ibuprofen (0.8 – 3.4) (Thiele-Bruhn and Aust, 2004; Xia et al., 2005; sigmaaldrich.com, 2016). Flunixin's low water solubility and affinity to organic carbon contribute to its slow movement through the soil. It continued to elute in high concentrations during the last week of the experiment, suggesting that flunixin would have continued eluting past week 13.

Flunixin meglumine is a non-steroidal, anti-inflammatory drug (NSAID) approved for use on cattle and horses. There is very little information on the environmental occurrence or behavior of flunixin. One study measured the occurrence of common drugs at a horse track, detecting flunixin in the lagoon ( $12 \text{ ng mL}^{-1}$ ), in the dust ( $5.8 \text{ ng g}^{-1}$ ), and on the floor of the

stalls (a maximum of  $251.1 \text{ ng g}^{-1}$ ) in surprisingly high concentrations (Barker, 2008). In that study, flunixin was detected in the highest concentrations of eight compounds measured by GC-MS (Barker, 2008).

No studies report the presence or threat of flunixin from cattle manure despite the fact that flunixin regularly exceeds residue levels in the meat and milk of cattle (Smith et al., 2015; Leavens et al., 2014). In 2014, dairy cows accounted for 59% of flunixin violations (USDS FSIS, 2015). Multiple studies have found that dairy cows are still excreting flunixin in their milk after the 36-h holding period at concentrations above the regulatory limits (Smith et al., 2015; Kissell et al., 2013). Additionally, the metabolite 5-hydroxyflunixin is excreted for longer periods of time than its parent compound in milk (Smith et al., 2015). This metabolite was not measured in this experiment, but it would be valuable to know whether 5-hydroxyflunixin is just as mobile in soil as its parent compound. Flunixin appears to be mobile in soil and resistant to degradation, but there are no other studies confirming this information. Ultimately, the resistance to degradation, high mobility, and regular use by the dairy industry make flunixin a likely contaminant to the environment.

#### Absence of Spiked CECs in the Effluent from the Chemical-Treated Columns

Most of the studied compounds did not move through the 1-meter long soil columns. In particular, there was no flush of hormones from the chemical treatment. Estrogens have  $K_{OW}$  values in the mid-3.5 range, meaning they are fairly hydrophobic and unlikely to move through the soil profile (Xia et al., 2005; Clarke and Smith, 2011). Other column studies have not seen movement of estrogens or progestogens through the soil (Salvia et al., 2014). One study concluded that non-extractable sorption to the soil particles was the primary pathway for removal of estrogens from the soil solution (Colucci and Topp, 2002). The major threat of



hormones would come from direct runoff of dairy farms or WWTF effluent into a water source. However, multiple studies have concluded that estrogens are unlikely to be a threat to wildlife from organic fertilizer application to agricultural fields due to estrogen's high sorption rates (Colucci and Topp, 2002; Lucas and Jones, 2006).

Additionally, there was no flush of the common human contaminants caffeine, acetaminophen, penicillin, or trichlorocarbide from the chemical treated column. This may partly be explained by the degradation characteristics of these compounds in soil. Penicillin has been found to be unstable in the soil, with a half-life of less than 2 days in a variety of soils (Salvia et al., 2014). Additionally, acetaminophen had a  $t_{1/2}$  of 4.6 days in a soil with a pH of 8.2 (Salvia et al., 2014). Another study found acetaminophen to be completely degraded after 14 days (Pino et al., 2015). Caffeine is found to rapidly mineralize to CO<sub>2</sub> in agricultural soils, with less than 20% of the compound extractable after just 6 days in a silt loam (Topp et al., 2006). Up to 65% of trichlorocarbide was found to degrade in soils due to biotic activity or volatilization (Clarke and Smith, 2011). Although these compounds are frequently detected in biosolids (Topp et al., 2008; Clarke and Smith, 2011; Borgman and Chefetz, 2013), their movement through the soil profile is limited by their rapid degradation.

Although some tetracycline antibiotics were detected, there was no pattern in leaching that would suggest the antibiotic had originated in the treatment. Oxytetracycline (OTC) was measured in just two of the five chemical-treated columns. One column had OTC detectable once (week 12); whereas the second column had two consecutive weeks of detectable OTC (weeks 8 and 9). Regardless, the maxima were 0.02 and 0.06 µg, respectively. Although these compounds may have originated with the treatment, the lack of consistency among replicates makes it impossible to determine whether this was tetracycline already present in the soil,

or if a maximum of 0.06 µg of OTC (yielding a recovery rate of 0.0006%) was able to move through the chemical-treated soil columns.

In one soil column experiment (diameter = 25 cm; length = 60 cm), investigators did not detect any oxytetracycline in the leachate after 4.2 mg was applied to each column (Kay et al., 2005a). Another study found 10 mg of tetracycline to degrade completely in just 14 days in 5 g of artificial soil (7:1:2 fine sand:sphagnum peat:kaolin clay) (Pino et al., 2015). The conclusion from this experiment is clear: compounds in the tetracycline family do not travel (or travel in miniscule amounts) through soil. They are unlikely to be a significant threat to the soil environment or groundwater.

#### Absence of CEC Leaching from the Manure-Treated Columns

There was no flush of CECs from the manure treatment despite detections of estrone, estriol, progesterone, 17 $\alpha$ -hydroxyprogesterone, sulfadimethoxine, sulfathiazole, and tetracycline in the applied manure (Table 2.5). The manure-treated columns experienced a delayed elution of bromide, sulfate, and copper, which suggests that transport of other compounds may also have been delayed. This additional time in the soil would have allowed for increased degradation of the organic compounds.

The absence of CECs in the leachate reduces one of the primary concerns arising from dairy manure application to irrigated agricultural lands. In this experiment, none of the CECs traveled through the manure-amended soil columns. Furthermore, the results from the chemical-treated columns suggest that compounds with concentrations one hundred times larger are still unlikely to move through the soil. Specifically, the threat of tetracyclines or hormones in dairy manure moving through soil and contaminating underlying groundwater is unlikely.

However, this study does not cover all aspects of CEC contamination in the environment. Common crops have been shown to uptake antibiotics (including tetracyclines and sulfonamides) from soil treated with animal manure and store those antibiotics in edible parts of the plant (Du and Liu, 2012; Kumar et al., 2005). Another study found endocrine disruption in lizards that lived near manure-amended agricultural fields (Verderame et al., 2016). Finally, multiple studies have shown that the organic matter content of the manure decreases leaching of antibiotics, but increases the runoff of those compounds (Burkhardt and Stamm, 2007; Kay et al., 2005b). Thus, contaminants in dairy manure do not have to move vertically through the soil profile to cause environmental harm.

We conclude that most of the CECs in dairy manure that threaten the environment are generally immobile in the soil environment. Namely, estrogens, progestogens, tetracyclines, zinc, and copper all eluted in near-zero concentrations or not at all from the soil columns. Additionally, the sulfadimethoxine and sulfathiazole originally detected in the manure did not travel through the soil profile. Thus, it is unlikely that the subsurface profile or groundwater is threatened by a single dairy manure application.

### Leaching of CECs from the Untreated Soil

Some compounds leached from all columns regardless of treatment. There was no apparent flush or peak in elution from the chemical treatment. Additionally, the fertilizer treatment did not leach significantly less of these compounds than the other three treatments, which would be expected if the compounds were associated with the treatments. This suggests that these compounds did not originate in the treatments applied to the columns. Instead, it is very likely that the measured compounds were already in the soil.

A total of 20 out of the 25 CECs studied were detected at some point throughout the

duration of the study. Many compounds were detected in only one column per treatment, including  $17\alpha$ -E2,  $17\beta$ -E2, estrone, ethinyl estradiol, enterodiol, equol, formonentin, oxytetracycline, and tetracycline. Alternatively, some compounds were prevalent in nearly every column, including caffeine, estriol, flunixin,  $17\alpha$ -hydroxyprogesterone, progesterone, sulfadimethoxine, and sulfamethoxazole. Only five compounds were never detected in the study: coumestrol, biochanin A, chlortetracycline, trichlorocarbide, and acetaminophen. Appendix B lists the cumulative average of each CEC detected by treatment, the range of cumulative values, and the number of columns per treatment each CEC was detected.

There was mechanical disturbance of the soil during the sampling, transport, and preparation of the column experiment. The sides were scraped by the plastic PVC piping during the sampling of the cores, and the top and bottoms of the soil columns were scratched during the set-up of the experiment. This may have broken apart soil aggregates, releasing previously protected compounds into the soil solution (McCarthy et al., 2008; Darrouzet-Nardi and Weintraub, 2014).

The compounds that were found randomly and evenly throughout the soil columns include hormones ( $17\alpha$ -estradiol,  $17\beta$ -estradiol, estriol, estrone, progesterone, and  $17\alpha$ -hydroxyprogesterone), phytoestrogens (enterodiol, equol, and formonentin), and human contaminants (penicillin, ibuprofen, and caffeine). The following graphs list each compound by week and include all 20 columns. There is no designation of treatment in the graphs, because there were no differences among treatments.

This data was too scattered and limited for any kind of statistical analysis, but there are many important patterns to note. Estriol was detected the most frequently (16 of 20 columns) of the four estrogen species (Fig. 3.17). Estriol is the most water soluble of the

studied estrogens because of its three hydroxyl function groups, making it most polar. It has the lowest  $K_{OW}$  of the estrogen species at 2.81 (Clarke and Smith, 2011). Thus, estriol would be expected to be the most mobile estrogen species in soil.

None of the hormones were detected after week 5. One study found estrone to have a half-life ranging from 5 – 25 days, depending on the soil. The same study concluded that  $17\beta$ -estradiol had a half life of 12 – 23 days (Lucas and Jones, 2006). These half-lives were significantly decreased if animal manure or urine was introduced into the soil microcosm. If the hormones appeared after mechanical disruption the soil, then they may have been nearly degraded by the end of week 5. Thus, it would have been more surprising to find estrogens later than 35 d after the start of the experiment.

Progesterone and its metabolite  $17\alpha$ -hydroxyprogesterone were detected in all twenty columns during some point of the experiment (Fig. 3.18). Notably,  $17\alpha$ -hydroxyprogesterone eluted primarily during weeks 4 and 5, which is after progesterone eluted (weeks 2 and 3). It makes sense that a metabolite would elute immediately after its parent compound.

Hormones are a predominant concern due to their potent endocrine disruption on many species at very low concentrations (Segner et al., 2013; Hanselman et al., 2003). However, the estrogens and progestogens detected in the soil leachate did not originate from the added manure, biosolids, or chemical additions. Whatever hormones were detected in the leachate seemed to come from the soil itself. Steroid hormones are produced by all mammals, and their presence is frequently detected in the environment (Snow et al., 2013). The ubiquitous presence of hormones in the environment makes it very possible that the detected hormones came from the soil itself.

Phytoestrogens were also frequently detected throughout the soil columns, most

without a discernable pattern among treatments (Fig. 3.19). Formononetin is the exception, as 6 in 9 of its detections were from the biosolids treatment. Formononetin is an isoflavone that is produced by red clover, red potatoes, and some legumes. In a grassland-red clover mixed field study, formononetin was detected in the highest concentrations in the topsoil as well as in the drainage water (Hoerger et al., 2011). This was expected as red clover primarily produces the phytoestrogens formononetin and biochanin A. However, equol was detected in 18% of the topsoil samples and 72% of the drainage water samples. They found this to be unexpected, because equol is an isoflavane that is a degradation product of two phytoestrogens that form in the intestinal tracks of mammals (Hoerger et al., 2011). Equol is mostly likely to be found in animal manure or biosolids, but was surprisingly found even in the subsoil (up to 30 cm) in the reported field experiment (Hoerger et al., 2011). The soil used in this experiment had been under agricultural use for decades, growing a variety of vegetables. It is very likely that the detected phytoestrogens – formononetin, enterodiol, and equol – were in the soil previous to the start of this experiment.

Caffeine was detected in at least one column per week, except weeks 2, 8, 9, and 10 (Fig. 3.20). It was detected in 19 of the 20 soil columns. Although caffeine was detected throughout the duration of the experiment, there was no discernible pattern among treatments. There was no peak from the chemical treatment, which would suggest that the caffeine had traveled from the top of the column. Additionally, there was no difference among treatments in total caffeine measured or in the weeks of elution. This suggests that the caffeine was previously in the soil.

