ABSTRACT

Title of Document: EFFECTS OF ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF POULTRY LITTER ASSOCIATED CONTAMINATES ON THE SEXUAL DEVELOPMENT OF *XENOPUS LAEVIS*

Amy X. Chen, Alma C. Gonzales, David Hu, Richa Kalsi, Tanya S. Kapoor, Hae Min Park, Samuel C. Park, Alexander M. Proctor, Alexander K. Ridgway, Andrew M. Taverner, and Nikola B. Vujcic

Directed By: Faculty Research Assistant, Steven D. Turley, College of Agriculture and Natural Resources

Poultry litter contains high levels of natural sex hormones, nitrogen, phosphorous, and trace amounts of heavy metals. Poultry litter runoff from poultry and farming operations in the Delmarva region can have serious impacts on frog development in the Chesapeake Bay Watershed. In this study, we investigated potential effects of litter compounds on *Xenopus laevis* development when exposed to environmental levels (0.35 and 0.70 g/L) of litter solution. We found that despite rapid hormone degradation, poultry litter solution still affected *X. laevis* development. Hormones were also more persistent in the lower poultry litter concentration, leading to even greater effects. Slowed growth and increased female gonadal abnormalities were observed after exposure to 0.35 g/L but not to 0.70 g/L of litter solution, and increased male gonadal abnormalities were observed after treatment to both litter concentrations. The developmental impacts examined in this study may have greater environmental impacts on frog reproduction and survival.

EFFECTS OF ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF POULTRY LITTER ASSOCIATED CONTAMINATES ON THE SEXUAL DEVELOPMENT OF *XENOPUS LAEVIS*

By

Team KERMIT

Amy X. Chen, Alma C. Gonzales, David Hu, Richa Kalsi, Tanya S. Kapoor, Hae Min Park, Samuel C. Park, Alexander M. Proctor, Alexander K. Ridgway, Andrew M. Taverner, and Nikola B. Vujcic

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Advisory Committee:
Mr. Steven D. Turley, Mentor
Dr. Dennis T. Burton
Dr. Daniel J. Fisher
Dr. Pamela J. Lanford
Dr. Mary Ann Ottinger
Ms. Marybeth Shea
Dr. Lance T. Yonkos

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Chapter 1: Introduction

1.1 Motivation

The Delaware-Maryland-Virginia (Delmarva) (glossary) area is one of the leading poultry producing regions in the United States. Increasing costs of synthetic fertilizers and the large surplus of poultry litter—chicken waste mixed with bedding—have resulted in the increased use of poultry litter (glossary) as fertilizer in agricultural lands (Dutta, Inamder, Sims, & Collins, 2010). Poultry litter is an excellent natural fertilizer, containing high concentrations of nitrogen, phosphorous and potassium. It provides organic matter, which improves soil structure and nutrient and water retention (He, Endale, Schomberg, & Jenkins, 2009). However, poultry litter also contains high levels of endocrine disrupting chemicals (EDCs) (glossary), which include natural sex hormones such as estradiol (glossary), estrone (glossary) and estriol (glossary), and trace amounts of heavy metals, such as lead, copper and arsenic (He et al., 2009).

Although much attention has focused on anthropogenic EDCs, such as pesticides, herbicides, PCBs (glossary), dioxins (glossary) and alkyl-phenols (glossary), less attention and research have been directed toward natural hormones in surface waters. The environmental load of natural hormones can potentially have a serious impact on aquatic organisms. The two main sources of hormone contamination include runoff from soil amended with animal manures, including poultry litter, and municipal wastewater effluents. Shore, Harel-Markowitz, Gurevich, and Shemesh (1993) observed greater hormone concentrations in streams receiving

runoff from poultry litter amended farms than those receiving effluent from sewage treatment plants.

Over the last decade, research has indicated that amphibians are particularly sensitive to EDCs (MacKenzie, Berrill, Metcalfe, & Pauli, 2003). The majority of the amphibian research has examined the effects of exogenous EDCs like herbicides and pesticides, manufacturing chemicals, plastic products such as bisphenol A (glossary), and surfactants (glossary), such as octylphenol (glossary), and nonylphenol (glossary). Most hormone research with amphibians has been performed with pure chemicals such as estradiol. Very little amphibian research has been conducted on the complex mixture of natural hormones, nutrients, and heavy metals associated with poultry litter runoff. These components will hereafter be referred to as poultry litter associated contaminants (PLAC) (glossary).

Our research was initiated to fill the voids in current EDC research. We analyzed the potential synergistic effects of PLAC on *X. laevis* development using environmentally relevant poultry litter concentrations. Our study also used only one initial dose of poultry litter solution, which was allowed to degrade to simulate a natural runoff event. Most studies re-dose continually with fresh exposure solution, creating an artificially high concentration of active chemicals. As the poultry and agriculture industry are essential and deeply rooted in the Chesapeake Bay (glossary) area, it is necessary to examine and understand the potential implications of poultry litter usage on the Bay and its inhabitants.

1.2 State of the Poultry Industry

Chicken is the number one source of meat consumed by Americans, with the 45 billion dollar broiler rearing industry producing upwards of 16.7 million kg of poultry per year (American Meat Institute, 2011; United States Department of Agriculture (USDA), 2010a; USDA, 2010b). Compared to other livestock, poultry has the highest production densities (Kellogg, Lander, Moffitt & Gollehon, 2000 as cited in PEW Environmental Group, 2011). Over the past 50 years, the poultry industry has transformed dramatically, shifting from traditional farms to large-scale concentrated feeding operations (CAFOs) that focus on efficiency and economic production. Today, the typical CAFO can produce approximately 605,000 birds per year (MacDonald, 2008 as cited in PEW Environmental Group, 2011). The number of chickens produced annually has increased by 1,400 percent from 581 million birds in 1950 to 8.9 billion birds in 2007 (The PEW Environmental Group, 2011). Conversely, the number of broiler farms have decreased by 98 percent from 1.6 million in 1950 to only 27,000 in 2007 (State-EPA Nutrient Innovations Task Group, 2009 as cited in The PEW Environmental Group, 2011).

Increased poultry demand and production has led to increased poultry waste production. Poultry operations generally dispose of litter waste by spreading it on open fields or croplands as its nutrients can be beneficial to plant growth (PEW Environmental Group, 2011). According to the USDA, poultry manure generally contains two to four times more nutrients compared to other livestock manures (Gollehon et al., 2001 as cited in The PEW Environmental Group, 2011). However, poultry litter management can be particularly challenging as excess nutrients and

heavy metal additives can travel from poultry feed to poultry litter and run off into local streams, rivers, and other bodies of water. Fields fertilized with manure produce twice as much nitrogen runoff and higher phosphorous runoff compared to non-manure fertilized fields (USDA, 2011). In fact, the United States Environmental Protection Agency (USEPA) (2010a) estimated that 19 percent of excess nitrogen and 26 percent of excess phosphorous in runoff were derived from animal manure. USDA researchers also found farms produce 690 million kilograms of excess nutrients, with more than half coming from poultry (Kellogg, et al., 2000 as cited in The PEW Environmental Group, 2011). Runoff and surface waters receiving agricultural runoff have also been found to contain significant concentrations of estradiol (Dutta, Inamdar, Tso, Aga & Sims, 2010). Estradiol is an endocrine disrupting compound (EDC) that may potentially induce feminization (glossary) in aquatic organisms. The concoction of nutrient and hormonal runoff solution can significantly alter the environment and impact wildlife that depend on it for survival.

1.3 Current State of Amphibians Globally

In 2004, a global assessment found that amphibian populations have been more threatened and have declined faster than that of either bird or mammal populations (Stuart et al., 2004). While the majority of declines have been due to habitat loss and overutilization, 48% of the declines were from other causes (Stuart et al., 2004). Houlahan et al. (2000) also found that 61 of the 936 amphibian populations surveyed were extinct and declines were greatest in North America, Australia, and New Zealand. One possible explanation for the decline is exposure to endocrine disruptors such as estradiol, a prevalent hormone in poultry litter, which can induce

intersex (glossary) and mixed sex (glossary) characteristics, inhibit reproduction, and affect population growth. Hu, Smith, and Carr (2008) linked estradiol with increased female sex ratios (glossary) in *Xenopus laevis* (African clawed frog) (glossary).

1.4 State of the Chesapeake Bay

On a more local scale, poultry production and amphibian health may be particularly problematic in areas surrounding the Chesapeake Bay. The Delmarva region surrounding the Chesapeake Bay is one of the top poultry producing areas in the United States (The PEW Environmental Group, 2011). In 2009, Delaware and Maryland alone produced 523.4 million broilers, roughly six percent of the total poultry produced nationwide on less than 0.5 percent of its landmass (The Delmarva Poultry Industry, 2009 as cited in The PEW Environmental Group, 2011). According to Gang (2000), the two states together generated 1.2 million cubic meters of litter, enough to fill an Olympic sized swimming pool 477 times. Agriculture, excluding poultry rearing, also provides a major source of economic revenue for the region. Agriculture is the largest commercial industry along the Eastern Shore of the Delmarva region (Maryland State Archives, 2013). Unfortunately, much of the poultry production and farming activity occur along areas surrounding a key water source: the Chesapeake Bay.

The Chesapeake Bay is the largest estuary in the United States, spanning from New York to Virginia, and has a watershed of 166,000 km^2 (CBF, n.d.). It provides a unique ecosystem to a variety of terrestrial and aquatic wildlife, housing more than 3,700 plants and animals (Chesapeake Bay Program, 2007). With the growing population, agricultural development, and sewage wastewater discharge, the Bay has

become increasingly polluted with excess nutrients and chemicals. Land application of animal waste is responsible for about one quarter of the total nutrient pollution affecting the watershed (Land, 2012). In the 1970s, the USEPA enacted a series of programs and regulations targeted at mitigating and preventing pollution, habitat destruction, and wildlife decline. However, according to the 2012 State of the Bay report, pollution remains one of the key issues facing the health of the Chesapeake Bay, with nitrogen levels, phosphorous levels, water clarity, and toxin levels being the main concerns (CBF 2012).

Pollution may have caused the decline in health of organisms that call the Bay their home. For instance, Blazer et al. (2005 as cited in Team FISH, 2010) correlated human land use with increased rates of intersex and testicular oocytes (glossary) in bass. Hormones such as estradiol may be a contributing cause of the problem. Yonkos, Fisher, and Van Veld (2005) detected estradiol in Maryland Eastern Shore streams and rivers. While most research to-date has focused on the health of fish populations, the health of amphibian populations has not been as thoroughly documented. Amphibians are particularly sensitive to endocrine disruptors and may also be threatened by exposure to estradiol and other poultry litter compounds.

According to the Chesapeake Bay Program (2007), "The Bay has sustained the region's economy and defined its traditions and culture since Captain John Smith sailed its waters 400 years ago." However, the Bay has been ravaged by human activity and active measures must be taken to reduce and prevent further degradation, and restore it to a more pristine state. The first step towards improvement is to gain a

better understanding of the potential implications of poultry litter usage on the Bay and its ecosystem.

1.5 Current State of Poultry Litter and EDC Research

Pesticides, herbicides, PCBs, dioxins, alkyl-phenols, and other anthropogenic EDCs have been a primary focus of research, while natural hormones in surface waters have not been as thoroughly studied. The environmental load of natural hormones (glossary), hormones that are released from animals, can potentially have a serious impact on aquatic organisms. Most of the laboratory and field studies evaluating the endocrine disrupting potential of poultry litter associated hormones have involved fish. The presence of the egg yolk protein precursor, vitellogenin (glossary), in males is a robust indicator of an exposure to an estrogenic stimulus and has been shown to be predictive of subsequent reproductive and histopathological (glossary) effects (Colborn, vom Saal, & Soto, 1993; Tyler, Jobling & Sumpter, 1999).

Lahnsteiner, Berger, Kletzl, and Weismann (2006) reported that estradiol concentrations of 1 ng/L drastically reduced sperm fertility and induced vitellogenin in male rainbow trout. Seki, Yokota, Maeda, and Kobayashi (2005) reported that exposure to 8.7 ng/L of estradiol caused intersex in medaka (*Oryzias latipes*). Routledge et al. (1998) demonstrated that estrone and estradiol exposure led to the demasculinization (glossary) of male rainbow trout (*Oncorhyncus mykiss*) at a 25 ng/L concentration.

Over the last decade, research has indicated that amphibians are particularly sensitive to EDCs (MacKenzie et al., 2003). The majority of the amphibian research

has examined the effects of exogenous EDCs like herbicides and pesticides, manufacturing chemicals, plastic products such as bisphenol A, and surfactants, such as octylphenol, and nonylphenol (glossary) (Colborn et al., 1993; Hayes et al., 2002; Huang, Matthews, Fertuck, & Zacharewski, 2005; Levy, Lutz, Kruger, & Kloas, 2004; Mackenzie et al., 2003). In many of these studies, estradiol was used as a positive control, causing gonadal abnormalities and altered sex ratios in leopard frogs (*Rana pipiens*) at concentrations as low as 1 ng/L. Wolf et al. (2010) performed the most comprehensive study on the effects of estradiol exposure on amphibian gonadal development. The group found that estradiol may significantly alter sex ratios and cause gonadal abnormalities in *X. laevis*; however, like most estradiol-*X. laevis* research, Wolf et al. focused on studying the effects of pure estradiol. Very little amphibian research has been conducted on PLAC.

1.6 Research Question and Objectives

The present study sought to investigate the effects of environmentally relevant concentrations of simulated poultry litter runoff on the gonadal development of *X. laevis*. The objectives were to determine potential correlations between exposure to poultry litter runoff during early developmental stages and (a) the presence of intersex or mixed sex gonads, (b) the presence external deformations, (c) male to female sex ratios, (d) mortality rates, (e) snout-vent length (glossary), and (f) wet weight (glossary) of *X. laevis.* Additionally, the present study analyzed the natural composition and decomposition of poultry litter, focusing specifically on estradiol, estradiol's degradates, nitrogen, phosphorous, and heavy metal compound levels over the duration of the test period.

1.7 Outline of Research

In the present study *X. laevis* tadpoles were exposed to (a) a high poultry litter concentration hereafter referred to as the high PLAC exposure, (b) a low poultry litter concentration hereafter referred to as the low PLAC exposure, (c) a positive control of pure estradiol, and (d) a negative control of well-water for 120 days. Afterwards, the juvenile frogs (glossary) were allowed to grow for an additional 20 days. Post exposure, the juvenile test subjects were sacrificed and gonadal tissues were examined microscopically for abnormalities. Water quality analysis was also performed and limb and growth abnormalities were recorded. The poultry litter solution was allowed to degrade over the 120-day period to mimic natural conditions, and the solution composition was monitored and analyzed.

Chapter 2: Literature Review

2.1 Poultry Litter Usage

2.1.1 Poultry Litter Background

Chickens generate approximately 726 million kilograms of poultry litter per year (Yonkos, 2005; Lange et al., 2002 as cited in Hanselman, Graetz, & Wilkie, 2003). The term poultry litter is used to describe the composite mixture of waste that is collected at the base of chicken houses, consisting of chicken excrement and bedding material, typically sawdust, wood shavings, or rice hulls (Mitchell & Donald, 1995; Ritz & Merka, 2009; Fulhage, 2006; Kelleher et al., 2002; Maryland Department of the Environment (MDE), 2009). Poultry litter can also contain feathers, beaks, and excess animal feed that falls into the bedding (Mitchell & Donald, 1995; Kelleher et al., 2002). According to Mitchell and Donald (1995), 0.5 to 0.7 kilograms of litter are produced per kilogram of poultry market weight. Per flock of poultry, the average amount of poultry litter produced ranges from 0.6 to 1.8 metric tons (Bolan et al., 2010).

