ABSTRACT

Title of Document: INVESTIGATING COMPOSTING AS A METHOD FOR REDUCING ESTROGENICTY IN POULTRY LITTER AND BIOSOLIDS

Kirsten Marie Hammett, Master of Science, 2015

Directed by: Assistant Professor, Lance Yonkos
Department of Environmental Science and Technology

Biosolids and poultry litter contain the natural estrogens 17β-estradiol and estrone, which can be transported to receiving waters via runoff when these materials are used as fertilizers. Estrogens are of concern because of their ability to act as endocrine disruptors and feminize fish. In this study, In-Vessel Aerated and Turned composting was investigated for its efficacy at mitigating estrogen concerns in BS and PL. Pre- and post-composted, BS and PL samples were investigated for total estrogenicity and estrogen species concentrations. In addition, conversion of estrone to 17β-estradiol was investigated by measuring the creation of deuterated 17β-estradiol from a deuterium-labeled estrone stock within aqueous PL mixtures. Data from these studies indicates that there may be efficacy in composting BS and PL prior to land application and suggest that estrone is capable of converting to the more potent 17β-estradiol species as a result of entering microbially active environments.

INVESTIGATING COMPOSTING AS A METHOD FOR REDUCING ESTROGENICTY IN POULTRY LITTER AND BIOSOLIDS

By

Kirsten Marie Hammett

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Advisory Committee:
Dr. Lance Yonkos, Chair
Dr. Gary Felton
Dr. Luke Iwanowicz

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LIST OF ABBREVIATIONS

BLYES……Bioluminescent Yeast Estrogen Screen

BS……...Biosolids

CAFOs……Concentrated Animal Feeding Operations

DDT……...Dichlorodiphenyltrichloroethane

D4-E1……..…….....Deuterated Estrone

D4-E2……..…….....Deuterated 17β-estradiol

EDC…….Endocrine Disrupting Chemical

EEQ…….…….....17β-estradiol Equivalents

ELISA…….....…....Enzyme-Linked Immunosorbent Assay

E1…….……..…….…..Estrone

E2……..…….…...……...17β-estradiol

EE2……...……...Ethnynlestradiol

IVAT……..In Vessel Aerated and Turned

LC-MS/MS……Liquid Chromatography Tandem Mass Spectrometry

MGD……..……...Millions of Gallons per Day

N…..……...Total Nitrogen

P…..……..Total Phosphorous

PFRP……...Process to Further Reduce Pathogens

PL…….……...……...…..Poultry Litter

PL-A…….……Poultry Litter Aerated

PL-A/T……Poultry Litter Aerated and Turned

PL-T…….....Poultry Litter Turned

PPCPs ……Pharmaceuticals and Personal Care Products

S/D……...…. Sterile and Dark Conditions

TO……...……Testicular Oocytes

USEPA…….……United States Environmental Protection Agency

Vtg……..Vitellogenin

WHO……..World Health Organization

WWTP……...….Waste Water Treatment Plant

CHAPTER ONE:

EFFICACY OF COMPOSTING AT REDUCING/ELIMINATING CONTAMINANTS OF CONCERN

INTRODUCTION

Endocrine Disruption and Endocrine Disrupting Chemicals

An endocrine disrupting chemical (EDC) is defined as "an exogenous substance or mixture that alters function(s) of an endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (WHO, 2002). Many of the most widely studied EDCs work by binding to receptor proteins that normally bind to naturally occurring steroids and thus act as hormone agonist or antagonist (Petersen et al., 1993; McEwen, 1981; Sumpter, 2005). In recent years, research has shown that the endocrine system is integrated closely with not only organism development but also the central nervous system, reproduction and the immune system (Kronenberg et al., 2007). Since World War II, large numbers and quantities of EDCs have entered the environment (Colborn et al., 1993; Guillette, et. al., 1995). These man-made chemicals include plasticizers, flame retardants, pesticides and pharmaceuticals, many resistant to degradation, ensuring persistence and broad environmental transport to aquatic systems (Colborn et al., 1993; Hamid and Eskicioglu, 2012). Many EDCs also have the capacity to volatilize allowing long range atmospheric transport (Eisenreich, et al., 1981; Rapaport et al., 1985). Endocrine disrupting chemicals have been found in virtually all surface (freshwater and marine), ground and precipitation (Colborn et al., 1993; Hutchinson et al., 2006). Because of their wide spread abundance, they have the potential to act both directly and indirectly on a wide range of species.

Organisms can be exposed to EDCs indirectly during prenatal and early postnatal development via maternal or directly through environmental contact (Mclachlan et al., 1975; Tomihara et al., 2015). Lipophilic properties of EDCs allow them to accumulate and be stored in body fat, which is consequently mobilized during egg development, pregnancy and lactation (Colborn et al., 1993).

Various studies have shown that EDCs are associated with abnormal function of organs/tissues such as the thyroid, mammary glands, liver, kidney, brain and bone (Colborn et al., 1993; Vandenberg et al., 2012). Birds, fish, shellfish, turtles and mammals have all shown evidence of negative impacts as a result of exposure to EDCs (Guillette et al., 1995). A common example of a chemical causing adverse effects in bird embryonic survival is the contamination of dichlorodiphenyltrichloroethane (DDT) in bald eagles in the Great Lakes region. Eggshell thinning and cracking was induced by the presence of DDT in the water where the eagles were actively feeding on contaminated fish (Faber and Hickey, 1973). Restrictions in DDT production and subsiding environmental levels have led to improved reproductive success and increases in bald eagle populations (Faber and Hickey, 1973; Grier, 1982).

The American alligator has also shown signs of negative effects as a result of EDC exposure (Guillette et al., 2000). A pesticide spill in 1980 caused a large amount of pesticide runoff to enter the surrounding Lakes in Florida. A study conducted by Guillette et al., (2000) found that alligators living in the contaminated lake, Apopka in the northwest region of Orlando, had decreased clutch viability and increased juvenile mortality. Reproductive health in the contaminated area remains low compared to other

lakes in the region that did not experience runoff from the pesticide spill (Guillette et al., 2000).

Much of the research conducted on endocrine disruption effects has focused on aquatic species (Sumpter 2005). The aquatic environment is the ultimate sink that receives a large amounts of intentional effluents from waste water treatment plants (WWTPs) and unintentional runoff from both agriculture and urban surroundings. A majority of the research conducted focused on estrogenic compounds, which have been shown to cause adverse effects in fish species (Sumpter 2005; Kolpin et al., 2013; Sumpter and Jobling, 2013). These effects can range from the induction of vitellogenin (Vtg) to intersex or testicular oocytes (TO) present within male fish species (Blazer et al., 2012). Vitellogenin is defined as the female egg-yolk protein that is normally only detected in female fish (Sumpter and Jobling, 2013; Yonkos et al., 2010). Vitellogenin is a precursor lipoprotein to the essential egg yolk proteins that are the source of energy and nutrients during embryonic and larval development. Therefore, Vtg only naturally occurs in significant amounts within mature female fish during ovarian recrudescence. However, Vitellogenesis (production of Vtg) has been shown to occur when male of immature fish are exposed to environmental estrogens and estrogenic chemicals and is therefore now commonly used as a biomarker for estrogen exposure (Kime et al., 1999). Intersex is described as the presence of gametic cells from the opposite sex being found in the gonad of the fish being examined (Gercken and Sordyl, 2002; Blazer et al., 2007). Among types of intersex being observed in fish, TO (the presence of oocytes within otherwise normally developing testicular tissue) is the most common form found in male fish. Evidence of intersex in fish was first reported in English Rivers when sport fishermen were unable to

identify the sex of wild roach that were caught downstream of WWTPs (Sweeting, 1981; Tyler and Jobling, 2008). Both increased Vtg levels and the detection of oocytes in male fish have commonly been used as bioindicators of exposure to estrogen (Sumpter 2005; Blazer et al., 2012). For example, aqueous exposures of poultry litter (PL)-amended field runoff was found to induce production of Vtg in adult male fathead minnows (Yonkos et al., 2010). Field studies have also used Vtg as a biomarker of estrogenic exposure in wild fish populations (Blazer et. al., 2012). Blazer et al., (2012) collected smallmouth bass from tributaries of the Potomac River with known point sources of discharge from animal farming and non-point sources with high human populations over a two year period and investigated for the presence of TO and Vtg induction (Blazer et al., 2012). Passive water samplers were also deployed at collection sites to analyze for total estrogenicity (Blazer et al., 2012). Analysis showed that the amount of land application of animal manures as well as the volume of WWTP discharges correlated positively with estrogenicity in a regional receiving waters as well as Vtg induction (mean concentration and percentage of males induced) and TO severity in regional fish populations (Blazer et al., 2012). Land application practices may also influence seasonal observations of intersex (Blazer et al., 2012). Although the presence of Vtg and TO are well studied, the effects of these characteristics on reproduction and overall fish health are not well known (Sumpter 2005). The discovery of TO within regions that lack point sources of discharge suggest that estrogens may be entering receiving waters via the land application of waste material such as biosolids (BS) and PL, causing feminization. Once these materials are applied to land, rain events have the ability to mobilize the material and cause significant runoff into aquatic habitats.

Most of the potent and relevant estrogenic compounds that have been found in nature are excreted animal steroids, meaning that they have come from human and animal waste (Hamid and Eskicioglu, 2012). These natural estrogens have been found to be significantly more potent than non-steroidal estrogen agonists such as industrial compounds or synthetic chemicals like plasticizers including organochlorine aromatic compounds (Legler et al., 1999; Hamid and Eskicioglu, 2012; Khanal et al., 2006). Estrogenic compounds that do make their way into receiving waters have the ability to bind to estrogen receptors within aquatic species and cause unintentional effects (Guillette et al., 2000).

Poultry Litter and Biosolids

It is well established that hormones such as estrogens persist in BS and PL (Clark and Smith 2011; Yonkos et al., 2010; Dutta et al., 2010; Khanal et. al., 2006). With recent increases in concentrated animal feeding operations (CAFOs), there has consequently been an increase in animal waste production. In the United States, animal manure from CAFOs is not required to be treated given that it is not directly transported to receiving waters (Khanal et. al., 2006). With a rise in population, waste generation by WWTPs has also increased. Despite the various treatment methods influent receives at WWTPs, BSs still emerge with potent concentrations of estrogens (Khanal et al., 2006).

There are two environmentally important free estrogens, 17β-estradiol (E2) the most bioactive form, and estrone (E1), a less active but generally more abundant form. Both of these natural estrogens are found in both BS and PL. Conventional treatment processes at WWTPs have been shown to eliminate approximately 85-99% of E2, while

E1 removal is not as efficient eliminating 25-80% and comparing influent to effluent products (Khanal et. al., 2006). The removal of these estrogens occurs during biodegradation while the estrogens are sorbed to organic materials within the sewage sludge (Khanal et. al., 2006). The levels of estrogens (ng/L) found in BS and PL, even after processing, are enough to cause adverse effects in fish populations (Sumpter 2005).

Although BS and PL have the ability to impact the environment negatively, they are also a valuable resource as fertilizer and soil amendment when land applied. They contain important nutrients such as phosphorous and nitrogen that aid in maintaining soil quality and recycling nutrients (Shiralipour et al., 1992). Using these waste products as fertilizers limits the need for inorganic fertilizers and reduces the amount of waste material being transported to landfills. Research pertaining to the land application of BS and PL has mainly focused on eutrophication as a result of nutrients, however, less is understood about other contaminants of concern including estrogens and their chemical behavior in receiving waters.

Best management practices have shown efficacy in reducing the amount of estrogens entering aquatic habitats via runoff (Yang et. al., 2012; Dutta et. al., 2010; Fisher et al., 2012). For example, time of application of litter amendments can affect the amount of estrogen leaching from PL when applied to pastures (Nichols et. al., 1997). A study conducted by Nichols et. al., (1997) found that rain events occurring directly after application caused the most amount of E2 to run off when compared to rain events that occurred seven days post application. The study also found that adding alum to litter decreased the amount of E2 runoff by 42-46% (Nichols et. al., 1997). One of the pioneering studies to measure E2 and testosterone in soil and runoff, conducted by

Finlay-Moore et. al., (2000), using enzyme immunoassay found that depending on application rate, E2 has the ability to increase in concentration post application. This study showed early evidence that estrogens entering the environment have the ability to change in species and possibly become more potent.

Estrogens and Estrogenicity

Environmental estrogens (E1 and E2) exist in both hydrophilic conjugated forms (with glucuronide and/or sulfate attachment groups) as well as more hydrophobic free forms (Dutta et. al., 2010). There are more than a dozen known conjugates of each free form (E1 and E2) (Figure 1.1). Because conjugated estrogens are more water soluble (and generally more abundant), they are more prone to be transported in runoff following agriculture applications (Dutta et al., 2012; Hutchins et al., 2007). Once conjugated species reach receiving waters, they encounter a robust microbial community which is capable of deconjugating and returning glucuronidated and sulfated conjugated estrogens to their more potent parent forms (Andaluri et al., 2010). Estrogens can exist in aqueous samples in a variety of forms. Free E2, is particularly bioactive, binding avidly to the estrogen receptor while others, like free E1, have only limited estrogen receptor binding capabilities (Hutchins et al., 2007). Conjugated estrogens (e.g., glucuronidated and/or sulfated E2 and E1) are incapable of binding to the estrogen receptor, and therefore are largely lacking in estrogenicity (Hutchins et al., 2007). However, their abundance and water solubility, coupled with the potential for microbial deconjugation, introduce the possibility of estrogenic activity actually increasing for a period of time after introduction to natural waters (Figure 1.1) (Hutchins et al., 2007).

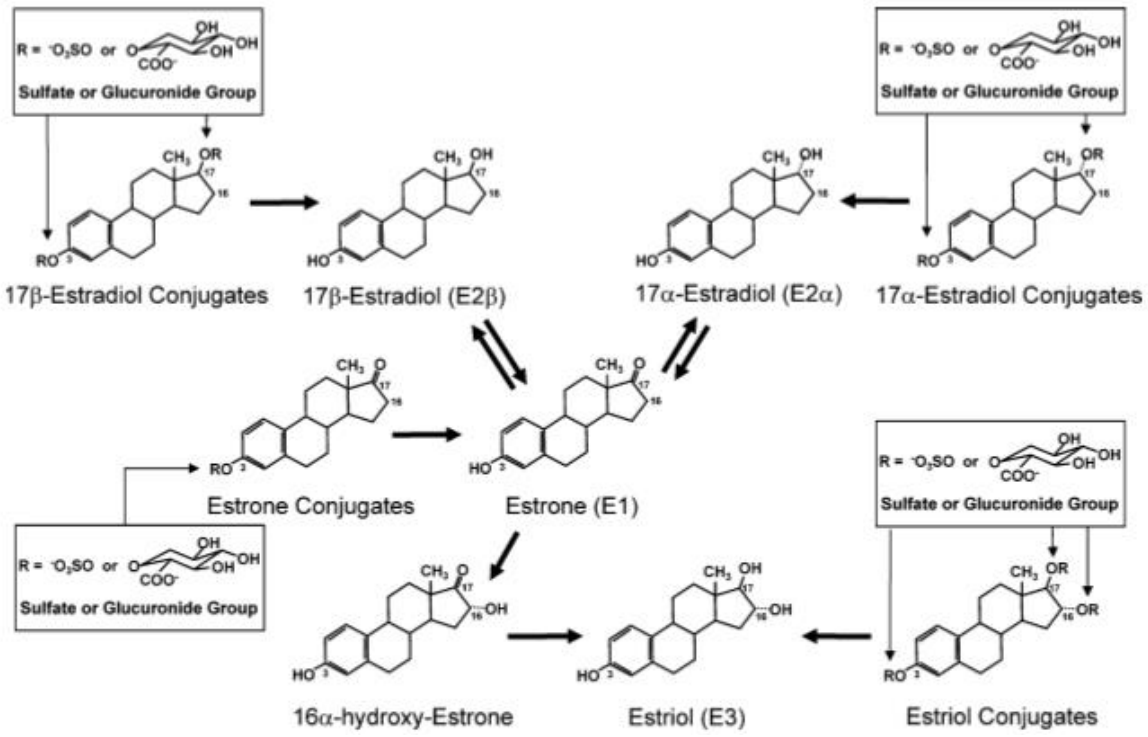

Figure 1.1. Image from Hutchins et al., (2007) illustrating the structure of estrogens and possible pathways of estrogen species conversion and deconjugation. Path demonstrates conjugated estrogens (sulfated or glucuronidated estradiol and estrone) deconjugating to the biologically available potent estradiol species and less potent species estrone. Transformation from estrone to estradiol and vice versa then degrades to less potent estradiol species. Transformation/conversion is a result of estrogen species entering microbially active environments.

Once waste material such as BS and PL enter receiving waters via land application and runoff, two dynamic and related microbial processes begin to occur. Initially, the microbial community becomes increasingly competent at deconjugating estrogens to free bioactive forms, leading to an increase in estrogenicity. This is followed by the microbial community targeting the now abundant free estrogens for transformation and degradation. First is conversion of E2 to E1, leading to a decrease in estrogenicity, followed by cleaving the steroid ring structure and permanent destruction of the estrogen

molecule (Hutchins et al., 2007). The relationship between these two processes determines the abundance and persistence of individual estrogen constituents and, more importantly, the collective potency in the aquatic environment (Hutchins et al., 2007).

While it is generally accepted that E2 transforms to the less potent E1 via microbial action (Dutta et. al., 2010), there is preliminary evidence suggesting that some portion of E1 might actually be converted to the more potent E2 (Hutchins et al., 2007). Because E2 is up to 100× more estrogenic than E1, only a modest conversion in this direction could dramatically increase the risk of endocrine disruption (Dutta et al., 2012).

In addition to natural estrogens entering receiving waters, a synthetic estrogens 17α- ethnynlestradiol (EE2) has also been found in waterways (Hamid and Eskiciogly, 2012). This compound, widely used as an oral contraceptive and in hormone replacement therapy, is ingested and then enters WWTPs once excreted (Hamid and Eskiciogly, 2012). 17α-ethinylestradiol has the ability to act on estrogen receptors with similar affinity as E2, making EE2 a contaminant of significant concern (Hamid and Eskiciogly, 2012).

It is estimated that the average WWTP in the United States produces 240 kg (dry weight) of solids for every million liters of influent water treated (Yang et al., 2012). This results in eight million tons (dry weight) of BS being produced annually in the U.S. with about 50% being land applied (Yang et. al., 2012). Being in close proximity to the Delmarva Peninsula and within the Chesapeake Bay region, the issue of possible environmental risk associated with land application of BS and PL is of growing concern. The Delmarva region is one of the top producers of poultry and is estimated to produce 377,000 tons of poultry litter annually in Maryland alone, with about twice that amount

being produced in the Delmarva Peninsula (Moyle, J.R. Personal communication, April 2015). Being located in the Bay area, there are an abundance of WWTPs in the region to ensure proper waste water treatment prior to it entering receiving waters that may harm the Bay. It is estimated that Maryland produces approximately 700,000 tons of BS annually (Maryland Department for the Environment, 2009). It is economically impractical to transport waste materials over long distances, therefore local farms receive a robust amount of both BS and PL. Treating waste water and producing poultry are both necessary and important activities that have the consequence of generating an abundant amount of waste material (BS and PL). Currently, these waste materials can either be sent to be buried in landfills or repurposed as fertilizer containing valuable nutrients and applied to land. It is clear that repurposing BS and PL as fertilizer has significant benefits, however it is also now known that local aquatic environments are receiving estrogen contaminated runoff from these land application practices (Hamid and Eskiciogly, 2012).

Biosolids and PL also contain ammonia, which is readily dissolved in water during rain events and transported to aquatic environments. Many fish species are sensitive to ammonia levels as low as 1 mg/L especially when pH levels are greater than 8.00 (USEPA 1999). Once ammonia reaches toxic levels, it inhibits the fish's ability to respire normally across the gills and can result in death (Randall and Tsui, 2002). Ammonia levels within BS and PL can vary greatly depending on the age and origin of the material being land applied (Randall and Tsui 2002).

Composting

With known endocrine disrupting and possible toxic effects from the land application of BS and PL, investigating methods to mitigate these consequences has become increasingly important. Composting is a process in which microorganisms decompose organic material in an aerobic biological controlled environment (USEPA, 2013). When used appropriately, composting aids in accelerating the natural process of decomposition by controlling moisture content, temperature and material mixture. Once composting is complete, raw waste materials are transformed into a useful and stable product that is beneficial to plants. During the composting process, microbes within the material raise the temperature through respiration. This increase in temperature encourages the destruction of pathogens, viruses, and invertebrates. Because microbes within the material are continuously using oxygen, it is important that the material has a source of oxygen, either through natural convection of forcing air through the material. Forcing air through the material also aids in maintaining appropriate temperatures where microbial activity is most efficient. Microbes require above a 5% oxygen content for proper respiration, although they can continue activity with less efficiency at lower levels. Turning of compost piles also aids in exposing outer surfaces to the interior where the microbial activity is most intense. Composting is an important process both for proper waste disposal, but also a business opportunity to produce a highly valuable soil conditioner.

In-vessel, aerated and turned (IVAT) composting is a method of composting that begins with material being contained inside a structure that is used to shield the material from environmental factors such as precipitation. An IVAT composting system is very

effective, however it requires a high level of management and can be costly to maintain because of its complexity. It is designed to handle large volumes of waste material, which helps in controlling odor and runoff. The structure that contains the material has a positive pressure forced-air source below to push air through the compost to maintain proper oxygen levels. In-vessel, aerated and turned composting is an existing technology that is already being used as a method to reduce pathogens and improve BS material for land application (Figure 1.2). Until this project, IVAT composting has not been investigated to determine its effects on estrogen content and estrogenicity within the material being composted.

Figure 1.2. Image of existing IVAT composting trench, illustrating the process on an industrial scale, located at the Baltimore City Composting Facility, which is operated by Veolia North America. Trench is approximately 6.1 m X 6.1 m X 106.7 m and contains a mixture of biosolids, wood chips, and wood ash under positive pressure aeration.

Composting has previously been shown to improve the quality of manure by decreasing odor, stabilizing nitrogen, and decreasing pathogen load (USEPA, 2013). Hakk et. al., (2005) showed a decrease (but not elimination) of E2 and testosterone in composted PL. This study provides support that total estrogenicity may decrease as a result of composting both BS and PL prior to land application. Research has also found that the composition of the material and resulting microbial community has a significant influence on how well hormones are degraded during the process (Layton et al., 2000). For example, E2 within two different types of starting material (mineralized plants and an industrial wastewater source) composted over a 72 day period showed differing degrees of estrogen degradation (Layton et al., 2000). Estrogen within the plant mineralized material degraded by 84% while sludge degraded by just 4% (Layton et al., 2000). This study suggests that composting processes may vary in their ability to degrade hormones based on the origin of the material. This is particularly important when studying hormones within BS and PL because the original material is different between the two products. Poultry litter contains a variety of natural estrogens and has not previously been subjected to waste management practices (Khanal et al., 2006). Biosolids, however, can contain natural estrogens along with synthetic estrogens such as previously mentioned EE2 (Khanal et al., 2006; Sumpter and Jobling, 2013; Pauwels et al., 2008). Biosolids have previously been treated via processes to further reduce pathogens (PFRP) (Khanal et al., 2006). Depending on the age and the technology employed by a specific WWTP, these processes can include lime stabilization or anaerobic digestion. In addition to these materials containing estrogens, BS also contain pharmaceuticals and personal care products (PPCPs) such as antibiotics as well as flame retardants, and pesticides (Wu et

al., 2009; Clarke and Smith, 2011). Biosolids are also monitored for persistent organic compounds and heavy metals that can negatively affect the environment (Azevedo-Silveira et al., 2003). In contrast, PL will only contain natural estrogens (E1 and E2) and lack any synthetic estrogens. Poultry litter will also contain trace amounts of antibiotics but many less species of PPCPs in general. Both BS and PL will have measureable (although differing) amounts of ammonia. These differing characteristics of BS and PL establish the need for further investigation into more commercially viable and effective methods for composting each to reduce the risk of endocrine disruption and other environmental effects.

In summary, land application of both PL and BS are valuable as sustainable solutions to maintaining soil quality, recycling nutrients, limiting reliance on inorganic nitrogen and phosphorus, and reducing the need to landfill human and animal wastes. These products provide a number of cost savings to regional farmers. However, land application is not without risks, both to human health and the environment. Poor application practices can threaten water quality (e.g., eutrophication), pathogens can threaten human (e.g., *E. coli, Salmonella sp.*) and animal (e.g., avian influenza) health, and residual contaminants-of-concern (e.g., metals, persistent organic pollutants, pharmaceuticals, and steroids) can accumulate and be transported to receiving waters with the possibility of unknown dangers. These issues are of particular interest in Maryland because of the dense regional poultry production industry (primarily on Delmarva) and the abundance of BS generated at regional waste water treatment facilities designed to minimize Chesapeake Bay nutrient pollution. To address these concerns, this

research aimed to quantify the effectiveness of IVAT composting at reducing or eliminating estrogens (natural and synthetic) in both BS and PL.