Caffeine is frequently detected in many environmental studies including horse stalls (Barker, 2008) and urban streams (Campanha et al., 2014). In fact, it is detected so frequently

in the environment that environmental studies classify caffeine as a “human indicator” instead of its drug classification (a stimulant) (Petrie et al., 2014). It was not surprising to find caffeine in the effluent throughout the duration of the experiment.

Although the soil columns had been taken from a field that had never received animal manure, the field had been exposed to anthropogenic contamination during its decades of production. Most notably, irrigation water used on the fields could have easily introduced any of the detected CECs into the soil profile. The irrigation water in southern Idaho is surface water, and thus exposed to runoff of upstream agricultural productions, livestock operations, and wastewater treatment facilities. Previous studies have documented a variety of antibiotics, testosterone, estrogens, plasticizers, and organic industrial contaminants in irrigation water (Fisher and Scott, 2008; Casey et al., 2004; Du and Liu, 2012; Garcia et al., 2001).

## Limitations

Unfortunately, there was some evident cross contamination apparent in the MS spectra. This was primarily due to the unexpectedly high concentrations of sulfadimethoxine, sulfamethazole, sulfamethoxazole, and flunixin during the weeks that they peaked in the chemical columns. These chemical concentrations from the chemical-treated columns were over two orders of magnitude higher than previously run calibration curve standards. Although the standards did not leave ghost peaks in the subsequent samples, I had not tested concentrations as high as the peak chemical-treatment effluent. One option to prevent the cross contamination may be to dilute the samples. However, doing so would have caused compounds with low concentrations to be diluted below the limits of detection (such as sulfathiazole). In retrospect, a blank should have been run after each chemical-treatment sample to clean the tubing in the HPLC-MS before running another sample.

Due to their high concentrations, sulfadimethoxine, sulfamethazole, sulfamethoxazole, and flunixin left ghost peaks in the chromatograms of the samples run after the chemical samples. This contamination exhibited a very clear pattern of decreasing concentration with each subsequent sample run. The contaminated values were less than 1% of the original detected amount in the chemical-treated samples. Weeks 5 and 10 showed high contamination for sulfadimethoxine, and weeks 7, 8, and 10 experienced cross contamination from flunixin.

It is very unlikely that the compounds discussed in “Leaching of CECs from the Untreated Soil” are the result of cross-contamination. First, in those samples, there was no high peak and subsequent steady decrease in the samples run consecutively. This would indicate contamination during the HPLC-MS analysis. Secondly, the compounds that elute from all treatments primarily stop after weeks 4 or 5. If there were contamination in the procedure itself (such as poorly washed glassware), then it would be expected that this contamination would continue throughout the duration of the experiment. In fact, if contamination were occurring during SPE concentration or sample preparation, then it would be likely that those compounds would increase throughout time, as contamination built up in the materials.

Additionally, this experiment was unable to measure the threat of environmental contamination by surface runoff. Some of the studied CECs, such as acetaminophen, have been shown to leave via surface runoff of biosolids, but not move downwards through the soil profile to reach the groundwater (Gottschall et al., 2012). Surface runoff and vertical leaching are both important vectors that contribute to the introduction of CECs into the environment, and both must be carefully studied to fully understand the potential impacts of repeated annual organic fertilizer applications to agricultural fields.



## Conclusion

This study used undisturbed soil columns from southern Idaho to evaluate the mobility of chemicals of emerging concern through the soil profile. The manure-treated columns had significantly more sulfate move through the soil, whereas very little of the metals in manure were transported. The annual loading of copper and salts to the soil from yearly manure amendments could threaten plant and soil organism health.

Overall, the soil serves as an adequate sponge for adsorbing many of the contaminants present in organic fertilizers. In particular, tetracyclines, hormones, and most human pharmaceuticals did not move through the soil profile through a combination of sorption and high degradation rates. However, six organic compounds were mobile due to their negative charge in the soil. Specifically, sulfonamide antibiotics, flunixin, and ibuprofen may pose a threat to groundwater contamination by vertical leaching through the soil profile.

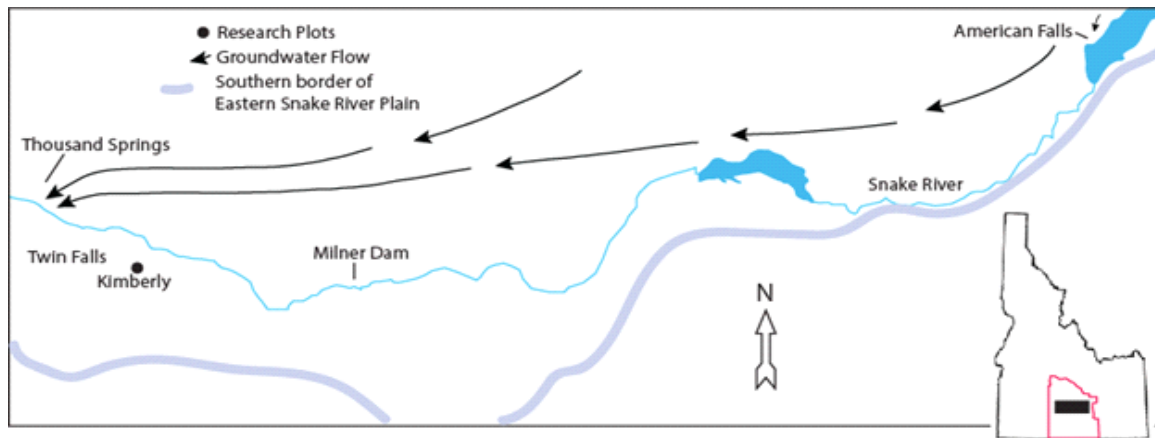


Figure 3.1 Map of Snake River in Southern Idaho. Red cutout outlines the Magic Valley. Undisturbed soil columns were taken from research plots near Kimberly, Idaho in May/June 2014.

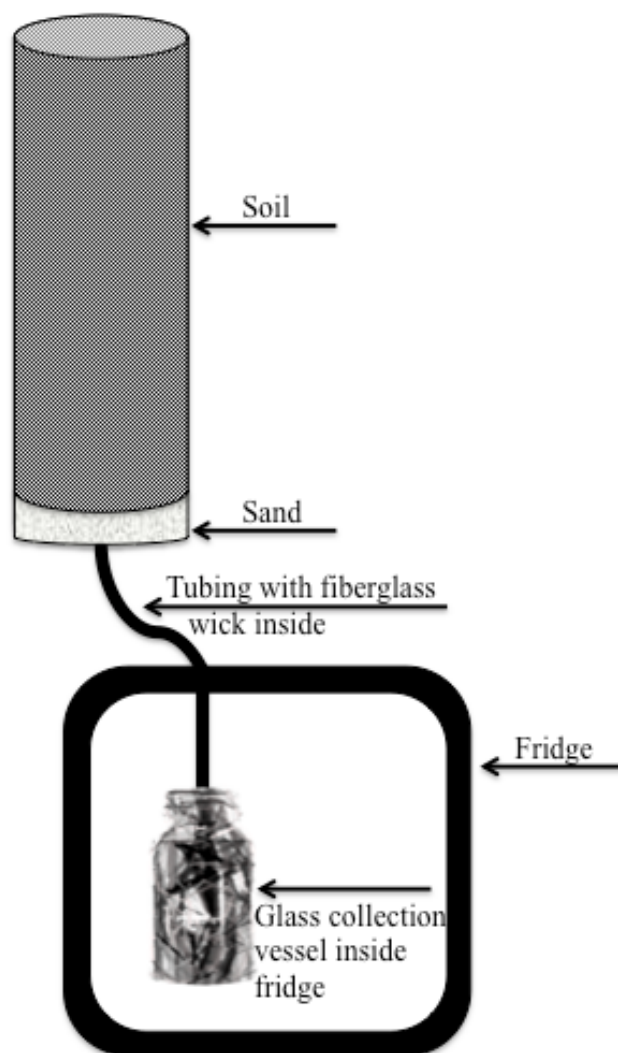


Figure 3.2 Schematic of leaching column and collection vessel configuration.

Table 3.1 Selected Elemental Analyses in Biosolids and Manure Reported on a Dry Weight Basis

Name	Dairy Manure	Biosolids
Date Received	8/18/15	7/9/15
Water Weight (%)	51.1	88.6
Total N ( $\text{g kg}^{-1}$ )	24.1	47.8
Available $\text{NH}_4\text{-N}$ ( $\text{g kg}^{-1}$ )	1.6	14.5
Available $\text{NO}_3\text{-N}$ ( $\text{mg kg}^{-1}$ )	10.0	30.5
P ( $\text{g kg}^{-1}$ )	9.0	30.7
K ( $\text{g kg}^{-1}$ )	40.4	3.4
Cu ( $\text{mg kg}^{-1}$ )	127	1229
Zn ( $\text{mg kg}^{-1}$ )	348	1040
pH	8.6	8.3

Total nitrogen was determined by combustion with a LECO CN638. Ammonium and nitrate concentrations were extracted with 2 M KCl solution and analyzed colorimetrically. Total metal concentrations were determined with nitric and perchloric digestion (Gavlak et al., 2005).

Table 3.2 Model Parameters for Bromide

Treatment	Parameter	Estimate	SE	<i>p-value</i>
Biosolids	m	0.56	0.26	0.0988
	L	9.82	7.68	0.2701
	B	1.14	0.35	0.0305
Chemical	m	0.43	0.01	<0.001
	L	2.29	0.02	<0.001
	B	11.57	0.78	<0.001
Fertilizer	m	0.42	0.00	<0.001
	L	2.06	0.00	<0.001
	B	50.09	0.00	<0.001
Manure	m	0.44	0.01	<0.001
	L	2.83	0.04	<0.001
	B	13.81	3.01	0.0004

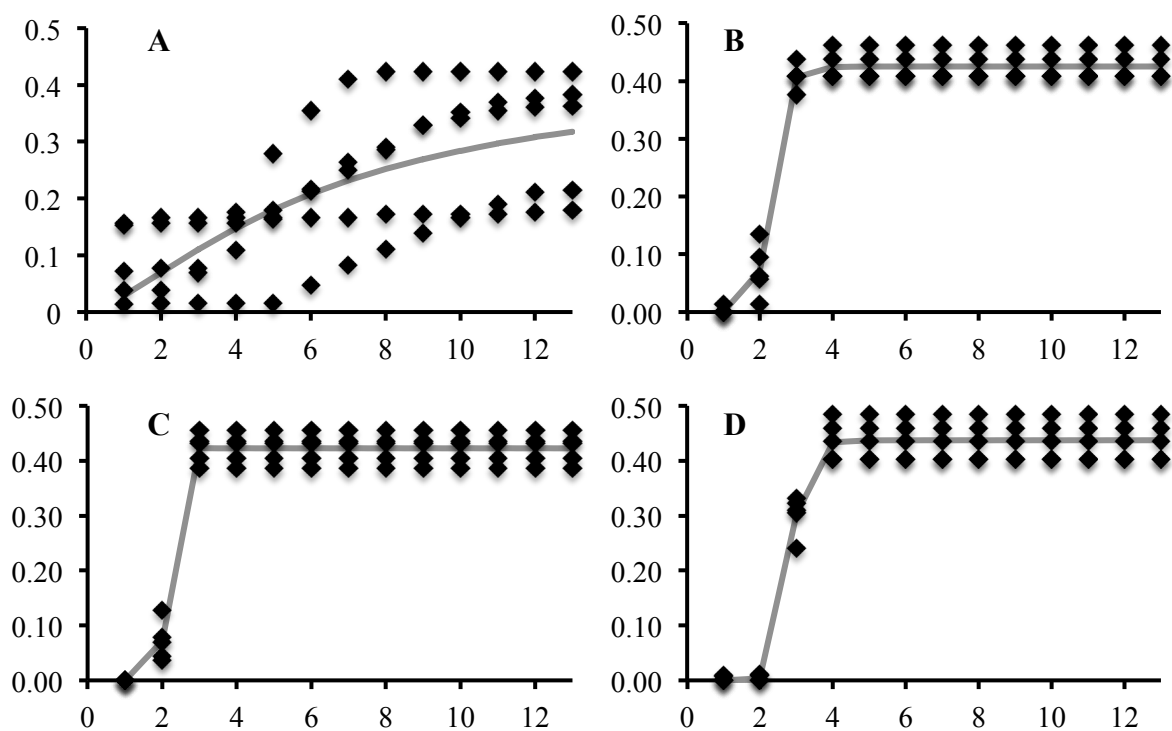


Figure 3.3 Cumulative leaching of bromide (mmol) by week (scatter points) and the model fit for each treatment (gray line): A) Biosolids; B) Chemical; C) Fertilizer; D) Manure.