Poultry litter is removed after five or six flocks of broilers, which occurs about once a year (Moore, Daniel, Sharpley & Wood, 1995). Two types of poultry litter that are often used for fertilization are broiler litter and caged layer manure (glossary) (Mitchell & Donald, 1995). Broiler litter contains more of the bedding material than caged layer manure, which typically has higher moisture content (Mitchell & Donald, 1995). According to Mitchell and Donald (1995), the moisture content of the litter is the most important characteristic to consider when it is applied. Higher moisture

content can drastically retard the decomposition rate and reduce the tendency of the poultry litter to stabilize (Kelleher et al., 2002). Moisture content in manure averages 70 to 77 percent weight when it is excreted, but moisture content of poultry litter that has dried in the bedding surface only averages to 20 percent of the weight (Mitchell & Donald, 1995). The amount of moisture in poultry litter is also directly correlated to the amount of nitrogen released as a form of ammonia, a volatile compound that contributes greatly to pollution (USDA, 2012).

Disposal of poultry litter is handled in one of three different ways. The methods of disposal are composting, anaerobic digestion and direct combustion (glossary) (Kelleher et al., 2002). Prior to application, poultry litter is treated by allowing it to compost in storage such that the decomposition of nutrients can take place (Moore, et al., 1995; Bolan et al., 2010). Composting poultry litter as a fertilizer is the most utilizable method of disposal because it provides beneficial nutrients and organic matter that improve the quality of cropland soil (Harmel & Patterson, 2008). Land application of poultry litter is beneficial for plant growth because of its high nutrient content (Gupta & Charles, 1999; Harmel et al., 2008). Anaerobic digestion (glossary) of poultry litter involves the breakdown of the organic matter within the waste by microbial organisms, leading to the formation of methane gas, carbon dioxide, and other inorganic byproducts that can be used as fuel for boilers in place of natural gas or fuel oil (Kelleher et al, 2002). Direct combustion of poultry litter also has the potential to be used as a fuel for heat and energy generation if the waste is burned in a combustion facility with sophisticated gas cleanup technology (Kelleher et al., 2002).

Best management practices state that the timing of poultry litter application should be during the active growth of the crop, or right before planting (Bolan et al., 2010). This means that poultry litter application takes place primarily during the spring and summer months of the year. By adhering to this schedule, the production of crops will be maximized (Bolan et al., 2010).

Poultry litter acquisitions and applications are regionally related, and typically the application occurs within 10 to 20 km from the production site (Moore, et al., 1995). This is because it is more difficult to transport poultry litter in comparison with chemical fertilizers (Chesapeake Bay Foundation (CBF), 2012). Land application amounts are regulated by each state and farmers are required to restrict application rates based on a Nutrient Management Plan (NMP) (glossary) that they develop. NMPs are meant to audit the amount of nutrients that are introduced for fertilization purposes. For the state of Maryland, the requirements of an acceptable NMP are defined in the MDE Code of Maryland Regulations (COMAR) chapter 15.20.07 and 15.20.08 (MDE, 2009). Although forcing farmers to draft nutrient management plans is ultimately intended to reduce water pollution, the application rates of poultry litter and other fertilizers is determined by the agronomic requirement of each crop, and does not directly consider water quality concerns (Land, 2012).

During heavy rainfall after poultry litter application, nutrients and hormones in poultry litter are washed into major bodies of water, distorting the normal hormone balance (Moore et al., 1995; Lange et al. 2002). Moreover, excessive application of poultry litter on fields can lead to increased levels of hormones, nitrogen, phosphorous, pathogenic microorganisms (glossary), and heavy metals seeping into

the land and eventually into groundwater and downstream surface water (Moore et al., 1995). Several studies have shown that the increase in hormone levels in water has significantly affected the reproductive systems of aquatic organisms (Kolpin et al., 2002). Because poultry litter has been shown to contain high levels of 17β-estradiol (hereafter referred to as estradiol) and is one of the most commonly used agricultural fertilizers, it is crucial to study the effects of poultry litter on aquatic wildlife.

2.1.2 Benefits of Poultry Litter Use in the Delmarva Region.

Economic Feasibility. Poultry litter is a highly economical alternative to synthetic fertilizers. Synthetic fertilizers are manufactured using ammonium sulfate and ammonium phosphate, which are derived from natural gas (Haber-Bosch, 2013). Over the last two decades, natural gas prices have been quite volatile, reaching record highs and lows (Price, 2012). This instability in the fossil fuel market translates directly to variable production costs for synthetic fertilizers. The cost of poultry litter remains relatively stable, especially in the Delmarva region. Furthermore, the Delmarva region is the nation's leading supplier of poultry litter, providing over 726 million kilograms a year (Yonkos et al., 2005; Lange et al., 2002 as cited in Hanselman et al., 2003). Unrivaled poultry litter production rates coupled with difficulty in long-distance transportation result in one of the most used fertilizers in the Delmarva region.

Nutrient Quality. Poultry litter is a rich source of essential macronutrients, including nitrogen, phosphorus, and potassium, typically in a 3-3-2 ratio (Gaskin, Harris, Franzluebbers & Andrae, 2009). One metric ton of poultry litter contains 30

kg of nitrogen, 30 kg of phosphorous, and 15 kg of potassium (Funderburg, 2009). The use of poultry litter is advantageous to that of synthetic fertilizer due to the slow conversion of nitrogen from its organic to inorganic form. Directly upon application, nearly 90% of the nitrogen in the litter is organic and unusable by plants. The remaining 10% is composed of inorganic nitrate and ammonium (Gaskin et al., 2009). While plants uptake the inorganic nitrogen, microorganisms in the soil slowly convert the organic nitrogen to inorganic forms. This gradual conversion ensures an even nutrient distribution throughout the season (Gaskin et al., 2009). Poultry litter also contains a variety of micronutrients such as copper and zinc, which are beneficial to crop growth in small amounts (Gaskin et al., 2009).

Soil Conditioning. Poultry litter's composition is a major contributor to its soil conditioning properties and leads to large increases in crop yields when compared to synthetic fertilizers (Comis, 2010b). The carbon from poultry litter degrades in the soil, improving overall quality and water retention ability (Causarano, Franzluebbers, Reeves & Shaw, 2006). Causarano et al., (2006) also noted that previous researchers have established that poultry litter can increase carbon content in the ground by almost threefold. This drastic rise in organic supplementation accounts for large boosts in crop yields and may reduce the length of fallow periods (Causarano, et al., 2006). In a similar 2010 study from Mississippi State University, researchers discovered that poultry litter increased cotton output by 12% over synthetic fertilizers (Comis, 2010b).

2.1.3 Chicken Feed Makeup, Benefits, and Issues.

General animal feed practices have changed considerably in the past 60 years as large-scale operations have supplanted small family-owned and -operated farms (Sapkota, Lefferts, McKenzie, & Walker, 2007). To improve the efficiency of poultry growth and laying capacity, prevent disease, and improve feed-conversion rates, poultry feed is supplemented with a variety of ingredients including antimicrobials (glossary), metal compounds, antioxidants, emulsifiers (glossary), binders, pH control agents, and enzymes. These supplementary ingredients may release biological, chemical, and other etiologic agents that may decrease the quality and safety of the food and negatively impact environmental and human health.

Antimicrobials (which include antibiotics) are common feed additives used to help prevent disease and improve growth and feed utilization (Australian Chicken Meat Federation, n.d.). Low dose antibiotics are also common additives used in chicken feed. Antimicrobial growth promoters (AGPs) (glossary) first became popular in the mid-1950s when it was discovered that antibiotics such as procaine penicillin and tetracycline given in subtherapeutic doses (<50mg antibiotic per kg feed) in animal feed could improve the feed-to-weight ratio for poultry, swine, and beef cattle (Marshall & Levy, 2011; Kelley et al., 1998; Banerjee, 2010). In the U.S., it is estimated that the nontherapeutic use of antibiotics is equal to or as much as eight times the quantity administered for therapeutic purposes (WHO, 1997; FDA, 2009 as cited by Marshall & Levy, 2011).

This practice was used without issue for years, but has come under fire due to health concerns. Microbiologists and infectious diseases experts found that farm

workers and animals at farms using AGPs had more resistant bacteria in their intestinal flora than those at farms that not using AGPs (Levy, Fitzgerald & Macone, 1976 as cited in Marshall & Levy, 2011). Studies over three decades continued to confirm a quantitative and qualitative relationship between the use of AGPs in feed and the mounting problem of drug resistant bacterial infections in humans (Marshall & Levy, 2011).

The majority of studies done in this area have focused on genera of bacteria that are normal gut flora, and it has been found that all of these are capable of producing pathogens dangerous to humans after AGP treatment of feed (Marshall & Levy 2011). An important study in 2007 dealt with gentamicin (glossary), which is not approved for AGP use in the US, but is the most popular antibiotic used for broiler chicken (glossary) production (Marshall & Levy 2011). Gentamicin is used to prevent early chicken mortality. It has been found that poultry workers were 32 times more likely to carry gentamicin resistant *E. coli* than non-poultry workers (Price et al, 2007 as cited in Marshall & Levy, 2011). This occupationally exposed population was also at a significantly higher risk for carrying multi-drug resistant bacteria (Price et al, 2007 as cited in Marshall & Levy, 2011).

The use of low dose antibiotics for a large number of animals in concentrated animal feeding operations (CAFOs) (glossary) serves to increase the selection density of the animals producing resistant bacteria (Marshall & Levy, 2011). This leads to an ecological imbalance favoring the selection for resistance genes. Animals that are fed low-dose antibiotics produce feces that carry resistant microbes which then spread resistance in the animal's native environment due to phenomena such as gene linkage

on bacterial plasmids and transposons (Marshall & Levy, 2011). Scientists estimate that 75% to 90% of antibiotics used in food animals are excreted unmetabolized directly into the environment (Marshall & Levy, 2011). This all leads to the spread of resistance to local microbiota (Kelley et al., 1998).

The resistance conferred by the AGPs in the feed is not limited to the AGPs given to the animals. It can also lead to multi-drug resistance, which means that resistance is developed to antibiotics that were never introduced into the feed (Marshall & Levy, 2011). This poses a great risk to human and animal health, and as a result, recommendations are in place by the US Department of Health and Human Services, and the FDA Center for Veterinary Medicine such as the *Guidance for Industry: The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals*, and the *Judicious Use of Antimicrobials for Poultry Veterinarians*. These final guidance documents have the goal of eliminating the use of antimicrobial drugs for growth promotion purposes.

Antibiotics added at nontherapeutic levels have increased the antibiotic resistance in both commensal and pathogenic bacteria in the animals, animal-based food products, and water, air, and soil samples collected near animal feeding operations (Aarestrup et al., 2000; Bager et al., 1997; Gorbach, 200; Wegener, 2003; Hayes et al., 2003; White et al., 2001; Chapin et al., 2005; Chee-Sanford et al., 2001; Gibbs et al., 2006; Jensen et al., 2002 as cited in Sapkota et al., 2007). It is believed that 60-80% of all antibiotics produced in the United States are used in feed, and many of the antibiotics are similar to those administered to humans in clinical trial settings (Mellon et al., 2001; 2007; FDA, 2004 as cited in Sapkota et al., 2007). The

antibiotic-resistant bacteria can be transferred up the food chain and can potentially affect human health. *Enterococcus faecium*, a strain of antibiotic-resistant bacteria derived from poultry, can be isolated in human feces up to two weeks post ingestion (Sorensen et al., 2001 as cited in Sapkota et al., 2007). Prior to the FDA's approval of fluoroquinolone (glossary) use in poultry feed in 1995, no fluoroquinolone-resistant bacteria (*Campylobacter jejuni)* were detected in poultry or human stools. In 1992, only 1.3% of stool contained *C. jejuni.* Following fluoroquinolone's approval in 1995, the rate has increased to 10.2% in 1998 (Smith et al., 1999 as cited in Sapkota et al., 2007).

In addition to antibiotics, metal compounds such as Roxarsone (4-hydroxy-3-nitrobenzenearsenic-acid) (glossary)—composed primarily of arsenic bonded to carbon atoms—are also added to feed to improve feed efficiency and promote chicken growth (Chapman & Johnson, 2002 as cited in Sapkota et al., 2007). Chapman & Johnson (2002 as cited in Sapkota et al., 2007) noted Roxarsone is added in concentrations of 22.7 g/ton to 45.4 g/ton. When combined with ionophores (glossary), Roxarsone can also act as a co-coccidiostat to control intestinal parasites (Chapman & Johnson, 2002 as cited in Sapkota et al., 2007). When ingested, Roxarsone degrades into inorganic arsenite (As^{III}) and inorganic arsenate (As^{V}) in animal digestive tracts and animal waste (Arai et al., 2003 as cited in Sapkota et al., 2007; Stolz et al., 2007 as cited in Sapkota et al., 2007). As^{III} and As^{V} are classified as human carcinogens, as chronic exposures through ingestion have resulted in skin, lung, bladder, and prostate cancers, as well as hypertensive heart disease and nephritis

(World Health Organization, 2001 as cited in Sapkota et al., 2007; U.S. EPA, 1998 as cited in Sapkota et al., 2007).

In a study in 2006, arsenic — commonly used as rat poison — was detected in 55 percent of chicken from grocery stores and 100 percent of chicken from fast food restaurants. Exposure to trace amounts can cause cancer and has been linked to cardiovascular disease and diabetes (Brogan, 2013). Such diseases are more likely to affect African Americans and Hispanic Americans, populations that rely more on poultry for their protein intake (Food and Water Watch, 2010a).

Arsenic fed to chickens also ends up in poultry litter. Estimates state that 10,000 kg of arsenic each year is added to farms and ultimately washes into the Chesapeake Bay (Food and Water Watch, 2010a). A study commissioned by the Maryland House of Delegates found that arsenic does not degrade in soils and concluded that the practice of using arsenic in chicken feed is unsustainable (Brogan, 2013).

The poultry industry, however, believes that Roxarsone is safe to use. In fact, Tyson Foods and Perdue — prominent poultry companies — only stopped using Roxarsone in 2004 and 2007, respectively, under public pressure. Poultry farmers use the drug to treat coccidiosis (glossary), a disease that causes anemia and diarrhea in poultry. The FDA approved Roxarsone use in 1944, but poultry farmers increased their use after discovering that Roxarsone helps chickens gain weight faster and improves flesh color. As stocking densities increased in poultry farms and poultry diseases spread quickly, feeding all chickens Roxarsone became standard practice. The FDA has not reevaluated its initial approval even though new studies on

Roxarsone's effects have been conducted (Food and Water Watch, 2010a).Despite legislation introduced in 2010, arsenic-based additives have not been banned nationally (Food and Water Watch, 2010a). In 2013, Maryland became the first state to ban arsenic-based additives on its farms (Brogan, 2013).