Four BS samples (from unique WWTPs) and one PL sample from a poultry broiler operation were acquired in the winter of 2014/2015. Biosolid samples were chosen from WWTPs with varying influent sources/volumes and different treatment processes. Upon arrival, samples from all material were collected for analysis prior to composting. Seven IVAT pilot-scale bins were constructed for composting both BS and PL upon their delivery. Both BS and PL material was IVAT composted over approximately 120 and 150 days (respectively). A Solvita test, which measures compost maturity, was utilized to evaluate compost maturity upon completion in spring 2015. Estrogenic activity (estrogenicity) and estrogen analytes (E1, E2 and conjugates) in water soluble fractions were quantified in both pre- and post-composted material using three methods: (1) quantifying Vtg induction in an in vivo, whole fish exposure model using male adult fathead minnows (*Pimephales promelas)* by means of a "sandwich" Enzyme-Linked Immunosorbent Assay (ELISA), (2) measuring total estrogenicity via an in vitro Bioluminescent Yeast Estrogen Screen (BLYES) Assay, and (3) quantifying estrogen analytes using liquid chromatography tandem mass spectrometry (LC-MS/MS). Because previous studies conducted at the Wye Research and Education Center indicated that an aqueous mixture in the order of 0.5 g/L of PL in water was environmentally relevant concentration, aqueous samples were prepared at a concentration of 0.5 g/L (dry weight equivalent) for all water soluble fractions under investigation (Fisher et al., 2012).

Toxicity levels were also examined in pre- and post-composted material to determine the effectiveness of IVAT composting at reducing frank toxicity. Standard 7-

day toxicity bioassays were performed using larval fathead minnows in a dilution serious according to USEPA methods (USEPA 2002). Mixtures of pre- and post- composted BS and PL material were generated with maximum concentrations based on preliminary ammonia results. Depending on ammonia concentrations, dilutions series were prepared to a concentration of up to 6.4 g/L (dry weight equivalent) with exposures renewed daily. Endpoints included survival and growth of fish.

The second portion of this research was investigating the conversion of E1 to E2. Briefly, a deuterium-labeled E1 (E1-d4) was introduced to aquaria including control and PL treatments. The E1-d4 control treatment was investigated to determine the conversion phenomenon in isolation of other influences. This allowed assessment of the role of hydrolytic reactions. Sterile environments of E1-d4 and E1-d4 plus PL treatments were maintained in dark conditions, which controlled for microbial and photolytic degradation. Water soluble fractions from all treatment groups were also investigated using the three methods previously stated.

Objectives

The overall goal of this study was to investigate IVAT composting as a method for reducing contaminants of concern within BS and PL materials. This research provides insight into a feasible and reliable method for mitigating contaminants of concern that will result in decreasing environmental risk through endocrine disruption in receiving waters. The two objectives for assessing the efficacy of IVAT composting were: (1) to quantify estrogens/estrogenicity in pre- and post- composted BS and PL from four distinct WWTPs and one PL broiler operation using the three methods previously

described and (2) to determine the effectiveness of composting to reduce frank toxicity. An additional objective discussed in chapter two was to determine the nature and rate of change in BS- and PL- associated estrogens once in aqueous solutions.

MATERIALS AND METHODS

Introduction

For this project, aqueous mixtures of four BS samples and one PL sample were generated for the dual purposes of determining abundances of soluble estrogens/estrogenicity using three methods (LC-MS/MS, BLYES and a whole fish exposure model) and to explore changes in estrogens/estrogenicity over time once in aqueous solutions. In the fish model, exposures of ten days allowed sufficient time for Vtg induction and amplification as a result of exposure to estrogens within the aqueous mixtures of both BS and PL. These experiments give insight as to the behavior of the estrogens within these materials once they enter receiving waters.

Material Source

Biosolid samples (2,000 kg per sample) were obtained from four distinct regional WWTPs located in Maryland. One PL sample (20,000 kg) was also obtained from a whole house cleanout of a conventional broiler operation. Biosolid samples were from Back River WWTP located in Essex, Kent Narrows-Stevensville-Grasonville (KNSG) WWTP on Kent Island, and Broadneck and Bay Ridge WWTPs in Anne Arundel County. Back River is a high capacity WWTP (treating 180 million gallons per day) with

complex inputs including industrial/commercial influents (Table 1.1). Back River uses anaerobic digestion as its process to further reduce pathogens (PFRP). The Back River WWTP is a modern facility, located in the city and handles an abundant amount of city generated influents. KNSG is significantly smaller in size, also uses anaerobic digestion but in contrast has mostly residential inputs. Bay Ridge, an older waste water treatment facility with minimal industrial inputs, uses lime stabilization as its means to further reduce pathogens. Broadneck is slightly newer but similar in size to Bay Ridge and also uses lime stabilization (Table 1.1). Samples were obtained from both Broadneck and Bay Ridge prior to lime stabilization, KNSG and Broadneck WWTPs utilize both chlorine and sulfur dioxide as a means of disinfection and dechlorination (respectively).

Table 1.1. Four biosolid samples obtained from four distinct regional waste water treatment plants and their corresponding characteristics. Delivery Date = date upon which material was delivered to the Wye Research and Education Center located in Queenstown, Maryland. Upon arrival, material was assigned an identification (BS-A, BS- B, BS-C or BS-D). PFRP = Process to further reduce pathogens. MGD = million gallons per day.

IVAT Composting

Identification	Delivery Date	Source	Influent	PFRP
BS-A	1-20-2015	Back River Baltimore, MD	Complex, industrial - 180 MGD	Anaerobic digestion
BS-B	1-20-2015	KNSG Kent Island, MD	-Mostly residential - 1 MGD	Anaerobic digestion
BS-C	1-21-2015	Broadneck Annapolis, MD	-Mostly residential - 6 MGD	Lime stabilization
BS-D	1-21-2015	Bay Ridge Annapolis, MD	-residential - 7.68 MGD	Lime stabilization

Seven composting bins were constructed using pressure-treated lumber at College Park, MD and transferred via truck and trailer to the Wye Research and Education Center in Queenstown, MD. Bins were approximately 1.5 m^3 (approximately 4.9 ft^3) in size and were designed to hold approximately two tons of compost per bin. Bins were constructed with two swinging doors in the front for accessing material and a removable metal lid to load material. Each bin was designed to have positive pressure forced-air blown up through the bottom corner of the bin (as per IVAT protocol) to aid in maintaining proper oxygen levels for ongoing microbial activity. Blowers were attached to the bins using flexible aluminum ducting (Figure 1.3).

A B

Figure 1.3. Image of constructed composting bin with swinging doors open illustrating a biosolid as it appeared within the structure (A). Image of blower mechanisms with aluminum ducting attached air blower to bottom corner of composting bin (B). Blower was equipped with a programmable timer that turned on and off on an adjustable schedule.

In-vessel aerated and turned composting was conducted on all four BS samples and PL material. The original PL material was divided into four sub-categories of treatments; aerated (A), aerated and turned (A/T), turned (T) and piled (not composted). For the first 14 days post arrival of the aerated PL (PL-A and PL-A/T) treatments were on a two minute on, 18 minute off blowing cycle (10% blowing time). Both turned treatments were turned every day, five days per week for the first ten working days. Turning was accomplished by opening doors and dumping material on a concrete pad, then reloading. First, the contents of the bin being turned were dumped onto a concrete surface through the front doors via the excavator. The material was then mixed using a front end loader and returned to its corresponding bin. After the initial turning period (10 of 14 days), the PL bins were no longer turned for the remainder of the composting period.

Upon arrival, BS samples were augmented with a carbon amendment composed of 40% virgin wood chips, 40% ash/peanut hull mix and 20% recycled "tailings" (provided by Baltimore City Composting Facility). Biosolid compost bins were monitored daily for oxygen content and temperature. Unlike the PL turning regime, the BS bins turning process was controlled due to consequences of cold temperatures. Water was added to all compost bins throughout the process to maintain a moisture content of 40-60%. All three PL treatments (T, A/T, and A) were composted for a total of 166 days while BS's were composted for a total of 132 days (Figure 1.4).

All samples were analyzed for nutrient content prior to and post-composting to determine the effects of composting on the utility of these materials in land application. Total nutrient analysis was conducted on samples that had been preserved at 4° C by

AgroLab located in Harrington, DE. Samples were analyzed for ammonia, total nitrogen, total phosphorus, moisture and dry matter (Table 1.2). When using both BS and PL as fertilizers, total nitrogen and total phosphorus levels are typically analyzed to assess the usability of the products to aid in agricultural growth rates.

Figure 1.4. Poultry litter piled on arrival (A), in a pilot-scale IVAT composting bin (B) and being turned (C,D).

Table 1.2. Nutrient analysis for pre- and post-composted biosolids and poultry litter. Nutrient analysis was conducted by AgroLab located in Harrington, DE. All analyses are reported as percentage on a dry basis. BS = biosolid. N = nitrogen.

Sample	**Treatment**	**Ammonium**	**Total N**	**Phosphorus**	**Dry Matter**
Poultry Litter	Pre	0.33	3.79	4.16	39.18
	Piled	0.252	3.95	7.94	40.13
	Turned	0.294	3.50	5.25	53.88
	Aerated	0.341	3.76	4.7	66.88
	Turned/Aerated	0.332	3.83	5.04	59.9
BS-A	Pre	0.444	4.99	5.29	25.81
	Post	0.335	3.16	3.67	47.7
BS-B	Pre	0.501	4.69	5.1	23.57
	Post	0.32	2.90	5.62	43.9
BS-C	Pre	0.395	4.57	4.03	13.72
	Post	0.325	2.95	4.47	64.1
BS-D	Pre	0.152	3.37	4.34	18.83
	Post	0.22	2.66	4.06	81.82

All samples were tested for material maturity via a Solvita test according to manufacturer's instructions at the completion of composting. The Solvita test, developed by Woods End Research Laboratory, Mt. Vernon, Maine, measures compost maturity by

determining carbon dioxide and ammonia emissions (Brewer and Sullivan, 2008). Briefly, approximately 250 mL of the material to be assessed was placed in a cup, followed by the addition of deionized (DI) water until the material appeared "shiny" in consistency according to the saturated paste protocol. The two "paddles" provided with the test kit were then placed inside the cup and the top was sealed. The paddles change color over the course of four hours depending on the emissions of carbon dioxide and ammonia. After incubation, with the paddles in the sealed cups, the colors on the paddles were compared to standards. All treatments were also tested for pH using a portable-hand-held pH meter (Table 1.3).

Table 1.3. Solvita test and pH results for post-composted biosolid and variously treated poultry litter material. PL-T = poultry litter turned. PL-A = poultry litter aerated. PL-A/T = poultry litter turned/aerated. BS = biosolid. Data was not available for piled poultry litter. Low carbon dioxide emissions (mature compost) resulted in a color value of eight while high carbon dioxide emissions resulted in a color value of one. Low ammonia emissions (mature compost) resulted in a color value of five while high ammonia emissions resulted in a value of one.

Treatment	**Ammonia Emissions**	**Carbon Dioxide**	**pH**
BS-A	5	5.5	4.5
BS-B	5	5.5	5.1
BS-C	5	4	6.4
BS-D	5	2.5	6.4
PL-T	1	7	8.9
PL-A	2	4	8.1
PL-A/T	2	6	8.4

Sample Collection

Samples were collected from all four BS's and PL material upon delivery at the Wye Research and Education Center, put in sealed five gallon buckets (three buckets per treatment) and transferred back to College Park. Material was then homogenized within each bucket using a large screen, during which all large particles were crushed and mixed into the total mixture. After all buckets were homogenized, material was then separated and portioned into amber jars and either stored at -20° C or 4° C depending on its final purpose (moisture content analysis, LC-MS/MS, BLYES, larval toxicity tests, or adult whole fish exposures). A 100 g portion was taken from each bucket and put in 250 mL amber jars and stored at 4° C for moisture content analysis. This resulted in three replicates (one from each bucket) for moisture content analysis. One 100 g sample was also taken from each bucket and stored at -20° C for total analyte (in addition to the soluble fraction) analysis (LC-MS/MS). Three 250 g portions were placed in 500 mL amber jars and stored at -20° C for long term storage. Finally, three 250 g portions were also stored at 4° C for generation of aqueous mixtures for BLYES analysis, adult male fish exposures and larval fish exposures. These samples were not frozen to preserve the microbial community.

Upon completion of composting, samples were again collected at the Wye Research and Education Center and transported back to College Park for further processing. At this time, only one five gallon bucket was taken from each bin. Again, the material was homogenized using a large screen and large particles, such as wood chips and feathers, were removed. The samples were then stored in either one L glass jars or 2.5 gallon plastic buckets at -20 °C or 4° C depending on purpose as previously stated.

Preparing Aqueous Media

Since the water soluble contaminants within BS and PL are what is environmentally available to aquatic organisms, aqueous fractions were prepared. Moisture content was determined by measuring mass of triplicate samples before and after 24 hours of drying at 102° C. Water soluble fractions were generated by aqueous extraction of dry weight-equivalent amount of both pre- and post-composted material (Table 1.4). The nominal amount for all samples was 2.0 g/L and was prepared in 20 L carboys with dechlorinated city water that had been aged for several days prior to experiments. Carboys were shaken and allowed to sit overnight, allowing the soluble material to dissolve while larger particles settled out. The next day, carboys were decanted into 100 L aquaria without resuspension of the settled material. The samples were then diluted 4 fold by adding 60 L of dechlorinated and aged water, making the total exposure volume 80 L within the 100 L aquaria. This dilution resulted in a final concentration of 0.5 g/L for all exposures.

Pre- and post- composted material was investigated over a ten day exposure period. The aqueous mixtures of pre-composted PL was invested for estrogenic activity on arrival in December 2014. Water from the 100 L aquaria was sampled on days 0, 2, 5, 7 and 10. The four BS materials were investiged for estrogenic activity in aeuous mixutres on arrival in January 2015. Water from aquaria was sampled on days 0, 3, 6, and 10. All post-composted material (BS and PL) were examined for aqueously extractible estrogens in June of 2015 and sampled on days 0, 3, 7, and 10. Along with all pre- and post- composted material, control environments not containing BS or PL were sampled

on corresponding days. All treatments were held static (without aqueous additions or filtration) with aeration over the course of the fish exposures.

Table 1.4. Moisture content for pre- and post-composted biosolid and poultry litter samples. All samples reported in g/L. Total mass was calculated by dividing the goal dry weight (0.5 g/L) by the proportion dry weight. BS = biosolid.

Sample	Treatment	Proportion moisture	Proportion dry	Target dry weight	Total mass
Poultry Litter	Pre	0.44	0.56	0.5	71.43
	Piled	0.61	0.39	0.5	102.56
	Turned	0.48	0.52	0.5	76.92
	Aerated	0.36	0.64	0.5	62.5
	Turned/Aerated	0.44	0.56	0.5	71.43
BS-A	Pre	0.75	0.25	0.5	160
	Post	0.55	0.45	0.5	88.89
BS-B	Pre	0.70	0.20	0.5	200
	Post	0.59	0.41	0.5	97.56
BS-C	Pre	0.853	0.148	0.5	270.27
	Post	0.4	0.6	0.5	66.67
BS-D	Pre	0.778	0.222	0.5	180.18
	Post	0.23	0.77	0.5	51.95

Aqueous Sample Collection and Preparation

Two 1.0 L samples were collected from each 100 L aquaria including a control tank on sample days (previously described). Samples were then filtered (0.7 µm GFF) and pH adjusted (25% sulfuric acid) to a pH of 4.0 $\pm$ 0.2. Biosolids and PL samples were pre-filtered (2.7 µm GFF) to reduce obstruction of the final filter (0.7 µm GFF). Filtering and acidifying minimizes bacterial load and aids in sterilization for further processing. During the entirety of this project, samples were collected and immediately extracted (within 30 minutes) eliminating the need for lower pH adjustment (2.0 $\pm$ 0.2) and sample storage. Samples were then separated into two 1-L pre-cleaned bottles. Samples intended for estrogen quantification (LC-MS/MS) received 100 µl of an estrogen standard mix using a gas-tight glass 100 µL Hamilton syringe for accuracy. The estrogen standard mix consisted of known amounts deuterated labeled E1, E2 and EE2 that were used to quantify estrogen species recoveries. Samples intended for total estrogenicity (BLYES) did not receive the standard mix. The Hamilton syringe was thoroughly rinsed with a triple methanol rinse between uses to minimize contamination. The estrogen standard mix was kept at -20° C in the dark to prevent estrogen degradation.

Solid Phase Extraction

Both 1-L samples were extracted using an Oasis HLB^{TM} (hydrophilic/lipophilic balanced 500 mg LP) cartridges (Part Number: 186000115) for solid phase extraction (SPE). The media within the cartridges binds to analytes within the aqueous samples being vacuum filtered through a 12-port manifold vacuum. Suction lines were cleaned prior to and post extraction with a methanol rinse (flow rate of 5-10 mL/min) followed by

a DI rinse at the same rate for approximately three minutes per solvent. New cartridges were labeled appropriately (treatment/date/purpose) then prepared following two different procedures depending on final purpose (BLYES or LC-MS/MS). Cartridges were pre-conditioned for LC-MS/MS with 6 mL of methanol followed by a 10 mL nanopure water. The nanopure water flush was stopped while approximately 2 mL of water was left above the media surface. Cartridges intended for BLYES received 5 mL ethyl acetate, 5 mL 50:50 methanol: dichloromethane, 5 mL methanol, and two aliquots of 10 mL nanopure water (flow rate and psi same as previously described). As before, approximately 2 mL of nanopure water was left in the cartridge for extraction.

Once the cartridges were pre-conditioned, samples were extracted using the previously cleaned suction lines, with weighted ends inserted directly in sample bottles and the other ends attached to appropriately labeled cartridges associated with each sample. Using a 12-port vacuum manifold, samples were extracted through the needle valves at the flow rate of 7 mL/min (+/- 2 mL/min). Samples took approximately 3 – 4 hours to extract the entire 1 L sample. Once the samples were extracted, the cartridges were allowed to dry on the vacuum manifold for approximately one hour at maximum airflow prior to being place in tin foil and stored at -20° C until further processing.

Solid Phase Extraction Elution

Following SPE, samples were eluted to glass centrifuge tubes labeled to coordinate with previously extracted cartridges. Labeled tubes were placed in the 12-port vacuum manifold below the correct labeled HLB cartridges. For LC-MS/MS, cartridges were eluted with two 5 mL aliquots of 90/10 ethyl acetate/methanol (flow rate same as

previously described). Tubes were then evaporated to less than 1 mL in total volume using a TurboVap exhausting ultra-pure nitrogen at a temperature of 35° C. While tubes were evaporating, cartridges were washed with two 5 mL aliquots of acid wash solution (5% methanol + 2% acetic acid + 93% nanopure water) followed by two 5 mL aliquots of base wash solution (5% methanol + 2% ammonium hydroxide + 93% nanopure water). The cartridges were then dried on the vacuum manifold for at least one hour while the glass tubes were drying in the TurboVap. Evaporated glass tubes were then placed back in vacuum manifold with corresponding cartridges. Cartridges were then eluted with two 5 mL aliquots of 98/2 methanol/ammonium hydroxide followed by a final evaporation in the TurboVap until completely dry (approximately two hours). The concentrated extract was then reconstituted in 0.5 mL methanol using a calibrated glass 1 mL pipette, vortexed and transferred to a prelabeled 2 mL amber glass vial via a glass pipette. Cartridges intended for BLYES analysis were first eluted with 6 mL methanol followed by 6 mL of 50:50 methanol dichloromethane (flow rate as previously described). The tubes were then removed and placed in the TurboVap until completely evaporated. Samples were then reconstituted in 0.5 ml methanol, vortexed and transferred to 2 mL amber glass vials using a glass pipette. All samples were stored at -20° C until further processing.

Estrogen Analysis via LC-MS/MS

Natural and conjugated estrogens were quantified using a method developed and validated by Elizabeth Mullin, PhD candidate at the University at Buffalo, Chemistry Department. Estrogens were quantified using one-shot liquid chromatography tandem mass spectrometry (LC-MS/MS). Because both BS and PL have complex matrices, it was

necessary to derivatize the natural estrogens within the samples to improve detection limits using electrospray ionization (ESI) mass spectrometry. Briefly, the estrogens within the matrices were transformed into derivatives of their original compounds so they may be quantified. Derivatization was achieved using two agents, dansyl chloride and pyridine-3-sulfonyl chloride. Samples were evaporated to dryness under nitrogen gas and reconstituted in 100 µL of 0.1 M bicarbonate buffer and 100 µl of 1 mg/mL derivatization agent in acetone. Samples were mixed and then allowed to react in a 60° C water bath. Derivatized samples were then reconstituted in 0.25 mL 50:50 nanopure water:acetonitrile. Each derivatized estrogen compound was optimized using triple quadrupole mass spectrometry. Liquid chromatography was then used to achieve separation and quantify estrogen species.

Estrogenicity Analysis via In Vitro BLYES

Estrogen standards were prepared in a sterile, round bottom, 96-well plate in a dilution series using a 5% methanol mix. Standards ran were 5.0 ng/mL down to .004 ng/mL. Final growth media (95 µL) was then put in a sterile, black 96-well plate for sample dilutions. Once samples were loaded in triplicate (5 µL), the standards were added to the black well plate in the first two rows (100 µL). The yeast was then added (100 µL) to standards and all samples, making the final volume within each well 200 µL. The plate (lid on), was then placed (within plastic Ziploc bag with a moist paper towel) inside the incubator for a total time of five hours (same specifications as previously described). Once the five hour incubation was completed, the lid was removed and the plate was read on SpectraMax L plate reader.

BLYES Limitations

The BLYES method, although effective in many laboratory situations has several notable limitations. The goal with this method is to measure total estrogen analytes (E1 and E2), however what is actually being measured is total estrogenicity or estrogen activity. Previous studies have shown that plastic materials have the ability to leach and produce estrogen-like effects (Sonnenschein and Soto, 1998). For example, it was shown that bisphenol-A (BPA) (an estrogenic substance) is released from polycarbonate flasks upon autoclaving (Krishnan et al., 2013). The study also found BPA from the flasks was able to bind to the estrogen receptor within the yeast strain used in the BLYES analysis (Krishnan et al., 2013). Although measures are taken throughout sample preparation to avoid plastic leaching contamination, it is possible that estrogen agonists are being introduced to the samples being analyzed and therefore a false positive may result. Also, the complex matrices (BS and PL aqueous solutions) that are being analyzed offer their own intricate issues. These aqueous solutions have suspended particles within the water column that can potentially interfere with the ability of the estrogen analytes to bind to the estrogens receptor within the yeast strand. Suspended particles may have the ability to block potential binding sites that estrogens would otherwise bind. Although the solutions are filtered twice prior to extraction, particles may precipitate from concentrated samples after extraction. The assay is also constrained by analytical detection limits. Because the assay has natural background noise due to the yeasts bioactivity, it is only possible to quantify total estrogenicity amounts to a level of 0.2 ng/L. Total estrogenicity amounts less than 0.2 ng/L are therefore unable to be quantified. In contrast, it is also possible for

the yeast to become oversaturated with molecules that are able to bind with the estrogen receptor within the yeast. If a sample has more estrogen than the yeast is able to bind, it may result in the assay reporting less total estrogenicity than what is the real amount. If oversaturation is suspected, the sample may be diluted.

Lastly, this assay employs live yeast organisms, which have precise culturing requirements. The growth media contains a finite amount of nutrients for the yeast, so allowing longer than necessary incubation may result in yeast death. Dead yeast may still contribute to optical density observations, however dead yeast cells will have inactive estrogen receptors therefore estrogens will not bind as expected. Lastly, yeast media contains nutrients that are not only suited for yeast growth but also bacterial growth. For this reason, this assay is easily contaminated by naturally occurring bacteria within a laboratory setting.

Estrogenicity Investigation via In Vivo Adult Male Fish Model

Test Species

Male fathead minnows (*Pimephales promelas)* are one of the most commonly used test species for investigating endocrine disruption effects (Eidem et al., 2006). The Organization for Economic Co-operation and Development (OECD) oversees that endocrine disrupting chemicals being investigated are relevant and transferable between laboratories. Currently, fathead minnows are included in the OECD's list of appropriate organisms to test for endocrine disruption. It is well established that male fathead minnows produce Vtg as a response to estrogen exposure (Hutchinson, T.H., et al., 2006,

Yonkos et al., 2010). Vitellogenin is now commonly used as a bioindicator of exposure to natural and synthetic estrogens as well as estrogen mimicking compounds (Eidem, et al., 2006). Fathead minnows are easily cultured within a laboratory setting and are sexually dimorphic, making them an ideal test species for this study. The male adult fathead minnows used for this study were approximately 5-7 months in age at start of experiment and were provided by Aquatic BioSystems, Inc, Fort Collin CO. Fish consistently arrived in good condition with lengths ranging from 49-87 mm.

Fish Exposures

Groups of adult male fathead minnows (n~10) were exposed in all-glass 100 L aquaria with static aeration for a 10 day exposure. During exposures, environments were monitored daily for temperature, dissolved oxygen, pH and ammonia levels (mg/L). Temperature remained at 22 $\pm$ 1 ° C, pH 7.5 $\pm$ 1 and dissolved oxygen was greater than 5 mg/L throughout all exposures. Ammonia content varied depending on treatment, but consistently increased in levels throughout the exposures. Ammonia content never exceeded 7.4 mg/L by day ten of any exposure. Fish were fed TetraMax^{TM} once daily.