Table 3.3 Soil Column Bulk Density

	Bulk density (g/cm <sup>3</sup> )	Water weight
Top <sup>a</sup>	1.12 <sup>a</sup>	23.18%
Middle <sup>b</sup>	1.34 <sup>b</sup>	21.09%
Bottom <sup>b</sup>	1.32 <sup>b</sup>	20.35%

The average bulk density of the top, middle, and bottom sections of the soil columns after the experiment. There was no significant difference among treatments (data not shown). Letters represent significant difference from each other; sections in the same group are not significantly different from each other.

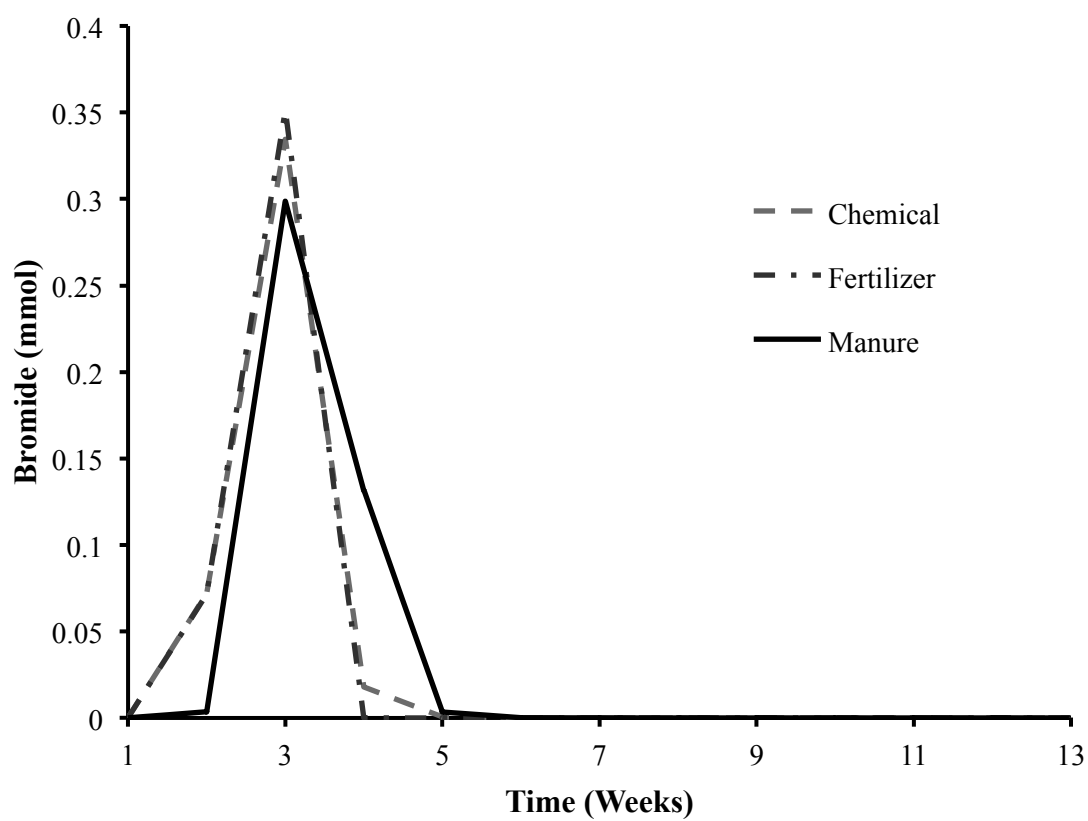


Figure 3.4 Predicted models for bromide (mmol) leaching from each treatment weekly.



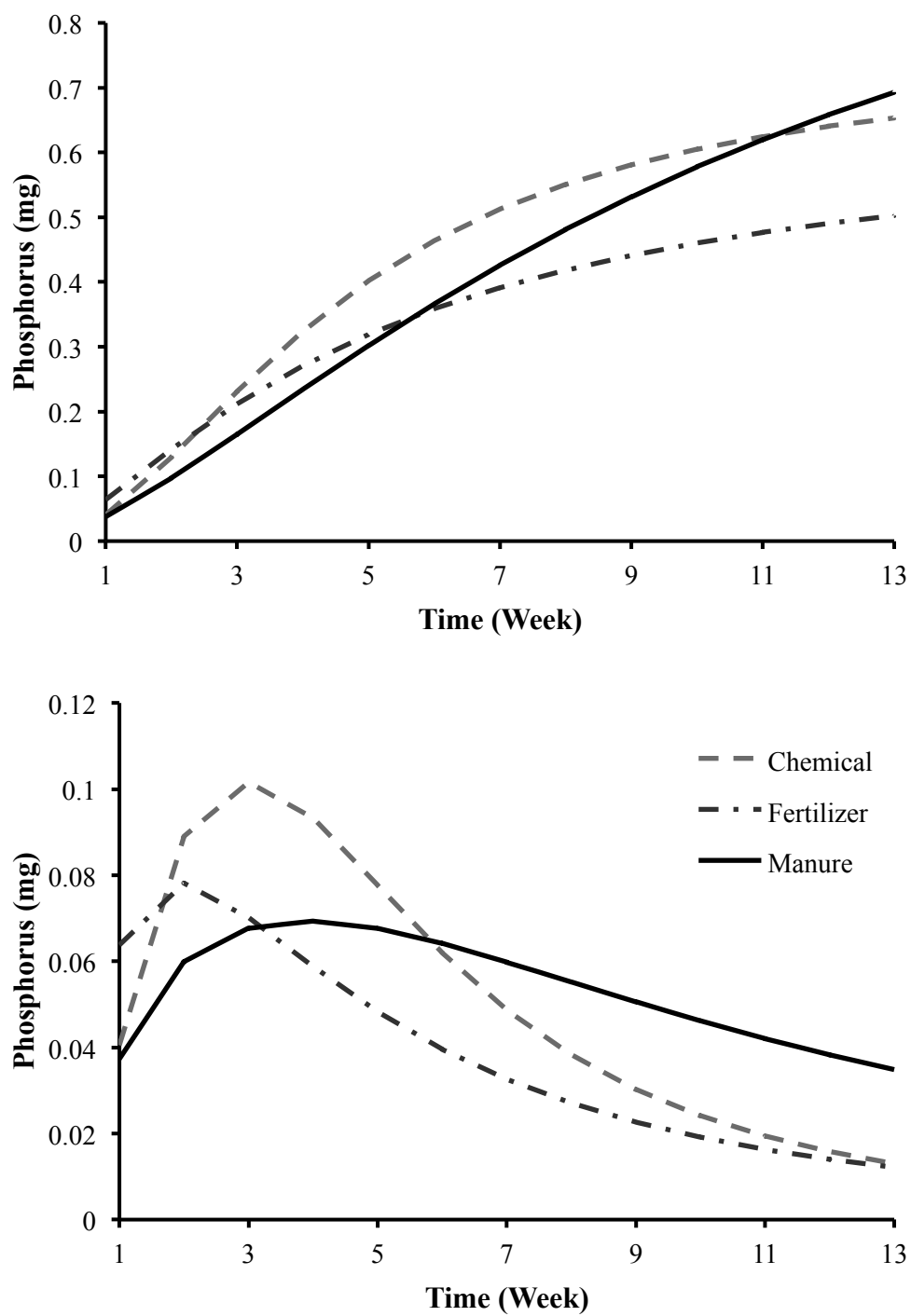


Figure 3.5 Predicted models for phosphorus (mg) leaching from each treatment: Cumulative model (top) and weekly (bottom).

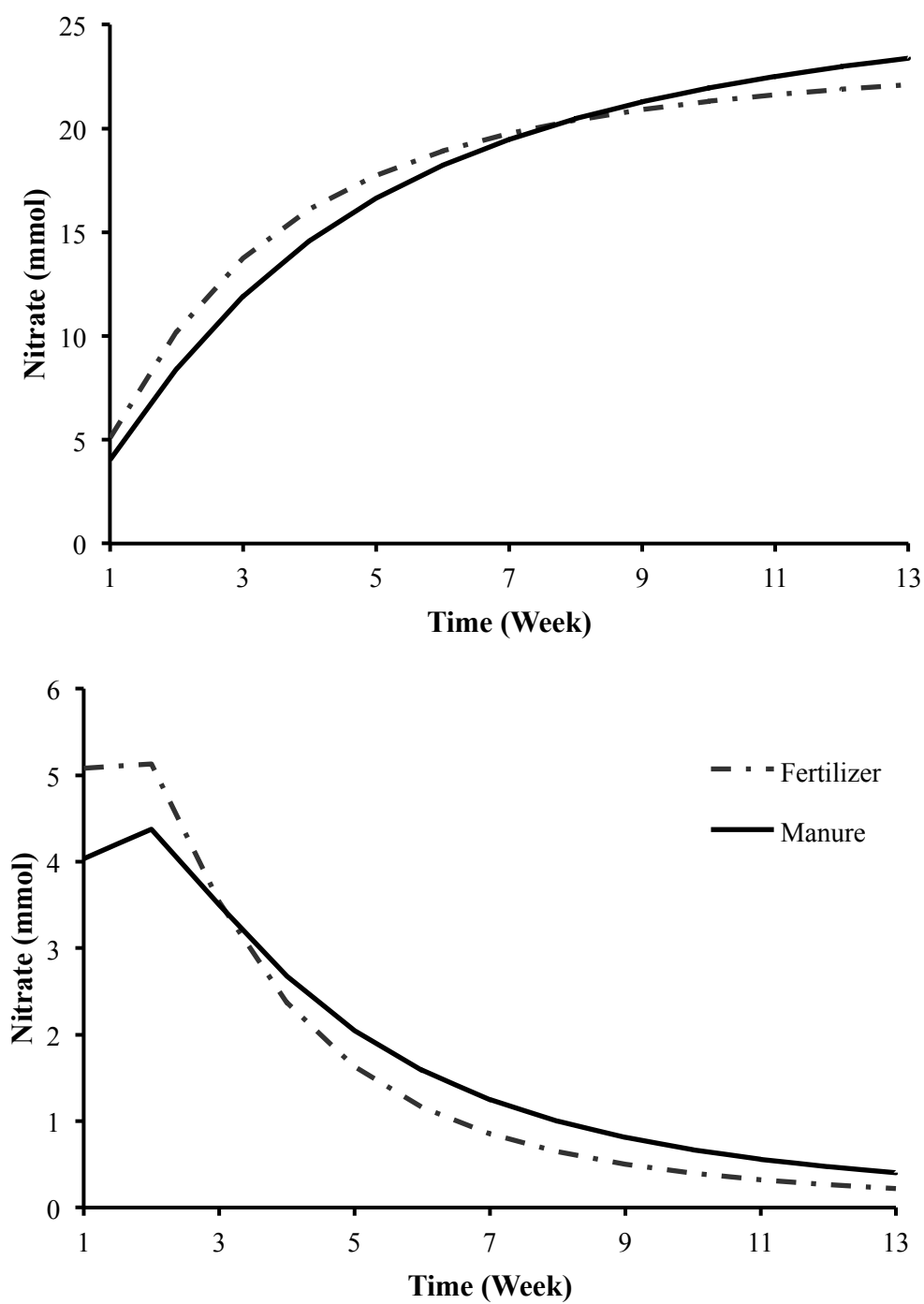


Figure 3.6 Predicted models for nitrate (mmol) leaching from fertilizer and manure treatments. Chemical treatment was not included because the data did not fit the model. Cumulative model (top) and weekly (bottom).