In addition to Roxarsone, other metallic compounds mixed such as copper, manganese, magnesium, zinc, and selenium are mixed into feed for their nutritional benefits (Scheideler, 2008; Sapkota et al., 2007). For instance, zinc is essential for eggshell formation and assists in immune system functionality (Scheideler, 2008). Manganese is also essential for eggshell formation and perosis (glossary) prevention (Ward, n.d.; Holder & Huntley, 1978; Rizk, Stake, & Simmons, 1980). Magnesium is important for chick hatchability and viability (Christensen et al., 1964 as cited in Hossain & Bertechini, 1998a). Selenium also provides a number of benefits including, but not limited to, improved feature quality and feed conversion efficiencies (Choct, Naylor, & Reinke, 2010). Copper is closely associated with iron metabolism and collagen and elastin (glossary) formation (Scheideler, 2008). Therefore, a deficiency in copper may lead to microcytic hypochromic anemia (glossary) or cardiovascular lesions (glossary) and aortic ruptures (glossary) (Scheideler, 2008).

Trace amounts of heavy metals can also pass through to poultry litter. On average, poultry litter contains 37 ppm of arsenic, 20 ppm of cadmium, 390 ppm of copper, 655 ppm of manganese, 35 ppm of lead, and 377 ppm of zinc (Kunkle, Carr, Carter & Bossard, 1981). A similar study that focused specifically on copper concentrations found that poultry manure contained a minimum of 25 ppm, maximum

of 1,350 ppm, and average of 438 ppm of copper (Hopkins & Ellsworth, 2005). The study also found that the typical copper loading (glossary) for poultry manure is 4.1 kg-copper/hectare/year (Hopkins & Ellsworth, 2005). The actual composition of individual poultry litter samples depend on the number of flocks raised, the type of rations fed, the base materials used, the frequency of cleaning, the application of ammonia control and other chemicals, and numerous other factors (Kunkle et al., 1981). The USEPA (1995a) currently sets the pollutant limits for metals in biosolids used in land application at 75 ppm arsenic, 85 ppm cadmium, 4,300 ppm copper, 840 ppm lead, 57 ppm mercury, 75 ppm molybdemum, 420 ppm nickel, 100 ppm selenium, and 7,500 ppm zinc.

Another study measured samples from ten different farms to determine concentrations of copper, zinc, manganese, and arsenic at different soil depths with a history of poultry litter application (Codling et al., 2008). The results yielded higher concentrations at depths closer to the surface for all metals (Codling et al., 2008). At the shallowest depths, the quantitative values for copper, zinc, manganese, and arsenic concentrations ranged from 7.7-32.1 mg/kg, 5.7-25.9 mg/kg, 12.3-71.1 mg/kg, and 0.41-3.05 mg/kg, respectively (Codling et al., 2008).

When poultry litter is applied as an agricultural fertilizer, metals can contaminate surface water or leach into groundwater. The exact concentrations of trace metals from agricultural runoff in surface and ground water have not been studied as most research to date has focused on the effects of agricultural runoff on trace element levels in soil.

Several farming techniques have been developed that may help mitigate the risks of trace metals. These suggestions include: (a) Spread manure over as many acres as possible, (b) Avoid putting excess trace elements in feed rations, and (c) Use high trace metal removal rate crops such as alfalfa (glossary) or genetically engineered plants (Hopkins & Ellsworth, 2005).

2.1.4 Current Chicken Feed and Maintenance Practices.

In order to properly maintain broilers, there are several requirements and regulations that farmers need to meet. Regulations include building, labor, management and equipment requirements necessary for a successful broiler chicken operation. There are also waste management and nutrient management regulations.

Poultry houses must be above ground on a level surface and have a smooth, level area at the end of the building for a mechanical loader (Muser, Nottingham, Rhodes, & Timmons, 2011). The building must be properly insulated, have sufficient ventilation, and be accessible by a well-graveled roadway (Muser et al., 2011). Most houses are 15 to 20 m wide and 183 m long (Muser et al., 2011). These houses must have stationary generators so that the mechanical aspects of the house remain operational (Muser et al., 2011).

Farmers must have a Maryland NMP for broiler chicken operations (Muser et al., 2011). This plan includes soil and manure analysis, description of the farming operation, map of the farm, amount of litter produced, and other various important records (Muser et al., 2011). Poultry farmers must manage all waste materials and ensure that waste does not adversely affect the environment (Muser et al., 2011).

Although poultry processing companies market poultry products, their poultry raising operations are outsourced to small- and mid-sized farms in the Delmarva area. These independent growers are then contractually obligated to bear the costs for waste management and removal while the poultry processing companies own the poultry. The ruling of *Tyson Foods, Inc v. MDE* states that MDE surpasses their enforcement authority by regulating agriculture operations (Sorisio, 2003).

2.1.5 Poultry Litter Induced Pollution

Growing public concern over poultry litter pollution is evident in both federal and state regulations. Federally, the Clean Water Act (CWA) (glossary) classifies agricultural runoff and agricultural storm water discharges as nonpoint source pollution. In spite of enacting new regulations, Congress allocated no funds to execute pollution management programs (Sorisio, 2003). A study by Kaplan, Johansson, and Peters (2004) noted that when only considering transportation costs, manure testing costs, soil testing costs, and NMP development costs, the regulations could generate $830 million in losses (1.6% of baseline returns) for the poultry industry when "40% of agronomic nutrient requirements are met with manure nitrogen and phosphorus."

The EPA also mandates that best management practices (BMPs) should be adhered to on a statewide basis and that each state's government must develop a nonpoint source pollution (NPS) management plan. In November, 2012, the EPA issued an updated list of key components for an effective state NPS management plan in an attempt to improve the quality of state-enforced programs. It is expected that each state revise and update their NPS plans every five years (USEPA, 2012a). The

EPA expresses that although each state is responsible for devising an appropriate NPS management plan for its wetlands, the EPA is responsible for relaying information between state and federal programs and ensuring complete understanding of the BMPs in both state and federal organizations (USEPA, 2012a). As of 2004, 35 states have enacted programs to control water pollution from animal feeding operations. Of these state programs, 34 limit manure nutrient-application rates prior to federal controls and 27 require animal producers to develop manure-management plans (EPA, 2002 as cited in Kaplan, Johansson, & Peters, 2004). Specifically, Maryland state legislature has also taken an active interest in limiting pollution. The Maryland General Assembly passed the Water Quality Improvement Act (WQIA) (glossary) which mandates that all farming operations with gross incomes of $3,500 or greater are required to to develop NMPs.

Additionally, the WQIA required those owning more than 4 hectares of land to complete an educational course in nutrient application every three years (Sorisio, 2003). In 2009, the MDE adopted a permit system in an attempt to further mitigate nonpoint source pollution from Animal Feeding Operations (AFOs) and CAFOs. The new plans force operators to take into account all animal manure and waste nutrients associated with animal production and to maintain proper management practices involved with the storage, stockpiling, and handling of manure (MDE, 2009). These stricter requirements reflect the increasing amount of regulation that is being enforced on the poultry industry.

2.2 Compounds in Poultry Litter

Poultry litter contains a variety of compounds including nitrogen, phosphorous, heavy metals, and hormones. Its over-application may cause environmental damage due to the presence of excess nutrients. The use of poultry litter as a fertilizer should be carefully monitored and planned across consecutive growing seasons.

Excess nutrients contribute to poor environmental and organism health. Among many sources leading to the Chesapeake Bay, poultry litter runoff is one of the nonpoint sources that substantially contributes to nutrient enrichment and poses greater risk than point sources (CBF, 2004b). The concentration of nutrients in the Chesapeake Bay is related to seasonal events and fertilizer application. The bay has a high nitrogen:phosphorous ratio of nutrient input during spring runoffs, and a lower nitrogen:phosphorous ratio of nutrient input during summer runoffs (Fisher, Peele, Ammerman & Harding, 1992).

The Chesapeake Bay has been subjected to increased nutrient pollution over the years (Fisher et al., 1992). Nutrients from the 17,000 km^2 watershed end up in the bay; in 2011 alone, 106 million kilograms of nitrogen and 21.7 million kilograms of phosphorus entered the bay (USEPA, 2011; Chesapeake Bay Program, 2007). The excess nutrients ultimately alter the bay ecosystem. The over-enrichment promotes algae bloom of phytoplankton or slime, reduced sunlight available for submerged aquatic vegetation, and reduced dissolved oxygen in the water (Fisher et al., 1992). Eventually, the submerged aquatic vegetation dies from lack of sunlight and bacteria consume a large quantity of dissolved oxygen to facilitate the decay process. The lack

of food source, habitat, and oxygen essentially decrease the water quality and cause reduction of benthic organisms (glossary), waterfowl, and redhead ducks at the bay (CBF, 2004a).

Furthermore, excess nutrients in the Chesapeake Bay promote growth of toxin-producing algae that are harmful to aquatic organisms and humans. In the summer of 1997, an outbreak of *Pfiesteria piscicida*, a toxin-producing dinoflagellate (glossary), persisted in Chesapeake Bay tributaries (Gilbert et al., 2001). According to Glasgow et al. (2001), approximately 50,000 juvenile Atlantic menhaden were killed during the outbreak. Furthermore, the *P. piscicida* outbreak caused fishermen and researchers to suffer from short-term memory loss, nausea, flu-like symptoms, breathing difficulties, rashes, and lesions (Boesch, 1999). Silbergeld, Grattan, Oldach, and Morris (2000) reported several case studies of individuals with varying degrees of neurological, immunological, and musculoskeletal difficulties after acute exposure to the toxin.

Since the 1997 outbreak in Maryland, similar outbreaks of *P. minimum* and *A. anophagefferens* occurred in the subsequent years (Gilbert et al., 2001). By 2001, forty new species of toxic dinoflagellates were found over the span of fifteen years (Glasgow et al, 2001). Based on water quality and nutrient content, many have concluded that elevated nutrient loading increases the susceptibility of blooming. Gilbert et al. (2001) found that the peak bloom occurred at areas with high quantities of dissolved organic carbon and dissolved organic nitrogen. Glasgow et al. (2001) claimed that nutrient enrichment of nitrogen and phosphorus stimulated toxic *Pfiesteria* strains. Fesperman and Wheeler (1997) specified that nutrient rich runoff

from chicken farms was the leading cause of toxic microorganism growth. As harmful algae blooms in the Chesapeake Bay seem to be increasing in frequency and diversity (as well as being correlated with nutrients and disrupted ecosystem), it is imperative to monitor key causes of nitrogen and phosphorus loading into the waterway.

2.2.1 Endocrine Disrupting Compounds

Out of the various contaminants that can threaten aquatic ecosystems, hormones are the most concerning (CBF, 2012). During heavy rainfall after poultry litter application, hormones in poultry litter are washed into major bodies of water, distorting the normal hormone balance (Moore et al., 1995; Lange et al. 2002). Moreover, excessive application of poultry litter on fields can lead to increased levels of hormones seeping into the land and eventually into groundwater and downstream surface water (Moore et al., 1995). Hormones act as endocrine disrupting compounds, or EDCs, which are broadly defined as exogenous compounds that have the ability to interfere with the biosynthesis, secretion, transport, binding, or biodegradation of natural hormones in an organism. EDCs ultimately affect organisms' endocrine systems and their functions. (Burkholder et al., 2007; Crisp, et al., 1998; Colucci, Bork & Topp, 2001). Existing literature holds that EDCs originate mainly from wastewater treatment plant discharges and waste from animal feeding operations (Bevacqua, 2011).

Natural hormones are bioactive at extremely low levels (Yonkos, 2005; Young et al., 2004). Though steroid-hormone EDCs include estrogens (glossary), androgens (glossary), and gestagens (glossary), the present study primarily focuses on

estrogens, as they are the most prevalent in broiler chicken litter (Lange, et al., 2002; Bevacqua, 2010). In fact, concentrations of estrone, estradiol, and estriol as low as 40 ng/L to have deleterious effects on aquatic species (Yonkos 2005). The predicted no effects concentration (PNEC) for estradiol and estrone are even lower at 1 and 3 ng/L, respectively (Young et al. 2004).

Lange et al. (2002) investigated the yearly excretion of estrogens and androgens in broiler chickens. This study found that the average female broiler chicken excretes 0.34 mg of estrogens and 0.7 mg of androgens per year, while the average egg-laying chicken excretes 7.1 mg of estrogens and 3.4 mg androgens per year. Other studies have found strikingly different results in poultry litter hormone concentrations. A single kilogram of broiler litter yielded between 33 μg to 904 μg of estradiol (Hanselman et al., 2003). Dutta**,** Inamdar**,** Tso, and Aga (2012) also investigated hormone concentrations in poultry litter, shown in Table 1.

Table 1:

Hormone Concentrations in Poultry Litter

Hormone	ng/g
Estrone	54.15
17β-Estradiol	4.95
17α-Estradiol	2.68
Estriol	8.13
17β-Estradiol-17S	74.25
17β-Estradiol-3S	3.37
17α-Estradiol-3S	3.1
Estrone-3S	28.95

Note. Various Hormone Concentrations in Poultry Litter. Adapted from "Concentrations of Free and Conjugated Estrogens at Different Landscape Positions in an Agricultural Watershed Receiving Poultry Litter" by S. K. Dutta, S. P. Inamdar, J. Tso, and D. S. Aga, 2012, *Water, Air and Soil Pollution, 223.* Copyright 2013 by Team KERMIT

2.2.1 Ecological Impact of EDCs

Estrogenic hormones are excreted to the environment in the urine and feces of all species of farm animals (Knight, 1980 as cited in Hanselman et al., 2003). Hanselman et al. (2003) analyzed hormone levels in solid waste of cattle and swine. Hanselman et al. found up to 239 μg/kg in cattle waste and 1215 μg/kg in swine waste

From nationwide reconnaissance data from the U.S. Geological Survey, estradiol and estrone concentrations were found to be 200 and 112 ng/L respectively, in a network of 139 streams in 30 states impacted by animal wastes (Kolpin et al. 2002 as cited in Hanselman et al., 2003). In runoff from Bermuda grass plots fertilized with liquid dairy manure, estradiol rates reached a maximum of 41 ng/L (Dyer et al. 2001 as cited in Hanselman et al 2003). Estradiol concentrations were determined to range from 20-2,530 ng/L in soil from grazed and ungrazed pastures fertilized with broiler litter (Finlay-Moore et al. 2000 as cited in Hanselman et al. 2003). Peterson, Davis, and Orndorff (2000 as cited in Hanselman et al. 2003) observed lower concentrations of estradiol ranging between 6-66 ng/L in five spring samples from aquifer systems in northwest Arkansas.

Many studies researched hormone concentrations in direct runoff from applied farmland. In one study, researchers observed varying concentrations of hormones following a series of consecutive rain events in Watkinsville, GA (Jenkins et al., 2006). Poultry litter application occurred in July 2000, but the major rainfall event did not occur until February of the following year (Jenkins et al., 2006). Values for estradiol concentrations in the soil were observed at 2.8-3.8 mg/ha, while testosterone concentrations were at 0.06-0.13 mg/ha (Jenkins et al., 2006). Concentrations in the

water runoff were measured six times corresponding to six rain events that occurred between February 23, 2001 and July 25, 2001 (Jenkins et al., 2006). Estradiol concentrations were observed at a minimum of 9.0 ng/L and maximum of 196.3 ng/L (Jenkins et al., 2006). Values of testosterone concentrations were generally lower than the corresponding estradiol values (Jenkins et al., 2006).