Plasma Collection

Plasma was collected on day ten according to methods previously described by Ankley et al (2001). Adult male fish were anesthetized in 100 mg/L buffered tricaine methanesulfonate (MS-222, Sigma Chemical, St. Louis, MO). Blood was collected from anesthetized fish through an incision into caudal sinus into 70 µl heparinized microhematocrit tubes. Post blood collection, fish were decapitated for euthanasia.

Microhematocrit tubes containing plasma were left on ice for no longer than 15 minutes before being centrifuged at 3,000 x g for 5 minutes. The plasma was then discharged into a pre heparinized and aprotinated 1.5 mL cryovial. The cryovials were then "snap" frozen using liquid nitrogen and stored at -40° C until analysis.

Vitellogenin Induction-ELISA

Briefly, fathead minnow plasma was introduced to a 96 well high binding plate and incubated for approximately 18 hours. During this time, Vtg within the plasma sample bound to the individual wells within the plate. After the incubation period, a primary wash was completed with a blocking buffer followed by the introduction of a monoclonal anti-carp antibody which cross reacts with fathead minnow Vtg. After the plate was washed for a second time, a secondary antibody (goat anti-mouse antiglobulin conjugated with horseradish peroxidase) was added, which binds to the primary antibody. Another wash was followed by the addition of a streptavidin-alkaline phosphatase (S-AP). A final wash was followed by the developer (100x 4-nitrophenyl phosphate at 100 mg/mL) which changed in color intensity as a response to the presence of Vtg. The plate was then read on a SpectraMax M2 plate reader and Vtg within the plasma is quantified.

Toxicity Tests

A toxicity test was preformed to determine the chronic toxicity within pre-and post- composted BS and PL. Newly hatched larval fathead minnows were used in a seven-day static renewal toxicity test for short-term chronic toxicity using a dilution series (USEPA 2002). Exposure concentrations were determined based on preliminary

ammonia results and did not exceed 6.4 g/L (dry weight) (Table 1.5). A 100% concentration was prepared based on dry weight equivalents from previously stored (at 4° C) samples and filtered to 0.7 µm. A dilution series was prepared daily, renewing approximately 90% of exposure water within each treatment (four replicates per treatment each containing ~250 mL of aqueous solution). Treatments were monitored daily for fish mortalities and water chemistry (pH, ammonia, temperature, and dissolved oxygen). Fish in each treatment chamber were fed ~200 µL of a concentrated suspension of newly hatched (less than 24 hours old) brine shrimp (*Artemia)* twice a day. At the completion of the test, all dead larval fish were discarded and the surviving fish from each replicate were euthanized in MS-222. Euthanized fish were placed in pre-weighed foil and dried at 75° C for a minimum of 24 hours to determine dry weights for each replicate of each treatment. Survival was analyzed as the endpoint.

Table 1.5. Toxicity bioassay concentrations for pre- and post-composted aqueous sample extracts for biosolids and poultry litter. Concentrations were determined based on preliminary ammonia analysis conducted on all material upon arrival at College Park, Maryland. All values are reported in g/L with 0.00 column indicating control environments. Each concentration consisted of four replicates.

Treatment	0.00	0.11	0.2	0.35	0.63	1.12	2.00	3.6	6.4
BS-A Pre	X	X	X	X	X	X	X	X	
BS-A Post	X					X	X	X	
BS-B Pre	X	X	X	X	X	X	X	X	X
BS-B Post	X					X	X	X	X
BS-C Pre	X	X	X	X	X	X	X		
BS-C Post	X					X	X	X	
BS-D Pre	X	X	X	X	X	X	X	X	X
BS-D Post	X					X	X	X	X
PL-Pre	X				X	X	X	X	X
PL-A	X				X	X	X	X	X
PL-T	X				X	X	X	X	X
PL-A/T	X				X	X	X	X	X
PL-Pile	X				X	X	X	X	X

Toxicity Test Deviations

Typically, chronic toxicity bioassays are run static without aeration for a total of seven days. However, because of the complex nature of these aqueous samples, dissolved oxygen in several of the more concentrated treatments (both pre- and post- composted BS and PL) dropped below 3.0 ppm. Aeration was introduced to treatments below 3.0 ppm throughout the exposures to prevent mortalities from suffocation due to lack of oxygen. In addition, a standard toxicity assay typically consists of extracting a sample directly from the environmental source and creating a dilution series. Because that was not possible with this model, a sample had to be generated based on preliminary ammonia results and creating appropriate concentrations for each sample (pre- and post-composted). Also, because pre- and post-composted samples were available at different times, all toxicity tests were not run in one test, but run as they became available and time permitted.

Glassware Preparation

All glassware received careful cleaning to ensure lingering detergents, impurities and estrogens had been washed-out. First glassware was cleaned with soap (Decon Labs-Contrad liquid detergent) and water. Following a triple DI rinse, glassware was then rinsed with 10% nitric acid, triple DI rinsed and rinsed with methanol. Glassware was then given a final triple DI rinsed and allowed to dry. All glassware that was able to be baked at 300° F for three hours was, while more fragile glassware was not. In addition to this, all glassware intended for BLYES analysis was autoclaved.

Statistical Analysis

Kruskal-Wallis One Way analysis of variance (ANOVA) on Ranks followed by Dunn's All Pairwise Multiple Comparison was conducted on both total estrogenicity (BLYES) results as well as mean plasma vitellogenin induction concentrations (ELISA).

RESULTS

IVAT Compost Process

Valuable compounds within PL (total nitrogen (N) and phosphorous (P)) for soil conditioning were analyzed in materials prior to and after composting. Total N concentrations were largely unchanged during the composting process, despite significant material volume loss (≥ 20%). Total P remained relatively constant with composting (total increases/decreases of < 10%) with the exception of the piled treatment, which increased > 60%. Ammonium levels were also comparatively unchanged in pre- and post-composted material (Table 1.2).

Moisture content is an important aspect to composting, which aids in facilitating the chemical reactions and nutrient transport required for the microbial growth and activity. All BS samples had a decrease in moisture content from pre- to post-composted material despite adding water throughout the process, which is expected with any composting process (Table 1.4). In the case of BS-D, the loss of moistures was the most substantial. On arrival, PL moisture content was 44%. During composting, PL-A showed moderated moisture loss due to evaporation. The non-aerated bin (PL-T) and the aerated and turned bin (PL-A/T) remained constant with a final moisture content value of 48%

and 44% (respectively). This is on the low end of acceptable beginning moisture content. Typically, an initial moisture content of 50% to 60% is desirable. The piled treatment remained uncovered throughout the process, which resulted in an increase in moisture content (60%) due to precipitation (Table 1.4).

Another important measure of compost activity is temperature. Temperatures in all BS and PL composting bins were measured at approximately a 50 cm depth inside the material. Biosolid samples A and B reached their peak temperature of approximately 80.0° C at around day 20, with sharp decreases thereafter until day 30, when insulation and heating mechanisms were employed. Once the bins were heated, temperatures remained between 25° C and 50.0° C for the remainder of the process. Biosolids C and D did not have as large of an initial increase in temperature, only reaching 32.0° C and 25.0° C (respectively) at day 20. Biosolids C and D then steadily increased to 55° C and 65° C (respectively) until approximately day 60, where they begin to show slightly erratic temperature changes, but with an overall decrease until composting completion (Figure 1.5).

On arrival, PL had a temperature of 26.7 °C. In the piled treatment, this increased to a maximum of 37.2 °C within one week before dropping over the following month to a stable temperature of 25.6 °C. Aeration appeared to initially assist composting bins in increasing temperatures. The aerated bin (PL-A), reached 55.6 °C at day 3, with its peak temperature of 75 °C at day 7. The temperature then decreased for the remainder of the initial two week period. The turned, without aeration bin, was slower to reach a maximum temperature of 52.3 °C at day 14. However, it was able to maintain a higher temperature for a longer period of time with a temperature of 21.1 °C at day 40. Poultry

litter A/T reached 55.6 °C at day 3 and a maximum temperature of 67.8 °C at day 5. Both PL-A and PL-A/T dropped significantly over the initial 40 days to < 7.5 °C (Figure 1.6). Overall, the temperatures indicate the composting process was incomplete with microbial activity peaking early and then quickly dissipating in the aeration treatments.

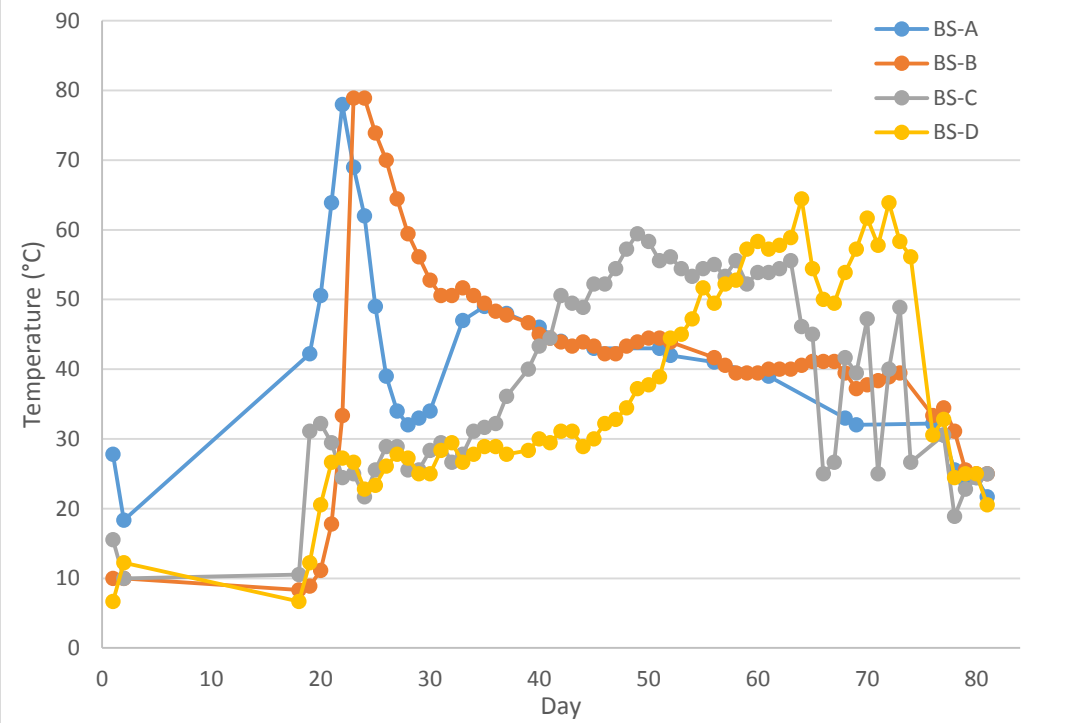

Figure 1.5. Temperatures (°C) over 80 days of composting for all four biosolid samples. From days 0 to 20, temperatures were measured by inserting a thermometer approximately 50 cm within the compost piles. After day 20, temperatures were automatically recorded via a daily monitoring device.

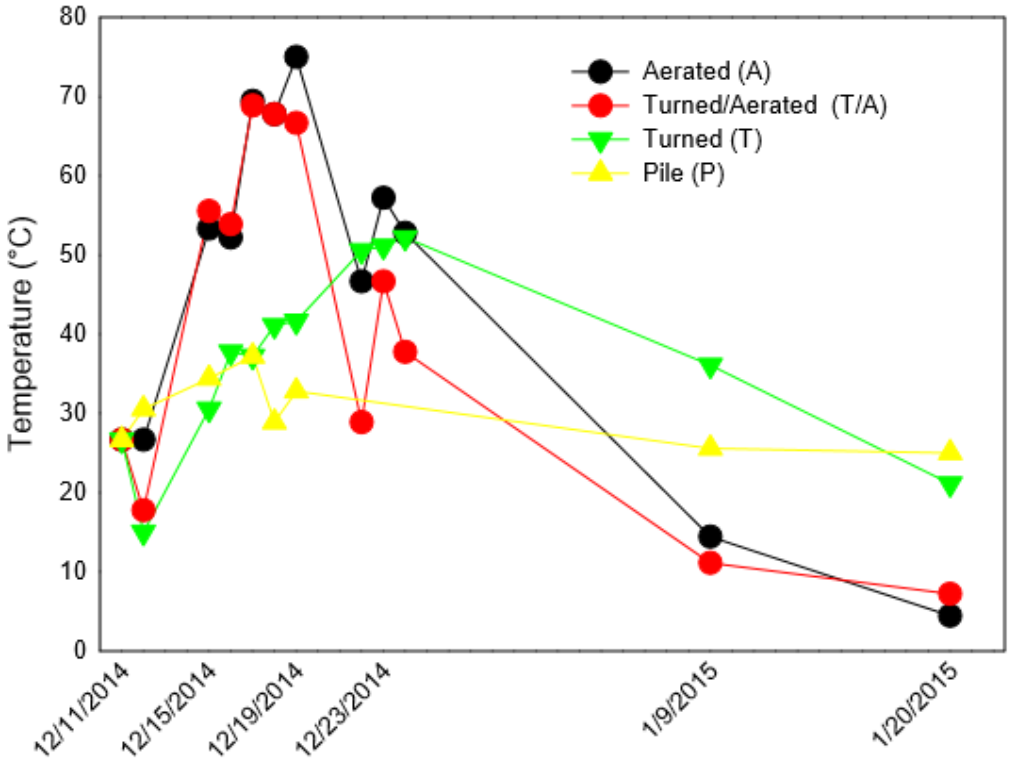

Figure 1.6. Temperatures (°C) over 80 days of composting for all poultry litter treatments. Temperatures were measured by inserting a thermometer approximately 50 cm within the compost piles.

Solvita Test

Ammonia values for Solvita tests indicate microbial efforts to consume material in the presence of nitrogen. The Solvita test indicated ammonia emissions for all BS material post-composted had a numerical value of 5 (on a scale of 1-5), indicating low emissions and mature compost (Table 1.3). Carbon dioxide emissions for BS material ranged from 2.5 to 5.5, with none reaching the goal numerical value of 8 (indicating

mature compost). This may be a result of a less than optimal composting process. Ammonia emissions for post-composted PL treatments ranged from a value of 1 to 2, indicating relatively high levels of ammonia emissions and immature compost. In contrast, post-composted PL carbon dioxide emissions results ranged from 4 in the aerated treatment to 7 in the turned treatment. With a value of 8 being low carbon dioxide, the composting process did appear to decrease carbon dioxide levels for PL. Data is not available for the piled treatment.

Estrogen/Estrogenicity Analysis of Aqueous Biosolid Mixtures

All results presented were analyzed on aqueous samples and therefore represent the soluble components of both BS and PL material. This soluble material is considered to demonstrate what will come off after land application and runoff events.

Aqueous Estrogenicity Analysis -BLYES

Based on an average standard curve, the limit of quantification (LOQ) for BLYES analysis was determined to be 0.4 ng/L while the detection limit (DL) was 0.2 ng/L. Any values that were found to be less than the DL (0.2 ng/L) were recorded as one half the DL (0.1 ng/L). All samples were assessed without further dilution than what already occurs according to the BLYES protocol. All aqueous solution analysis was conducted on a concentration of 2.0 g/L of BS or PL mixtures.

The aqueous solution of BS-A prior to composting was the most estrogenic according to BLYES analysis, climbing to a peak concentration of 5.5 ng/L on day six before dropping to 3.2 ng/L on day ten (Figure 1.7). This material was clearly higher in

estrogenicity before composting than after when estrogenicity never exceeded 1.0 ng/L over the 10-d exposure.

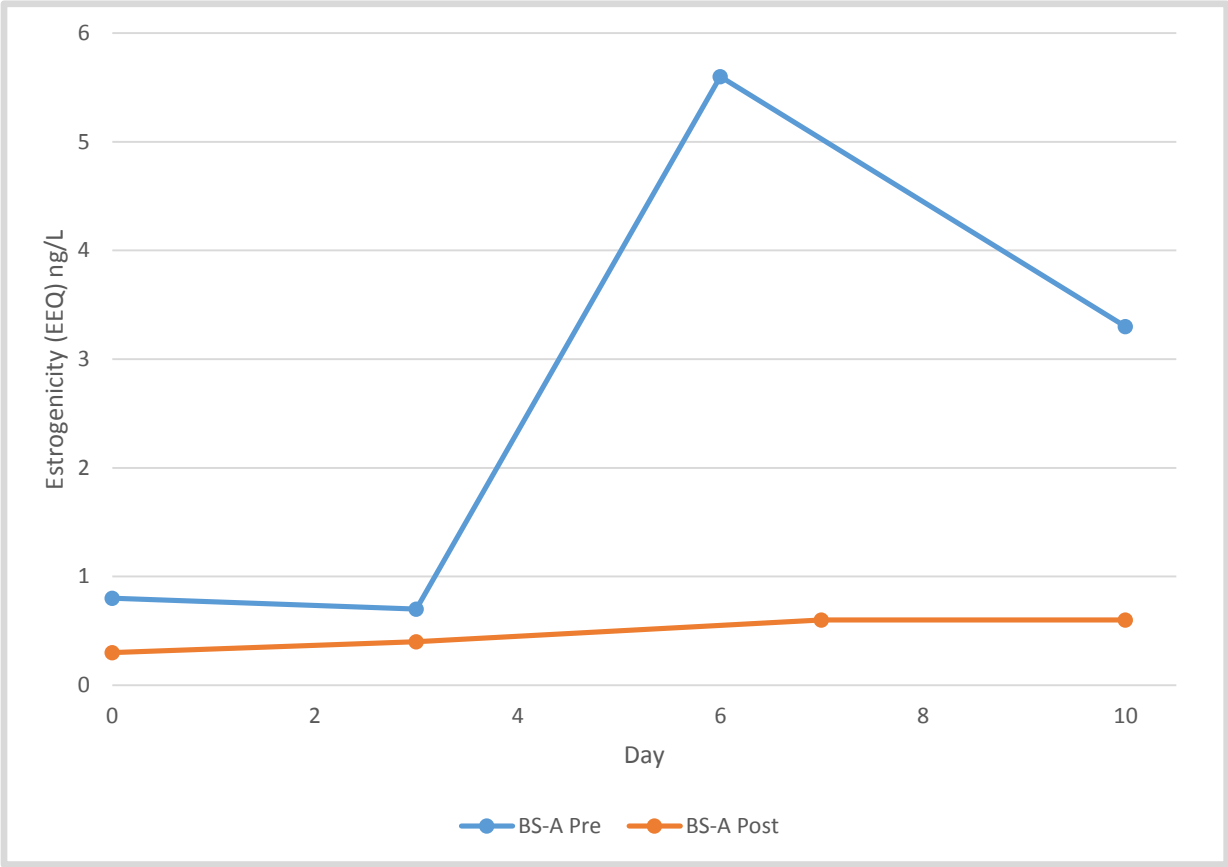

Figure 1.7. Estrogenicity over 10-d exposure period analyzed via BLYES for pre- and post- composted BS-A (estrogenicity values reported in 17β-estradiol (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L).

Biosolid-B appeared to have a slight decrease in pre- composted estrogenicity between days zero and three while post-composted estrogenicity increased over the ten day exposure period, although never exceeding 0.4 ng/L (Figure 1.8).

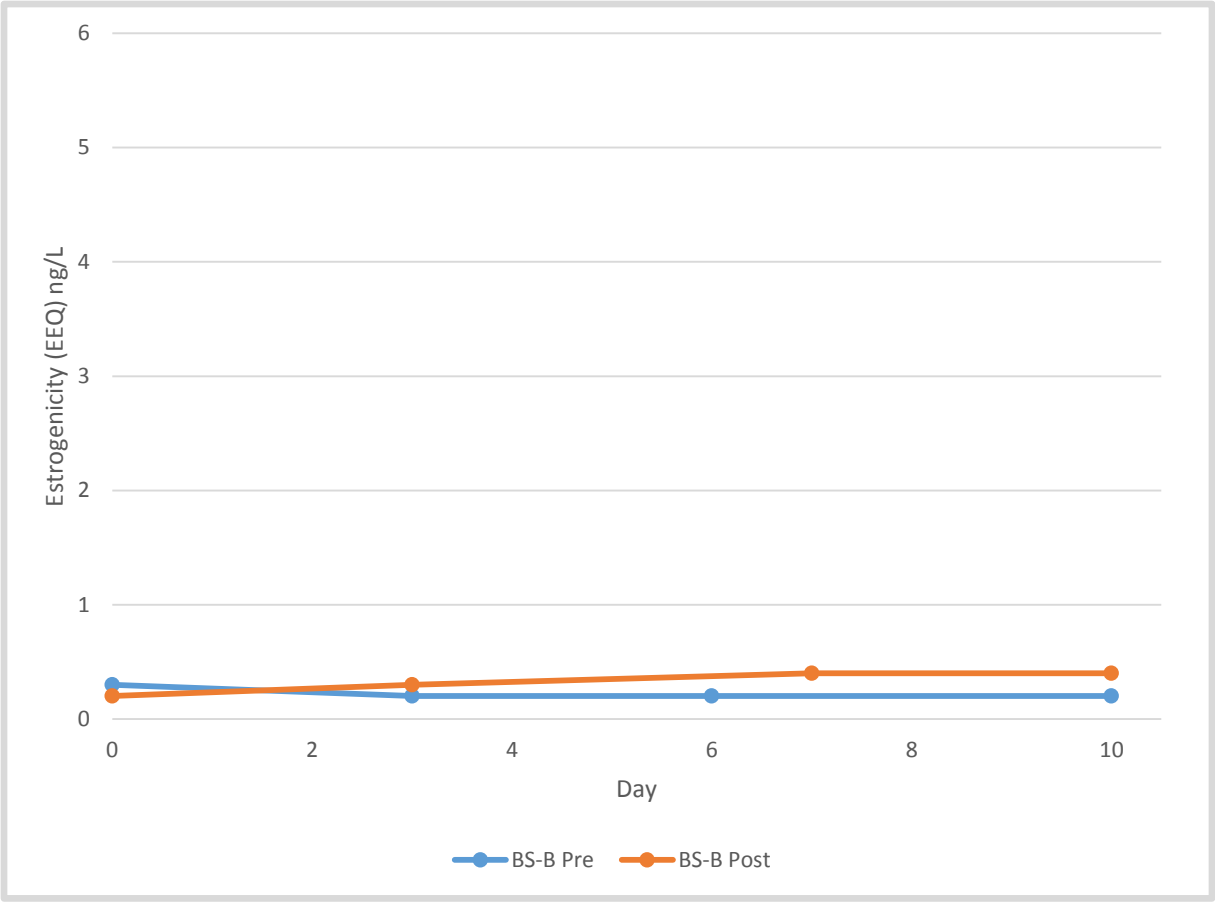

Figure 1.8. Estrogenicity over 10-d exposure period analyzed via BLYES for pre- and post- composted BS-B (estrogenicity values reported in 17β-estradiol (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L).

At day ten, post-composted material was higher in estrogenicity than pre-composted material. However, because estrogenicity in aqueous mixtures of pre-composted BS-B never exceeded the LOQ at any time, apparent trends cannot be stated with confidence. Both pre-composted and post-composted BS-C showed similar overall trends, increasing throughout the exposure but neither exceeding 0.6 ng/L (Figure 1.9). Analysis of pre-composted BS-D indicated a peak estrogenicity of 2.1 ng/L on day 6.

Post-composted BS-D remained below 0.5 ng/L throughout the ten day exposure (Figure 1.10).

Biosolid-A had the highest estrogenicity prior to composting (10 day mean of 2.6 ng/L; peak 5.5 ng/L) followed by BS-D (mean of 1.1 ng/L; peak 2.1 ng/L). BS-C (mean of 0.4 ng/L; peak 0.6 ng/L) and BS-B (mean 0.2 ng/L; peak 0.4 ng/L. After composting, estrogenicity in BS-A and BS-D averaged 0.5 ng/L and 0.4 ng/L, respectively. These represent compost-related reductions in estrogenic activity of >80% and 64% in BS-A and BS-D, respectively. Estrogenicity in BS-B was detectable but below quantification limits on arrival and showed little evidence of change from composting. Estrogenicity in BS-C was slightly higher, but also showed little evidence of change from composting (Figure 1.11).