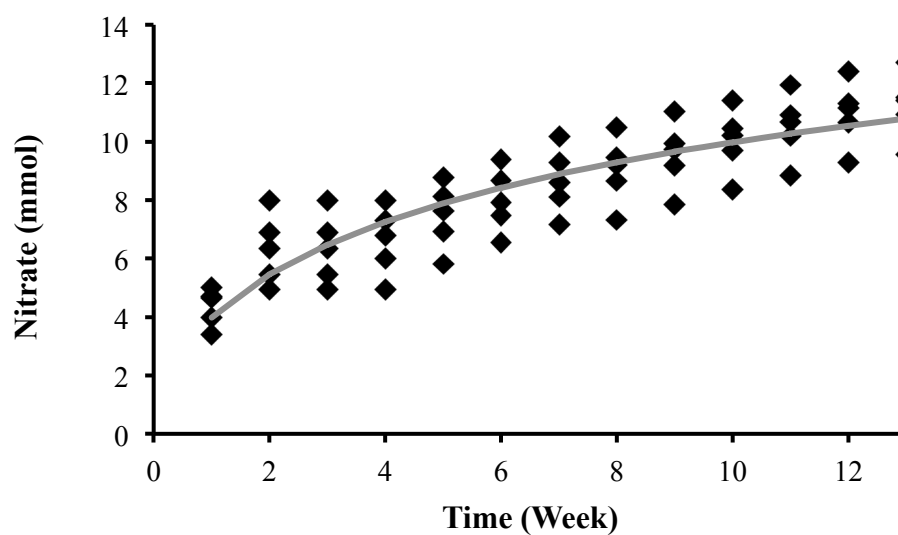


Figure 3.7 Scatter plot of cumulative nitrate (mmol) leaching from the chemical treatment with the attempted model; data is nearly linear, which resulted in a poor fit for the model.

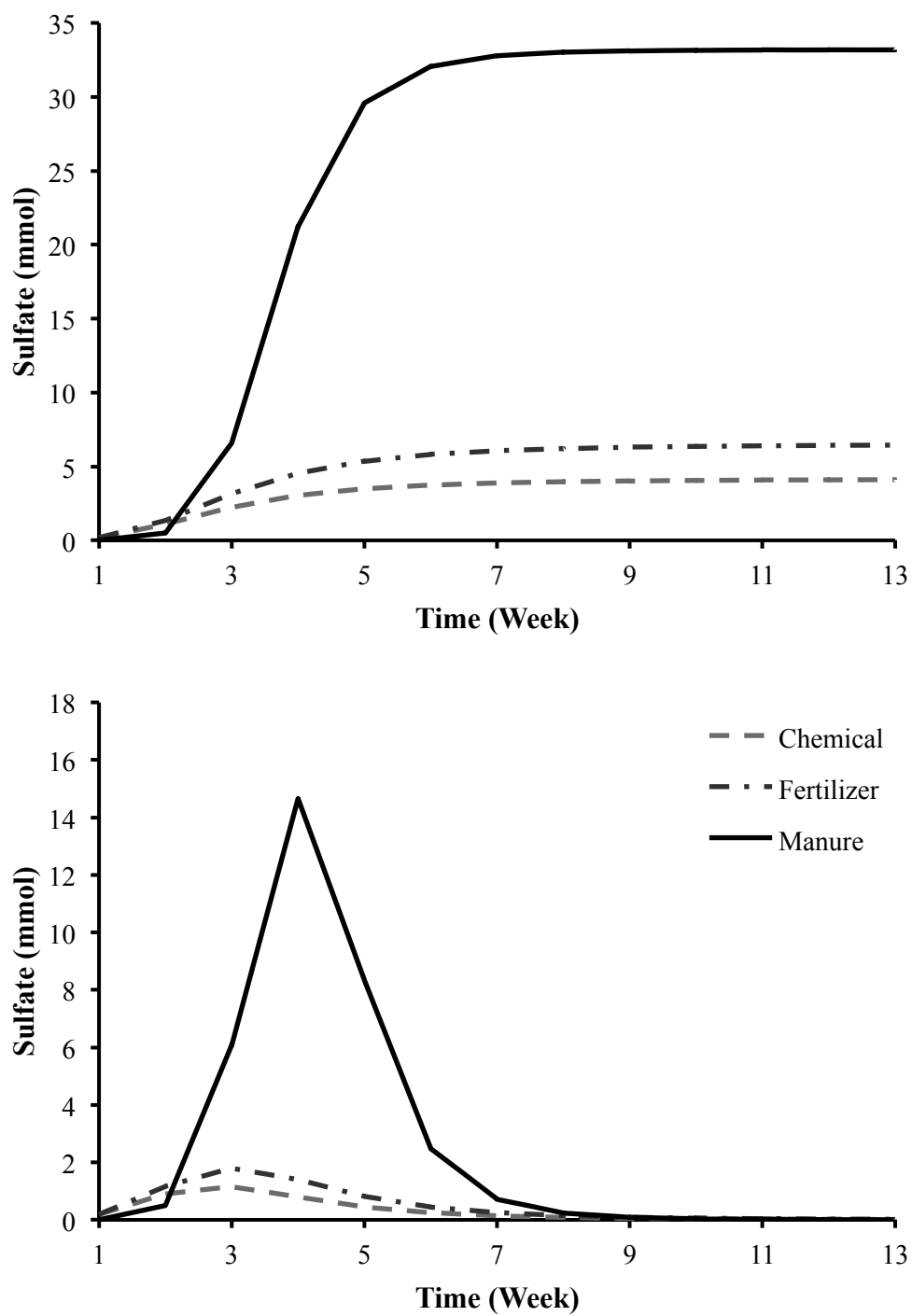


Figure 3.8 Predicted models for sulfate (mmol) leaching from each treatment: Cumulative model (top) and weekly (bottom).

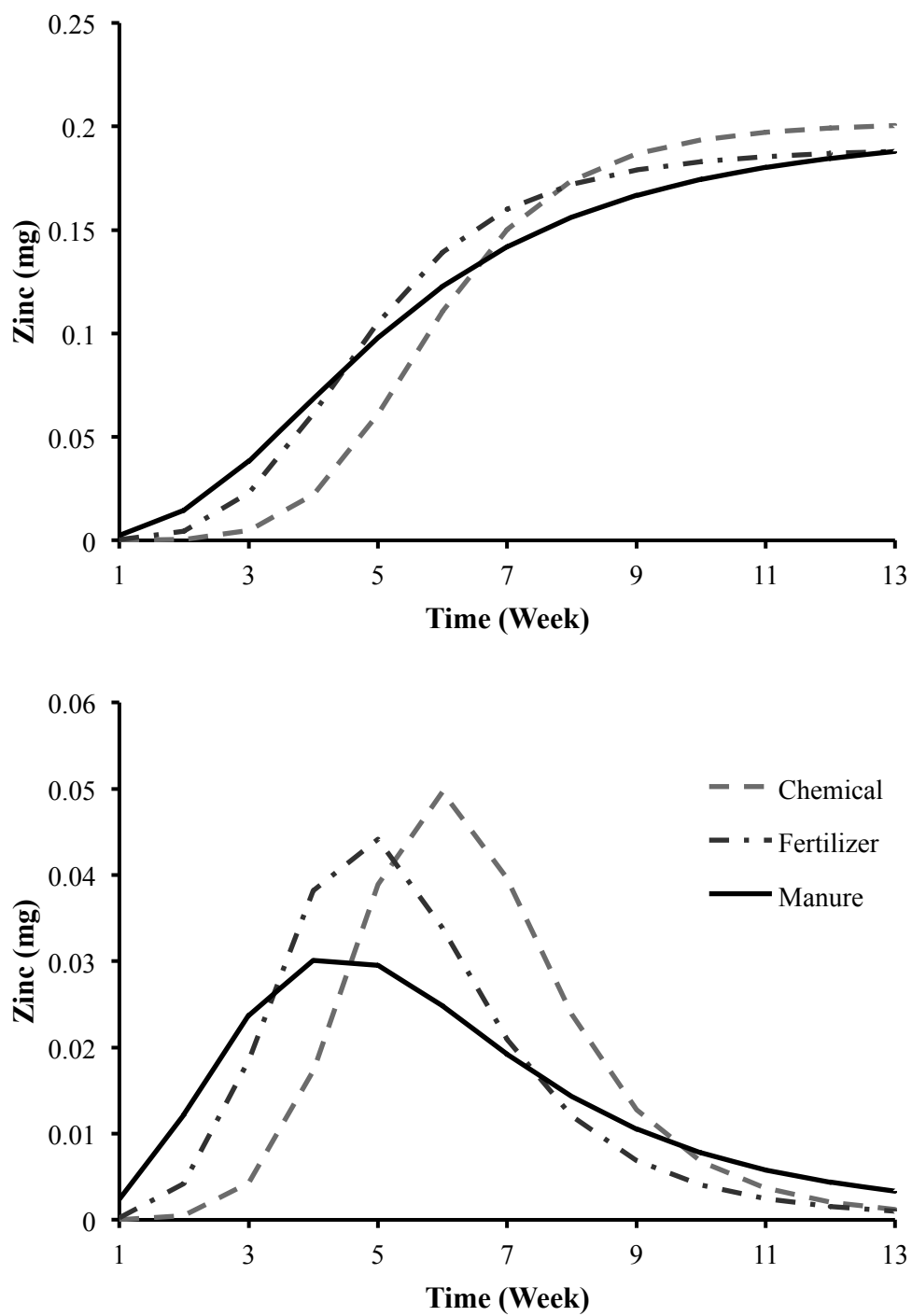


Figure 3.9 Predicted models for zinc (mg) leaching from each treatment: Cumulative model (top) and weekly (bottom).

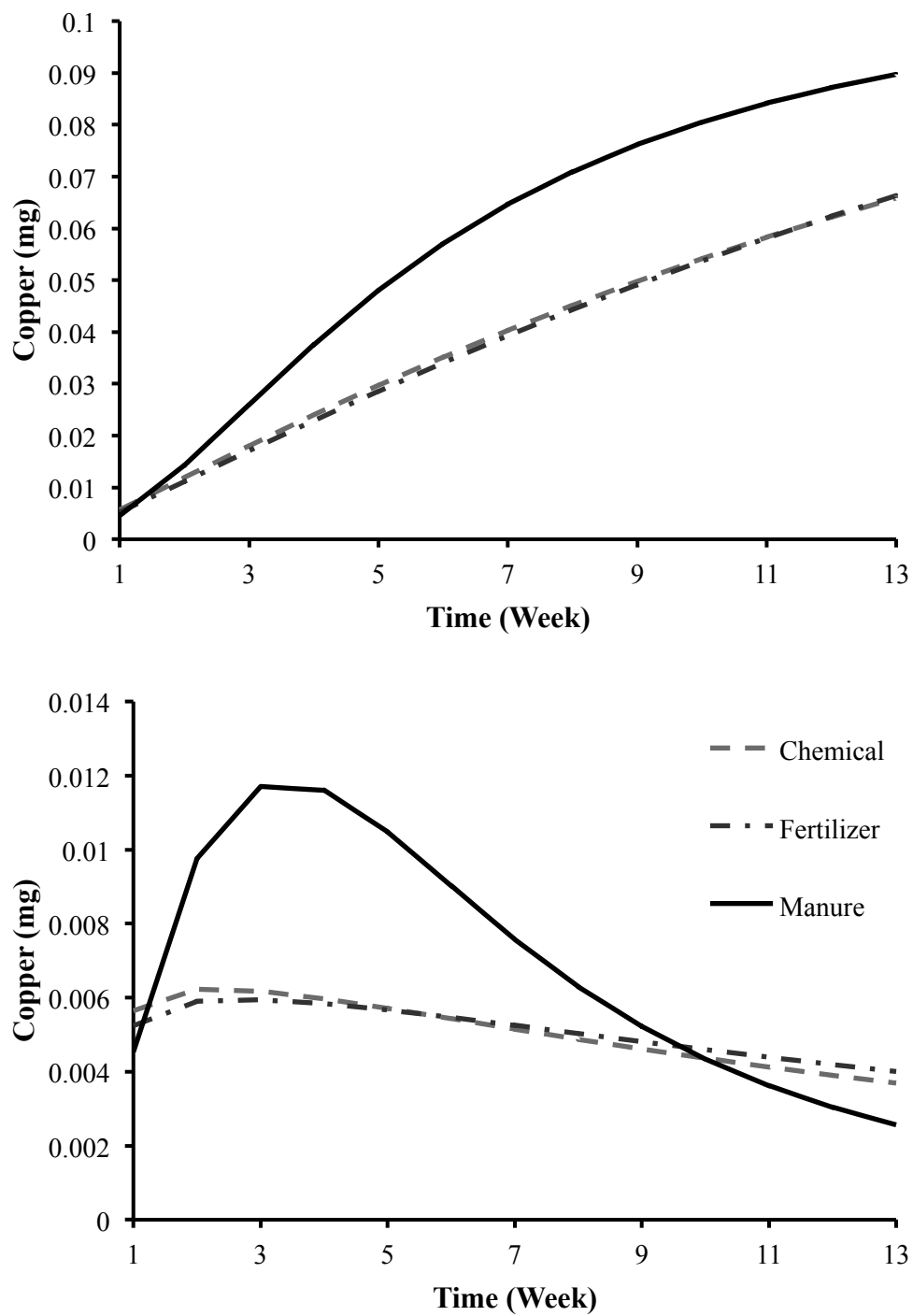


Figure 3.10 Predicted models for copper (mg) leaching from each treatment: Cumulative model (top) and weekly (bottom).