Moreover, estradiol has been found at alarmingly high concentrations in the Delmarva area. Yonkos et al. (2005) found that estradiol concentrations ranged from 19 to 75 ng/L in Maryland Eastern Shore streams and rivers that receive agricultural runoff. Dorabawila and Gupta (2005) analyzed concentrations of estradiol in a number of Chesapeake Bay tributaries along the Eastern Shore of Maryland. Samples were taken from different locations along the Wicomico, Manokin, and Pokomoke rivers, as well as three separate ponds, three sewage treatment plants, and four coastal bays in the area (Dorabawila & Gupta, 2005). Measurements may be artificially high since they were calculated using ELISA kits (see Poultry Litter Compounds Detection Methods section). Concentrations ranged from 1.9-6.0 ng/L in the river samples, 1.7-7.6 ng/L in the pond samples, and 2.3-3.2 ng/L in the coastal bay samples (Dorabawila & Gupta, 2005). Values as high as 71.2 ng/L were observed from the sewage treatment plant samples (Dorabawila & Gupta, 2005). Of these concentrations, the higher river values were collected downstream on the Wicomico River, which is considered one of the most polluted rivers in Maryland (Dorabawila & Gupta, 2005).

Impact of EDCs on Amphibians. Amphibians tend to be among the first organisms to be affected by environmentally induced stress (Kloas et al. 2002). There

has been a significant increase in the quantities of deformed frogs found in North America since the 1990s, suggesting that wetland environments may be in peril (Gardiner et al., 2003). Simultaneously, there has been a global decrease in the number of amphibian species (Gardiner et al., 2003).

Recently, a lot of research has involved all-trans retinoic acid (glossary) and its effects on frog development. Retinoic acid is a metabolic derivative of Vitamin A involved with limb development in most vertebrate species. The biggest concern is that a compound in the environment could interfere with the retinoic pathway. So far, it appears that elevated exposure to retinoic acid in the mid-blastula stage had the most teratogenic effects on *R. pipiens, R. clamitans,*and *X. laevis* (Degitz, Kosian, Makynen, Jensen, & Ankley, 2000). Most of the deformities seen were in the hind limbs, and not the forelimbs as seen in this study.

In addition to limb malformations, EDCs can cause problems with sexual differentiation in frogs. The EDC estradiol is a potent feminizer in amphibians, and has been shown to cause partial to complete feminization of male frogs. In a study by Mackenzie et al. (2003), North American frog species *R. pipiens* and *R. sylvatica* were exposed to estradiol at concentrations between 1 µg/L and 100 µg/L. Even in the lowest concentration, the majority of male frogs were completely feminized, with remaining frogs exhibiting intersex characteristics (Mackenzie et al., 2003). Wolf et al. (2010) have found that estradiol causes feminization of male frogs, a higher female to male ratio, and intersex characteristics at a concentration of 200 ng/L. Exposure to other estrogens, such as ethinylestradiol, results in feminization or testicular oocytes in amphibian species as well (Mackenzie et al., 2003).

The enzyme aromatase (glossary) allows androgens to be converted into estrogens (Olstead et al., 2009). Studies have shown that several aromatase inhibitors may function as EDCs, and can have adverse effects on amphibians. In one such study, *R. pipiens* and *R. sylvatica* were exposed to the aromatase inhibitor flavone at a concentration of 1 µg/L, resulting in an increase in intersex characteristics (Mackenzie et al., 2003). Exposure of *X. tropicalis* to the aromatase inhibitor fadrozole (glossary) at a concentration of 16 µg/L resulted in the complete masculinization of genotypically female frogs (Olmstead et al., 2009). At lower concentrations, fadrozole caused reduced body mass in female frogs (Olmstead et al., 2009).

Impact of EDCs on Fish. In a multi-study review, Mills and Chichester (2005) surveyed existing literature to determine individual hormones' effects on fish populations. While they looked at runoff in general, they examined many estradiol derivatives that are also present in poultry litter (Mills & Chichester, 2005). The study also found that exposure effects differed among Medaka, carp, goldfish, fathead minnows, and guppies (Patyna et al., 1999; Oshima et al., 2003; Kramer et al., 1998; Gimeno et al., 1998; Bjerselius et al., 2001; Kinnberg et al., 2003; Bayley et al., 1999 as cited in Mills & Chichester, 2005). For example, while exposure to estradiol was found to reduce egg production in the fish species Medaka and fathead minnows, no effects were seen in guppies (Patyna et al., 1999; Oshima et al., 2003; Kramer et al., 1998; Kinnberg et al., 2003; Bayley et al., 1999 as cited in Mills & Chichester, 2005). Intersex gonads were seen in Medaka and carp, while reduced male gonadal tissue was observed in Medaka, goldfish and carp (Patyna et al., 1999; Oshima et al., 2003;

Bjerselius et al., 2001; Gimeno et al., 1998; as cited in Mills & Chichester, 2005). For all species studied, the earlier in development the organisms were exposed to estradiol, the higher the mortality rate and the higher the chance for irreversible damage, such as intersex gonads (Patyna et al., 1999; Oshima et al., 2003; Kramer et al., 1998; Bjerselius et al., 2001; Gimeno et al., 1998; Kinnberg et al., 2003; Bayley et al., 1999 as cited in Mills & Chichester, 2005). Male Medaka exposed to estriol and estrone exhibited intersex gonads, and the female to male ratio was unnaturally high (Patyna et al., 1999; Oshima et al., 2003 as cited in Mills & Chichester, 2005).

The same review paper found that exposure to ethinylestradiol, a xenoestrogen (glossary), resulted in high mortality, even when exposed as reproductive adults (Metcalfe et al., 2001 as cited in Mills & Chichester, 2005). In general, egg production decreased or ceased completely when exposed to ethinylestradiol. An anomaly was observed in fathead minnows, which experienced increased egg production after an exposure of only 0.1 ng/L of ethinylestradiol. Interestingly, physical deformities in fathead minnow eggs were only seen in high (16 ng/L) exposures. Delayed maturation and testicular growth were observed in rainbow trout, sandy goby, and zebrafish. Intersex gonads were also observed in adult male Medaka. The review paper also includes a research study on the effects of testosterone exposure on young Medaka at a high concentration of 100 ng/L (Mills & Chichester, 2005). After an exposure of only six days, intersex gonads were observed.

Of the estradiol-derived hormones in poultry litter, little research has been done on 17α-estradiol compared to 17β-estradiol. In an effort to remedy this disparity,

Shappell, Hyndman, Bartell, and Schoenfuss (2010) exposed fatheaded minnows of both sexes to both hormones for a 21-day period. The study found that males in both types of estradiol treatments showed higher vitellogenin (glossary) levels, supporting the research of Yonkos et al. (2010). However, 17β-estradiol was found to be eight to nine times more potent than 17α-estradiol. Fish exposed to either type of estradiol tended to be less aggressive in nest-protection than their control counterparts (Shappell et al., 2010).

Researchers have also conducted field studies by collecting wild fish from or raising fish in locations downstream from runoff sources (Blazer, Iwanowicz, Kolpin, Alvarez, & Focazio, 2007; Yonkos et al., 2010). The U.S. Geological Survey completed two studies researching gonadal development of fish in the Potomac River. smallmouth bass in the Potomac River that exhibited skin lesions also displayed mixed sex characteristics (Blazer et al., 2007). Random sampling over two years showed a marked increase in fish with testicular oocytes if the fish prematurely hatched before their usual spawn time (Blazer et al., 2007). As more cross sections were sampled (ranging from five to ten per organism), testicular oocytes were more accurately assessed (Blazer et al., 2007). Blazer et al. (2007) collected smallmouth bass from the Shenandoah River, the South Branch Potomac, and the Conococheague Creek over two years and noted the types of fertilizers and pesticides used if the land around the collection areas served as farmland. Areas downstream of wastewater treatment plants or fields and agricultural operations tended to have fish populations with greater incidences of testicular oocytes (Blazer et al., 2007). Fish populations

with higher incidences of testicular oocytes exhibited lower sperm motility and increased sperm deformities (Blazer et al., 2007).

Another study raised fathead minnows in various concentrations of poultry litter and tested vitellogenin levels to detect feminization (Yonkos et al., 2010). Increased poultry litter concentrations directly correlated to increased vitellogenin levels and increased rates of intersex fish. Furthermore, some poultry litter solutions found in the environment were concentrated enough to produce the same effects (Yonkos et al., 2010).

Impact of EDCs on Mammals (Including Humans). In addition to fish and amphibian species, EDCs also affect the reproductive health of mammals. Research on the effects of EDCs on *X. laevis* and other wildlife may be indicative of an increased risk to humans. The European Workshop on Endocrine Disrupters concluded that chemicals that mimic sex hormones, corticosteroids, and thyroid hormones are likely the cause of most of the endocrine effects leading to sexual deformities (Harrison, Holmes, & Humfrey, 1997). Some EDCs can persist in organisms due to their stability and nonpolar lipophilic nature (Damstra, Barlow, Bergman, Kavlock, & Van Der Kraak, 2002). These EDCs will bioaccumulate in organisms and biomagnify up the food chain (Damstra et al., 2002). Another study found that human fetal exposures to high levels of estrogenic compounds can lead to an increased risk of male gonadal disorders (Toppari et al., 1996). Humans are exposed to estrogenic compounds in food, plastics, pesticides, and other chemicals (Harrison, et al., 1997).

Male reproductive health is declining worldwide (Toppari et al., 1996; Boisen et al., 2004, 2005). The USA, Finland, Denmark, and the UK have had higher rates of testicular cancer, prostate cancer, hypospadias (glossary), and cryptorchidism (glossary) in recent years (Harrison et al., 1997; Giwercman, Carlsen, Keiding, & Skakkeback, 1993). Since 1940, there is evidence of lower semen quality and perhaps of falling sperm count, although the latter is difficult to quantify (Harrison et al., 1997; Giwercman et al., 1993). Because male reproductive health decline is not restricted to one geographic area, the cause is likely the result of a more global phenomenon, rather than genetic factors (Giwercman et al., 1993; Lottrup et al., 2005).

Phthalates, another group of well-known EDCs, have also been implicated in negatively affecting human health. Exposure to phthalates (glossary) in young boys has been shown to lower androgen levels and decrease anogenital distance, both of which are signs of feminizing effects (Lottrup et al., 2005). The results from human studies are in agreement with results from rodent studies, suggesting phthalates may negatively affect the male reproductive system (Lottrup et al., 2005).

There is also evidence that EDCs can have an effect on female reproductive health. Diethylstilbestrol (DES) (glossary), a synthetic, estrogenic substance, has been linked with increased breast cancer rates in mothers given the drug during pregnancy (Giusti, Iwamoto, & Hatch, 1995). Additionally, daughters of mothers given DES are at higher risk of clear-cell cervicovaginal cancer, vaginal epithelial changes, reproductive-tract anomalies, and premature births (Giusti et al., 1995). Giusti et al. (2005) also hypothesize that, since DES is an estrogenic substance, it may be a good

model for the effects of other estrogenic substances, particularly estradiol, in humans. There is also increasing concern about exposure to EDCs during fetal, neonatal, and childhood development, as this is when "programming" of the endocrine system occurs (Damstra et al., 2002). Thus, EDCs secreted in maternal milk or transferred to the offspring transplacentally are particularly concerning (Damstra et al., 2002).

The majority of present research has focused on the effects of high concentrations of estradiol on mammals while little research has been done linking a low environmental dose of estradiol to the aforementioned effects. For example, in a study by Adler and Nelson (1988), female rats were ovariectomized (glossary) and implanted with Silastic or polyethylene implants containing estradiol. The Silastic implants delivered a larger dose of estradiol than what a female rat would experience normally, while the polyethylene implants delivered a physiologically normal amount of estradiol (Adler & Nelson, 1988). Regardless, both experimental groups experienced vaginal cornification, which declined after a number of weeks (Adler & Nelson, 1988). After the peak cornification, the rats became less sensitive to estradiol, and vaginal cornification could not be induced again (Adler & Nelson, 1988).

One study has shown that a male mouse fetus positioned between two female mouse fetuses is exposed to more estradiol than usual, resulting in enlargement of the prostate and behavioral changes as an adult (vom Saal, 1981 as cited in vom Saal, et al., 1997; Even, Dhar, & vom Saal as cited in vom Saal et al., 1997). Further research by vom Saal et al., (1997) supports this finding; a low dose of estradiol in fetal male mice results in an enlarged prostate. However, higher estradiol exposures result in decreased prostate size (vom Saal, et al., 1997). Additionally, it was found that when

mice were exposed to estradiol neonatally, there were increased rates of prostatic intraepithelial neoplasia (PIN) (glossary), which is the precursor lesion for prostate cancer (Ho, Tang, de Fraustro, & Prins, 2006). Ho et al. (2006) also found that the neonatal exposure to estradiol led to changes in DNA methylation (glossary) patterns, which might cause predisposition for prostate cancer.

There is also evidence EDCs play a role in weight gain and adipocyte development (glossary) (Heindel & vom Saal, 2009 as cited in Furst et al., 2012; Janesick & Blumberg, 2011 as cited in Furst et al., 2012; Newbold et al., 2009, as cited in Furst et al., 2012). This is supported in a study by Furst et al. (2012), in which the amount of fat male pigs had was linked with the amount of estradiol their mothers received during pregnancy (Furst et al., 2012). Furst et al., (2012) speculate that EDCs could be responsible for the current obesity epidemic in children and adults. Further, childhood obesity is linked with an increased risk of severe obesity in adults, and with an increased risk of cardiovascular disease (The, Suchindran, North, Popkin, & Gordon-Larsen, 2010 as cited in Furst et al., 2012; Raghuveer, 2010 as cited in Furst et al., 2012).

2.2.2 EDC Degradation.

The persistence of an EDC is a major determining factor of its potential effect on the environment. While estradiol has a relatively short half-life (approximately 13 hours), Ying and Kookana (2005) found that in sandy loam, estradiol's half-life is extended to seven days under aerobic conditions and 24 days under anaerobic conditions (Nagpal & Meays, 2009). Bera et al. (2011) studied the sorption of testosterone and estradiol in poultry litter. According to this study, testosterone

desorbs more quickly in the presence of poultry litter. Further estradiol desorbs less readily in the presence of poultry litter (Bera et al., 2011). From this, it can be inferred that poultry litter causes estradiol to stay longer in the soil and thus degrade more quickly than if it were to run off into a stream.

Most organisms excrete estrogens in their less bioactive, conjugated form, as the conjugated form is more soluble in water (Shrestha, Casey Hakk, Smith, & Padmanabhan, 2012). Poultry excrete about 69% of their estrogens as conjugates (Shrestha et al., 2012). The main conjugates of steroidal estrogenic hormones are glucuronides and sulfides (Shrestha et al., 2012). These conjugated forms will typically deconjugate into more active forms (Kumar, Johnson, Nakada, Yamashitam, & Tanaka, 2012). In river water, it was found that estrone-3-glucuronide deconjugates into estrone, a less estrogenic and more persistent form, in nearly a stoichiometrically one-to-one conversion (Kumar et al., 2012). Estradiol-3-glucuronide deconjugates into estrone and estradiol, though the mass balance suggests that other degradation products exist (Kumar et al., 2012). Finally, it was found that estrone-3-sulfide and estradiol-3-sulfide were far more stable than glucoronides, showing slowed degradation in activated sludge and raw sewage and little to no degradation in river water (Kumar et al., 2012).