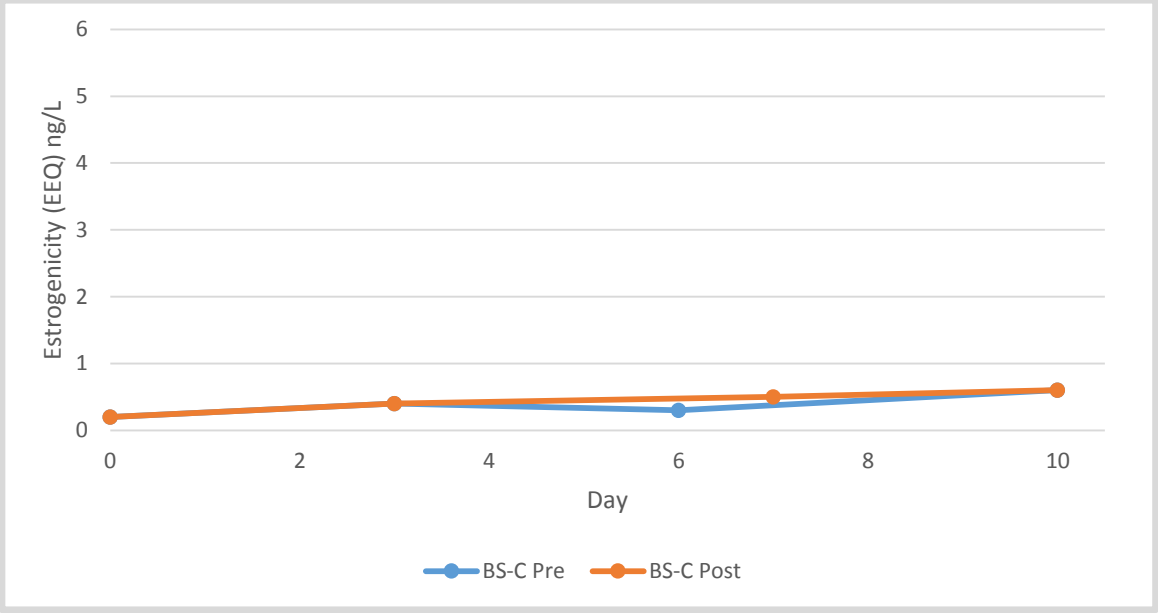

Figure 1.9. Estrogenicity over 10-d exposure period analyzed via BLYES for pre- and post- composted BS-C (estrogenicity values reported in 17β-estradiol (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L).

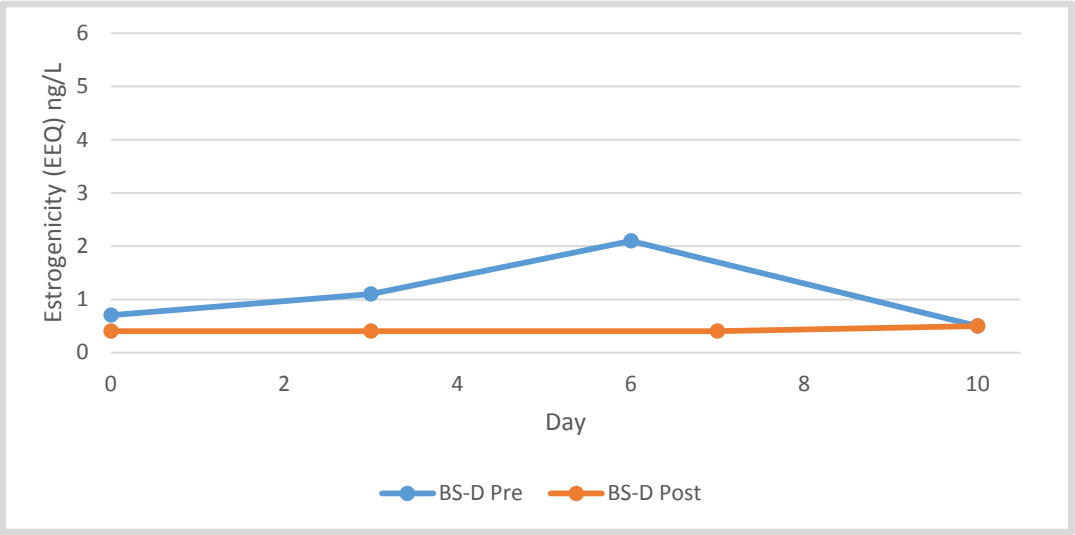

Figure 1.10. Estrogenicity over 10-d exposure period analyzed via BLYES for pre- and post- composted BS-D (estrogenicity values reported in 17β-estradiol (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L).

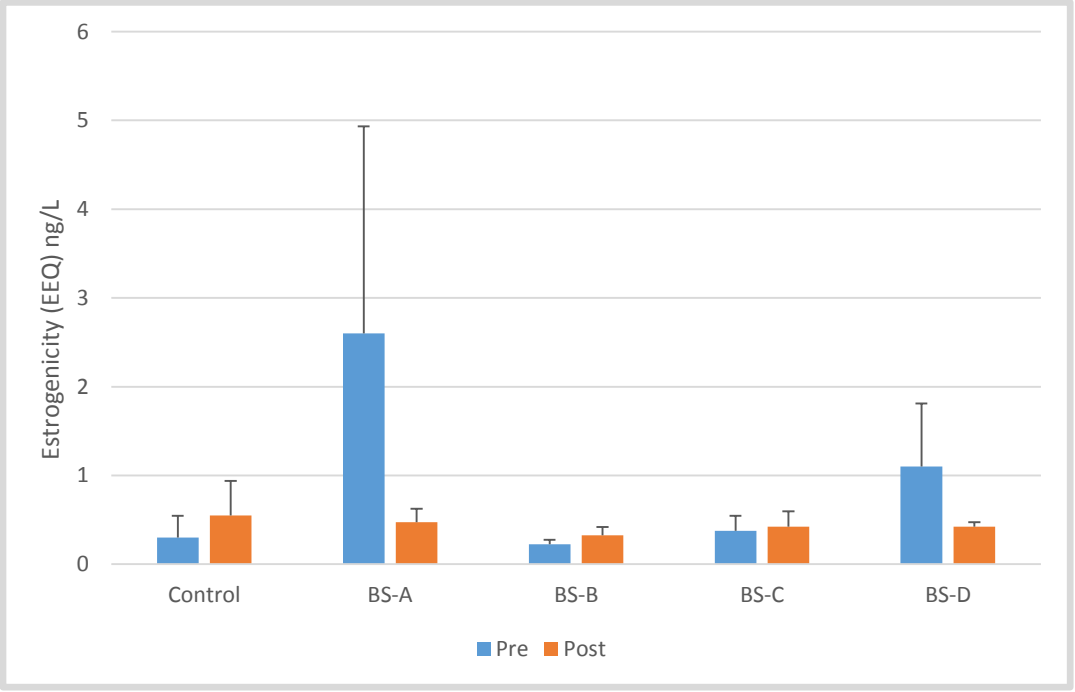

Figure 1.11. Mean estrogenicity over 10-d static exposure analyzed via BLYES for pre- and post- composted biosolid samples (estrogenicity values reported 17β- estradiol equivalents (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L). Error bars indicate standard deviation.

Vitellogenin Induction-ELISA

The LOQ for the Vtg ELISA analysis was determined to be 16 ng/mL based on an average standard curve. The DL was determined to be 8 ng/mL, with any values less than 8 ng/mL being reported as half the detection limit (4 ng/mL). All samples were analyzed at a dilution of 1:1000. If samples needed verification, analysis was replicated. In this situation, if analysis was verified, the initial values were reported because of possible freeze/ thaw degradation of the Vtg within the plasma samples.

All fish from control exposures as well as pre- and post-composted BS-C had Vtg levels below the DL. Several fish within pre-composted BS-A, BS-B and BS-D treatments had elevated Vtg levels (3/12, 2/12, and 1/12 fish respectively) with induction most apparent in BS-A (Figure 1.12). Only one fish exposed to any of these materials post-composted had detectable Vtg, this from the BS-D treatment. Induction was also reported as a proportion (%) of fish expressing below detect (≤ 8.0 ng/mL), medium (8-25 ng/mL) or high (≥ 25 ng/mL) plasma Vtg concentrations (Figure 1.13). High amounts of Vtg was considered significant Vtg induction when compared to low or medium induction values.

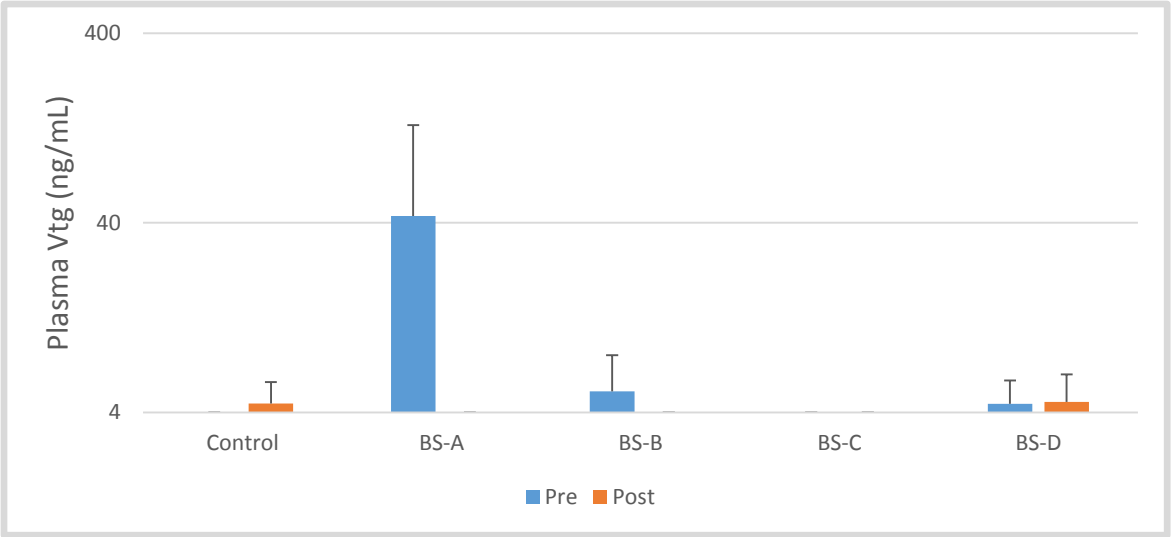

Figure 1.12. Mean plasma vitellogenin (Vtg) concentration of mature male fathead minnows (*Pimephales promelas)* following 10-d static exposures to aqueous fractions of pre- and post-composted biosolid samples. All values below the detection limit of 8 ng/mL were reported as 4 ng/mL. Error bars indicate standard deviation.

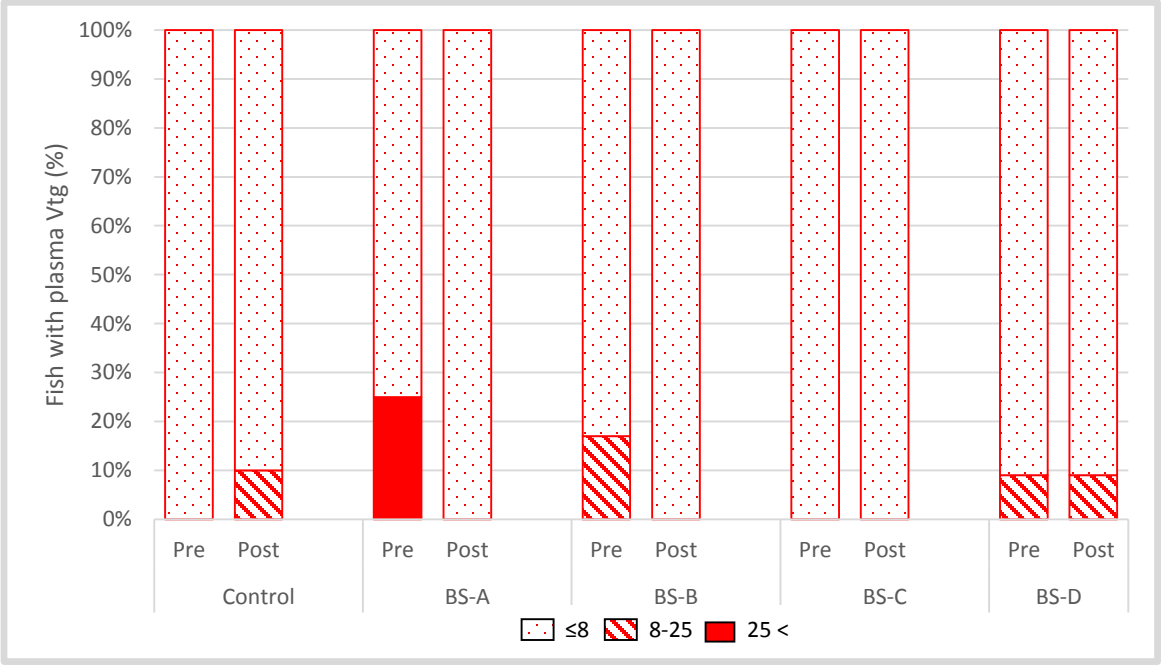

Figure 1.13. Proportion (%) of mature male fathead minnows (*Pimephales promelas)* expressing below detect (≤ 8 ng/mL) medium (8-25 ng/mL) and high (> 25 ng/mL) plasma vitellogenin (Vtg) concentration following a 10-d static exposures to aqueous fractions of pre- and post-composted biosolid samples.

Aqueous Estrogen Analysis-LC-MS/MS

Due to the complex matrix of the aqueous samples there were significant complications in quantifying both E2 and E1. Percent recoveries for pre-composted samples were widely variable, ranging from 1% to 65%. Biosolid-D had consistently low recovery rates in both pre- and post-composted samples ranging from 2% to 6% in pre-composted and 14% to 38% in post-composted material. Because recovery rates for all samples were so variable, all data was reported without incorporating percent recoveries. For all pre-composted BS samples, E2 was consistently not detected. All post-composted BS samples were below the LOQ for E2.

The LOQ for E1 was 1.7 ng/L, any values below this were reported as 1.0 ng/L. Pre- composted BS-A contained quantifiable E1, reaching its peak at day six with a value of 93 ng/L. Post-composted BS-A had consistently low levels of E2, flirting with LOQ throughout the entire exposure period (Figure 1.14). Pre- and post-composted BS-B did not have significant levels of E1 (Figure 1.15). Biosolids C and BS-D did not show any significant trends, however both did have E1 concentrations of 10 ng/L at various time points throughout the aqueous collection for both pre- and post-composted material (Figures 1.16, 17). Trends E1 (LC-MS/MS) data agreed well with estrogenicity results for BS-A, which had significant aqueously extractible estrogens within the material. Biosolid-A was the only sample to achieve E1 levels above 10.0 ng/L at any point throughout the 10-d exposure, reaching a level of 93 ng/L at day six in pre-composted material. Post-composted BS-A material never rose above 10 ng/L, indicating that composting may have been effective in lowering total E1 levels.

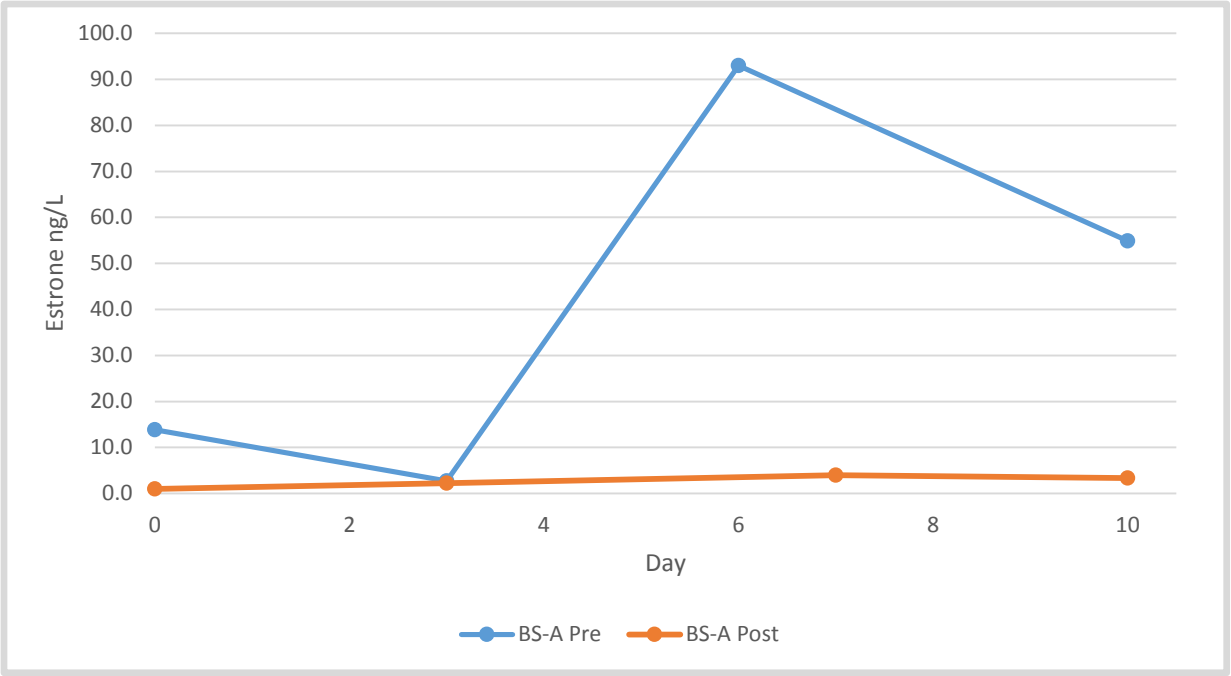

Figure 1.14. Estrone over 10-d exposure period analyzed via LC-MS/MS for pre- and post- composted BS-A (estrone values reported in ng/L; values below the detection limit of 1.7 ng/L were reported as 1.0 ng/L).

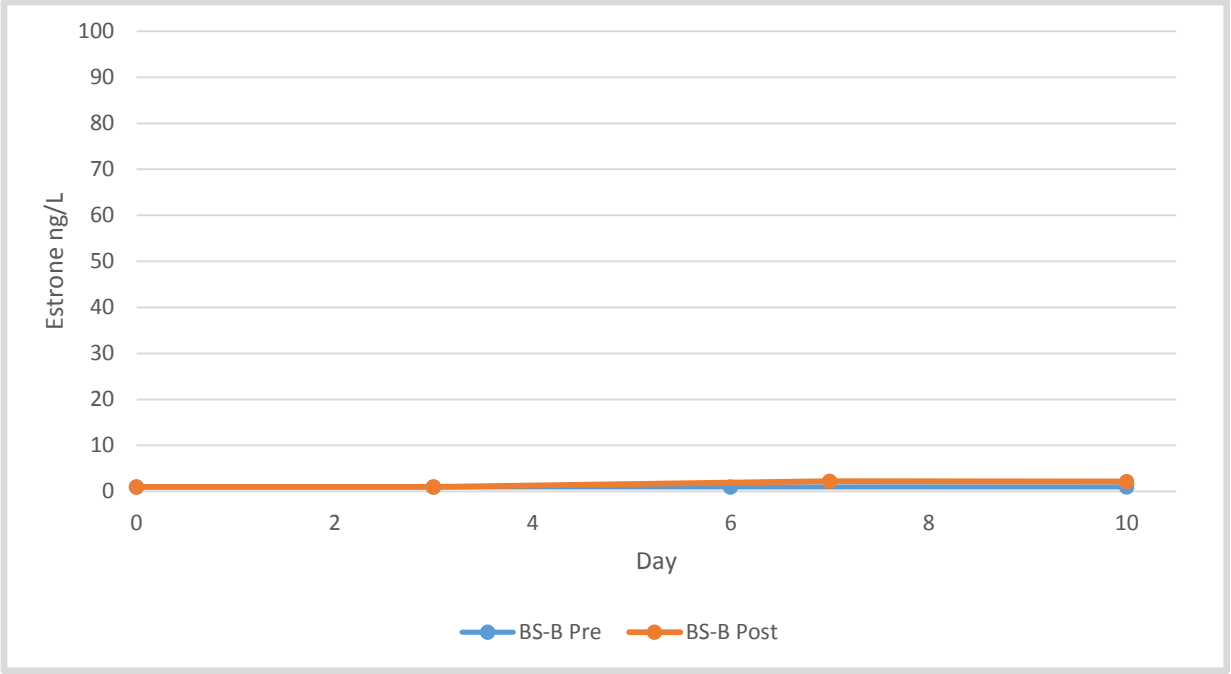

Figure 1.15. Estrone over 10-d exposure period analyzed via LC-MS/MS for pre- and post- composted BS-B (estrone values reported in ng/L; values below the detection limit of 1.7 ng/L were reported as 1.0 ng/L).

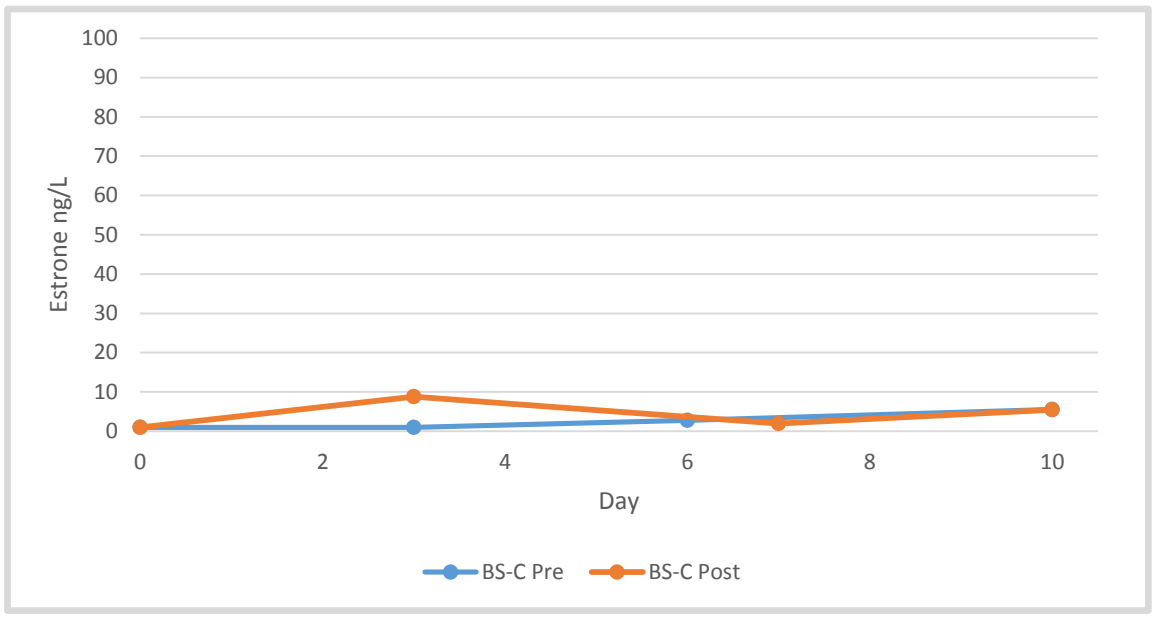

Figure 1.16. Estrone over 10-d exposure period analyzed via LC-MS/MS for pre- and post- composted BS-C (estrone values reported in ng/L; values below the detection limit of 1.7 ng/L were reported as 1.0 ng/L).

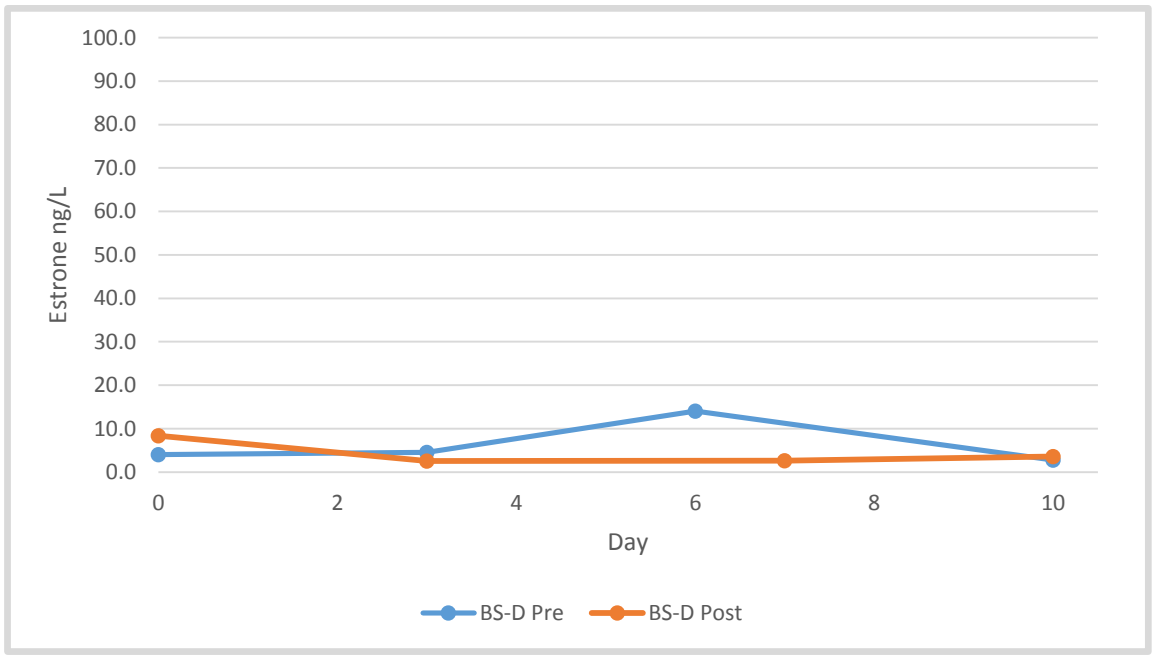

Figure 1.17. Estrone over 10-d exposure period analyzed via LC-MS/MS for pre- and post- composted BS-D (estrone values reported in ng/L; values below the detection limit of 1.7 ng/L were reported as 1.0 ng/L).

Poultry Litter

Estrogen/Estrogenicity Analysis of Aqueous Poultry Litter Mixtures

As stated previously, all results are based on the analysis of the water soluble fractions of both BS and PL material. This was meant to represent the aqueous fractions that are environmentally relevant post a runoff event.