Table 3.4 Model Parameters of Compounds that Eluted from Chemical Treatment

Compound	Parameter	Estimate	SE	<i>p</i> -value
Sulfamethazine	m	29.32	7.73	0.02
	L	5.12	0.13	<0.0001
	B	4.69	0.49	0.00
	s	-2.60	0.24	0.00
	Sm	17.19	5.47	0.03
Sulfadimethoxine	m	494.89	51.85	0.00
	L	5.96	0.17	<0.0001
	B	3.62	0.37	0.00
	s	29.81	2.72	0.00
	Sm	110.66	35.60	0.04
Sulfamethoxazole	m	49.73	6.13	0.00
	L	5.54	0.37	0.00
	B	2.39	0.25	0.00
	s	3.44	0.31	0.00
	Sm	12.03	3.83	0.04
Sulfathiazole	m	0.44	0.06	0.00
	L	5.45	0.14	<0.0001
	B	5.49	0.66	0.00
	s	-0.04	0.00	0.00
	Sm	0.12	0.04	0.04
Flunixin	m	1694.46	135.33	0.00
	L	10.67	0.28	<0.0001
	B	5.13	0.26	<0.0001
	s	-40.13	3.66	0.00
	Sm	212.45	69.70	0.04
Ibuprofen	m	19.06	4.59	0.01
	L	2.23	0.05	<0.0001
	B	12.90	2.28	0.00
	s	-0.89	0.08	0.00
	Sm	10.25	3.24	0.03

Table 3.5 Total Eluted and Recovery Rates of CECs from Chemical Treatment

	Average Eluted ( $\mu\text{g}$ )	Standard Error ( $\mu\text{g}$ )	Average Recovery Rate	Standard Error
Sulfamethazine	30.26	17.35	0.30%	0.17%
Sulfadimethoxine	472.06	89.85	4.72%	0.90%
Sulfamethoxazole	45.12	10.62	0.45%	0.11%
Sulfathiazole	0.44	0.14	0.004%	0.001%
Flunixin	1275.98	138.50	12.76%	1.39%
Ibuprofen	19.07	10.22	0.19%	0.10%



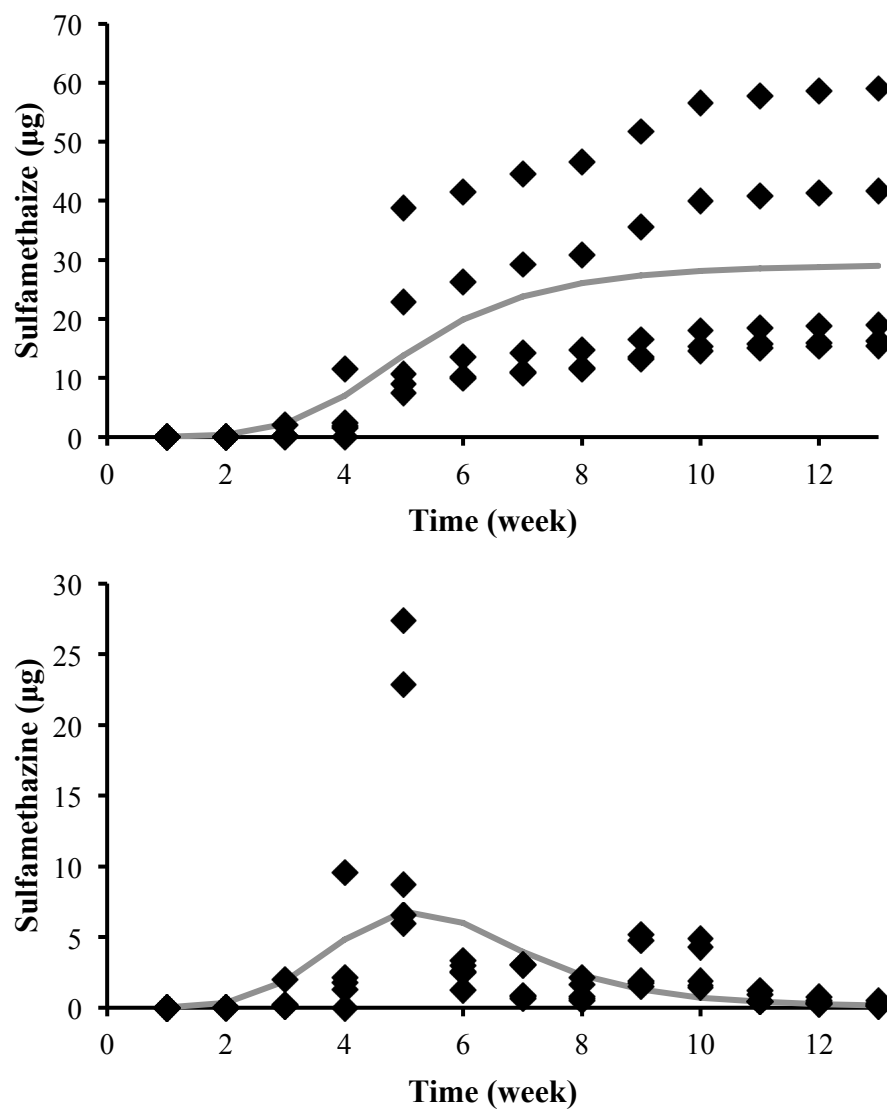


Figure 3.11 Predicted models for sulfamethazine ( $\mu\text{g}$ ) leaching from the chemical treatment: Cumulative model (top) and weekly (bottom).

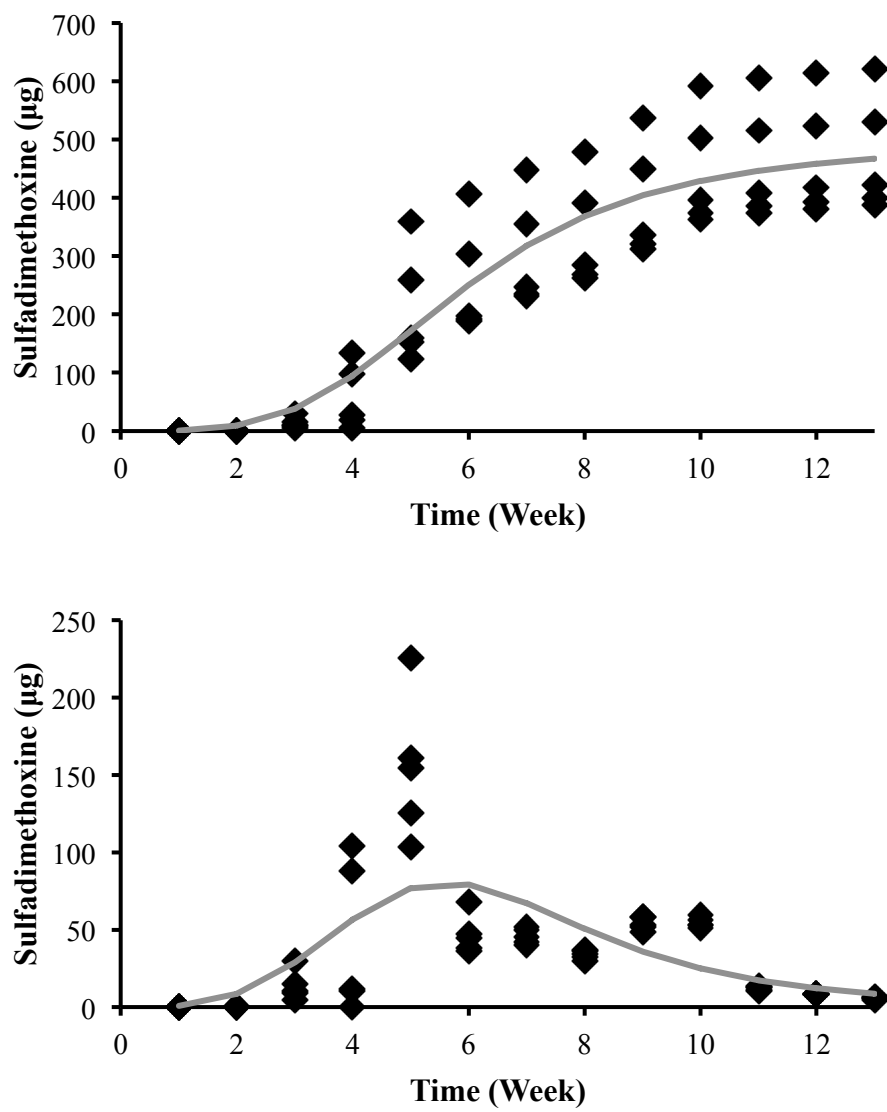


Figure 3.12 Predicted models for sulfadimethoxine (µg) leaching from the chemical treatment: Cumulative model (top) and weekly (bottom).

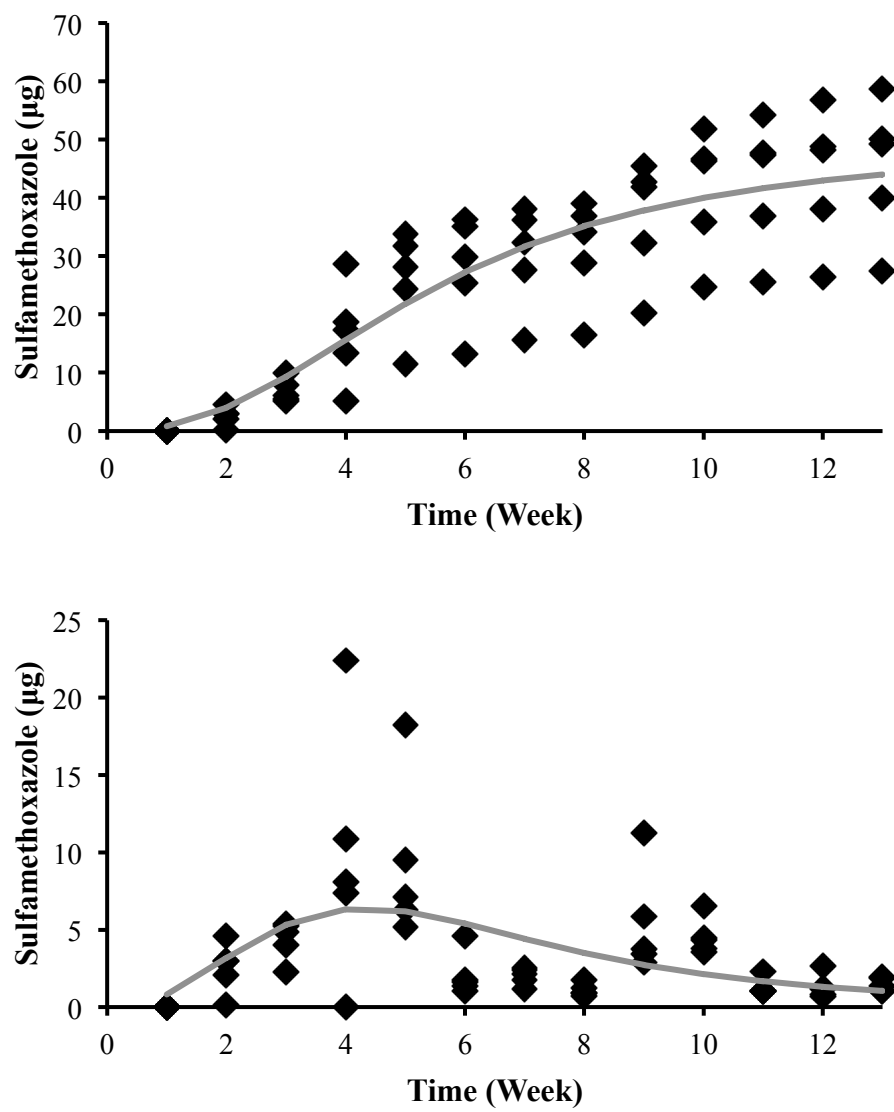


Figure 3.13 Predicted models for sulfamethoxazole (µg) leaching from the chemical treatment: Cumulative model (top) and weekly (bottom).