Other major elements that affect EDC persistence in the environment are microbes. Many studies have shown microbes are important in the degradation of estradiol into its primary metabolite, estrone (Hanselman et al., 2003; Fan, Casey, Hakk, & Larsen, 2007; Kumar et al., 2012; Xuan, Blassengale & Wang, 2008). Although estrone is less estrogenic compared to estradiol, it also persists much longer

than estradiol in the environment (Lee et al., 2003 as cited in Dutta et al., 2012; Hutchins et al., 2007 as cited in Dutta, Inamdar, Tso, & Aga, 2012). D'Ascenzo et al. (2003) claim that estrone is the most important endocrine disruptor among natural estrogens.

A study by Yu, Roh, and Chu (2007) identified a number of bacteria which were able to degrade estradiol. They also identified three distinctive degradation patterns exhibited by the bacteria. Most bacteria (*Aminobacter* (strains KC6 and KC7), *Brevundimonas* (strain KC12), *Escherichia* (strain KC13), *Flavobacterium* (strain KC1), *Microbacterium* (strain KC5), *Nocardioides* (strain KC3), *Rhodococcus* (strain KC4), and *Sphingomonas* (strains KC8–KC11 and KC14)) were able to degrade estradiol into estrone (Yu et al., 2007). Strains KC6, KC7, and KC8 were able to degrade both estradiol and estrone, but the rate of estrone degradation was much slower than that of estradiol (Yu et al., 2007). Finally, only one strain, KC8, was able to quickly degrade estradiol and estrone as its only carbon source for energy (Yu et al., 2007). Another study noted that estradiol degradation was greatly reduced by the presence of sulfadimethoxine (glossary) (Xuan et al., 2008).

2.2.3 Poultry Litter Compounds Detection Methods

Currently, gas chromatography-mass spectrometry (GC-MS) (glossary) and liquid chromatography-mass spectrometry (LC/MS) (glossary) are the best techniques for quantifying contaminants in water (Comerton, Andrews & Bagley, 2009). Both techniques rely on four basic steps: pre-treatment, clean up, concentration, and analysis (Comerton et al., 2009). In the pre-treatment step, the sample is filtered,

preservatives are added, and the pH is adjusted (Comerton et al., 2009). Next, in the cleanup step, the substance for analysis is extracted and eluted, typically using solid phase extraction (Comerton et al., 2009). In the concentration step, a stream of nitrogen is used to evaporate the solvent and concentrate the sample (Comerton et al., 2009). In the final step, either GC-MS or LC/MS is performed to analyze the sample (Comerton et al., 2009). GC-MS is advantageous over LC/MS for analysis of non-polar, volatile compounds (Comerton et al., 2009). Additionally, GC-MS is less prone to error from water matrix effects (glossary) and in general has a lower limit of detection compared to LC/MS (Comerton et al., 2009). Therefore, GC-MS was chosen for this study over LC/MS because of the non-polar nature of estrogens.

When compared to other available types of assays, GC-MS consistently outperforms in detection of estradiol and associated compounds (Santen et al., 2007). A study by Shore and Shemesh (2003) found an estradiol concentration of 141 ng/L in raw sewage using radioimmunoassay (RIA) (glossary). D'Ascenzo et al. (2003) observed "…unexpectedly large amount[s of estradiol] raises the doubt that immunoassay techniques might overestimate E2 by cross-reactions." A study by Santen et al. (2007) compared detection levels of estradiol between GC-MS and a RIA. They found that most types of RIA tended to detect a combination of all available estrogenic metabolites, yielding incorrectly high results, while GC-MS only measured the compound under study (Santen et al., 2007). By using GC-MS rather than other assays, the present study attempted to avoid false-positive detection of estradiol after it degraded into other forms. A study by Lee, et al. (2006) found that GC-MS was able to detect concentrations of estradiol as low as 0.2 ng/L. Estradiol

used in the present study degraded over time, resulting in lower concentrations. Thus the use of GC-MS was beneficial to detect the point at which estradiol was completely degraded.

2.2.4 Nitrogen

Nitrogen exists in two forms: organic and inorganic. Organic nitrogen (glossary) is urea, proteins, or amino acids, while inorganic nitrogen (glossary) is ammonia, ammonium, nitrates, nitrites, nitrogen gas, or nitrogen oxides. Portions of inorganic nitrogen such as ammonium, nitric oxide, and nitrites are available nitrogen, nitrogen that is readily usable by plants (Nahm, 2003; Iowa State University, 2001). Nitrate and ammonium are the most readily usable forms of nitrogen by plants (Nahm, 2003).

The most important input farmers can control to increase crop yields on non-irrigated fields is nitrogen (Ribaudo, 2011). Consequently, farmers apply animal manures, one of several excellent sources of supplemental nitrogen, as fertilizers to aid plant development. As different sources of animal manure have distinct nitrogen contents, farmers consider various types of manure to control the amount of nitrogen applied to the field (Lory, 1999; Zublena, Barker & Carter, 1993). Poultry manure is frequently incorporated into many fertilizer programs because it has one of the highest concentrations of nitrogen and the highest turnover rate of nitrogen mineralization (glossary) (microbic conversion of organic nitrogen into inorganic and available ammonium and nitrate) (Nahm, 2003; Sylvia et al., 2005). Nitrogen is found in the form of uric acid and undigested proteins in poultry litter (Nahm, 2003). Pratt and Tewolde (2009) observed nitrogen values of 0.65-1.10 and 0.70-1.23 g/kg,

respectively, in till and no-till soil samples of fields fertilized with poultry litter.. Jenkins, Endale, Schomberg, and Sharpe (2006) measured in-soil nitrogen concentrations of 66.8-90.8 kg/ha in a field treated with poultry litter. After incorporation, 50% of the applied nitrogen is immediately available for use in the first growing season. Twenty percent of the original amount is then mineralized and available for use in the next season (CBF, 2008).

While poultry litter is a rich nitrogen source, not all of the available nitrogen may be absorbed by plants. Continual application of poultry litter that exceeds crop nutrient requirements leads to increased nitrogen concentrations in surface and ground water (Sharpley, 1997). To decrease potential environmental contamination, poultry manure is often supplemented with carbon-rich and nitrogen-deficient adsorbents such as sawdust, straw, wood shavings, rice hulls, and bedding, which help capture and immobilize the available nitrogen into less potent organic nitrogen (Nahm, 2003).

Agriculture is the largest source of nitrogen contamination to the environment in the United States (Ribaudo, 2011). The nitrogen can negatively impact the environment through five pathways: (a) ammonia volatilization (glossary), (b) soil erosion, (c) leaching, (d) runoff, and (e) denitrification (glossary). Over two years, up to 50% of the applied nitrogen may volatilize or pollute local waterways (CBF, 2004).

The first form of environmental nitrogen pollution is through ammonia volatilization. According to Bergström, Djodjic, Kirchmann, Nilsson, and Ulén (2007), nitrogen from ammonium accounts for up to 50% of the total nitrogen in the

poultry manure. Ammonium, along with other forms of nitrogen, can volatilize into ammonia and be lost to the environment (Mallin & Cahoon, 2003). If manure is not properly incorporated into soil, 15-35% of the nitrogen contained could volatilize (CBF, 2004b). Most of the aerosolized ammonia comes from livestock operations whose huge fans vent ammonia gases from waste out of buildings. Warm temperatures can also increase the rate of ammonia volatilization. Not only does the aerosolized nitrogen pollute the atmosphere, but it also dissolves into water sources. According to the CBF (2004b), up to 27% of nitrogen that reaches the Chesapeake Bay is from aerosolized ammonia. The aerosolized ammonia can also impact chickens in chicken houses, as atmospheric levels higher than 25 µL/L can cause reduced egg production, blindness, increase susceptibility to diseases, damage the respiratory tract, and decrease the ability to gain weight (Carlisle, 1984; Moore, Daniel, & Edward, 1999).

Alternatively, ammonia may further convert to nitrate and nitrite through the process of nitrification (Nahm, 2003). While ammonia is released into the air, nitrate and nitrite pollute the groundwater and decrease water quality through soil erosion, leaching, or surface runoff. In soil erosion, nitrogen attached to soil particles can be carried by wind or water to surrounding waters. As nitrate does not readily bind with soil, it is mobile and can leach or run off. Leaching occurs when heavy rainfall easily moves dissolved nitrate through the soil profile. The nitrate eventually ends up in the groundwater or in surface water via groundwater flow. In addition to moving downward into groundwater, nitrate from poultry litter can also move laterally

through surface runoff into nearby streams (Liebhardt et al., 1979 as cited in Mallin & Cahoon, 2003).

In the Sharpley (1997) study, nitrogen levels were measured in 10 different soils applied with poultry litter, each exposed to 10 successive rain events to simulate runoff. The highest concentrations of nitrogen were detected following the first rain event, at 3.90-5.93 mg/L, and decreased consistently by the tenth rain event, to 1.45-2.78 mg/L (Sharpley, 1997). The study also determined that the differing nitrogen adsorbing properties of soils contributes to the amount of nitrogen lost through runoff (Sharpley, 1997).

Nitrogen runoff can pose a series of potential problems. Excess nitrogen is known to cause eutrophication, which reduces water quality and depletes dissolved oxygen levels, inhibiting aquatic organism survival (Lory, 1999). According to Iowa State University, (2001), nitrate contamination in drinking water can also pose problems to humans, especially infants. According to Iowa State University, high concentrations of nitrate in drinking water can cause methemoglobinemia (glossary). When nitrate is introduced to infants within the first six months, bacteria in their digestive systems convert it to nitrite (Iowa State University, 2001). Nitrite interferes with oxygen transport and can cause severe developmental problems and even death (Iowa State University, 2001).

2.2.5 Phosphorus

Phosphorus helps to ensure proper mineralization of eggshells and prevent skeletal abnormalities in poultry (Bolan, Naidu & Anderson, 2012). Because the

uptake of phosphorus in poultry is generally inefficient, farmers supplement poultry feed with inorganic phosphorus additives.

However, there are rising concerns about the excessively high phosphorus content in manure. In a study on pig manure, Bergström et al. (2007) found a distinct correlation between the amount of additional phosphorus added to the feed and the amount of phosphorus excreted in manure. Poultry manure has the highest total phosphorus content in comparison to other manure produced by livestock (Bolan et al., 2010). Even when the fresh manure is mixed with carbonaceous bedding materials, phosphorus concentration usually remains relatively high.

Farmers use poultry litter as a fertilizer because phosphorous is beneficial for plant development. According to Asher and Loneragan (1967), plants need 20-30 μM of phosphorous for survival. However, the level of phosphorus applied to fields through poultry litter exceeds the amount necessary for plant development (Bolan et al., 2010).

Previously, the amount of poultry litter applied to fields was based on the amount of nitrogen needed by the crops (Bolan et al., 2010). Because poultry litter has a low nitrogen:phosphorus ratio, farmers who apply poultry litter at nitrogen-based amounts end up contributing a large excess of phosphorus to the environment, which then builds up in the soil (Maguire, Mullins, & Brosius, 2008). Until only recently, it was believed that soil holds phosphorus very well and does not leach into the ground water, but this has been refuted (Maguire et al., 2008). Research has also shown that crop yields do not change depending on whether a nitrogen- or phosphorus-based application is chosen, so it has been argued that nutrient

management guidelines should shift towards a phosphorus-based approach (Maguire et al., 2008). However, by adhering to a phosphorus-based application rate, the poultry litter:land ratio decreases, which results in a surplus of poultry litter that then must be disposed of by alternative means (Maguire et al., 2008).

The Maryland WQIA of 1998 was one of the first statutes that started mandating phosphorus-based poultry litter application (Maguire et al., 2008). Farmers were to file nitrogen-based NMPs by 2001 and then update them to a phosphorus basis by 2004 (Perez, 2010). A similar regulation was passed for Virginia in 1999, mandating the creation of NMPs with a nitrogen-based application rate by 2001 and a phosphorus-based application rate by 2005 (Perez, 2010).

The accumulation of phosphorus in agricultural soil poses a major problem to farmers. As phosphorus saturation from poultry litter increases, the soil's phosphorus retention capacity drastically decreases, leading to phosphorus pollution of nearby water sources (Hooda et al., 2001).

When high concentrations of phosphorus are applied to the field continuously, the excess phosphorus contaminates ground and surface water (Reddy, Rao & Takkar, 1999; Mallin & Cahoon, 2003; Bolan et al., 2010; Bergström et al., 2007; Lory, 1999). Runoff is a potent form of phosphorus contamination because it can transport large concentrations of soluble phosphorus that is harmful to aquatic organisms (Figure 1) (Lory, 1999). This process is accelerated when the field is inclined (Lory, 1999). Phosphorus can also contaminate subsurface water through leaching (Bergström et al., 2007; Lory, 1999). However, leaching is often prevented by sorption to soil and is only a small contributor to phosphorus transport into streams

(Bergström et al., 2007; Lory, 1999). Lastly, phosphorus can be transported through erosion by binding (glossary) to soil particles that break off during rain events (Figure 1) (Lory, 1999).

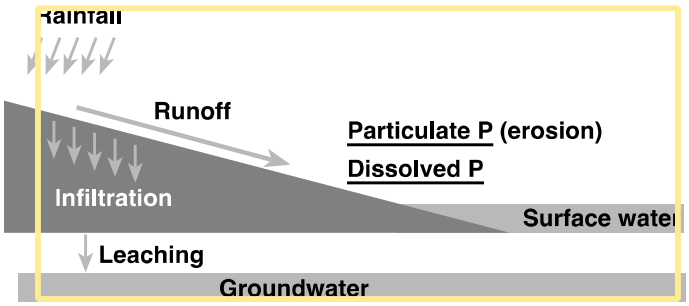

Figure ***1***: Pathways for Phosphorous Loss. From "Managine Manure Phosphorous to Protext Water Quality" by J.A. Lory, 1999

In the Sharpley (1997) study, the highest concentrations of phosphorus were measured following the first rain event at 0.95-2.13 mg/L and decreased consistently until the tenth rain event to 0.44-0.90 mg/L. Sharpley also determined that phosphorous absorption properties of different soil types affect how much phosphorus is lost through runoff. On cotton fields treated with poultry litter, 0.08-0.14 and 0.02-0.13 g/kg of phosphorus were observed in litter samples of tilled and no-till sites, respectively (Pratt & Tewolde, 2009). Another study looked to determine concentrations of phosphorus at different soil depths with a history of poultry litter application (Codling et al., 2008). Samples were analyzed from 10 different farms and tabulated (Codling et al., 2008). At the shallowest depths, the quantitative values for phosphorus concentrations ranged from 300-1,069 mg/kg (Codling et al., 2008). Finally in the Jenkins et al. (2006) study, poultry litter was applied to a field and the in-soil phosphorus concentration was measured at 27.9-32.4 kg/ha.