Aqueous Estrogenicity Analysis-BLYES

Detection and quantification limits were established as previously described. Estrogenicity in PL prior to any treatment remained between 1.0 ng/L and 2.0 ng/L from days 0 to 7 before increasing to 4.8 ng/L on day ten (Figure 1.18). Poultry litter from all other treatment groups (3 compost variants and the pile) showed a general trend of increasing until day six, then decreasing by day ten. Mean estrogenicity in pre-composted PL and post-composted material plus the piled treatment are given in Figure 1.19.

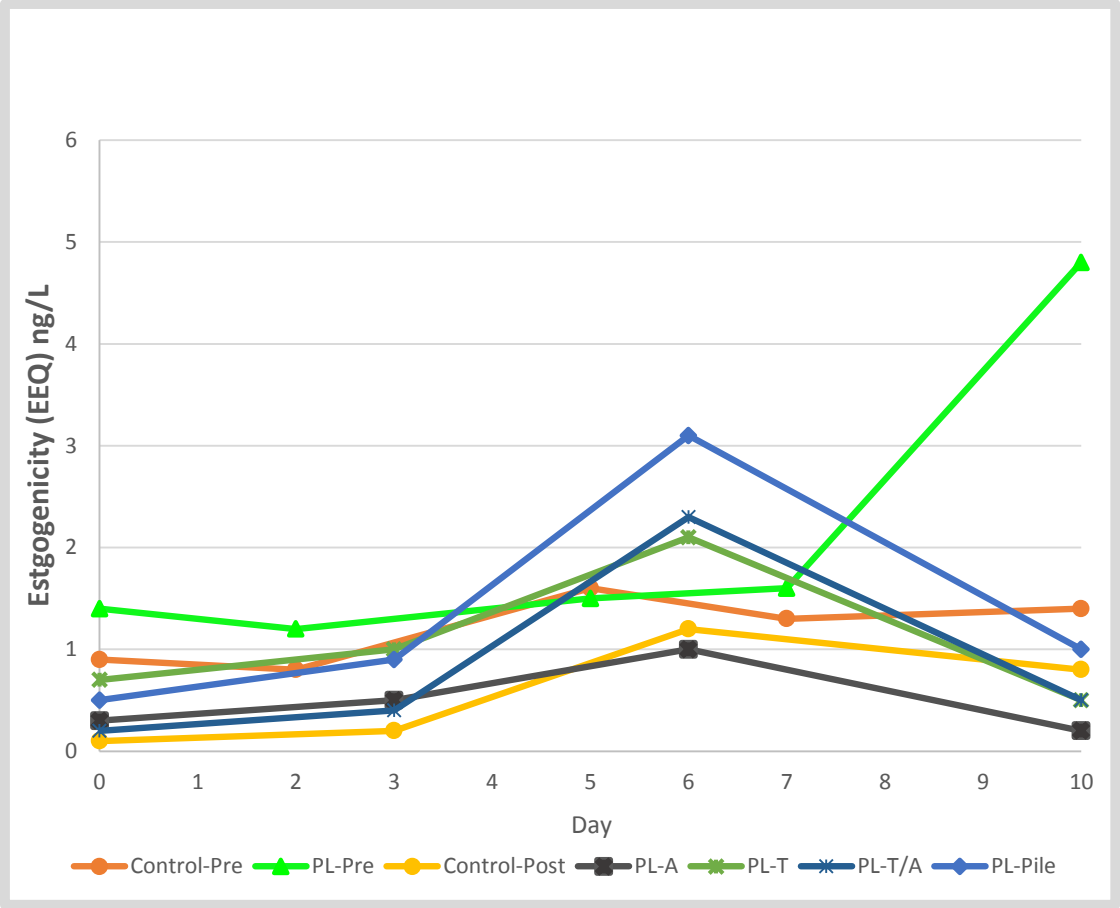

Figure 1.18. Estrogenicity following 10-d static exposures analyzed via BLYES for aqueous mixtures of poultry litter prior to and after various composting treatments: aerated (A), turned (T), aerated and turned (A/T), piled (P) (total estrogenicity values reported in 17β-estradiol equivalents (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L).

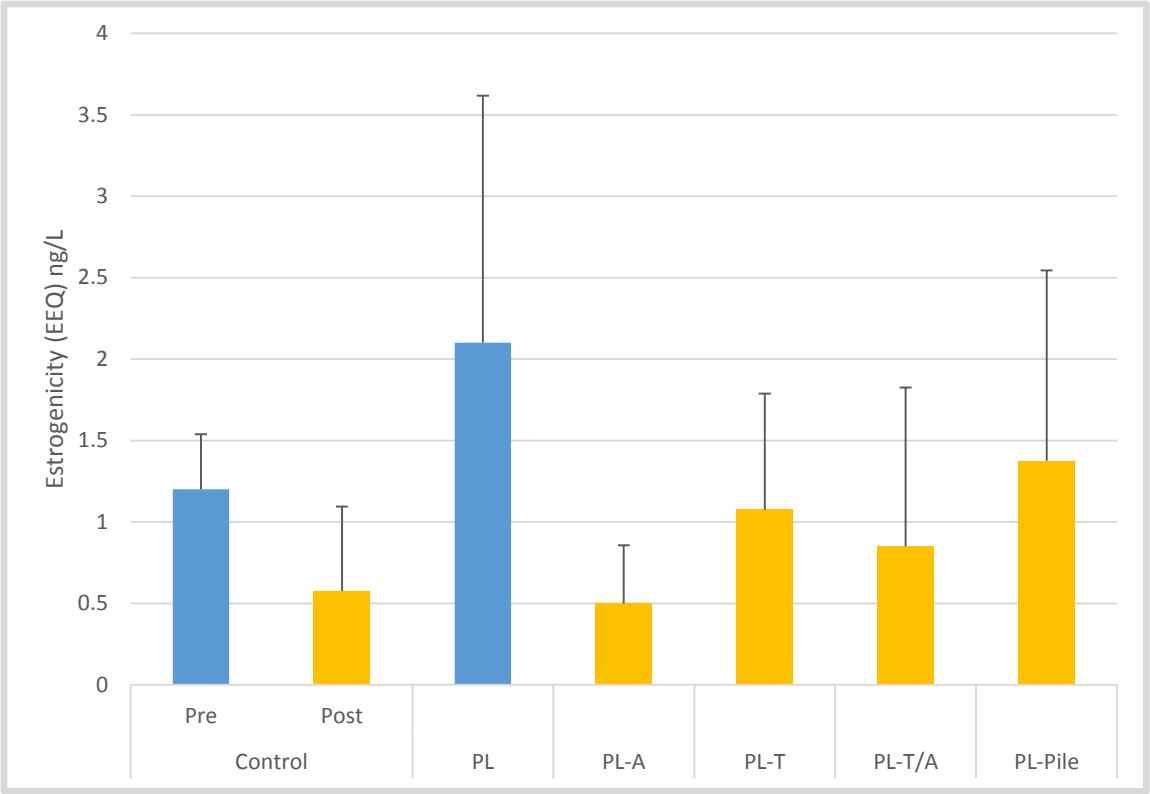

Figure 1.19. Mean estrogenicity following 10-d static exposures analyzed via BLYES for aqueous mixtures of poultry litter prior to and after various composting treatments: aerated (A), turned (T), aerated and turned (A/T), piled (P) (total estrogenicity values reported in 17β-estradiol equivalents (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L).

Vitellogenin Induction-ELISA

All fish within the pre-composted control treatment had no detectable levels of Vtg. Five out of 12 fish in the pre- composted PL treatment had significant Vtg induction, with the average of those induced being 110 ng/mL. The remaining seven fish were not Vtg induced. The overall average for Vtg induction in the pre-composted PL group was 48 ng/mL (those below detect reported as 4 mg/mL) (Figure 1.20).

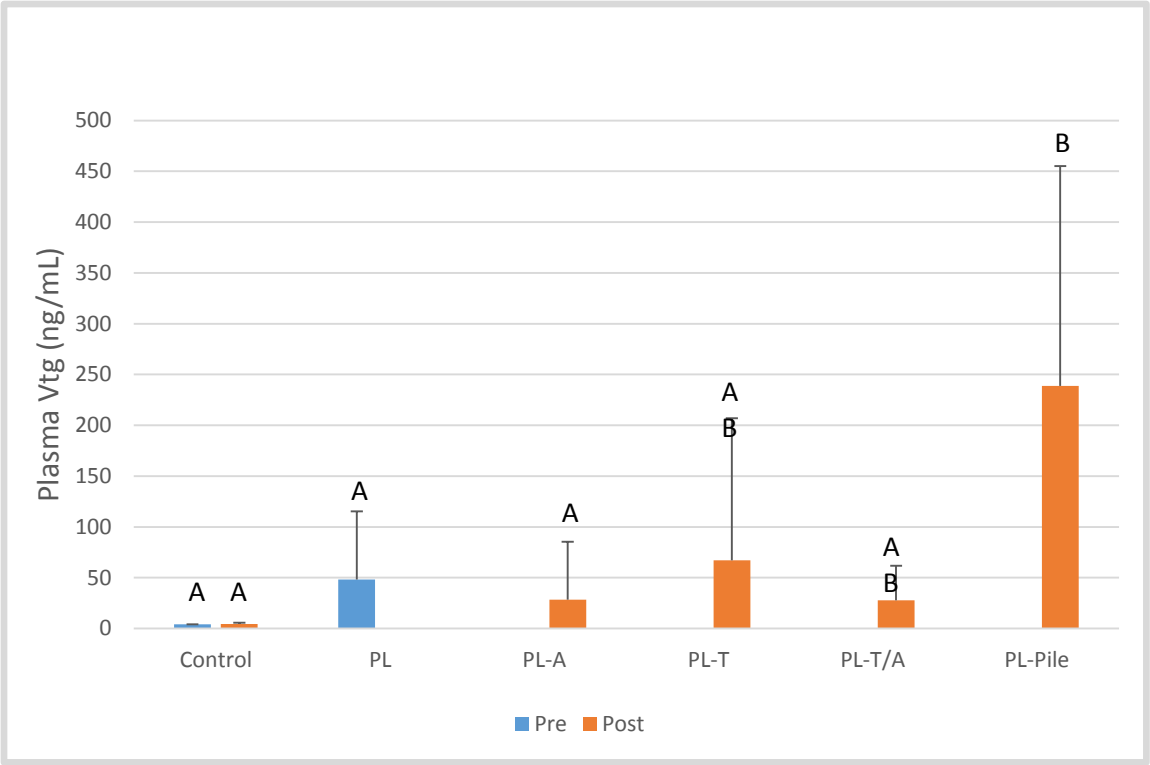

Figure 1.20. Mean plasma vitellogenin (Vtg) concentration of mature male fathead minnows (*Pimephales promelas)* following 10- day static exposures to aqueous mixtures of poultry litter prior to and after various composting treatments: aerated (A), turned (T), aerated and turned (A/T), piled (P). Columns labeled with the same letter were found to not differ significantly (Kruskal-Wallis One Way ANOVA on Ranks followed by Dunn's All Pairwise Multiple Comparison; $P<0.05$). Error bars indicate standard deviation.

The post composted PL control environment was the same as the BS post composted control group. Induction was also reported as a proportion (%) of fish expressing low (≤ 8 ng/mL), medium (8-25 ng/mL) or high (> 25 ng/mL) Vtg plasma concentrations (Figure 1.21).

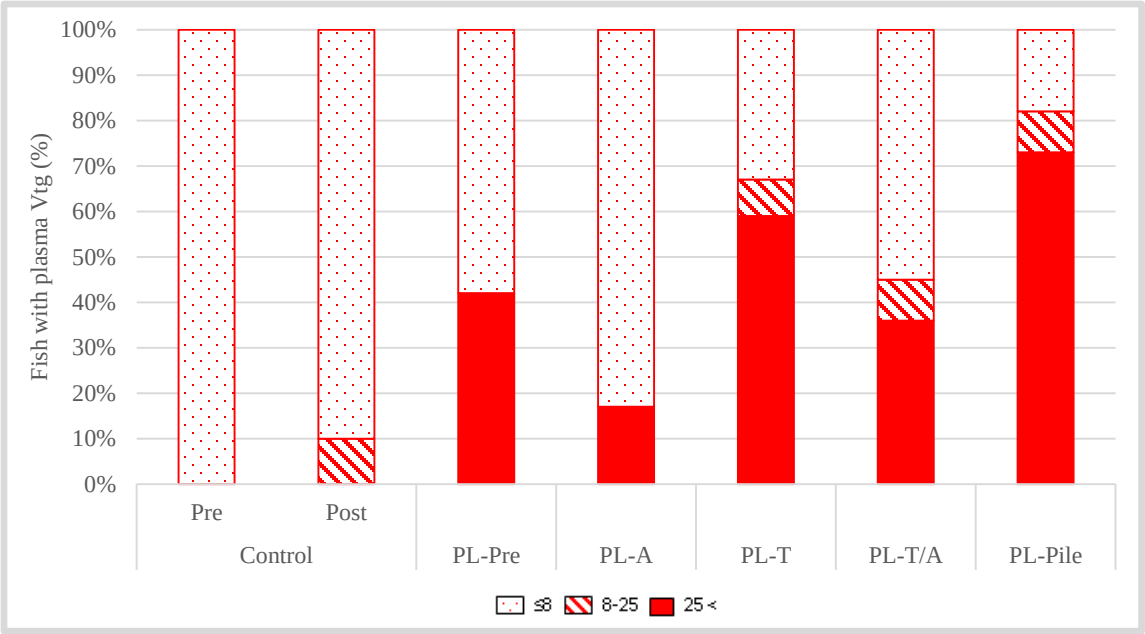

Figure 1.21. Proportion (%) of mature male fathead minnows (*Pimephales promelas)* expressing below detect (≤ 8 ng/mL), medium (8-25 ng/mL) and high (25 <) plasma vitellogenin (Vtg) concentrations following a 10-d static exposure to aqueous mixtures of poultry litter prior to and after various composting treatments: aerated (A), turned (T), aerated and turned (A/T), piled (P).

Aqueous Estrogen Analysis-LC/MS/MS

The previously stated difficulties for quantifying estrogens within BS samples also applied to PL aqueous samples. Similarly, E2 was below LOQ in all pre- and post-composted PL material with the exception of a single PL-piled treatment, which had a reported value of 40 ng/L at day 0 of aqueous sample collection. This value was deemed to have resulted from analytical complications and/or possible contamination. Estrone values ranged widely for all PL treatments, with day 10 of pre-composted PL having the highest value of 138 ng/L. There were no obvious trends with E1 levels throughout the 10-d exposure period for PL (Figure 1.22).

Generally, more abundant amounts of E1 were found in PL material both pre- and post-composted. Pre-composted PL had elevated E1 levels throughout the 10-d exposure rising from 17 ng/L to 137 ng/L. Piled PL and turned/aerated material showed similar quantities in E1, never rising above 30 ng/L. Turned PL material showed a general trend of decreasing from 75 to 8 ng/L over the exposure period. LC-MS/MS estrogen quantification data indicated that E1 levels may decrease in aerated composted and piled PL material.

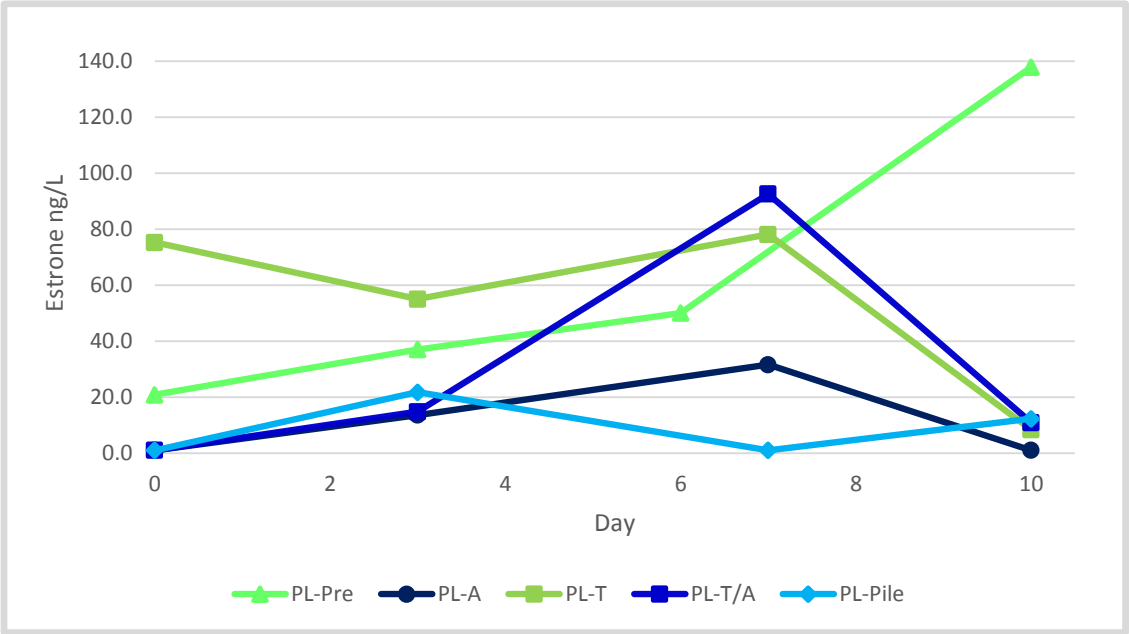

Figure 1.22. Estrone analyzed via LC-MS/MS following a 10-d static exposure to aqueous mixtures of poultry litter prior to and after various composting treatments: aerated (A), turned (T), aerated and turned (A/T), piled (P). Estrone values reported in ng/L; values below the detection limit of 1.7 ng/L were reported as 1.0 ng/L.

Toxicity of Aqueous Biosolid and Poultry Litter Mixtures

Toxicity analysis was conducted using the National Pollutant Discharge Elimination System (NPDES) Whole Effluent Toxicity (WET) spreadsheets made available through the Environmental Protection Agency (http://cfpub.epa.gov/npdes/docs.cfm?document_type_id=1&view=Policy%20and%20Guidance%20Documents&program_id=45&sort=name). The spreadsheet is used to calculate the chronic exposure effects of pollutants within aquatic habitats. Effects analyzed included no observable effect concentration (NOEC), lowest observable effect concentration (LOEC), and inhibition concentration 25% (IC25), with lower and upper 95% confidence intervals (Table 1.6).

All BS samples had an increase in IC25 values when comparing pre- composted to post-composted results. IC25 values also increased with PL treatments with the exception of aeration, which decreased (Table 1.6). Several treatment groups required aeration because of below acceptable levels dissolved oxygen within the exposure vessels. It was determined that because of aeration, some 6.4 g/L treatments experienced no lethality effects and therefore IC25 values were not calculated.

Table 1.6. Toxicity as indicated by exposures of larval fathead minnows (*Pimephales promelas*) to pre- composted and post-composted aqueous biosolids and poultry litter extracts (bioassays preformed according to USEPA 2002 standards). Exposure concentrations were generated each day (all seven days of exposure) by creating serial dilutions beginning with the top treatment that was prepared based on previously determined ammonia concentrations for each sample. NOEC = no observable effect concentration. LOEC = low observable effect. IC25 = inhibition concentration producing a 25% reduction in survival (endpoint measurement) when compared to control treatments. L 95% C.I. = Lower confidence interval. U 95% C.I. = Upper 95% confidence interval.

Sample	**Treatment**	**NOEC**	**LOEC**	**IC25**	**L 95% C.I.**	**U 95% C.I.**
BS-A	Pre	0.63	1.12	1.20	0.90	1.28
	Post	3.6	>3.6	>3.6	N/A	N/A
BS-B	Pre	3.6	6.4	4.18	4.00	4.44
	Post	6.4	>6.4	>6.4	N/A	N/A
BS-C	Pre	0.63	1.12	0.88	0.79	0.99
	Post	3.6	>3.6	>3.6	N/A	N/A
BS-D	Pre	3.6	>3.6	1.87	1.39	3.92
	Post	6.4	>6.4	>6.4	N/A	N/A
PL	Pre	<0.63	0.63	0.70	0.67	0.74
	Pile	0.63	1.12	1.31	1.13	1.43
	Turned	0.63	1.12	0.90	0.84	0.97
	Aerated	<0.63	0.63	0.21	0.18	0.25
	Turned/Aerated	1.12	2	1.22	0.91	1.31

DISCUSSION

Endocrine disrupting chemicals, including steroids such as estrogens, have received an increasing amount of attention over the past several decades (Colborn et al., 1993; Sumpter and Jobling 2013; Sumpter 2005; Fenner-Crisp et al., 2000; Khanal et al., 2006). It is also now well accepted that estrogens, are commonly found in both BS and PL (Andaluri et al., 2012; Hakk et al., 2011; Ciparis et al., 2012; Yang et al., 2012; Dutta et al., 2011). Once these materials are land applied as fertilizer, water soluble fractions that may contain estrogens have the ability to mobilize and be transported to receiving waters (Yonkos et al., 2010; Shore et al., 1995; Nichols et al., 1998; Herman and Mills 2003). As previously stated, estrogens are of great concern within receiving waters because of their ability to act as an endocrine disruptor and feminize and cause intersex in wild fish populations (Colborn et al., 1993; Yonkos et al., 2010; Sumpter 2005; Blazar et al., 2012; Iwanowicz et al., 2015). The purpose of this project was to investigate IVAT composting as a method to mitigate the estrogen concerns within both BS and PL. Previously, composting has been shown to decrease the water-soluble steroids E2 and testosterone within PL (Hakk et al., 2005). Composting has also been shown to induce microbial degradation of estrogens using activated sludge (Shi, et al., 2004). In-vessel aerated and turned composting is a commercially relevant method that could provide great benefits to facilities producing both BS and PL waste products. Four BS samples were acquired from four distinct WWTP and one PL sample was acquired from a poultry broiler and investigated over a 10 day period for estrogen content in both pre- and post-composted material. All material was analyzed using three different methods (LC-

MS/MS, BLYES and a whole fish exposure model –ELISA) to explore changes in estrogens/estrogenicity overtime once the material was in an aqueous solution.

Originally, both BS and PL materials were scheduled to arrive in the spring of 2014 to begin composting during the months of June, July, and August. Because of unforeseen complications, samples were not delivered until the winter of 2014/2015. This resulted in composting through the winter months of January, February and March. Cold temperatures during the winter months were a significant hindrance to the overall composting process. Although all materials were kept in the composting bins (previously described), the cold conditions resulted in both BS and PL material having difficulty reaching and maintaining appropriate temperatures necessary for successful composting (approximately 130° F (54° C)). Once it was determined that the environmental conditions were causing adverse effects on the experiment, BS bins were insulated and heated to aid in temperature maintenance. Although insulating and heating mechanisms did result in the increase of the internal temperatures within the BS material, the overall composting process was most likely effected by the cold temperatures. Heating the composting bins may also have resulted in loss of overall moisture.

A composting project of this scale requires daily monitoring and adjustments to correct for temperature, turning schedule, and moisture content. Because of logistical reasons, these daily adjustments were not always possible and therefore composting may not have reached its full potential. If this experiment were to be repeated, a person educated in the composting process should monitor the composting on a daily schedule and adjust turning and aerations regimes appropriately. These factors may have also contributed to a less than desirable ending compost product.

Previously, Changa et al., (2003) found a Solvita maturity test to be an inexpensive and relatively accurate way of measuring compost maturity via analyzing carbon dioxide and ammonia emissions. All three compost treated PL samples (aerated, turned, and aerated/turned) indicated high ammonia emissions according to the Solvita Test. High ammonia emissions are an indicator of immature compost. In contrast, post-composted BS samples analyzed via a Solvita test reported lower ammonia emissions, indicating a more mature compost. However, BS carbon dioxide emission values ranged from 2.5 to 5.5 (Solvita test) indicating that carbon dioxide was still present and compost was immature. Data is not available for piled PL material. In summary, the Solvita test indicated that neither post-composted BS or PL samples reached full compost maturity. This was most likely a result of issues described previously as well as time. Composting typically requires about 120 days to become mature material.

Biosolid samples were acquired from four different WWTPs with diverse influent properties. Biosolids acquired from the Back River WWTP, which is located in Baltimore, continuously stood out as being the most estrogenic upon arrival (analyzed via BLYES, LC-MS/MS and ELISA). Having significant city influents from various drainages and sewage systems this WWTP has complex inputs. These various inputs may contribute to the BS having relatively high estrogenicity when comparing its characteristics to the other samples acquired from less complex WWTPs (KNSG, Broadneck and Bay Ridge). In addition to having the most complex inputs of all the BSs acquired, Back River is also the largest WWTP, treating approixmately180 million gallons per day (22 times more influent than Bay Ridge, the second largest WWTP sampled. Because of dense human populations within city perimeters, Back River may

receive higher amounts of synthetic estrogen influents in the form of EE2 inputs through sewage systems. Since EE2 acts similarly to the natural estradiol hormone by binding to the estrogen receptor, this could contribute to higher overall estrogenicity levels and greater environmental risk (Hamid and Eskiciogly, 2012). Interestingly, the two smallest WWTP in terms of gallons treated per day (KNSG and Broadneck), had the lowest amounts of detectable estrogens within the material both pre- and post-composted. There was only trace amounts of estrogenicity in pre-composted material from KNSG, while Broadneck contained no quantifiable estrogenicity in either pre- or post-composted material. Biosolids acquired from Back River continuously stood out as being the most estrogenic in nature, which was reflected in estrogenicity, estrone quantification and Vtg induction. Material from the Bay Ridge WWTP indicated that there may be some estrogenicity found within the water soluble material, although LOQ limited the amount of significance found within pre- and post-composted material.