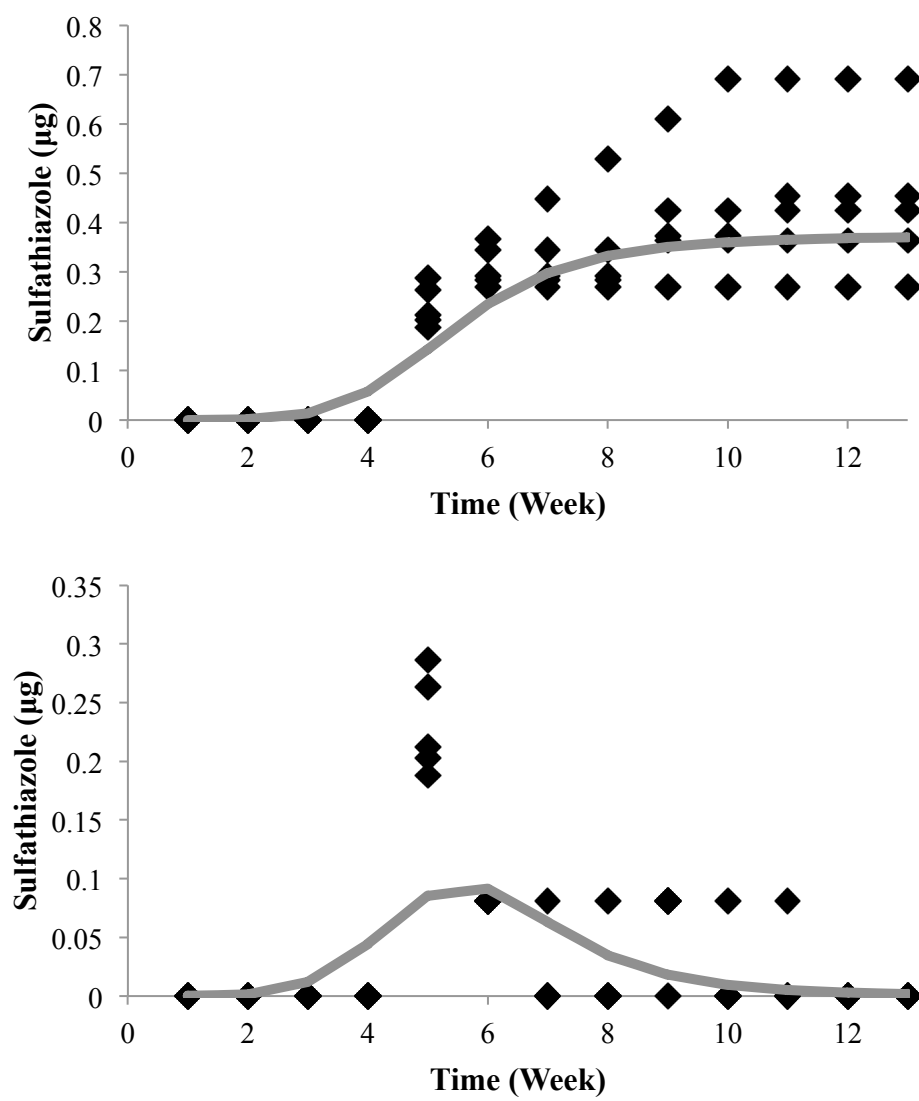


Figure 3.14 Predicted models for sulfathiazole ( $\mu\text{g}$ ) leaching from the chemical treatment: Cumulative model (top) and weekly (bottom).

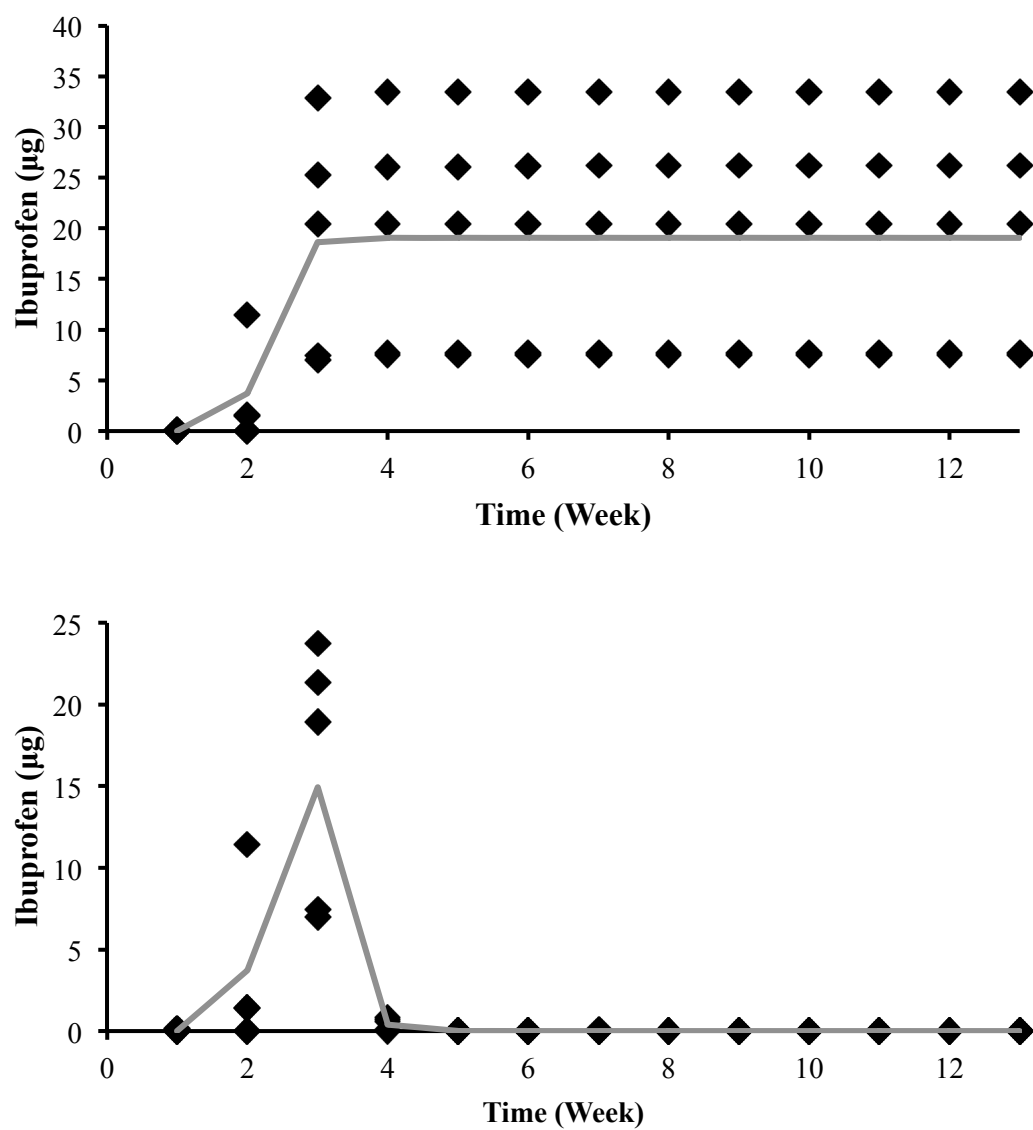


Figure 3.15 Predicted models for ibuprofen ( $\mu\text{g}$ ) leaching from the chemical treatment: Cumulative model (top) and weekly (bottom).

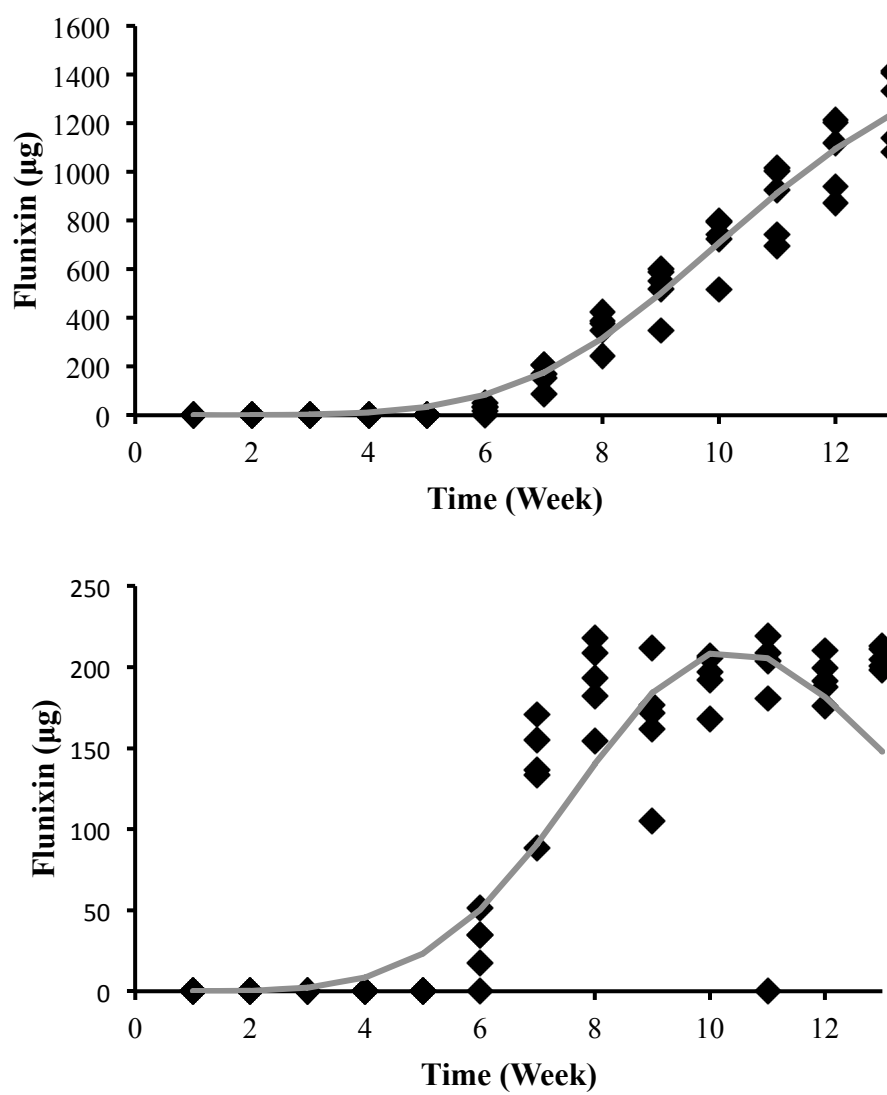


Figure 3.16 Predicted models by flunixin ( $\mu\text{g}$ ) leaching from the chemical treatment: Cumulative model (top) and weekly (bottom).