High levels of phosphorus in runoff can have serious detrimental effects. For example, phosphorus from agricultural runoff comprises 40% of total phosphorus

polluting the Baltic Sea (Bergström et al., 2007). Glasgow and Burkholder (2000) found 16,000 metric tons of phosphorus from poultry litter in the Neuse River watershed, North Carolina in 1998. This elevated level of phosphorus in water can cause eutrophication, decreased water clarity, and bad odor and taste (Moore & Edward, 2005; Moore et al., 1999; Lory, 1999). In a 2005 publication, Moore and Edward raised concerns about the water quality of affected sources of drinking water, such as Lake Eucha, Lake Spavinaw, and the Illinois River.

2.3 Conventional Till vs. No-till

Different soil preparation methods and practices can affect the level of runoff that leaches into the environment. Two common farming techniques are conventional tilling (glossary) and no-tilling (glossary). Conventional tilling is a practice in which the land is plowed for weed and pest control prior to planting and is used to prepare for seeding (Horowitz, Ebel & Ueda, 2010). Approximately 36 million hectares were tilled in 2009 (Horowitz et al., 2010). Fertilizers, manures, and supplemental organic matters can be easily incorporated during tillage to supplement the soil. Conventional tillage can be costly as it requires more diesel and labor than no-till farming (Sullivan, 2011). According to Horowitz et al. (2010), conventional tillage affects retention of organic matters and indirectly affects the environment. Conventional tillage can lead to soil compaction and loss of organic matter as aerobic microbes use the air mixed into the soil to break down nutrients (World Wildlife Fund, n.d.). In fact, a decrease in frequency and intensity of tillage could increase the retention of organic matters and is being supported by various policymakers (Horowitz et al., 2010).

The “no-till” method is slowly growing in popularity. No-till farming refers to a planting technique that does not use prior seedbed preparation and minimizes the disturbance of the soil (USEPA, n.d.b). Farm tools used in no-till operations are designed to manage cover crop residue left on the topsoil, which is buried or removed during tillage. Disc openers or row chisels are often used to insert the seeds into the seedbed with as little soil disturbance as possible (Phillips & Phillips, 1984 as cited in Morse, 1999). Fields are maintained with cover crops (glossary) to protect the soil between plantings. Cover crops used in no-till can also protect and feed the soil, prevent weed growth, and fix atmospheric nitrogen (Derpsch, Friedrich, Kassam, & Li, 2010). The cover crops are then killed using herbicides such as paraquat (Morse, 1999). The leftover residue is beneficial to insects, annelids, and fungi which increase the porosity of lower soil layers, making it easier for plant roots to spread (Derpsch et al., 2010). Cover crop residue increases water retention and reduces the amount of soil and nutrients that would erode under heavy rainfall (Morse, 1999).

No-till agriculture can also effectively retain more carbon matter, thus sequestering carbon and reducing greenhouse gas emissions (Horowitz et al., 2010). A reduction in tillage was estimated to increase sequestration by 0.33 metric tons whereas a strict no-till practice was estimated to increase sequestration by 1.58 metric tons per hectare per year over a 20-year period (Horowitz et al., 2010). In the Corn Belt, tillage has resulted in a decrease of 20 to 50% in sequestered soil carbon, which could lead to a rise in greenhouse gases, contributing to global warming (Lal, Reicosky, & Hanson, 2007). The highest carbon dioxide fluxes have been observed

after deep soil disturbance tilling, with less carbon dioxide loss after no-till farming (Reicosky & Lindstrom, 1993).

While no-till farming is on the rise, issues still exist in implementation. Conventional seeders cannot be effectively used on no-till plots due to the high density of the soil. This means that new tools for seed implantation must be developed and made available to farmers commercially. Injection (glossary) is a technique where supplements are mechanically incorporated to the soil. The sub-surface application of the poultry litter is found to decrease runoff and emission by 90% (Comis, 2010a). Liquid manures are easier to inject than dry manure, but mixing the slurry could be time consuming. Subsurfer, a non-pressurized system that can be used to inject dry poultry litter into the soil, is an example of equipment developed by the ARS for sub-surface fertilizer application. Initial testing has found the subsurface applicator is effective in transferring poultry litter into soil with minimal disturbance (Kleinman, Wolf, Sharpley, Beegle & Saporito, 2009). This application results in greater crop yields and decreased volatilization of ammonia. Despite its benefits, the injection method is time consuming and its production costs are double those of regular broadcast spreaders. Even with the injection method, however, the rate of leaching in no-till farming may be higher than that in tilled farming with heavy rainfall.

2.4 Xenopus laevis

2.4.1 *Xenopus laevis* as a Model Species

X. laevis is a common frog species used in research on the effects of EDCs (van Wyk, Pool, & Leslie, 2003). Although *X. laevis* is not native to the Chesapeake

Bay Watershed, previous studies have shown that this frog species can be used as a model organism because researchers can easily induce reproduction, thus yielding a large number of offspring (Field, Tomlinson & Wheeler, 2005). Using a large number of offspring from a single breeding pair can control for genetic differences and increase statistical significance of results. Additionally, *X laevis* is sensitive to hormones during development (Qin & Xu, 2006). In particular, *X. laevis* breeds and develops in standing water that could contain runoff contaminated by poultry litter (Wolf et al., 2010). Its aquatic lifestyle allows for maximum exposure to aqueous contaminants found in water, making a suitable candidate for this study. The African clawed frog also has a translucent integumentary system, which allows us to observe internal development (Descamps, Buytaert, de Kegel, Dirckx, & Adriaens, 2012). Finally, *X. laevis* completes metamorphosis in a relatively short time, which minimizes the duration of the testing period (Gilbert, 2000).

2.4.2 *Xenopus* Limb and Gonadal Development Staging

The *Xenopus* zygote begins to divide immediately once fertilized (stage 1) (Segerdell, Bowes, Pollet, & Vize, 2008). After 1.5 hours, the zygote is completely cleaved, creating two identical cells (Segerdell et al., 2008). Division continues into the formation of a blastula which is comprised of a single layer sphere of cells known as blastomeres that surrounds a vacant center (Segerdell et al., 2008). By 10-12 hours post-fertilization, the embryo reaches stage 10 (Segerdell et al., 2008). The cells reorganize into a three tissue layered structure called the gastrula (Segerdell et al., 2008). During this period, the ectoderm (glossary), mesoderm (glossary), and endoderm (glossary) are differentiated (Segerdell et al., 2008). Formation of the

neurula (glossary) is complete 19-20 hours post-fertilization (Segerdell et al., 2008; Bowes et al., 2009). The nervous system begins to form starting with the establishment of the neural tube, which will eventually develop into the brain and spinal cord (Gilbert, 2000). Organogenesis (glossary) continues with the formation of segmented somites that differentiate into muscles and skin (Gilbert, 2000). Limb development begins at stage 46, 4 DPF (glossary). The hind leg buds appear first, followed by forelimb buds several days later (Segerdell et al., 2008). As the tadpole grows, the tail starts to shrink and is only about half its total length by stage 62 (Segerdell et al., 2008). However, limb development and tail resorption rate is adversely affected by a variety of compounds that may be present in the environment, including heavy metals such as copper or organic compounds (Fort and Stover, 1997). Thyroid hormones signal for metamorphosis at stage 66, approximately 58 DPF (Gilbert, 2000).

2.4.3 Genetic Determination of Sex

Sex determination in amphibians is sensitive to endocrine disruption. If embryos or larvae are treated with estrogen, the result is generally male-to-female sex reversal (glossary) and treatment with estrogen synthesis inhibitors (like cytochrome P450 aromatase) leads to female-to-male sex reversal (Yoshimoto & Ito, 2011).

X. laevis sex determination is genetically based on a female heterogametic ZZ/ZW-type sex-determining system (Yoshimoto & Ito, 2011). In ZZ gonads containing somatic cells surrounding primordial germ cells (PGC) (glossary), gene product DMRT-1 transactivates target genes that lead to testis formation (Yoshimoto & Ito, 2011). In ZW gonads, gene products DM-W and DMRT-1 are co-localized in PGC-

supporting cells leading to the formation of either a DM-W/DMRT-1 heterodimer or a DM-W homodimer that can bind the DMRT-1 target gene during stage 48 and/or stage 50 of *X. laevis* development (Yoshimoto & Ito, 2011). This prevents the target gene from interacting with the DMRT homodimer, leading to transcriptional repression of the pathway that would lead to testis formation, thereby leading to ovary formation (Yoshimoto & Ito, 2011). Exposure to estrogenic compounds may potentially suppress certain pathways leading to normal gonadal formation (Yoshimoto & Ito, 2011). At stages 56-57, male germ cells in ZZ gonads migrate into the medulla (glossary) and differentiate into spermatogonia (glossary) (Yoshimoto & Ito, 2011). In ZW gonads, female germ cells, including oogonia (glossary) and oocytes stay in the cortex around the ovarian cavities (Yoshimoto & Ito, 2011).

2.4.4 Factors Affecting *X. laevis* Sexual Development

As mentioned earlier, EDCs have the ability to antagonize or interfere with the biosynthesis and biodegradation of natural hormones, and ultimately affect the endocrine system and its functions (Burkholder et al., 2007; Colucci, Bork & Topp, 2001). Additionally, tadpole malformations have been found to be associated with agricultural chemicals, though the results are less clear (Gardiner et al. 2003). A relationship between anti-estrogenic and anti-androgenic compounds and changes in gonadal development in amphibians has been observed in the laboratory (Cevasco et al. 2008).

Wolf et al. (2010) performed studies on the effects of estradiol on *X. laevis* at four concentrations: 0; 200; 1,500; and 6,000 ng/L, and determined 200 ng/L as the approximate EC50 (glossary). At the end of the experiment, it was found that

complete feminization and severe morphologic effects resulted from an exposure to concentrations greater than 200 ng/L (Wolf et al, 2010). Additionally, Wolf et al. (2010) hypothesized that frogs would be less likely to have complete feminization than reproductive abnormalities at concentrations lower than 200 ng/L. Specifically, EDCs were linked to partial or complete conversion from the male to the female phenotype, which includes testicular oocytes (Figure 2), ovotestes (glossary), sex reversal, hermaphroditism (glossary), altered sex ratios, feminization, retarded gonadal development, discontinuous gonads (glossary), and pigmentation changes in *X. laevis* and in other species of frogs (glossary). These characteristics are indicative of intersex and mixed sex frogs (Figure 3) (glossary).

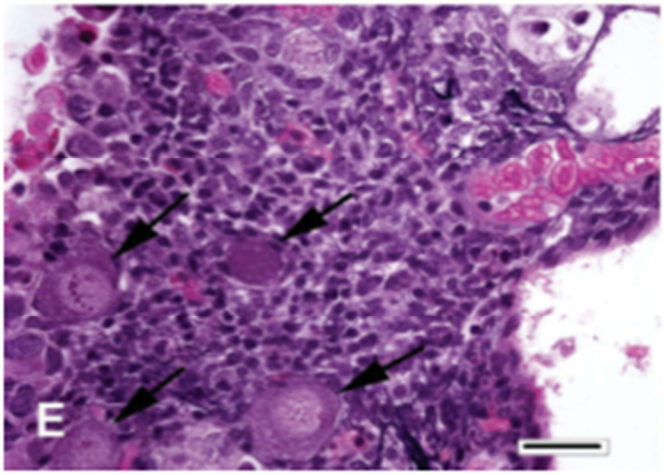

Figure 2: Microscopic Depiction of Testicular Oocytes in *X. laevis* exposed to 200 ng/L of estradiol. Arrows denote the testicular oocytes. From "Effects of 17β-Estradiol Epxosure on *Xenopus Laevis* Gonadal Histopathology," by J. C. Wolf, I. Lutz, W. Kloas, T. A. Springer, L. R. Holden, H. O. Krueger, and A. J. Hosmer, 2009, *Environmental Toxicology and Chemistry, 29*, p. 1098.

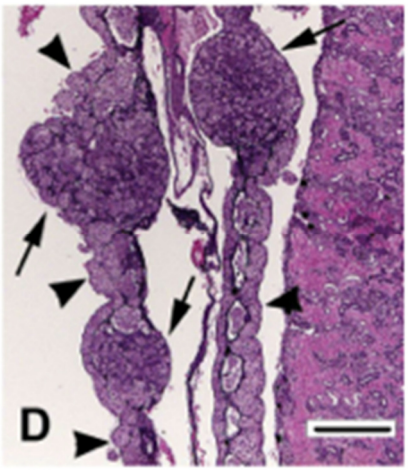

*Figure **3**:* Microscope Depiction of Mixed-sex Gonads in *X. laevis* exposed to 200 ng/L estradiol. Arrows indicate segments that resemble testicular tissue and arrowheads indicate segments that resemble ovarian tissue. From "Effects of 17β-Estradiol Epxosure on *Xenopus Laevis* Gonadal Histopathology," by J. C. Wolf, I. Lutz, W. Kloas, T. A. Springer, L. R. Holden, H. O. Krueger, and A. J. Hosmer, 2009, *Environmental Toxicology and Chemistry, 29*, p. 1098.

In a study conducted by Cevasco et al. (2008), adult *X. laevis* were exposed to EDCs with estrogenic and androgenic modes of action. The experiment showed clear-cut effects of EDCs on *X. laevis* gonad histomorphology (Cevasco et al., 2008). Females exposed to ethinylestradiol had the largest vitellogenic oocytes and greater numbers of atretic ooxytes (*Figure **4***) (Cevasco et al, 2008). Females exposed to tamoxifen (glossary) showed a striking decrease in the number of oocytes along with the appearance of male germ cells and spermatogenic nests (Cevasco et al, 2008). Males frogs exposed to ethinylestradiol (glossary) were found to have reduction of seminiferous tubule diameter, a distinct thickening of the interlobular connective tissue, testicular oocytes, and a much shorter average testis length compared to the control group (Cevasco et al., 2008). Additionally, oocytes scattered within males tissue was observed in frogs exposed to water from the Lambro River (Figure 5) (Cevasco et al., 2008).

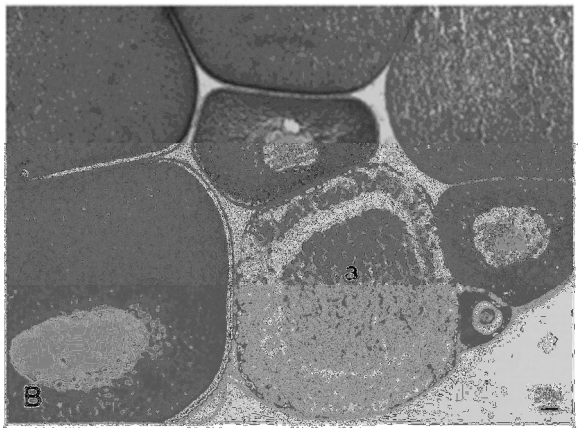

*Figure **4**:* Ovarian histology of female *X. laevis* exposed to ethinylestradiol with an atretic oocyte. From "Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: II. Effects on gonad histomorphology," by A. Cevasco, R. Urbatzka, S. Bottero, A. Massari, F. Pedmonte, W. Kloas, A. Mandich, 2008, *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology, 147*. p 247.