Estrogenicity (BLYES) results in PL material showed a similar trend for most composted treatments, increasing to their highest level by approximately day six and then decreasing for the remainder of the 10-d exposure. An exception to this trend was the pre-composted PL, which had a sharp increase in estrogenicity on the last day of aqueous sample collection. This same trend was also observed in both BS-A and BS-D. This data may indicate that estrogenicity levels within materials that are exiting WWTP facilities and poultry broilers may change over time as a result of entering microbially active aquatic environments. If material is sampled prior to land application for estrogen analysis, those results may not accurately represent what fish populations will actually

encounter. This could lead to unpredictable estrogen amounts entering receiving waters post runoff events and unknown endocrine disruption risk for fish.

Typically, poultry waste from poultry broilers is stored in large piles prior to land application. BLYES results indicated that estrogenicity may have increased in material that was piled versus compost treated (PL-A, PL-T, PL- A/T). As described earlier, microbes within PL may have the ability to deconjugate conjugated estrogens to less potent species (E2 and E1) (Hutchins et al., 2007). Microbial activity may also result in the transformation from the less potent E1 estrogen species to the more potent E2 (Dutta et al., 2010; Hutchins et al., 2007). Both of these processes would result in an overall increase in estrogenicity and environmental risk to receiving waters. Because E2 was not consistently found within the piled samples, the increase in estrogenicity was most likely due to the deconjugation of estrogens. Composting accelerates the degradation process, and therefore may have the potential to degrade parent estrogen species to estriol conjugates at a faster rate than piling. Because of a lack of replicates, statistical analysis were not performed.

Vitellogenin induction has become a popular bioindicator for estrogen exposure in many fish (Sumpter, 2005). Because of this, many assays have been developed to quantify plasma concentrations. Beidem et al., (2006) developed and validated an enzyme-linked immunosorbent assay (ELISA) that quantified Vtg in fish plasma. Previous to this study, a monoclonal mouse antibody was developed specifically for fathead minnow Vtg (Denslow et al., 1999). Eidem et al., (2006) purified Vtg from plasma with previously E2 treated fathead minnows using chromatography. The purity and identity of the Vtg was validated and a protein analysis was completed using an

amino acid analysis (Eidem, et al., 2006). The ELISA was then developed using a sandwich design. Within the plasma, blood constituents like albumin can also be found, introducing the possibility of matrix effects when conducting this assay (Eidem, et al., 2006). A minimum dilution factor of 1000x was previously selected to minimize matrix interference effects (Eidem, et al., 2006).

In this study, fish responded to estrogen exposure as expected, being Vtg induced when estrogenicity rose above approximately 2.0 ng/L. A 10-d exposure period in aqueous extracts was able to induce quantifiable Vtg in both pre-composted BS and pre- and post-composted PL samples, however pre-composted BS-A and PL piled treatments were the most robust in Vtg induction. There was one exception to Vtg induction results that differed greatly from what was expected. A single fish in the post-composted control treatment had significant Vtg induction of > 400 ng/mL. Initial and verification analyses of the plasma verified significant Vtg induction. Because of previously published data concerning natural background levels of Vtg, this fish was most likely female and not male (Palace et al., 2002). An adult female fathead minnow would have significant naturally occurring Vtg within their plasma (Palace et al., 2002). Unfortunately, the whole fish that the plasma was collected from was not preserved so determining its gender is no longer possible. The BLYES analysis of the control treatment did indicate estrogenicity levels of approximately 1.0 ng/L. Although unlikely, this low level of estrogenicity may have been enough to induce an especially sensitive male fish, offering another explanation. The fish with the elevated Vtg was deemed an outlier and the data was excluded from overall calculations.

Pre- and post-composted Vtg (ELISA) data for BS-A and PL treatments indicated that there may be efficacy in composting BS and PL prior to land application. Upon arrival (in pre-composted material), BS-A had enough estrogen to significantly induce 25% of fish exposed to a level greater than 25 ng/mL. In contrast, fathead minnows exposed to post-composted BS-A material did not result in Vtg induction above detection in any fish. Vitellogenin induction data combined with overall decrease in estrogenicity indicates composting could mitigate estrogen concerns within more potent (higher estrogenicity) biosolid samples.

As mentioned previously, the ELISA assay is limited by consequence of plasma containing components other than Vtg. The plasma collected from the fathead minnows was not purified for Vtg and therefore other proteins within the plasma can also bind to the high binding plate (Perez et al., 2007). This phenomenon could result in binding sites being occupied by proteins other than Vtg and therefore the assay reporting lesser amounts than the true Vtg value (Perez et al., 2007). In addition, as an effect of the assay, the plasma samples are frozen and thawed prior to the initial analysis. The plasma is stored without buffering solution, making the Vtg protein susceptible to degradation through freeze/thaw. This becomes a concern when samples are analyzed more than once, increasing the amount of freeze/thaw events. Because of this, when applicable, the first recorded value for Vtg results was reported.

The complex nature of working with environmentally relevant samples (BS and PL aqueous mixtures) posed significant analytical challenges (Perez, et al., 2007). Because estrogens can cause significant effects at very low concentrations, having an analytical technique with low quantification levels is of great importance. Because the

derivatization technique used is selective to phenol groups, phenolic compounds that are present within the matrix may also bind to the derivatizing agents. This unintentional binding may increase background signals and therefore an internal calibration method was necessary.

The complex nature of these samples also significantly affected the recovery rates. Recover rates were calculated by Elizabeth Mullin at Buffalo by determining the amount of standard that was recovered post processing that was introduced pre-processing. Percent recover was widely inconstant ranging from 2% to 97% depending on treatment and material. Besides the complex nature of the matrix, percent recovery of the deuterated estrogens within the aqueous samples could have been caused by methanol evaporation. All samples intended for LC-MS/MS analysis were reconstituted in 0.5 mL methanol and then shipped (overnight) to the University at Buffalo. During this time, some methanol could have evaporated, which may have resulted in increased recovery rates. Biosolid sample D had consistently low recover rates in pre-composted material, with a recovery rate never exceeding 5%. Post-composted material had higher rates ranging from 24.7% to 38.0%. In this sample (BS-D), composting may have aided in decreasing the matrix effect and increase quantification accuracy. Because recovery rates ranged so erratically, they were not incorporated into the overall calculations for the quantification data. Instead, only the raw E1 numbers were recorded. This method was also more consistent with the BLYES analysis, which did not have recovery rate information incorporated. Estradiol information was not included in this initial report due to complications in quantification methods. Estradiol quantification data (LC-MS/MS) was not found in a single pre-composted sample (BS or PL) at a LOQ below 2.2 ng/L.

However, post-composted data was received with estradiol numbers that were below the previous LOQ, making results incomparable.

As previously mentioned, the BLYES analysis is a measure of estrogenicity and therefore responds to any compound that is able to bind to the estrogen receptor within the yeast. Because of this, it is possible to report false positives due to the contamination within the aqueous samples being investigated. This fact became especially important when examining the pre- composted PL and its corresponding control exposure. The control exposure group reported total estrogenicity of slightly above 1.0 ng/L, which could be considered a significant amount. The fish within the control exposure did not indicate Vtg induction, demonstrating that what the BLYES was measuring may not have been estrogens within the exposure but contamination post sample collection. There was also a significantly large increase (4x) in estrogenicity and E1 in the pre-composted PL on the last day of collection (10-d). Such a large increase in estrogenicity was not seen during any other exposure experiment during this research. This large increase may have been a result of contamination during the collection and extraction process. Contamination may have been a result of improper glassware preparation. As previously mentioned, studies have shown that estrogen mimicking compounds such as BPA, may be capable of binding to the estrogen receptor within the yeast strain (Krishnan et al., 2013). Although, BPA contamination would not account for the E1 increases.

This experiment examined laboratory created concentrations of soluble extracts of waste materials that can only be hypothesized to mimic environmentally relevant concentrations that aquatic species experience following run off events. In order to directly and accurately examine estrogen content and activity within soluble extracts

following run off events, samples would need to be taken directly from the environment, which was not possible during this study.

Toxicity results indicated that by composting all BS samples (A, B, C and D), the inhibition concentration producing a 25% decrease (IC25) in survival values increased, indicating a decrease in frank toxicity. Toxicity results for PL litter materials indicated that IC25 values increased for piled, turned and turned/aerated treated PL, also indicating a decrease in overall frank toxicity. In contrast, the IC25 value for aerated PL decreased. Aerated treated PL showed the most significant decline in overall estrogenicity, which decreased environmental risk potential, however the IC25 value decreased. During the toxicity experiments, several of the higher concentrations of both pre- and post-composted BS and PL treatments were gently aerated due to low oxygen levels within the exposure vessels. This may have resulted in skewed IC25 values, increasing the concentration of the aqueous extracts the larval fathead minnows were able to survive in.

In conclusion, the composting portion of this project indicated that IVAT composting may be effective as a means of reducing contaminants of concern in the category of steroidal hormones (estrogens). Overall, data from BLYES, ELISA and LC-MS/MS generally did agree with one another, supporting the hypothesis that composting could be a means of mitigating estrogen effects in BS and PL material. Biosolid-A offered the most interesting results, being the most estrogenic upon its arrival. Poultry litter that was piled instead of composted showed evidence that estrogen activity may increase overtime. This is extremely relevant because piling PL is a standard practice for poultry broilers in the Delmarva Peninsula, which is in the Chesapeake Bay watershed. Because the actual composting process was not 100% successful and somewhat

incomplete, a definite answer as to how effective composting remains unanswered. However, this study does provide important evidence that when there are quantifiable amounts of estrogens, composting may be an important tool to incorporate during waste disposal procedures to decrease environmental risk via endocrine disruption in aquatic species.

CHAPTER TWO:

ESTROGEN CHEMICAL SPECIES CONVERSION

INTRODCUTION

It is generally accepted that E2 predominantly transforms to the less potent E1 via microbial action (Dutta et al., 2010). There is, however, evidence suggesting that some portion of E1 might actually be converted to the more potent E1 (Hutchins et al., 2007). Because E2 is up to 100x more estrogenic than E1, only a modest conversion in this direction could dramatically increase the risk of endocrine disruption (Dutta et al., 2012). Knowledge of this process is important for two reasons. First, where estrogenic media (e.g., WWTP effluent: PL or BS amended agriculture runoff) enters natural receiving waters, conversion to more potent estrogens over time suggests an increased risk to resident biota and perhaps the need for monitoring beyond simple "time-zero" sampling and analysis. Second, and more germane to this project, composting employs microbes to degrade organic material. While this process is likely effective at degrading both E2 and E1 to non-estrogenic constituents, there is a possibility that some portion of E1 is actually converted to E2, thus raising overall estrogenicity despite lowering total estrogen analytes.

The second portion of the current research project was designed to investigate the phenomenon of E1conversion to the more potent E2 species by measuring E2 generation over time in static "aqueous environments" spiked with E1. Several variables were investigated for their influence on estrogen conversion. These included the effects of fish being present or absent, the effects of complex organic media (in the form of aqueous

poultry litter) being present or absent, and the effects of sterilization. The main hypotheses being investigated were that, once in solution, E1 could convert to E2 and that the process would require the presence of a microbial community and be influenced by the nature and complexity of that community. Investigation of estrogen conversion was accomplished by quantifying the occurrence of deuterated E2 (E2-d4) following the introduction of a deuterium-labeled E1 (E1-d4) to the various aqueous environments. By deuterating E1, heavy hydrogen atoms are stably-attached to the steroid carbon backbone (Figure 2.1). Because deuterated groups are conserved during microbial transformation of estrogens, labeling of E1 serves as a way of quantifying the rate and nature of microbial estrogen transformation/conversion.

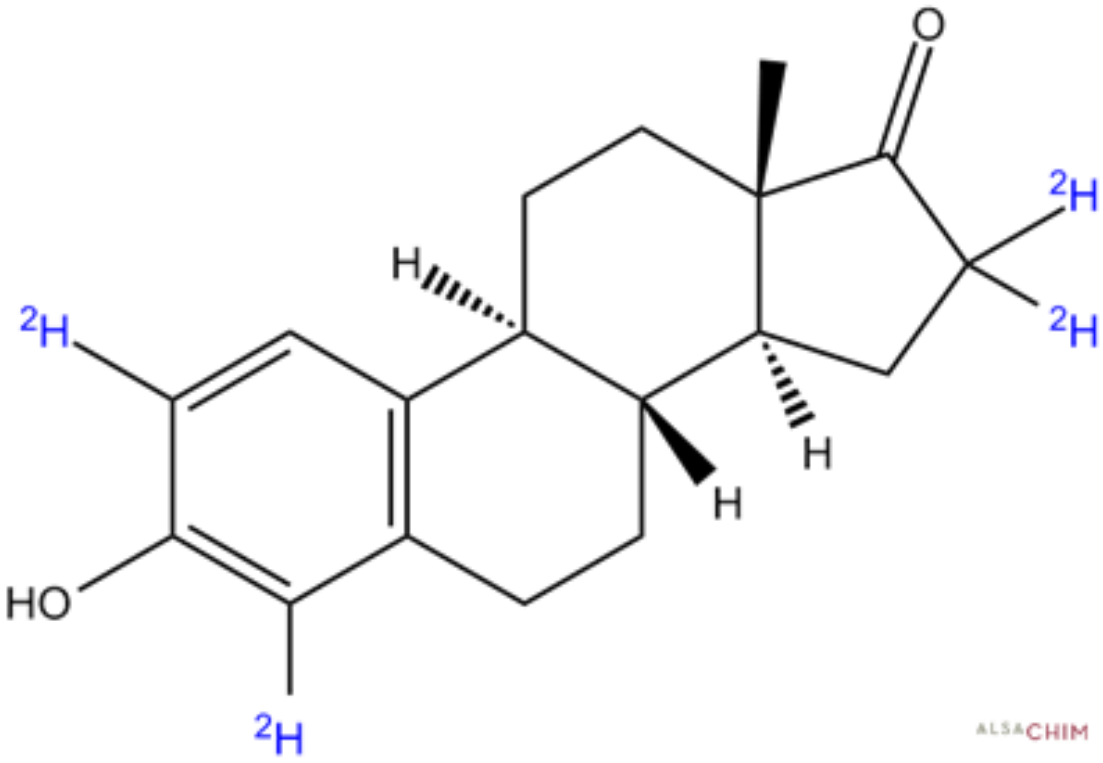

Figure 2.1. Deuterium-labeled estrone (E1-d4) indicating location of heavy hydrogen atoms.

METHODS

In all eight "aqueous environments" were employed (six 100 L all-glass aquaria and two 15 L glass carboys). All eight environments were held static without renewals over a 28 day period. Aquaria containing 80 L of water were maintained open to ambient air, continuously aerated, and illuminated on a 16-h-8-h light:dark cycle. Carboys containing 13 L of water were sterilized, sealed and maintained in the dark. Two of the glass aquaria contained control water (dechlorinated, aerated municipal water), one with adult male fathead minnows (for the first 14 d) and one without. Three aqueous environments contained control water spiked with E1-d4, two aquaria (one with and one without fish), and one sterile carboy. The remaining three aqueous environments (two aquaria with and without fish and one sterile carboy) were generated using control water augmented with PL and spiked with E1-d4 (Figure 2.2).

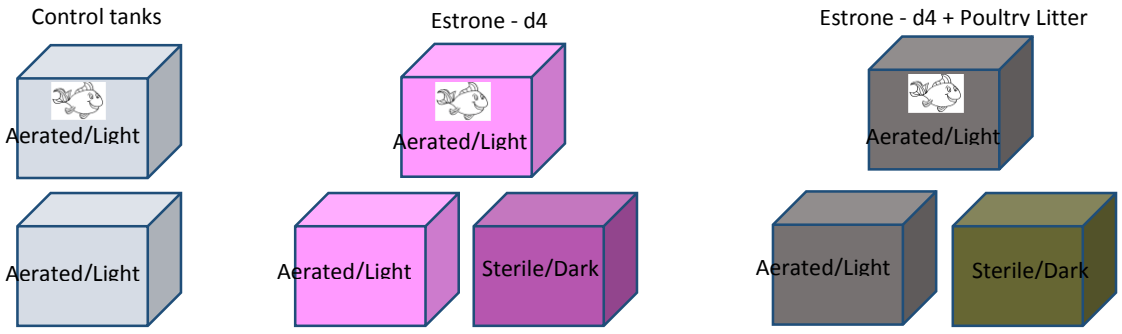

Figure 2.2. Graphic representation of the eight aqueous environments for estrogen chemical conversion study. = mature male fathead minnows (*Pimephales promelas*. Estrone- d4- indicates the presence of deuterated estrone.

Inclusion of fish in E1-d4 exposure treatments and augmenting E1-d4 treatments with PL allowed investigation of the conversion phenomena under increasingly complex "real world" conditions. Sterile replicates of E1-d4 with and without PL treatments maintained in the dark controlled for microbial and photolytic degradation, allowing assessment of the role of biotic hydrolytic reactions.

Prior to the experiment, 15 L carboys intended for sterile conditions were cleaned with soap and water, allowed to dry and sprayed with ethanol. They were then baked at 100° C overnight to limit bacterial contamination.

Water soluble fractions were generated by aqueous extraction of dry weight-equivalents for PL material. Similar to the composting study, the target amount for all samples was 2.0 g/L and was prepared in 20 L carboys with dechlorinated city water that had been aged for several days prior to experiment. Carboys were shaken and allowed to sit overnight, allowing the soluble material to dissolve while larger particles settled out. The next day, carboys were decanted into 100 L aquaria without resuspension of the settled material. The samples were then diluted 4 fold by adding 60 µL of dechlorinated and aged water, making the total exposure volume 80 L within the 100 L aquaria. This dilution resulted in a final concentration of 0.5 g/L for all exposures. Aerated/lighted conditions with and without fish, all received a 1 mL spike of E1-d4 at a concentration of 148 mg/L. The control aquaria did not receive E1-d4. Both sterile/dark with and without PL conditions received 60 uL of the E1-d4 and had a total volume of 13 L. Once the E1-d4 had been added to sterile/dark aquaria, they were again baked at 90° C overnight to reduce bacterial contamination. Sterile/dark aquaria were kept in a biological

hood to decrease contamination. A siphon system was installed at the opening of the 20 L glass carboy to decrease possible contamination while obtaining samples for analysis.

Aqueous Sample Collection

Over the 28 day exposure period two 1.0 L samples were collected at prescribed intervals (see sample schedule Table 2.1) for LC-MS/MS and BLYES analysis via methods described previously.

Day	E1-d4 N/F	E1-d4 W/F	Control N/F	Control W/F	E1-d4+ PL N/F	E1-d4+ PL W/F	E1-d4 S/D	E1-d4 + PL S/D
0	X		X		X		X	X
1	X	X			X	X		
3	X	X			X	X		
5	X	XX			X	XX		
7	X	X	X	X	X	X	X	X
10	XX	X			XX	X		
14	X	X	X	X	X	X	X	X
21	XX	XX	X	X	XX	XX	X	X
28	X	X	X	X	X	X	X	X

Table 2.1. Aqueous extract collection schedule for estrogen chemical species conversion study. X = Single sample collected (two 1.0 L samples). XX = Duplicate samples collected for replication purposes. E1-d4 = deuterated estrone. N/F= no fish. W/F = with fish. S/D = sterile/dark. Deuterated estrone was not introduced to control environments.

BLYES and ELISA Assays

All assays were performed as previously described with the exception of sample dilution. Several samples analyzed via both BLYES and ELISA needed further dilution than the composting experiment due to high levels of total estrogenicity as a result of

introduction of E1-d4. Introduction of E1-d4 resulted in elevated levels of total estrogenicity and high Vtg induction within fish.

Test Species and Plasma Collection

Adult male fathead minnows were again utilized for the estrogen conversion study for reasons previously described. Plasma was also collected as previously described. In contrast to the composting study, which fish were exposed in all environments for equal amounts of time, fish were exposed in various time periods. Fish within the control environment were present for days 0 to 14 (plasma collection on day 14). Fish within the E1-d4 without PL and E1-d4 with PL were exposed from days zero to seven, zero to 14 and seven to 14. Fish were separated within the aquaria via a netted enclosure that allowed for aqueous samples to penetrate the enclosures so all fish were exposed to aqueous extracts. Post day 14, all exposures lacked fish but were maintained static (with aeration) for the remainder of the 28 day exposure period to allow for further aqueous sampling.

RESULTS

Estrogen Analysis via LC-MS/MS

Quantification of E1-d4 and E2-d4 via LC-MS/MS showed E1 did convert to E2 in all non- sterile treatments (Figure 2.3-4).

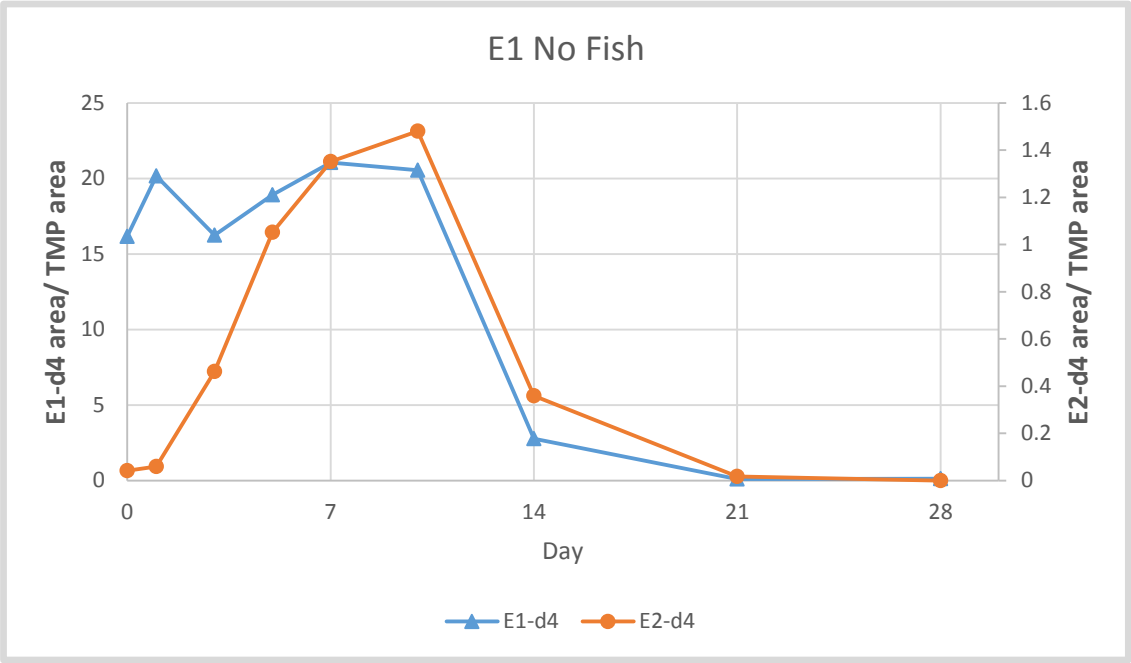

Figure 2.3. Occurrence and trends of deuterated estrogens (estrone and estradiol) in aqueous environment spiked with deuterated estrone without fish for estrogen chemical conversion study. All values are reported as area/TMP area due to a lack of deuterated estradiol standard. Deuterated estrone is reported on primary vertical axis and deuterated estradiol is reported on secondary vertical axis. E1-d4= deuterated estrone. E2-d4= deuterated estradiol.

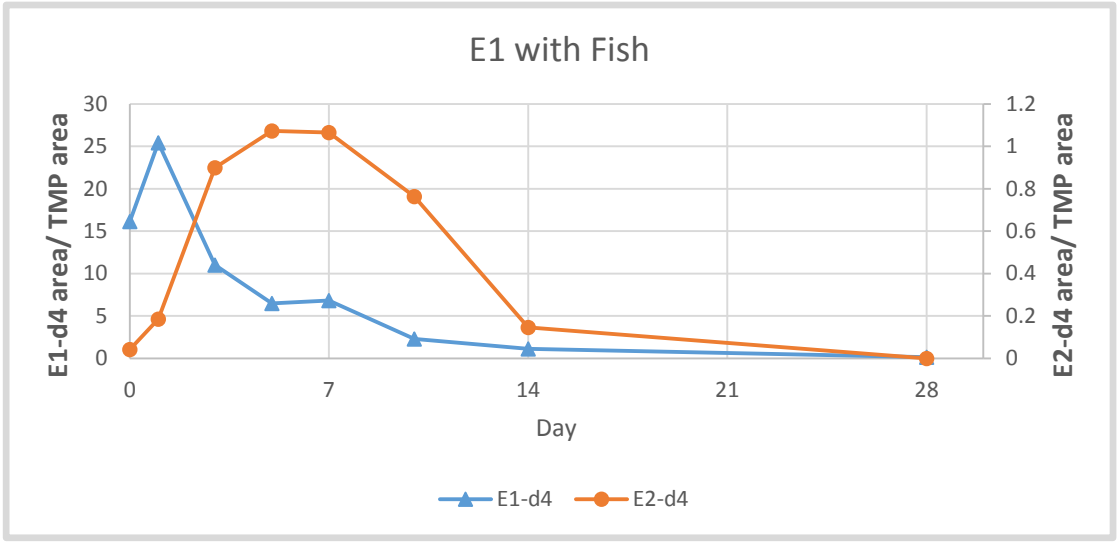

Figure 2.4. Occurrence and trends of deuterated estrogens (estrone and estradiol) in aqueous environment spiked with deuterated estrone with fish for estrogen chemical conversion study. All values are reported as area/TMP area due to a lack of deuterated estradiol standard. Deuterated estrone is reported on primary vertical axis and deuterated estradiol is reported on secondary vertical axis. E1-d4= deuterated estrone. E2-d4= deuterated estradiol. Data not available for day 21.