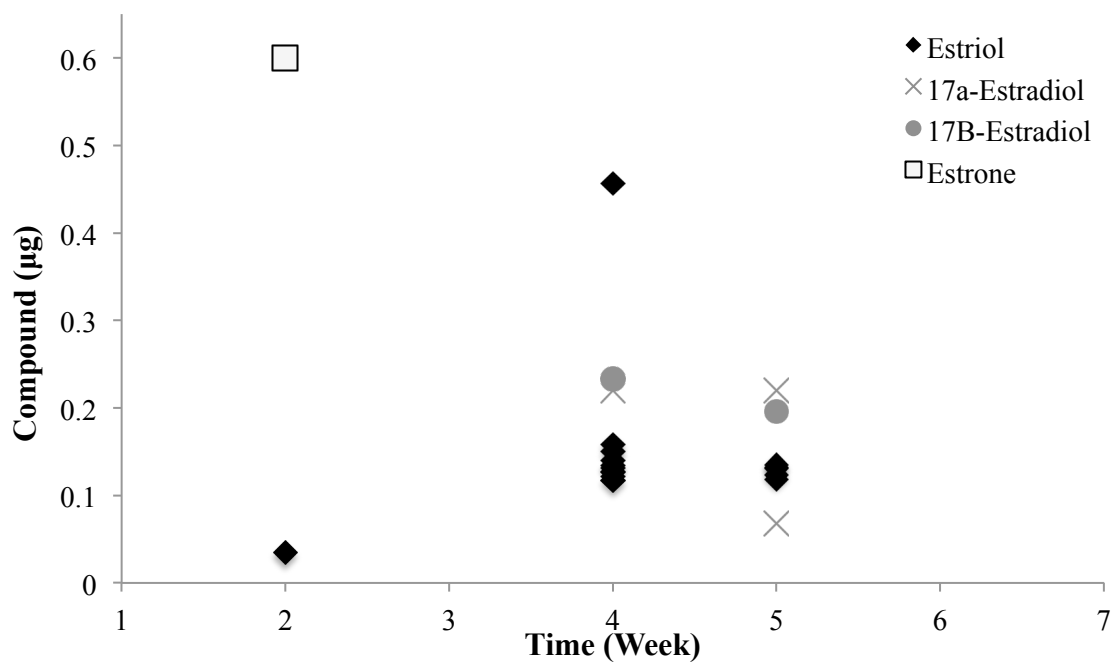


Figure 3.17 Estrogens plotted by species for all treatments; Estrogens were only detected weeks 2 – 5.

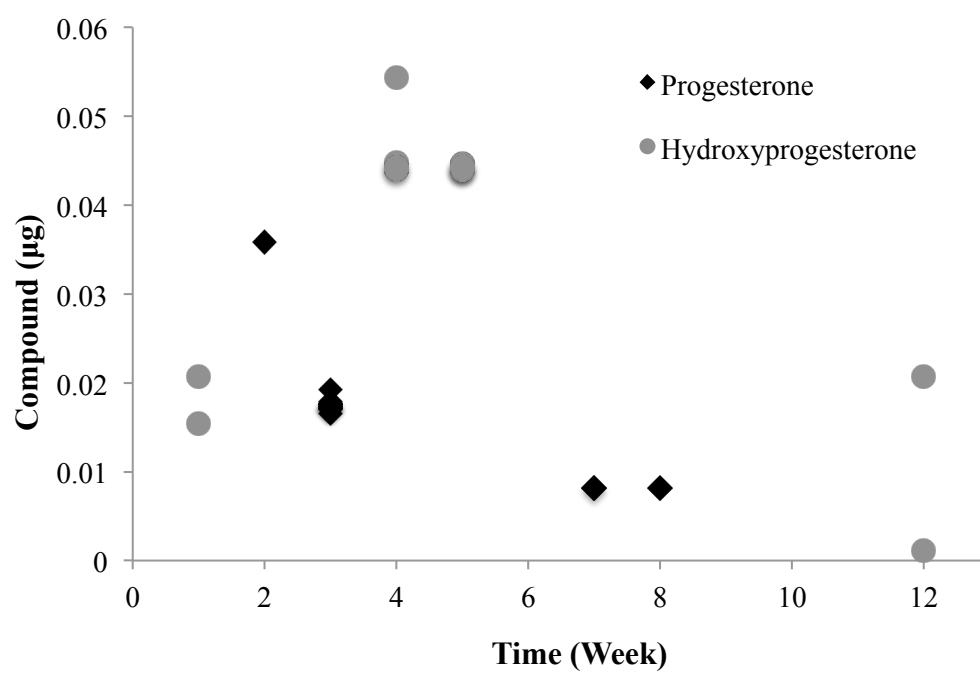


Figure 3.18 Progestogens plotted by species from all treatments.



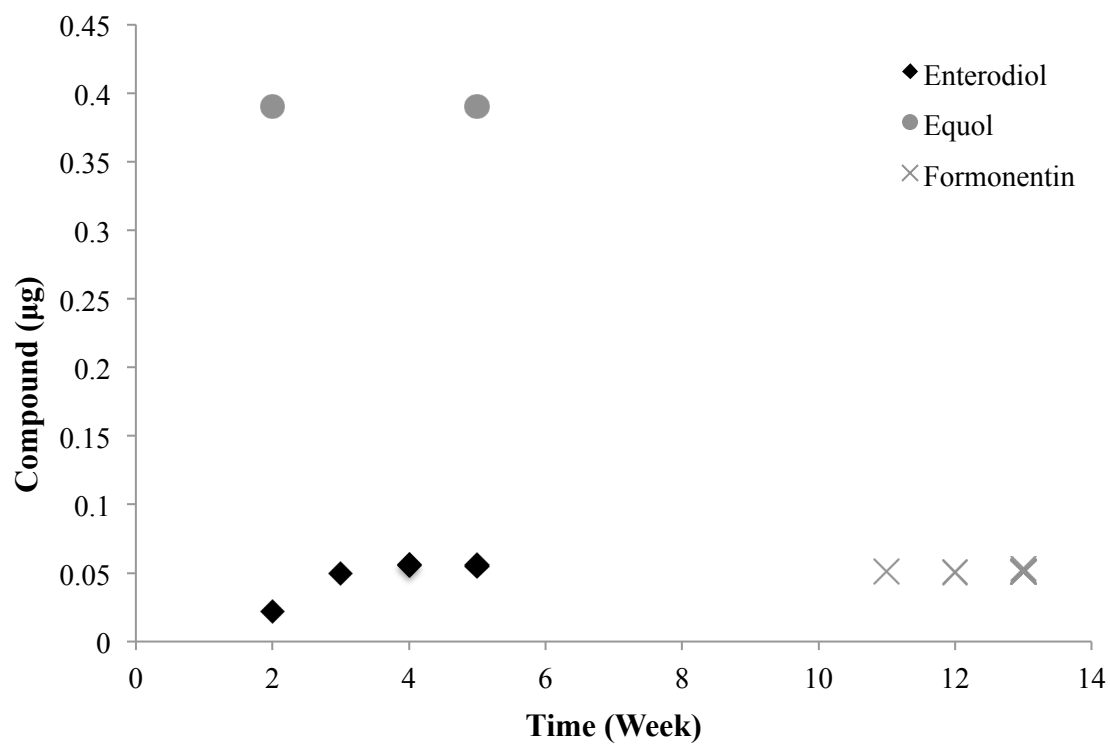


Figure 3.19 Phytoestrogens plotted by species from all treatments.

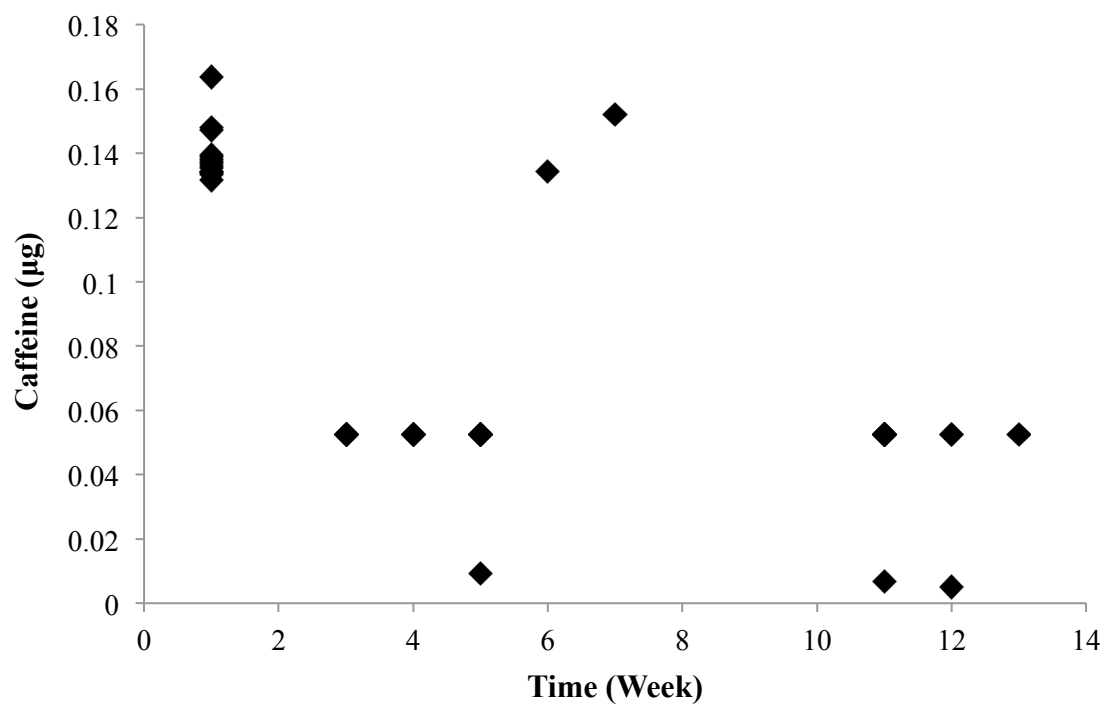


Figure 3.20 Caffeine detection in µg per week; It was detected in the effluent of all twenty columns.

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## Appendix A: Model Parameters in SAS

Table A1. Model Parameters for Copper				
Treatment	Parameter	Estimate	SE	<i>p</i> -value
Chemical	m	0.178	0.068	0.0201
	L	20.851	11.655	0.0953
	B	1.126	0.127	<0.0001
Fertilizer	m	0.206	0.000	<0.0001
	L	25.091	0.000	<0.0001
	B	1.133	0.000	<0.0001
Manure	m	0.111	0.014	<0.0001
	L	5.778	0.280	<0.0001
	B	1.801	0.086	<0.0001
Random	s	0.003	0.000	<0.0001
	Sm	0.030	0.001	0.0079
Contrast Values for 'm' Parameter				
		F Value	<i>p</i> -value	
Manure vs Chemical		0.95	0.3456	
Manure vs Fertilizer		47.15	<0.0001	
Chemical vs Fertilizer		0.16	0.6910	

Table A2. Model Parameters for Nitrate				
Treatment	Parameter	Estimate	SE	<i>p</i> -value
Chemical*	m	21.173	4.227	0.0074
	L	12.167	7.989	0.2024
	B	0.586	0.075	0.0015
Fertilizer	m	24.050	0.000	<0.0001
	L	2.460	0.000	<0.0001
	B	1.460	0.000	<0.0001
Manure	m	27.732	1.297	<0.0001
	L	3.679	0.223	<0.0001
	B	1.350	0.081	<0.0001
Random	s	0.829	0.076	0.0004
	Sm	2.010	0.658	0.0378
Contrast Values for 'm' Parameter				
		F Value	<i>p</i> -value	
Manure vs Fertilizer		8.06	0.047	

\*The parameters for the chemical treatment were not compared to the other treatments because the data was inappropriate for the model used.