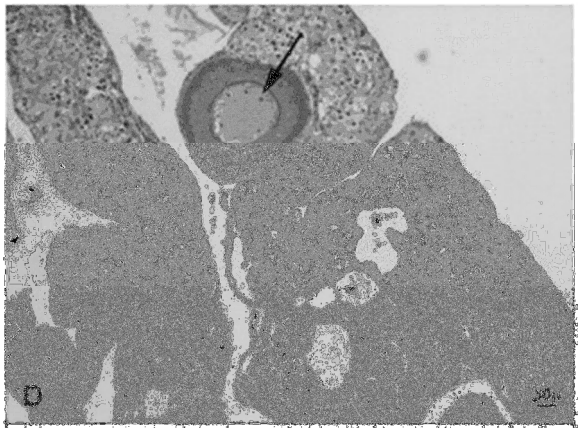

*Figure **5**:* Early vitellogenic oocyte in male tissue. Note cavitation in male tissue (*). From "Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: II. Effects on gonad histomorphology," by A. Cevasco, R. Urbatzka, S. Bottero, A. Massari, F. Pedmonte, W. Kloas, A. Mandich, 2008, *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology, 147*. p 247.

Sex ratios may also be distorted by temperature. Exposure to high temperatures during larval development has been shown to induce masculinization, while exposure to low temperatures has been shown to induce feminization in several amphibian species. (Hayes, 1998; Wallace et al., 1999). Jooste et al. (2005) found increased development of testicular oocytes when *X. laevis* at N.F. (glossary) stage 66 was exposed to atrazine (glossary) (1-25 mg/L) during development (glossary)

However, Hayes et al. (2006) found conflicting results and reasoned that the increased development of testicular oocytes may have developed after exposure to low temperatures.

Chapter 3: Methodology

3.1 Testing Design

3.1.1 Testing Guidelines

This study adhered to methods set out in the *Standard Guide for Conducting Whole Sediment Toxicity Test with Amphibians* published by the American Society for Testing and Materials (American Society of Testing and Materials [ASTM], 2006).

3.1.2 Pilot study

A preliminary study was performed to assess and amend test maintenance and protocols, water quality (specifically ammonia levels), tank density, juvenile frog preservation, feeding rates, and food type. The percent water change required to maintain adequate water quality with minimal disruption to the organisms was determined along with a feeding rate that would promote optimal growth and development. Due to high mortality at concentrations exceeding 1 g/L of poultry litter solution in the pilot study, test concentrations of 0.7 g/L and 0.35 g/L of poultry litter solution were chosen to ensure adequate survival in the primary study. Surviving juvenile frogs from the pilot study were preserved and used to perfect gonad excision techniques.

3.1.3 Acquisition of embryos

X. laevis embryos were obtained from breeding colonies at the University of Maryland - Wye Research and Education Center (UMD-WREC). A maximum of ten

adults were maintained in flow through (4 replacement volumes per day) circular polyethylene aquaria (0.91 m I.D. x 0.36 m high) with a water depth of 10 cm. UMD-WREC non-chlorinated deep well water (hereafter referred to as well water) held at 23.0 ± 0.5°C served as a culture medium. Breeding frogs were fed daily with Xenopus Express Premium Floating Food (Xenopus Express Inc., Brookville, FL). The colony was kept under a photoperiod of 16 h light: 8 h dark. 400 and 800 I.U. (glossary) of human chorionic gonadotropin (HCG) (glossary) was injected in the dorsal lymph sac (glossary) of the males and females, respectively, during the dark cycle in order to induce breeding (glossary). Amplexus (glossary) occurred 4-6 hours after injecting HCG (glossary); egg deposition occurred 9-12 hours following HCG injection.

3.1.4 Tadpole selection

A large number of tadpoles from one breeding pair were brought to the testing laboratory in the Biochemistry Building of the University of Maryland campus. The tadpoles were transported in well water that had been saturated with oxygen in sealed polyethylene containers. *X. laevis* embryos were maintained in well water until 12 DPF (Days Post Fertilization). At 12 DPF, the tadpoles were randomly loaded into each test replicate.

3.1.5 Poultry Litter Preparation and Treatment Solution Chemistry

Raw poultry litter was acquired from a Delmarva chicken house. Two five gallon buckets, lined with garbage bags, were filled and sealed. The poultry litter samples were stored in the dark at 4°C until the start of the test. Prior to the test,

poultry litter was pressed through a 1 mm stainless steel sieve to remove feathers and other large debris. This finer poultry litter was used to better mimic the particle size more likely to be carried away during field runoff.

Three 55-gallon plastic drums were filled with 189 L (50 gallons) each of non-chlorinated deep well water from the University of Maryland-Wye Research and Education Center (UMD-WREC). 387.50 g of sieved poultry litter was added to each drum to produce a stock solution of 2 grams of poultry litter per 1 liter of well water. The stock solutions were thoroughly homogenized using a 1-meter-long stainless steel paddle. To keep the stock solution homogenized throughout the test, they were stirred vigorously for 2 minutes each day of the test. The three stock solution drums were side-by-side, and maintained at 23.0°C with a 16 hour light:8 hour dark cycle. The stock solution was allowed to degrade naturally, with only gentle aeration. This stock solution was diluted with well water throughout the course of the study to prepare 0.35 g/L and 0.7 g/L of poultry litter solution for the low poultry litter exposure and the high poultry litter exposure, respectively. The stock solution was stored at UMD-WREC throughout the duration of the study. Each week, adequate amounts of control well water and poultry litter stock solution were transported from UMD-WREC to the on-campus laboratory to perform the renewals.

3.1.6 Preparation of Samples for Hormone Quantification

On test day 0, stock solution, and low PLAC (0.35 g poultry litter/L) and high PLAC (0.70 g poultry litter/L) test solution samples were prepared and allowed to homogenize for an hour. The samples were then filtered through 0.70 micron glass fiber filters. Filtered samples were then adjusted to a pH of 2 using 1 N sulfuric acid.

The samples were stored in 500 mL amber glass bottles in the dark at 4°C. On test days 7, 14, 21 and 28, low PLAC treatment samples were homogenized, filtered and pH-adjusted in the same procedures. On test days 7 and 14, high PLAC treatment samples were homogenized, filtered and pH adjusted in the same procedures. On test days 7, 14 and 21, 17β-estradiol (Sigma Aldrich) was dissolved in 5 mL of ethanol and diluted with deionized water to prepare a 2 mg/L positive control stock solution. The samples were further diluted with well water to produce a 200 ng estradiol/L positive control test solution. The positive control test solutions were filtered and pH-adjusted in the same manner as the poultry litter samples, and were stored in amber glass bottles in the dark at 4°C. All samples were shipped on ice to the University of Buffalo Chemistry Laboratory for GC-MS analysis of hormone concentrations.

On test day 28, samples from the poultry litter stock solution at the UMD-WREC and the poultry litter stock solution at the testing laboratory were collected for wet chemistry analysis. Equal volumes of poultry litter solution were taken from each aquaria. Aliquots from the high poultry litter exposure were combined and aliquots from the low poultry litter were combined for wet chemistry analysis. Samples were taken to ALS Environmental, Middletown, PA for analysis of ammonia, nitrate, nitrite and heavy metals including arsenic, copper, zinc, lead, and chromium.

3.1.7 Laboratory Setup

The tadpoles were divided into two experimental and two control groups: a high poultry litter exposure, a low poultry litter exposure, a positive control and a negative control. 0.7 g of poultry litter/L of well water was used for the high exposure and 0.35 g of poultry litter/L of well water was used for the low exposure. 200 ng of

pure estradiol/L of well water was used for the positive control and pure well water was used for the negative control. Each group had five replicates of 20 organisms for a total of 100 organisms per group. Each replicate was maintained in a 5-gallon aquarium (19 L) filled with 5 L of either control water or poultry litter solution initially. Starting at week two, the volume of solution in each aquaria was increased by 1 L per week over a period of 5 weeks to 10 L to accommodate *X. laevis* growth (see Appendix A). The temperature was maintained at 23 ± 2 °C with a 16 hour light to 8 hour dark cycle. Twenty-five percent water replacements were performed on Monday, Wednesday, and Friday of each week (see Appendix A). To account for evaporation, a smaller volume of water was removed than was added (see Appendix A). Gentle aeration maintained adequate water quality by reducing ammonia levels. Dissolved oxygen, pH, conductivity, and ammonia levels were measured periodically. A study by Lutz et al. (2008) noted that optimum parameters for water quality include pH optimally between 7.9 and 8.3, ammonia between 0 and 0.35 mg/L,, and conductivity 726-817 µg S/cm.

3.1.8 *X. laevis* Care and Maintenance

Starting at 5 DPF, tadpoles were fed twice daily with an aqueous suspension of Xenopus Tadpole Powder (Xenopus Express Inc., Brookville, FL). Feeding amounts were increased on a weekly basis to meet needs of growing tadpoles. Feeding amounts were also adjusted to accommodate for mortalities within each replicate, such that feeding volumes were equal in all replicates (see Appendix A). . Survival and behavioral observations were made daily. Typically metamorphosis normally occurs between 55-75 DPF (Gilbert, 2000). After metamorphosis, juvenile

frogs were fed - *ad libitum* with crushed Xenopus Express Premium Floating Food once per day. Debris, fecal matter, excess food and dead organisms were removed on a daily basis. At 131 DPF, the remaining frogs and tadpoles were taken back to UMD-WREC and placed in flow-through aquaria to promote more rapid growth and metamorphosis.

3.1.9 *X. laevis* Histology and Preservation

The frogs are classified as juveniles from the time they complete metamorphosis until they are sexually mature. At 152 DPF, we sacrificed the frogs by immersion in MS-222 (glossary). The frogs were then rinsed, and preserved in 40% formalin (glossary). A single, mid-line incision was made to allow for preservation of the internal organs. Snout-vent length (SVL), the distance between the snout and anus were measured for each frog. Juvenile frogs were blotted dry with paper towels and wet weights were measured. Gross observations of each frog's gonads were noted and any external abnormalities were photographed.

After preserved juvenile frogs were measured and weighed, paired kidney/gonad tissues were excised from each frog. The kidney/gonad tissues ranged from 5-8 mm long by 3-5 mm wide. The tissues were preserved in 10% buffered formalin in labeled glass vials. Prior to sending the tissue samples for histological slide preparation, the formalin was removed and replaced with a 70% ethanol solution.

The tissue samples were delivered to the Maryland DNR Cooperative Oxford Laboratory (Oxford, MD) for histological slide preparation. The samples were infiltrated and embedded to produce parrafin histology blocks. Transverse sections

across each tissue's long axis were systematically sectioned, with each 20th section being retained such that the longitudinal distance between retained sections was 100 microns. For each kidney/gonad block, 30 step sections were retained on three slides per frog tissue sample. The retained sections were stained with Mayer's hematoxylin and eosin (MHE) (glossary) to enhance histological analysis.

3.2 Analysis Procedures

3.2.1 Slide Analysis

For each frog, we received three slides containing 10 sections. All slides were examined as they contained enough gonadal sections to evaluate abnormalities. Observations made by Hecker et al. (2006) were used as comparison for gonadal observations. Each slide was independently examined by three people to ensure reproducible observations. When observations were conflicting, gonads were photographed at two magnifications for verification/identification of gonadal abnormalities. When multiple photographs were required to document the entire abnormality, the photographs were stitched using the freely available Hugin Panoramic Photo Stitcher Software with the following settings: Lens Type: Normal (rectilinear) and Focal Length: 35 mm. Frogs were classified as either having a specific abnormality or "not". Sex ratios were calculated based on gonadal observations.

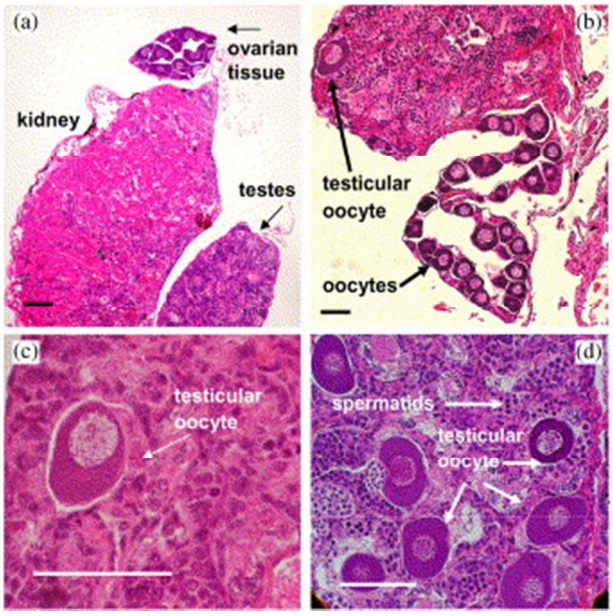

*Figure **6**:* Histology of Postmetamorphic *X. laevis*. (a) Intersex gonad; (b-d) Testicular oocytes (as indicated by arrows). From "Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*," by K. K. Coady, M. B. Murphy, D. L. Villeneuve, M. Hecker, P. D. Jones, J. A. Carr, … P. J. Giesy, 2005, *Ecotoxicology and Environmental Safety, 62,* p 167.

Statistical analyses were performed under the guidelines of the USEPA method for estimating the chronic toxicity of effluents and receiving waters (USEPA, 2002). All percent data, including survival, sex ratio and gonadal abnormalities, were arc-sine square root transformed before the statistical analyses were conducted. The null hypothesis that each group is equal is initially tested by the Dunnett's test. Dunnett's test consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison test for comparing each of the treatment means with the control mean. The assumptions upon which the uses of Dunnett's test are contingent are that the observations within the treatments are independent and normally distributed, with homogeneity of variance. The groups that do not meet the normality and/or homogeneity of variance assumptions are evaluated by non-parametric statistics such as Steel's Many-One Rank test or Williams test. The

statistical tests were performed using ToxCalc (TSS, 2006) at a minimum probability level of 0.05.

Chapter 4: Data Collection and Results

4.1 Amphibian Results

4.1.1 Survival

For this study, 100 organisms were exposed to each of the test treatments: negative control, positive control, low PLAC and high PLAC. All statistical analyses were determined based on control quantities. There was no statistical difference ($p>0.05$) between control survival and survival in both the low and high PLAC test solutions. Survival was 47% in the control treatment, compared to 52% survival in the low PLAC treatment and 49% survival in the high PLAC treatment. Survival in the positive control treatment was significantly ($p<0.05$) reduced compared to all of the other treatments, with only 19% survival.

4.1.2 Growth

Growth was measured postmortem and prior to dissection using a combination of snout vent length (SVL—measurement of body length from the tip of the nose or snout to the anus or vent, excluding the tail) (glossary) and wet weight (body weight) (glossary). Average SVL was significantly decreased ($p<0.05$) for both males and females in the low PLAC and positive control exposures. Average male SVL was 30.00 mm for the negative control, compared to 24.57 mm for the low PLAC and 23.60 mm in the positive control. Average female SVL was 29.75 mm for the

negative control, compared to 25.94 mm for the low PLAC and 24.42 mm for the positive control treatments. Compared to the controls, average high PLAC SVL was not statistically significant ($p>0.05$) for either males or females. Average high PLAC SVL for males and females were 29.05 mm and 31.08 mm, respectively.