In the E1-d4 without fish treatment, E1-d4 initially increases from day 0 to 10 where it then begins to degrade. Deuterated estradiol increases in the initial ten days as the more E1 is converting to the less potent species E2 as a result of the naturally occurring microbial community within the solution. Both E1-d4 and E2-d4 were complety degraded/undetectable by day 21 and remained undetectable at day 28. Similarly, E1-d4 with fish experienced an initial increase in E1-d4, however the degradation process occurred earlier than the treatment lacking fish, beginning the degradation process by the first day. Deuterated estradiol increased (initial conversion from E1) from day 0 until day 7 when it also began its degradation process. Both deuterated estrogens were undetectable by day 28 (Figure 2.3). There was no E2-d4 detected throughout the 28 day exposure period in the E1-d4 sterile/dark environment indicating a lack of transformation likely due the eradicated microbial community (Figure 2.5).

Deuterated estrone treatments with PL augmentation both with and without fish, showed similar trends, E1 conversion to E2, however and degradation were slightly faster in the environment containing fish. Both estrogens were undetectable by day 14 (Figures 2.5-6). Unlike the sterile/dark treatment lacking PL, there was estrogen conversion in the treatment containing PL. Deuterated estradiol was detected by day three of the exposure and remained detectable throughout all 28 days. Deuterated estrone never completed degraded but did decrease notably over time (Figure 2.7). All control samples had undetectable amounts of both E1-d4 and E2-d4 throughout the 28 day exposure.

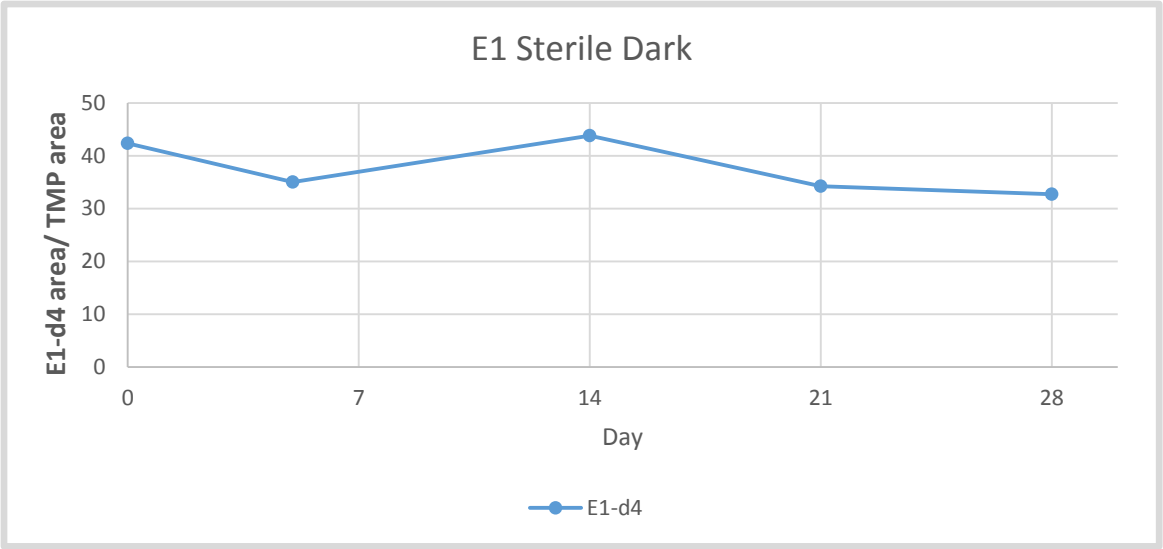

Figure 2.5. Occurrence and trends of deuterated estrogens (estrone and estradiol) in sterile/dark aqueous environment spiked with deuterated estrone for chemical conversion study. All values are reported as area/TMP area due to a lack of deuterated estradiol standard. Deuterated estrone is reported on primary vertical axis and deuterated estradiol is reported on secondary vertical axis. E1-d4= deuterated estrone.

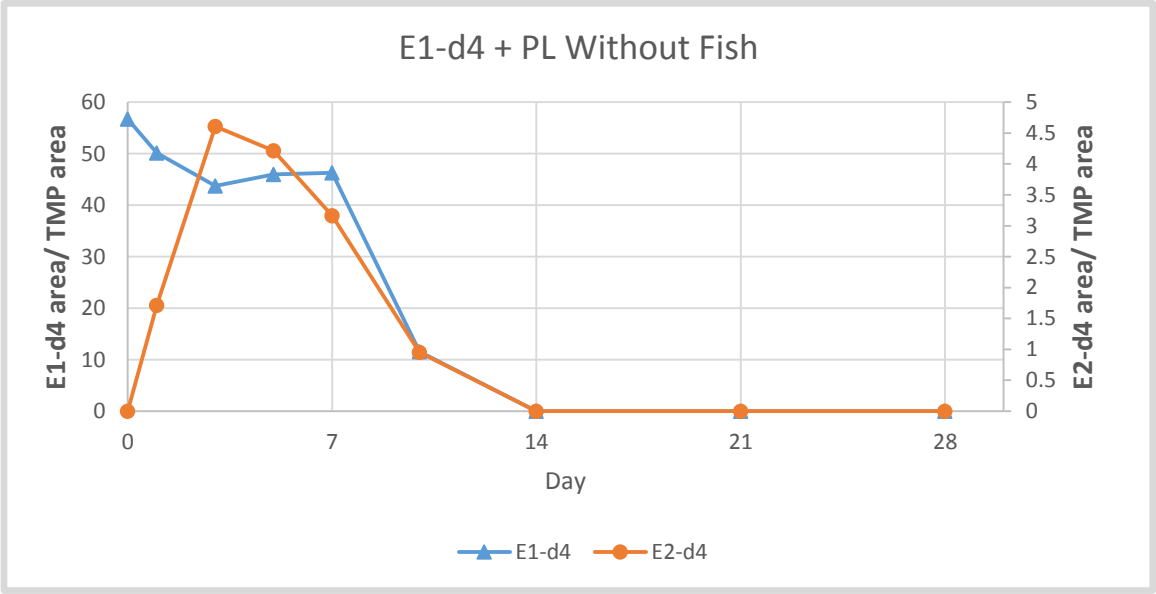

Figure 2.6. Occurrence and trends of deuterated estrogens (estrone and estradiol) in aqueous environment spiked with deuterated estrone plus poultry litter with fish for estrogen chemical conversion study. All values are reported as area/TMP area due to a lack of deuterated estradiol standard. Deuterated estrone is reported on primary vertical axis and deuterated estradiol is reported on secondary vertical axis. E1-d4= deuterated estrone. E2-d4= deuterated estradiol.

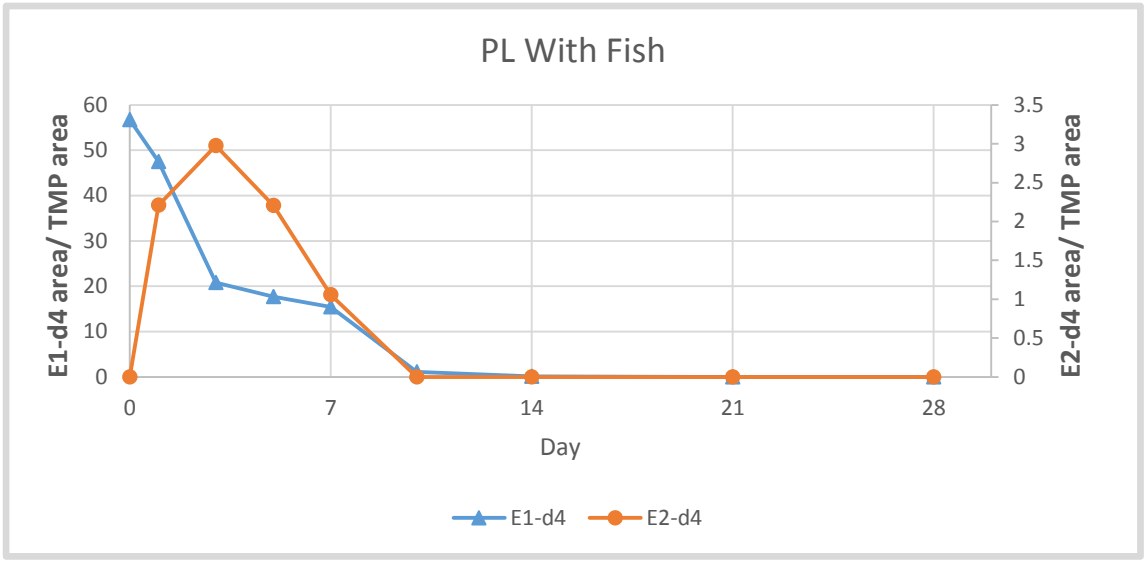

Figure 2.7. Occurrence and trends of deuterated estrogens (estrone and estradiol) in aqueous environment spiked with deuterated estrone plus poultry litter without fish for estrogen chemical conversion study. All values are reported as area/TMP area due to a lack of deuterated estradiol standard. Deuterated estrone is reported on primary vertical axis and deuterated estradiol is reported on secondary vertical axis. E1-d4= deuterated estrone. E2-d4= deuterated estradiol.

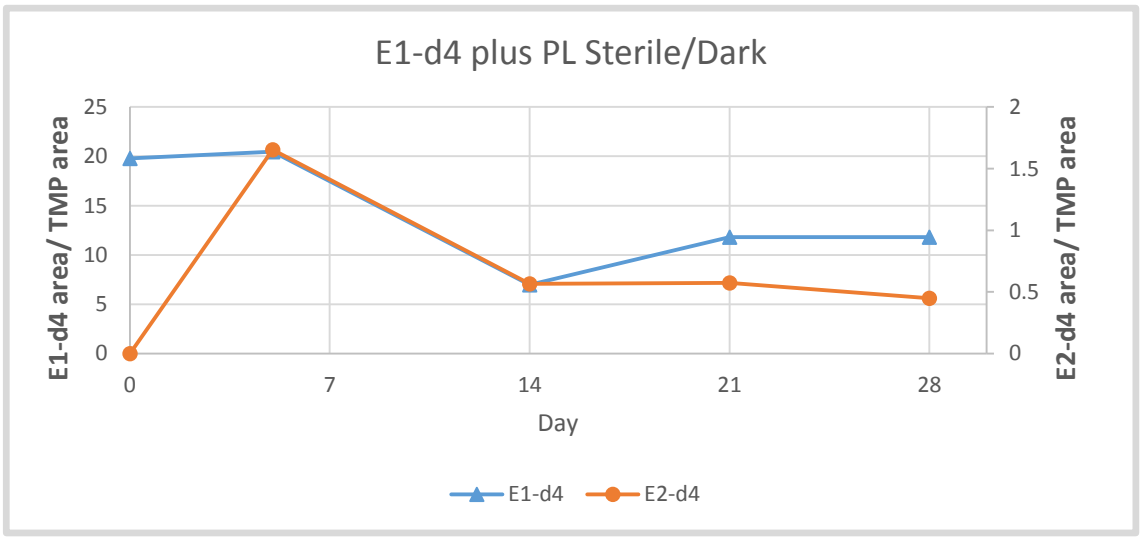

Figure 2.8. Occurrence and trends of deuterated estrogens (estrone and estradiol) in sterile and dark aqueous environment spiked with deuterated estrone plus poultry litter for estrogen chemical conversion study. All values are reported as area/TMP area due to a lack of deuterated estradiol standard. Deuterated estrone is reported on primary vertical axis and deuterated estradiol is reported on secondary vertical axis. E1-d4= deuterated estrone. E2-d4= deuterated estradiol.

Estrogenicity Investigation via In Vivo Adult Male Fish Model

Total estrogenicity results (BLYES) supported findings from LC-MS/MS. Total estrogenicity decreased in both non sterile environments over the 28 day period, however environments with fish decreased faster compared to without fish. By adding the component of fish, degradation occurred earlier. Total estrogenicity within the sterile/dark environment remained constant throughout the exposure (Figure 2.9).

The most abrupt degradation in total estrogenicity was observed in the E1-d4 plus PL with fish environment, showing degradation by day one. The E1-d4 plus PL treatment lacking fish had an initial increase in estrogenicity (100.0 to 180.0 EEQ) in the first three days and then began to degrade. Both environments had undetectable estrogenicity by day 14. The sterile/dark environment that contained PL remained consistent throughout the exposure (Figure 2.10). All control treatments had undetectable amounts of estrogenicity according to the BLYES assay. Average estrogenicity is reported in figure 2.11.

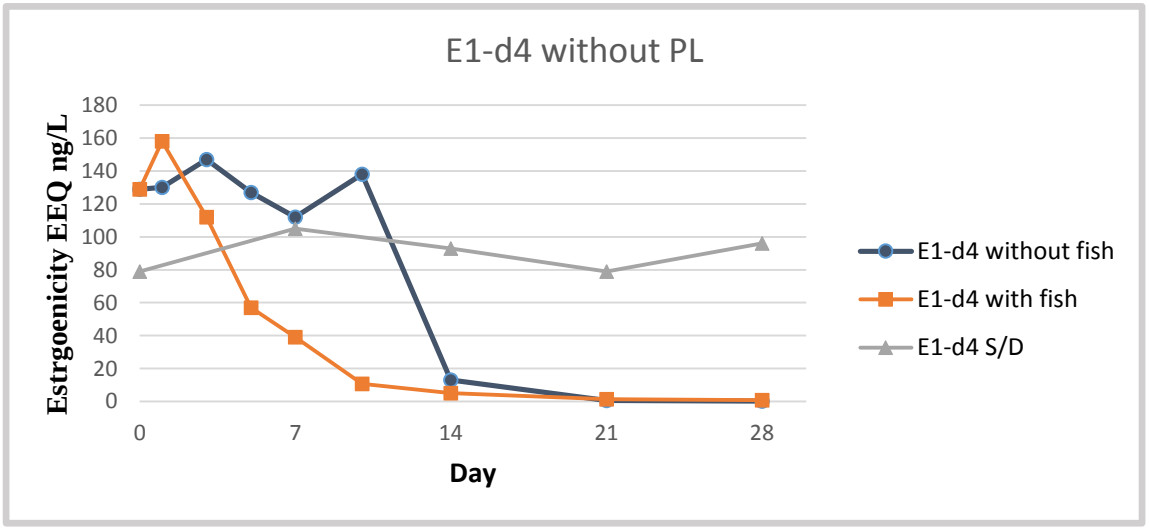

Figure 2.9. Estrogenicity over 28-d exposure period analyzed via BLYES for estrogen chemical conversion study (estrogenicity values reported in 17β-estradiol equivalents (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L). E1-d4= deuterated estrone. S/D-sterile/dark environment.

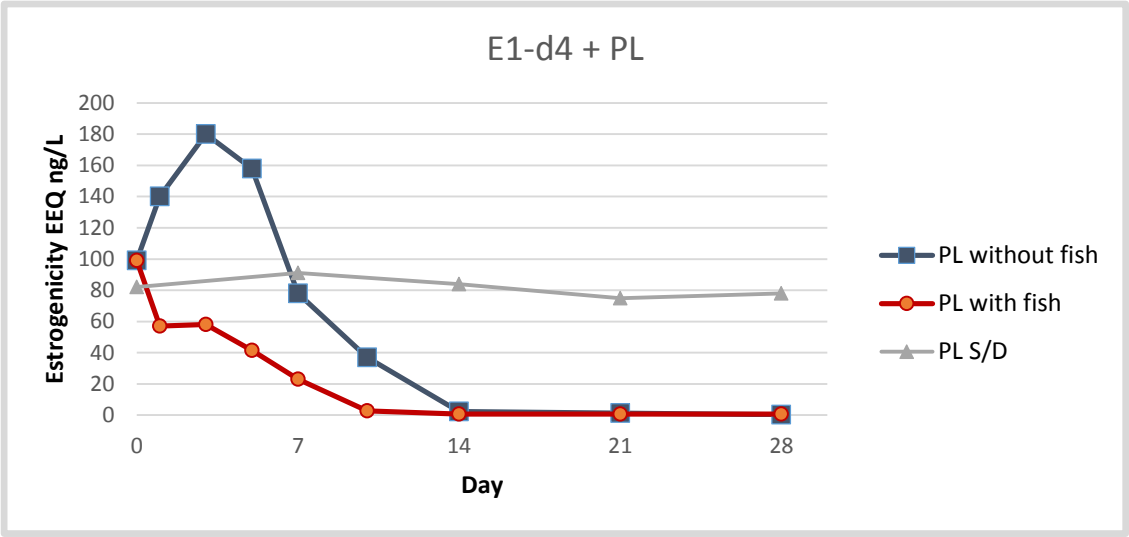

Figure 2.10. Estrogenicity over 28-d exposure period analyzed via in vitro BLYES for estrogen chemical conversion study (estrogenicity values reported in 17β-estradiol equivalents (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L). E1-d4= deuterated estrone. PL= poultry litter. S/D= sterile/dark.

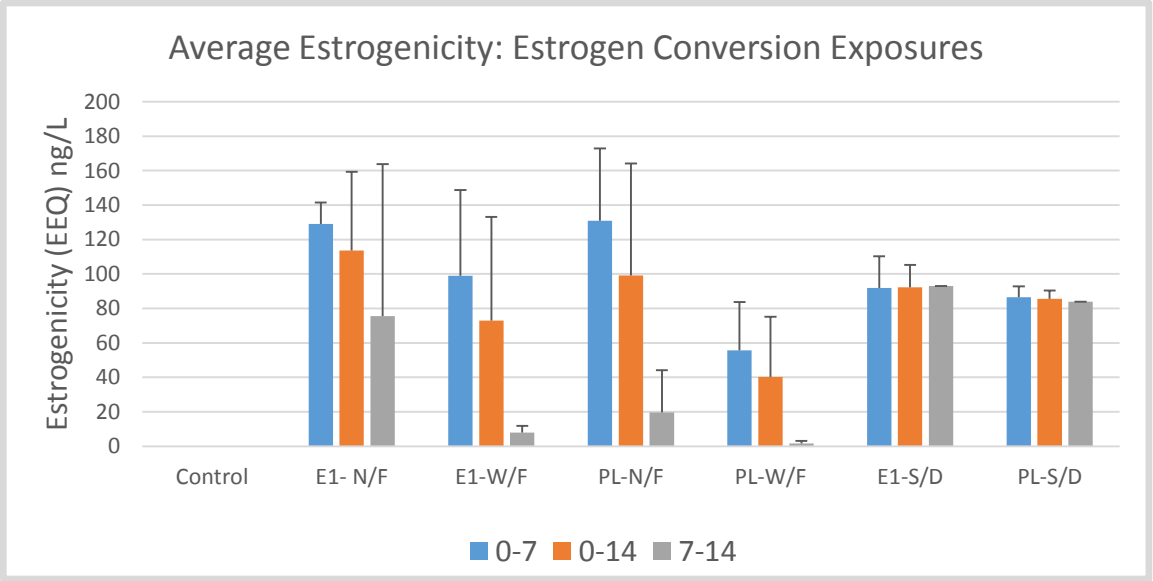

Figure 2.11. Mean estrogenicity over 14-d fish exposure period analyzed via BLYES for all eight aqueous conditions (total estrogenicity values reported in 17β-estradiol equivalents (EEQ); values below the detection limit of 0.2 ng/L were reported as 0.1 ng/L). Averages were calculated for days 0 to 7, 0 to 14 and 7 to 14. E1-N/F= deuterated estrone without fish. E1-W/F= deuterated estrone with fish. PL-N/F= deuterated estrone plus poultry litter without fish. PL-W/F= deuterated estrone plus poultry litter with fish. E1-S/D= deuterated estrone in sterile/dark environment. PL/S/D= deuterated estrone plus poultry litter in sterile/dark environment. Error bars indicate standard deviation.

Estrogenicity Investigation via In Vivo Adult Male Fish Model

Fish in all treatment groups (E1-d4 with and without PL) that were introduced at day zero had significant Vtg induction including those collected on days seven and 14 (Figure 2.12). Fish introduced on day seven were not Vtg induced (Figure 2.12). Fish within the control environment were introduced at day 0 and collected at day 14. Control fish had all below LOQ of Vtg (Figure 2.12). There was slight evidence in decreasing overall amounts of Vtg induction between fish exposure between days 0-7 and fish exposure days 0-14, although averages were not significantly different (Figure 2.12). There was also no significant differences in Vtg induction in fish within the PL augmented environments compared to those not in the PL augmented environments. Vitellogenin induction was also reported as a proportion of induction as previously done in chapter one. Induction was separated into three categories: high induction (> 25 ng/mL), medium induction (8-25 ng/mL), and low induction (≤ 8 ng/mL). Fish that were within aquaria spiked with the E1-d4 had high amounts of Vtg induction, with the exception of a single fish in the E1-d4 without PL 0-14 day group, which had low induction (Figure 2.13). Above 25 ng/mL induction indicates a significant amount of estrogen exposure, which was expected because of the E1-d4 spike.

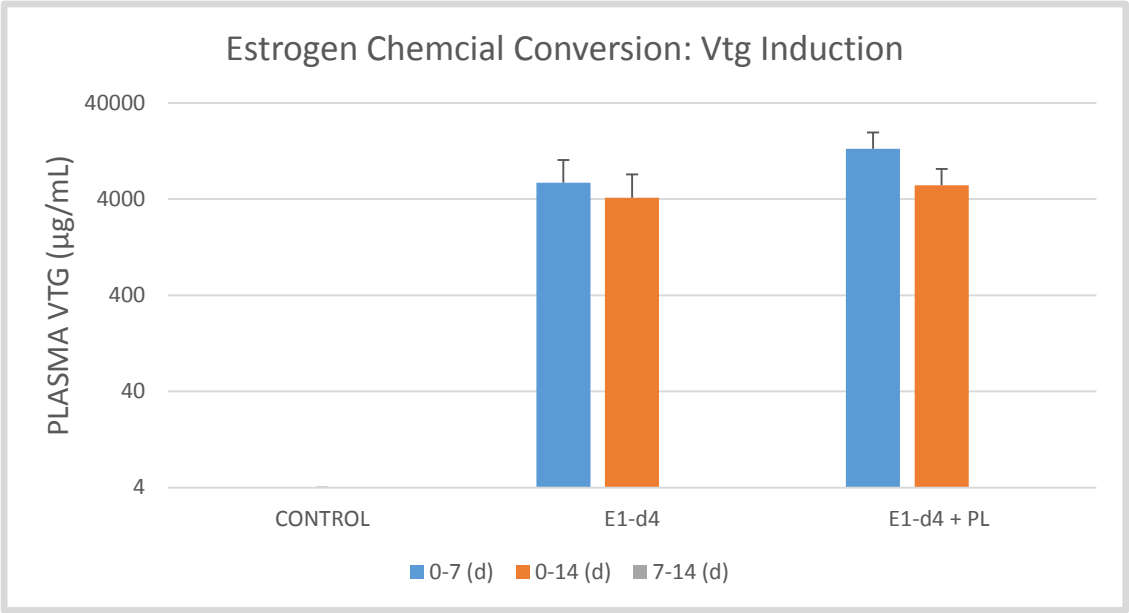

Figure 2.12. Mean plasma vitellogenin (Vtg) concentration of mature male fathead minnows (*Pimephales promelas)* following exposures in periods of 0-7 days, 0-14 days and 7-14 days to aqueous extracts. All values below the detection limit of 8 ng/mL were reported as 4 ng/mL. E1-d4 = deuterated estrone. E1-d4 + PL= deuterated estrone plus poultry litter. Error bars indicate standard deviation.