Table A3. Model Parameters for Phosphorus				
Treatment	Parameter	Estimate	SE	<i>p</i> -value
Chemical	m	0.747	0.138	<0.0001
	L	4.610	0.425	<0.0001
	B	1.872	0.233	<0.0001
Fertilizer	m	0.640	0.000	<0.0001
	L	5.018	0.000	<0.0001
	B	1.364	0.000	<0.0001
Manure	m	1.211	0.304	0.0013
	L	10.645	3.373	0.0070
	B	1.457	0.208	<0.0001
Random	s	0.063	0.003	<0.0001
	Sm	0.271	0.061	0.0006
Contrast Values for 'm' Parameter				
		F Value	<i>p</i> -value	
Manure vs Chemical		2.18	0.1615	
Manure vs Fertilizer		3.54	0.0810	
Chemical vs Fertilizer		0.60	0.4506	

Table A4. Model Parameters for Sulfate

Treatment	Parameter	Estimate	SE	<i>p</i> -value
Chemical	m	4.153	1.586	0.0202
	L	2.870	0.154	<0.0001
	B	2.923	0.501	<0.0001
Fertilizer	m	6.510	0.000	<0.0001
	L	3.060	0.000	<0.0001
	B	3.160	0.000	<0.0001
Manure	m	33.190	1.513	<0.0001
	L	3.678	0.013	<0.0001
	B	6.871	0.142	<0.0001
Random	s	0.505	0.027	<0.0001
	Sm	3.376	0.667	0.0002
Contrast Values for 'm'				
Parameter		F Value	<i>p</i> -value	
Manure vs Chemical		175.58	<0.0001	
Manure vs Fertilizer		311.26	<0.0001	
Chemical vs Fertilizer		2.21	0.1594	

Table A5. Model Parameters for Zinc

Treatment	Parameter	Estimate	SE	<i>p</i> -value
Chemical	m	0.203	0.047	0.0007
	L	5.807	0.168	<0.0001
	B	5.636	1.103	0.0002
Fertilizer	m	0.191	0.000	<0.0001
	L	4.76	0.000	<0.0001
	B	4.306	0.000	<0.0001
Manure	m	0.203	0.048	0.0008
	L	5.132	0.345	<0.0001
	B	2.717	0.501	<0.0001
Random	s	0.027	0.002	<0.0001
	Sm	0.103	0.019	<0.0001

Contrast Values for 'm' Parameter

	F Value	<i>p</i> -value
Manure vs Chemical	0.00	0.9952
Manure vs Fertilizer	0.07	0.7979
Chemical vs Fertilizer	0.07	0.7986



## **Appendix B: CEC Elution from the Biosolids Columns**

The biosolids-treated columns experienced problems with water flow, and this was verified by the movement of bromide through the columns. Instead of a clear peak, bromide leached in all 13 weeks from the biosolids-treated columns. The extended leaching of bromide from the biosolids treatment was likely due to slower water movement through the soil column. The biosolid treatment had an average bromide recovery rate of 62%, compared to the mid-80% recoveries of the other three treatments. The reduction in recovery rate and the additional four weeks of leaching time verify that the biosolids amendment caused a dramatic difference in the movement of water in those soil cores.

Despite the limited mobility of water and solutes, the biosolids treatment leached the most total phosphorus (P) of the four treatments (3.31 mg). Possibly, the increased organic matter in the biosolids added to the total phosphorus loss in the biosolids leachate. When biosolids are applied to fields, they are often applied to match the nitrogen requirements of the crop. This results in the frequent over-application of P (Lu et al., 2012). In this experiment, biosolids were applied at a rate to match the total nitrogen of the manure, which resulted in a nearly 75% increase in total P added to the biosolids-treated columns compared to the manure-treated columns. This partially explains why the total cumulative P leached from the biosolids columns was three times higher than P from the manure columns.

The EPA mandates that application of biosolids cannot exceed the agronomic rates of nitrogen, but their regulations do not regulate phosphorus loading in biosolids (EPA/832/R-93/003, 1994; Lu et al., 2012). The over-application of phosphorus can have severe environmental impacts through eutrophication. Eutrophication occurs when a body of water receives excess nutrients, resulting in massive algae blooms. When the algae die, the microbes

responsible for decomposition deplete the water source of its dissolved oxygen which kills fish, insects, waterfowl, and decreases the species diversity (EPA, 2013; Smith and Schindler, 2009). Some algae species produce toxins that threaten human health, forcing some cities in Ohio to boil their water during toxic algae blooms in Lake Erie (Michalak et al., 2013). Biosolids application to agricultural fields could contribute to excess phosphorus additions to the environment, increasing the possibility of eutrophication.

The biosolids treatment leached half as much nitrate as the manure or fertilizer treatments (12.6 mmol). It also leached slightly less sulfate, copper, and zinc compared to the other treatments. Likely, this decrease in solute elution was due to the decreased water flow in the columns.

Sulfathiazole was the only compound to leach from the biosolids-treated columns (Fig. 3.14). The biosolids contained  $63.22 \mu\text{g kg}^{-1}$  dry weight sulfathiazole, which lead to  $0.57 \mu\text{g}$  of sulfathiazole applied to each column (Table 2.5). Interestingly, sulfathiazole eluted significantly earlier from the biosolids column (peaking at week 2) than the chemical columns (peaking at week 5). Bromide was detected in the biosolids treatment leachate during week 1, suggesting that compounds were able to travel the length of the column. The high pH of organic fertilizers encourages the anionic form of sulfonamides (Thiele-Bruhn and Aust, 2004; Burkhardt and Stamm, 2007), which would make sulfathiazole more mobile in the soil environment. Another soil column experiment has shown that solution pH was the most important factor in mobility of pharmaceuticals, increasing the mobility of weakly acidic compounds (Borgman and Chefetz, 2013).

Additionally, the high organic matter content of biosolids has been shown to clog pores in soil (Burkhardt and Stamm, 2007). The clogging of smaller pores may have

promoted transport through macropores or along the sides of the PVC pipe. Furthermore, increased dissolved organic matter has been shown to decrease sulfonamide sorption ( $k_d$ ) to soil (Chu et al., 2013; Zhang et al., 2014). This was attributed to competition for sorption sites between sulfonamides and dissolved organic matter. If less sulfathiazole sorbed to the soil, then more is available in the soil solution, thus increasing mobility.

Sulfathiazole from the biosolids treatment had a much higher recovery rate (74.08%) than from the chemical treatment (<0.01%). This is likely due to both decreased sorption of sulfathiazole to the soil and the time difference in elution. Sulfathiazole began eluting from the chemical treatments during week 5, when the antibiotic would have experienced increased degradation, but the sulfathiazole originating from the biosolids eluted during weeks 1 – 4, experiencing fewer degradation half-lives. Additionally, organic matter in the biosolids likely increased mobility and decreased sorption of sulfathiazole to the soil. Thus, more of the sulfathiazole in the biosolids treatment was mobile and collected in the leachate.

Although the data from biosolids-treated columns cannot quantitatively be compared to the other three treatments, the data is not useless. Nearly three times as much phosphorus moved through the biosolids-amended columns compared to the manure-treated columns. Additionally, sulfathiazole leached from the biosolids and through the entirety of the soil profile, whereas the sulfonamides in the manure did not. A second soil column experiment is currently underway, and this time, we used a smaller application rate for the biosolids. Repeating the experiment will give insight as to whether the CEC leaching patterns observed above are indicative to biosolids applied on the soil or simply from the circumstantial pressure of clogged pores.

### Appendix C: Organic Compound Elution from Soil Columns

Table C1. Average Cumulative Amount ( $\mu\text{g}$ ) Leached from Each Treatment

	Chemical	Biosolids	Manure	Fertilizer
17 $\alpha$ -Estradiol	0.044	0.000	0.014	0.044
17 $\beta$ -Estradiol	0.039	6.733	0.000	0.000
Estrone	0.000	0.120	0.000	0.000
Estriol	0.169	0.139	0.110	0.105
17 $\alpha$ -Ethinyl estradiol	0.037	0.036	0.000	0.037
Progesterone	0.019	0.028	0.019	0.019
17 $\alpha$ -Hydroxyprogesterone	0.057	0.044	0.057	0.076
Coumestrol	0.000	0.000	0.000	0.000
Enterodiol	0.025	0.011	0.022	0.011
Formononetin	0.021	0.061	0.000	0.010
Biochanin A	0.000	0.000	0.000	0.000
Equol	0.078	0.000	0.078	0.000
Sulfamethazine	30.259	0.015	0.003	0.007
Sulfadimethoxine	472.060	1.368	0.857	0.740
Sulfamethoxazole	45.115	0.060	0.033	0.024
Sulfathiazole	0.441	0.422	0.000	0.000
Tetracycline	0.000	0.016	0.000	0.000
Oxytetracycline	0.015	0.010	0.000	0.000
Chlorotetracycline	0.000	0.000	0.000	0.000
Penicillin G	0.056	0.106	0.014	0.097
Flunixin	1275.981	9.533	1.386	1.679
Acetaminophen	0.000	0.000	0.000	0.000
Caffeine	0.200	0.100	0.229	0.274
Ibuprofen	19.073	0.019	0.052	0.060
3,4,4'-Trichlorocarbanilide	0.000	0.000	0.000	0.000

Table C2. Range of Cumulative Amounts (µg) Eluted from Replicates by Treatment				
	Chemical	Biosolids	Manure	Fertilizer
17 $\alpha$ -Estradiol	0.00 - 0.22	0	0.00 - 0.07	0.00 - 0.22
17 $\beta$ -Estradiol	0.00 - 0.20	0 - 33.66	0	0
Estrone	0	0 - 0.6	0	0
Estriol	0.12 - 0.45	0 - 0.28	0 - 0.27	0 - 0.12
17 $\alpha$ -Ethinyl estradiol	0 - 0.19	0 - 0.18	0	0 - 0.19
Progesterone	0.02 - 0.02	0.02 - 0.05	0.02 - 0.03	0.02 - 0.03
17 $\alpha$ -Hydroxyprogesterone	0.04 - 0.09	0 - 0.09	0 - 0.09	0.04 - 0.10
Coumestrol	0	0	0	0
Enterodiol	0 - 0.1	0 - 0.06	0 - 0.11	0 - 0.06
Formononetin	0 - 0.10	0.05 - 0.10	0	0 - 0.05
Biochanin A	0	0	0	0
Equol	0 - 0.39	0	0 - 0.39	0
Sulfamethazine	15.41 - 59.05	0.00 - 0.06	0.00 - 0.02	0.00 - 0.02
Sulfadimethoxine	387.55 - 621.11	0.91 - 1.95	0.41 - 1.45	0.15 - 1.33
Sulfamethoxazole	27.46 - 58.71	0.02 - 0.09	0.01 - 0.11	0.00 - 0.07
Sulfathiazole	0.27 - 0.69	0.24 - 0.73	0	0
Tetracycline	0	0.00 - 0.08	0	0
Oxytetracycline	0.00 - 0.06	0.00 - 0.05	0	0
Chlorotetracycline	0	0	0	0
Penicillin G	0 - 0.24	0 - 0.25	0 - 0.04	0 - 0.33
Flunixin	1083.45 - 1414.96	4.17 - 15.76	0.72 - 2.25	0.26 - 3.46
Acetaminophen	0	0	0	0
Caffeine	0.14 - 0.32	0.00 - 0.23	0.14 - 0.35	0.24 - 0.34
Ibuprofen	7.7 - 33.45	0 - 0.09	0 - 0.14	0 - 0.17
Trichlorocarbanilide	0	0	0	0

Table C3. Number of Replicates with Detectable Amount of Each CEC <sup>+</sup>				
	Chemical	Biosolids	Manure	Fertilizer
17 $\alpha$ -Estradiol	1	0	1	1
17 $\beta$ -Estradiol	1	1	0	0
Estrone	0	1	0	0
Estriol	4	4	4	4
17 $\alpha$ -Ethinyl estradiol	1	1	0	1
Progesterone	5	5	5	5
17 $\alpha$ -Hydroxyprogesterone	5	4	4	5
Coumestrol	0	0	0	0
Enterodiol	2	1	1	1
Formononetin	1	4	0	1
Biochanin A	0	0	0	0
Equol	1	0	1	0
Sulfamethazine	5	2	1	2
Sulfadimethoxine	5	5	5	5
Sulfamethoxazole	5	5	5	4
Sulfathiazole	5	5	0	0
Tetracycline	0	1	0	0
Oxytetracycline	2	1	0	0
Chlorotetracycline	0	0	0	0
Penicillin G	2	3	2	2
Flunixin	5	5	5	5
Acetaminophen	0	0	0	0
Caffeine	5	4	5	5
Ibuprofen	5	1	2	2
3,4,4'-Trichlorocarbanilide	0	0	0	0

+ Out of five replicates