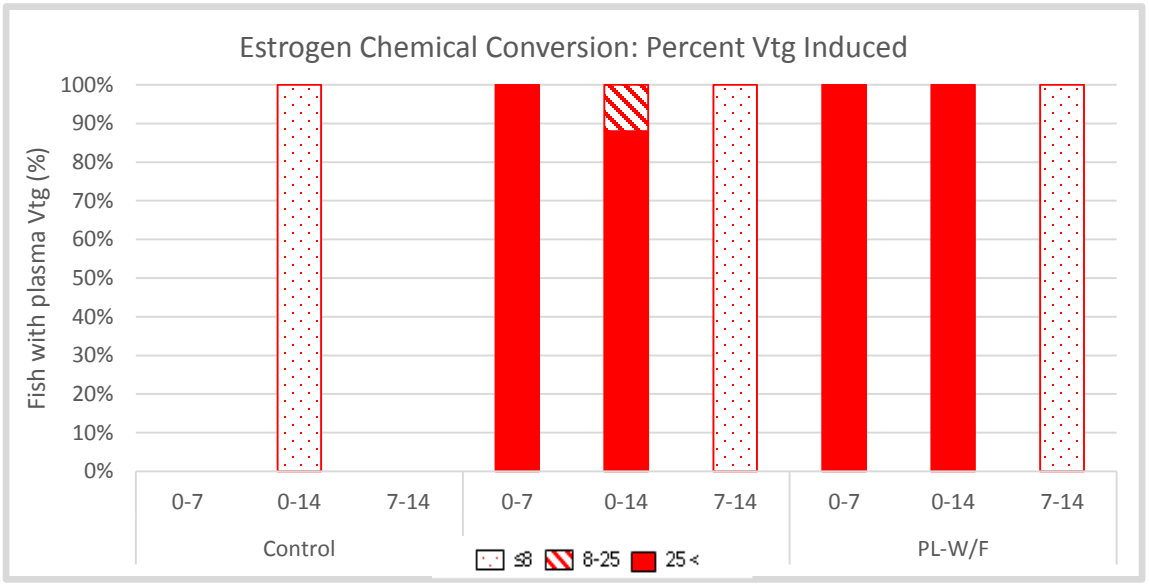

Figure 2.13. Proportion (%) of mature male fathead minnows (*Pimephales promelas)* expressing below detect (≤ 8 ng/mL) medium (8-25 ng/mL) and high (25 < ng/mL) plasma vitellogenin (Vtg) concentration following exposure periods of 0-7, 0-14 and 7-14 days static exposures to aqueous extracts. Error bars indicate standard deviation.

DISCUSSION

The second portion of this study was investigating the possible transformation of the parent estrogen species, E1 to the more potent species, E2. This transformation is significant because E2 is up to 100x more estrogenic than E1 (Dutta et al., 2012). Only a slight transformation of E1 to E1 could result in a dramatic increase of endocrine disruption risk to wild fish populations in receiving waters. Hutchins et al., (2007) stated that "free estrogens can undergo reversible transformations". Although it is generally accepted that E1 has the potential of transforming to E2, studies are lacking in showing the specifics of this significant transformation (Hutchings et al., 2007; Ying et al., 2006; Lucas et al., 2006; Gentili et al., 2002; D'Ascenzo et al., 2003). It is well established that both BS and PL contain a variety of contaminants of concern, including estrogens (Dutta et al., 2012; Topp et al., 2008; Clarke and Smith 2011; Hamid and Eskicioglu 2012). Because of the abundant use of both materials as a fertilizer source, it is important to understand the possible transformation of estrogens once within the environment. This study analyzed the transformation of E1 to E2 in aqueous fractions of differing environments for the creation of E2-d4. It is important to note that this study only examined the transformation of E1 to E2 in the presence of PL amended aqueous fractions and did not include BS material. Results of this experiment are depicted in area/ TMP area because an E2-d4 standard was not available and therefore estrogen quantification could not be reported in ng/L for E2. However, the estrogen quantification results indicated that there was transformation of the less potent species E1 to the more potent species E2 in all environments except the sterile/dark lacking PL treatment. This was clearly shown by the detection of E2-d4. Deuterated estrogens do not naturally

occur, therefore the only explanation for the detection of E2-d4 would be the conversion from E1-d4. This data, in addition to previously reported transformations, indicates that this estrogen conversion occurs as a result of estrogen species entering microbially active communities (e.g. environments with PL aqueous extracts and/or fish) (Hutchins et al., 2007).

All environments that demonstrated an E1 to E2 conversion had similar trends in the detection E2-d4 over the 28-d exposure period. Deuterated estradiol was detected early on, steadily rose in all environments (with the exception of sterile/dark without PL) and then began to degrade and became undetectable between days 14 and 28. Results indicate that by introducing the E1-d4 to more microbially robust communities, the transformation of E1 to E2 occurs at a faster rate. For example, the most robust microbial communities (PL with fish and PL without fish) experience the fastest transformation (E1 to E2) and degradation of estrogen species. The same process of transformation did occur in the environments without PL, but at a slower rate.

Deuterated estradiol was not detected at any time point in the sterile/dark community lacking PL, indicating a sufficient sterilization technique, eliminating the microbial community capable of estrogen transformation. In contrast, E2-d4 was detected in the sterile/dark environment containing PL. Because the environment lacking PL did not show signs of estrogen transformation, this indicates that the sterilization technique may not have been entirely effective for the PL treatment. Heating the glass carboys containing the aqueous solutions at 75 °C overnight may not have completely eradicated the bacterial community, allowing for some bacteria to remain active and cause transformation of E1. The addition of PL adds a naturally occurring bacterial community

that is more robust than an aqueous environment lacking the addition of PL. The sterilization technique utilized may have been effective at eradicating a portion of the microbial community, leaving another portion intact and able to convert estrogen species. If this project were to be repeated, autoclaving the glass carboys prior to and after the addition of the aqueous media would be a more adequate sterilization technique.

Estrogenicity data (BLYES) agreed with estrogen quantification data (LC-MS/MS) in all instances with the exception the sterile/dark environment containing PL. Within the environments lacking PL, estrogenicity amounts decreased (degraded) the fastest in the aquaria containing fish. With the addition of fish, a biological component, bacteria is being introduced to the community from the fish's natural activities. The earlier estrogenicity degradation is most likely a result of the fish's presence. A similar trend was observed in the environments containing aqueous PL. When PL and fish were present, BLYES results indicated estrogenicity levels below detection earlier than when fish were absent. Overall, it was observed that by introducing both PL and/or fish, estrogen transformation and degradation began to occur earlier in the exposure when compared to environments lacking PL and/or fish.

The amount of E1 that was introduced to the E1+d4 positive treatments was a quantity that wild fish populations would not normally encounter. Because of these elevated levels, it was expected that all fish within those treatments would be significantly induced for Vtg. Vitellogenin induction data (ELISA) was in agreement with what was expected. Interestingly, fish that were introduced to the environments at day seven (opposed to day 0) in all environments were not induced for Vtg. All fish that were introduced to E1-d4 positive environments at day 0 were significantly Vtg induced.

This indicates that the estrogen conversion and degradation process, under these conditions, most likely occurs in the first seven days post introduction of the E1-d4. This may indicate that wild aquatic species are most vulnerable to endocrine disruption (Vtg induction in male species) in the first days following a runoff event post land application of BS and PL. Both control treatments (with and without fish) had below detection values for all three analytical techniques (BLYES, LC-MS/MS and ELISA).

In conclusion, the second portion of this study provides evidence that the less potent estrogen species, E1 is capable of changing to the more potent E2 species as a result of entering microbially active communities. This realization becomes relevant when analyzing waste materials that are exiting WWTP as well as poultry broilers. Waste that is being utilized as fertilizer may have the ability to increase dramatically in estrogenic activity as a result of being introduced to the environment, especially receiving waters after run off events.

Appendix A

BLYES Protocol

Making Media

It is very important to maintain sterile technique throughout the BLYES process. The media is easily contaminated, which can result in skewed results. Final medial solution should not be stored longer than two weeks. Anything that the yeast touches must be soaked in a 10% bleach solution prior to being discarded.

Yeast Minimal Media (base medium)

Reagents:
50 mg – L-histidine
50 mg – adenine
20 mg – L-arginine-HCl
20 mg – L-methionine
30 mg – L-tyrosine
30 mg – L-isoleucine
30 mg – L-lysine-HCl
25 mg – L-phenylalanine
100 mg – L-glutamic acid
150 mg – L-valine
375 mg – L-serine
13.61 g – KH_2PO_4 (Potassium Phosphate (Monobasic Anhydrous))
1.98 g – $(NH_4)_2SO_4$ (Ammonium Sulfate)
4.20 g – KOH Pellets (Potassium Hydroxide)
0.20 g – $MgSO_4$ (Magnesium Sulfate)
1 ml – $FeSO_4$ solution (40mg/50ml water)
Methods: This may be done outside of biological hood.

1. Add 700 mL DI water to glass.
2. Add all above 16 reagents to the glass container and stir. NOTE: slight heat may be necessary to mix thoroughly.
3. Once thoroughly mixed, bring to 1L with DI water.
4. Autoclave using ‘Liquids setting- slow’
5. Wrap in parafilm to prevent contamination. Store in 4°C refrigerator.

Vitamin Solution

Vitamin solution is heat sensitive. Do not pour in final media while it is still hot.

Reagents:
20 mg – thiamine
20 mg – pyridoxine
20 mg – pantothenic acid
100 mg – inositol
50 mL – biotin solution (1 mg/50 mL H_2O)
Methods:

1. Add 400 mL DI water to glass container.
2. Add all of the above 5 reagents to the glass container and stir. NOTE: slight heat may be necessary to mix thoroughly.
3. Filter sterilize using a 0.2μm bottle-top filter into a sterile bottle. Filter sterilize in hood.
4. Wrap in parafilm. Store in 4°C refrigerator.

Glucose Solution

Reagents:
20 g – D-(+)-Glucose
Methods:

1. Add 80 mL DI water to glass container
2. Add 20 g D-(+)-Glucose to the glass container and stir. NOTE: slight heat may be necessary to mix thoroughly.
3. Bring to 100 mL
4. Autoclave using 'Liquids setting'
5. Store in 4°C refrigerator.

L-Aspartic Acid Solution

Reagents:
400 mg – L-Aspartic acid (Magnesium Salt)
Methods:

1. Add 100 mL DI water to glass container.
2. Add 400 mg aspartic acid and stir. NOTE: slight heat may be necessary to mix thoroughly.
3. Autoclave using 'Liquids setting'
4. Store in 4°C refrigerator.

L-Threonine Solution

Reagents:
1200 mg – threonine
Methods:

1. Add 50 mL DI water to glass container.
2. Add 1200 mg threonine and stir. NOTE: slight heat may be necessary to mix thoroughly.
3. Autoclave using 'Liquids setting'
4. Store in 4°C refrigerator.

Copper (II) Sulphate Solution

Reagents:
0.3745g – $CuSO_4$ (Copper (II) Sulfate)
Methods:

1. Add 50 mL DI water to glass container.
2. Add 0.3745g $CuSO_4$ to the glass container and stir. NOTE: slight heat may be necessary to mix thoroughly.
3. Filter sterilize using a 0.2µm bottle-top filter into a sterile bottle.
4. Store at room temperature.

Final growth YMM

Reagents
854.5 mL – Yeast minimal Media (base media)
10 mL – Vitamin solution from
100 mL – Glucose solution from
25 mL – L-aspartic acid solution
8 mL – L-threonine solution
2.5 mL – Copper (II) sulphate solution
Methods:

1. Place the 0.2µm bottle-top filter onto the glass bottle.
2. Add all 6 of the above reagents into the filter reservoir and filter through.

Growing Yeast

1. Remove BLYES aliquot from -80°C freezer from sterile glycerol freezer box. Thaw for approximately 5 minutes.
2. Sterilize all materials and interior of fume hood with isopropyl alcohol. Set aside when done.

3. Pour no less than 100 mL YMM into empty 250 mL bottle being careful not to put lids down, nor touching the insides of the lids. Replace lid of YMM and set aside.
4. Pipette the entire contents of the BLYES aliquot into the 250 mL bottle. Replace lid on 250 mL bottle.
5. Place 250 mL bottle into rotary shaker at 30°C and 150 RPM for **30-40 hours** prior to running assay.
6. Return YMM to 4°C refrigerator.

The used 2 mL pipette tip and empty 2 mL cryovial must be placed in 10% bleach solution prior to placing in biohazard waste container and discarded.

Running BLYES Assay

Methods:

1. Sterilize all materials and interior of fume hood with isopropyl alcohol. Set aside when done.
2. Pour 50 mL of the BLYES yeast from the growing yeast into each of the 2 50 mL conical tubes. Centrifuge the tubes at 2000RPM at 15°C for 10 minutes.
3. Remove from centrifuge, pour off supernatant.
4. Pour approximately 20 mL of YMM media into one conical tube, vortex and transfer 100 ul to sterile 96 well plate and check optical density. Optical density should be between .70 and .80. Dilute or reconstitute accordingly. Typically, two 50 mL conical tubes produces enough yeast to run 4 plates in one day.
5. Pipette 1 mL absolute methanol into a 50 mL conical tube.
6. Pipette (using the remaining 25 mL pipette tip) 19 mL YMM into the same 50 mL conical tube from Step 4. Steps 5&6 have created a 5% methanol growth media which will be used to make standards.
7. Preload standards with 5% methanol solution into the dilution plate according to the chart below. It is NOT necessary to discard tips, nor mix. Units are µL.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	270	150	240	150	150	150	150	150	150	150	150	200
B	270	150	240	150	150	150	150	150	150	150	150	200

8. Add 30 µL of the E2 standard into dilution plate wells A1 and B1 mixing 5x with the growth media. NOTE: Discard pipette tips after EACH well.
9. Using the 12 channel pipetter, move volumes from each dilution plate well according to the table below mixing 5x prior to the move and 10x after the move. Discard pipette tips after EACH transfer. Set mixing well aside when finished. **Do not move any contents from row 11 into row 12.**

	1	Move	2	Move	3	Move	4	Move	5	Move	6	Move	7	Move	8	Move	9	Move	10	Move	11	Move
A		150		60		150		150		150		150		150		150		150		150		0
B		150		60		150		150		150		150		150		150		150		150		0

10. Pour YMM media into a solution basin.
11. Pipette 95 µL YMM from solution basin (from Step 10) into 96-well black plate using the 12 channel pipetter. It is NOT necessary to discard tips, nor mix.
12. Pipette 5 µL of from sample vial into each well in triplicate. Tips must be changed between each well. Do NOT allow the lid of the 96-well black plate to touch anything. Do NOT transfer over the top of the 96-well black plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std2	Std3	Std4	Std5	Std6	Std7	Std8	Std9	Std10	Std11	Std12
B	Std1	Std2	Std3	Std4	Std5	Std6	Std7	Std8	Std9	Std10	Std11	Std12
C	s1	s1	s1	s2	s2	s2	s3	s3	s3	s4	s4	s4
D	s5	s5	S5	s6	s6	s6	s7	s7	s7	s8	s8	s8
E	s9	s9	s9	s10	s10	s10	s11	s11	s11	s12	s12	s12
F	s13	s13	s13	s14	s14	s14	s15	s15	s15	s16	s16	s16
G	s17	s17	s17	s18	s18	s18	s19	s19	s19	s20	s20	s20
H	s21	s21	s21	s22	s22	s22	s23	s23	s23	s24	s24	s24

13. Transfer 100 µL of standards from the dilution plate into the first 2 rows of the 96-well black plate using 50-300 µL 8 channel pipetter and 20-200 µL pipette tips. NOTE: Discard pipette tips after EACH well.
14. Transfer 100 µL BLYES media into each well of the 96-well black plate using the 50-300 µL 8 channel pipetter and 20-200 µL pipette tips. NOTE: It is NOT

necessary to discard tips after each transfer as long as the tips do not touch the liquid in the wells.

15. Place lid in completely on the 96-well black plate, place in rotary shaker for 4-6 hours at 30°C and 150 RPM.

BLYES Formula

A 2,000 fold concentration occurs as a result of extracting 1 L of sample, eluting and reconstituting in .5 mL of methanol. This is then followed by a 5 fold dilution during the BLYES assay when 5 µl of sample is diluted in 95 µl of YMM. Despite being diluted as a byproduct of the assay, many samples need further dilution due to high total estrogenicity levels. This is incorporated by multiplying by the factor by which the sample was diluted in order to be quantifiable. If the sample was not diluted, then a dilution is not integrated. The BLYES program (luminesce reader) has been programmed to report concentration in ng/mL so as a consequence, the final value must be multiplied by 1,000 to report in ng/L.

$$\frac{Concentration}{2{,}000}\ X\ 20\ X\ Dilution\ Factor\ X\ 1{,}000$$

Appendix B

Enzyme-Linked Immunosorbant Plasma Vitellogenin Assay (ELISA)

Buffers:

Dilution Buffer (PBSZ): Dissolve 1.604g trisodium phosphate, dodecahydrate (Sigma S-10010), 0.693g of monosodium phosphate, anhydrous (Sigma S-0751), 8.766g sodium chloride (S-9888) and 0.2g sodium azide (S-8032) in 900 mL 19MOhm water with gentle stirring. Once dissolved adjust to pH 7.6 with 1N HCl. (pH is already close to 7.6, so you'll only need a few drops to get there) Adjust final volume to 1L and store at 4 degrees C.

NOTE: To speed up pH adjustments in buffers **below**, 6N HCl may be used to pH adjust.

Wash Buffer (TBST): (Make 4L) Dissolve 4.844g Sigma 7-9 and 35.064g sodium chloride in 900 mL 18-MOhm water with gentle stirring. Once dissolved add 200 microliters tween-20 and adjust pH to 7.6 with 1N HCl. Adjust final volume to 4L and store at 4 degrees C.

Blocking Buffer: Dissolve 1.211g Sigma 7-9 and 8.766g sodium chloride in 900 mL 18-MOhm water with gentle stirring. Once dissolved add 10g bovine serum albumin (this takes a little time to dissolve) and 50 microliters Tween-20 and adjust pH to 7.6 with 1N HCl. Adjust final volume to 1L and store at 4 degrees C.

Carbonate Buffer: Dissolve 3.179g sodium carbonate and 0.19g magnesium chloride in 900 mL 18-MOhm water with gentle stirring. Adjust pH to 9.6 and adjust final volume to 1L. Store at 4 degrees C.

Procedure**:**

1. Allow 1 hour for plasma samples to defrost at room temperature. Less time is fine (allow at least half an hour).
2. Label plate templates. You will use two plates for dilution, the final plate is a different plate (96-well Easy Wash High Binding, non-sterile Costar 3369) which will be read by the plate reader.
3. Once plasma samples are defrosted, centrifuge @3000rpm for ~10min

	1	2	3	4	5	6	7	8	9	10	11	12
A	**STD 1**	**STD1**	**STD1**	**s1**	**s5**	**s9**	**s13**	**s17**	**s21**	**s25**	**s29**	**s33**
B	**STD 2**	**STD 2**	**STD 2**	**s1**	**s5**	**s9**	**s13**	**s17**	**s21**	**s25**	**s29**	**s33**
C	**STD 3**	**STD 3**	**STD 3**	**s2**	**s6**	**s10**	**s14**	**s18**	**s22**	**s26**	**s30**	**s34**
D	**STD 4**	**STD 4**	**STD 4**	**s2**	**s6**	**s10**	**s14**	**s18**	**s22**	**s26**	**s30**	**s34**
E	**STD 5**	**STD 5**	**STD 5**	**s3**	**s7**	**s11**	**s15**	**s19**	**s23**	**s27**	**s31**	**s35**
F	**STD 6**	**STD 6**	**STD 6**	**s3**	**s7**	**s11**	**s15**	**s19**	**s23**	**s27**	**s31**	**s35**
G	**STD 7**	**STD 7**	**STD 7**	**s4**	**s8**	**s12**	**s16**	**s20**	**s24**	**s28**	**s32**	**s36**
H	**STD 8**	**STD 8**	**STD 8**	**s4**	**s8**	**s12**	**s16**	**s20**	**s24**	**s28**	**s32**	**s36**

The first <u>three</u> <u>columns</u> of the plate are for STANDARDS. The following directions apply to the samples only, which begin on the fourth column. See the plate template for visual.

4. While plasma is in the centrifuge, prepare dilution plates. (Dilutions are based on amount of expected Vitellogenin in the plasma samples based on season and sex). Use 96-well Round Bottom Assay Plate (Costar 3360).
    a. Dilution Buffer is PBSZ. (*Store in a solution basin for convenience*)
    b. Dilutions
        i. 1:100 = 2µL plasma: 198µL dilution buffer *(first 96-well plate)*
        ii. 1:1000 = 20µL *1:100 dilution*: 180µL dilution buffer *(second 96-well plate – final dilution)*
        iii. 1:10000 = 2µL *1:100 dilution*: 198µL dilution buffer
        iv. 1:100000 = 20µL *1:10000 dilution*: 180µL dilution buffer

5. Prepare dilutions by adding the required amount of dilution buffer to sample wells.
    a. First dilution plate is 1:100 -- Add 198µL dilution buffer to each sample well.
    b. Second dilution plate is 1:1000 – Add 180 µL dilution buffer to each sample well.

6. Add plasma to the first dilution plate. Be sure to mix samples by pipetting up and down (to the FIRST stop) 10x.
    a. The first dilution plate is 1:100 -- Add 2µL of plasma sample to each sample well.
    b. Be sure to pipette from the *middle* of the sample – you do not want the material from the bottom
7. After finishing the first dilution plate, add the required amount of the first plasma dilution to the second dilution plate.
a. Add 20µL from each well on the first dilution plate to each corresponding well on the second dilution plate.
b. Mix 10x times before and after transferring samples.

The following directions are for the preparation of the standards on the second dilution plate. The standards are prepared as dilutions that go from all standard to all dilution buffer. (STD2 = row 2, STD3 = row 3)

8. In the standard wells (first three columns) on the second dilution plate, add 100µL dilution buffer to rows 2-8. The first row (STD1) will ONLY have standard. The eighth row (STD8) will ONLY have dilution buffer.

9. Prepare Standard in a 25 mL centrifuge tube
    a. Dilution of SMB Vtg = 1µL SMB Vtg : 2,199µL dilution buffer *Heather put in 2200 µL buffer then removed 1µL
    b. Dilution of LMB Vtg = 1µL LMB Vtg : 1,199µL dilution buffer
    c. Dilution of FHM Vtg = 1µL FHM Vtg : 1,259µL dilution buffer
After creating standard, vortex to homogenize before adding it to the plate.
10. Add 100µL of diluted Vtg to STD2 wells of the dilution plate. Only insert the smallest section of the tip into the dilution buffer when adding standard. Make sure there are no remaining drips of standard on the tip. Replace tips before mixing. This will increase standard accuracy.
11. Prepare 2-fold dilution of standards by pipetting 100µL of STD2 into STD3. Again, do not insert tips into dilution buffer when *adding* STD2 to STD3 dilution buffer and just insert tip at the surface. Replace tip before mixing STD3.
12. Repeat this step for remaining rows except STD8, which is all dilution buffer.

Preparation of the final plate (96-well Easy Wash High Binding, non-sterile Costar 3369). DO NOT touch the bottom of the VTG plate. That is where the plate is read.
13. In the final plate, add 50µL of the diluted Vtg standard to STD1 wells. Remember STD1 is ALL standard.
14. Add 50µL of each standard and diluted sample from the second dilution plate to the corresponding wells on the final plate.
15. Cover plates with a plate seal and incubate in 4 degrees C refrigerator overnight. (12-18 hours)
16. The next morning, remove block buffer and carbonate buffer from fridge and allow 1 hour to come to room temperature.

For each of the following steps, you can use same pipette tips for the entire plate, just be careful not to touch the sample or touch the tips to the sides of the walls

17. Wash plates in plate washer 5 times. (SkanWasher 400)
18. Add 300µL blocking buffer to plates
19. Incubate at RT for 1 hour
20. 5 minutes prior to end of incubation, prepare primary monoclonal antibody (mAb) [Mouse anti-striped bass VTG Monoclonal antibody à ND-3G2]
    a. 5 µL Striped bass mAB: 4995 µL block buffer (1:1000). Be careful when mixing the dilution because this gets foamy – swirl up and down gently.
    b. 5 µL Carp mAB: 4995 µL blocking buffer (1:1000).
21. Wash plates 5x in plate washer
22. Add 50 µL mAb to plates
23. Incubate at RT for 1 hour
24. 5 min prior to the end of incubation, prepare secondary antibody. [Biotin-SP-conjugate Affinipure Goat anti-mouse à Code: 115-065-003, LOT: 108597, 2.0mL]
    a. 5 µL 2Ab: 4995 µL blocking buffer (1:1000)
    b. if 2Ab is stored in glycerol, 10µL 2Ab: 4990 blocking buffer
25. Wash plates 5x in plate washer
26. Add 50 µL 2Ab to plates
27. Incubate at RT for 1 hour
28. 5 min prior to the end of incubation, prepare streptavidin-alkaline phosphatase (S-AP) [Streptavidin Alkaline Phosphate conjugate à Ref: SA1008, LOT: 894794A]
    a. 1 µL S-AP: 4999 µL blocking buffer (1:5000)
29. Wash plates 5x in plate washer
30. Add 50 µL S-AP to plates
31. Incubate at RT for 30 minutes
32. Take the developer out of the freezer to thaw in the dark. Turn on plate reader (Spectramax M2e) to allow it to warm up.
33. 5 min prior to the end of incubation, prepare developer (100x 4-nitrophenyl phosphate 100mg/mL)
    a. 50 µL 100x N-P: 4950 µL carbonate buffer (1:100). Keep buffer in dark. Not as sensitive to shaking.
34. Wash plates 5x in plate washer
35. Add 50 µL developer to plates. Use different tips to load developer into wells. If you forget to use new tips, continue on. It does not seem to make a huge difference, however CVs may decrease with new tips.
36. Incubate at RT for 30 minutes. – Put under tin foil. Developer is light sensitive.
37. Read plate on microplate reader using Vtg protocol.

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