In congruence with SVL data, average wet weights were significantly decreased ($p<0.05$) for both males and females exposed to the low PLAC and positive control. Average male wet weight was 3.99 g in the negative control, compared to 2.15 g and 1.88 g in the low PLAC and positive control, respectively. Average female wet weight was 4.94 g in the negative control, compared to 2.37 g and 2.08 g in the low PLAC and positive control, respectively. Compared to the negative control, average wet weights were not statistically significant ($p>0.05$) in the high PLAC exposure, measuring at 3.94 g and 4.56 g for males and females, respectively.

4.1.3 External Abnormalities

External abnormalities were documented post treatment. None of the treatments induced statistically significant ($p>0.05$) differences in external abnormality rates. 0% developed external abnormalities in the negative control, compared to 0% in the low PLAC, 0% in the positive control, and 4% in the high PLAC treatments. In particular, front limb deformities were observed in the high PLAC (Figure *7*).

Figure 7: Example of a front limb deformity. Copyright 2013 by Team KERMIT

4.1.4 Sex Ratios

There was no significant difference ($p>0.05$) in the percentage of females (sex ratios) between the control and low and high PLAC treatments. The control and high PLAC treatments each had 56% females. Though not statistically significant ($p>0.05$), the percentage of females in the low PLAC treatment was only 41%. The E2 positive control group had a significantly higher ratio of females to males, with 71% females, compared to the control sex ratio.

4.1.5 Gonadal Abnormality Rates

Gonadal abnormalities were determined by microscopically examining cross sectional slides of the surviving frogs' gonads. Low PLAC, high PLAC, and estradiol treatments all produced significantly increased ($p<0.05$) rates of gonadal abnormalities in males. In the negative control, 5.2% (one of 19) of male frogs exhibited gonadal abnormalities in the form of testicular oocytes, the presence of ovarian tissue in the testes of a male frog (Figure 8 andFigure ***10***). In the low PLAC exposure, 19.2% (five of 26) of male frogs showed abnormalities: four developed

testicular dysgenesis, the incomplete development of the testes (Figure 9 and Figure ***10***) and one developed testicular oocytes (19.2% total deformation rate). In the high PLAC exposure, 20.0% (four of 20) of male frogs developed abnormalities: two developed testicular dysgenesis (glossary) and two developed testicular oocytes. In the positive control, 40.0% (two of five) of male frogs developed abnormalities: both developed testicular oocytes (Figure 8). See table 2.

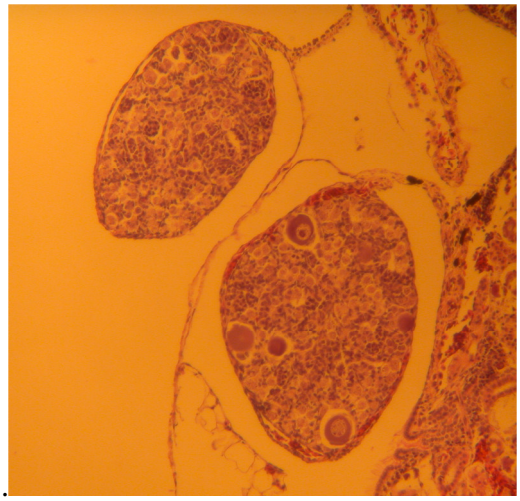

*Figure **8**:* Example of testicular oocytes. Copyright 2013 by Team KERMIT

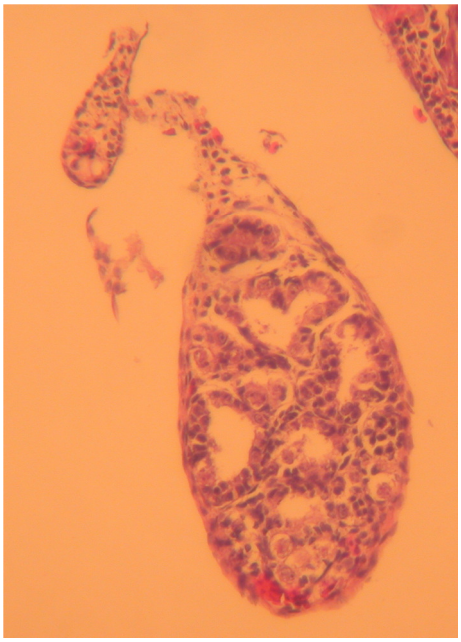

*Figure **9**:* Example of testicular dysgenesis. Copyright 2013 by Team KERMIT

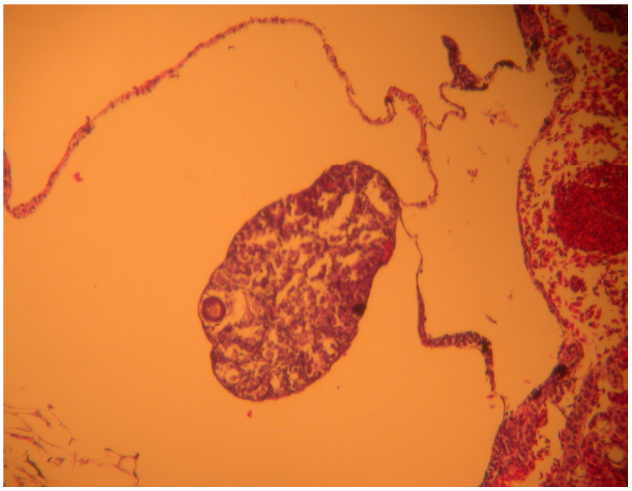

*Figure **10**:* Example of testicular dysgenesis and a testicular oocyte. Copyright 2013 by Team KERMIT

Post treatment, 0% (zero of 24) of the female frogs exhibited abnormalities in the control. The low PLAC and positive control treatments significantly increased ($p>0.05$) the presence of female gonadal abnormalities. In the low PLAC, 16.7% (three of 18) of the female frogs developed gonadal deformities: one frog displayed ovarian dysgenesis (the incomplete development of ovaries) (glossary), one frog developed both ovarian dysgenesis and a cyst (glossary), and one frog showed atresia (glossary) and minor testicular tissue (Figure 11 and Figure ***12***). In the positive control, 33.3% (four of 12) of the frogs developed gonadal abnormalities: three frogs developed atresia (absence or abnormal closure of oocytes) and one frog developed ovarian dysgenesis. The high PLAC solution did not significantly affect the rate of gonadal abnormalities in females. Only 12.0% (three of 25) of the female frogs developed abnormalities: two frogs developed male tissue with the presence of spermatocytes and one frog developed ovarian dysgenesis (Figure 14). See Table 2.

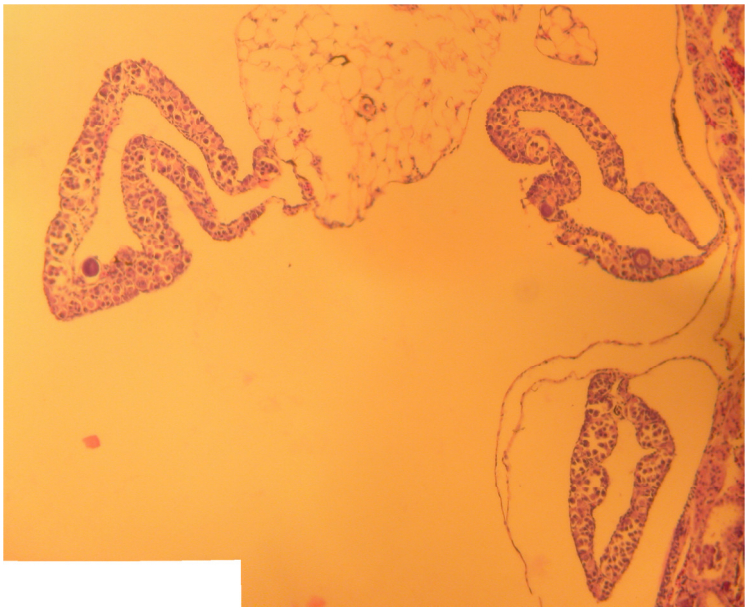

*Figure **11**:* Example of ovarian dysgenesis. Copyright 2013 by Team KERMIT

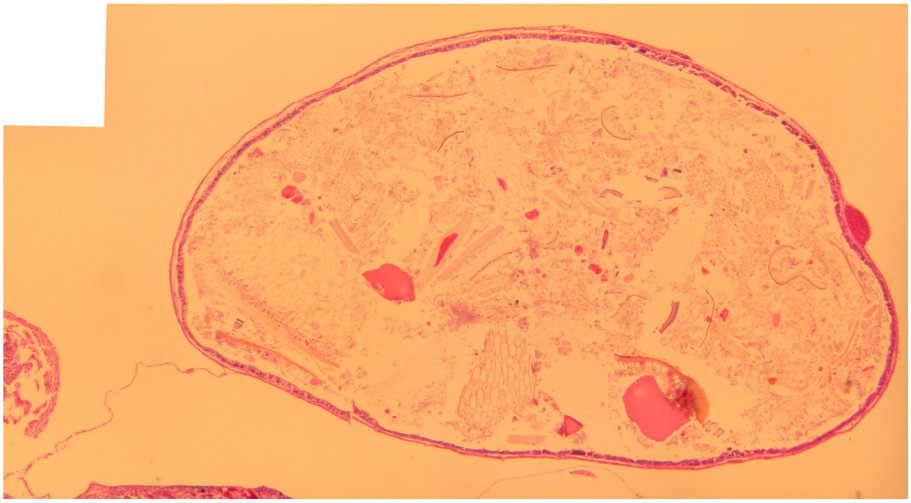

*Figure **12**:* Cyst. Copyright 2013 by Team KERMIT

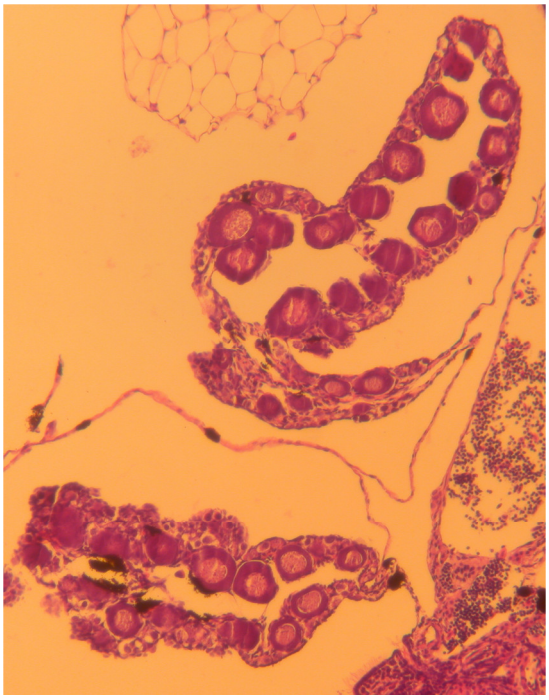

*Figure **13**:* Example of atresia. Copyright 2013 by Team KERMIT

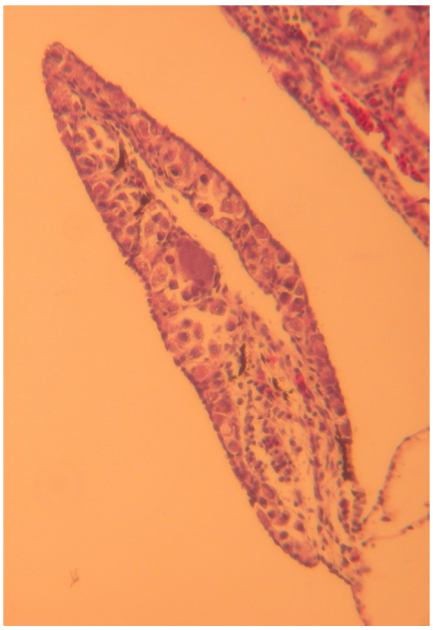

*Figure **14**:* Example of spermatocytes. Copyright 2013 by Team KERMIT

Looking at the overall population, the low PLAC and positive control induced statistically significant ($p<0.05$) increases in gonadal abnormality rates. In the control exposure, 2.3% (one of 43) of frogs developed abnormalities. Comparatively, 18.2% (eight of 44) and 41.2% (seven of 17) of the frogs developed abnormalities in the low

PLAC and positive control exposures. The high PLAC exposure produced a 15.6% (seven of 45) gonadal abnormalities rate and did not significantly affect ($p>0.05$) the gonadal abnormalities rate for the total population.

Table 2:

Summary of Frog Results for Four Treatments

Treatment	Survival	Sex	Sex Ratios	Mean SVL [range] (mm)	Mean Wet Weight [range] (g)	External Abnormalities	Gonadal Abnormalities
Control	47% (47/100)	Male	44% (19/43)	30.00 [21-45]	3.99 [1.1-7.5]	0% (0/19)	5.2% (1/19)
		Female	56% (24/43)	29.75 [20-39]	4.94 [0.9-8.6]	0% (0/24)	0% (0/24)
		Indeterminate	-	28.25 [22-36]	3.80 [1.3-7.5]	0% (0/4)	-
		Total	-	29.72 [20-45]	4.00 [0.9-8.6]	0% (0/47)	2.3% (1/43)
Low PLAC	52% (52/100)	Male	59% (26/44)	24.57 [17-33]	2.15 [0.7-4.9]	0% (0/26)	19.2% (5/26)
		Female	41% (18/44)	25.94 [22-23]	2.37 [1.2-4.7]	0% (0/18)	16.7% (3/18)
		Indeterminate	-	21.84 [15-29]	2.14 [0.2-3.5]	0% (0/8)	-
		Total	-	24.71 [15-33]	2.15 [0.2-4.9]	0% (0/52)	18.2% (8/44)
High PLAC	49% (49/100)	Male	44% (20/45)	29.05 [16-43]	3.94 [0.5-11.3]	0% (0/20)	20.0% (4/20)
		Female	56% (25/45)	31.08 [23-43]	4.56 [1.6-14.9]	8.0 % (2/25)	12.0% (3/25)
		Indeterminate	-	29.75 [23-36]	4.03 [2.3-6.5]	0% (0/4)	-
		Total	-	30.14 [16-43]	4.36 [0.5-14.9]	4.1% (2/49)	15.6% (7/45)
E2 Positive control	19% (19/100)	Male	29% (5/17)	23.60 [21-26]	1.88 [1.1-2.6]	0% (0/5)	40.0% (2/5)
		Female	71% (12/17)	24.42 [21-27]	2.08 [1.3-2.7]	0% (0/12)	33.3% (4/12)
		Indeterminate	-	24.50 [22-27]	2.20 [1.6-2.8]	50.0% (1/2)	-
		Total	-	24.21 [21-27]	2.04 [1.1-2.8]	5.3% (1/19)	35.3% (6/17)

Note. For a more detailed table of results, see Appendix B.
The indeterminate frogs data were excluded from the analysis.

4.2 Water Chemistry Results

4.2.1 Hormone Analysis

In this study, hormone concentration was analyzed to ensure environmental relevance and detect hormone breakdown. The GC-MS analysis included quantification of the following hormones: estrone, 17α-estradiol, 17β-estradiol, estriol, and ethinylestradiol as well as the following hormone-conjugates: estrone-3-glucuronide, 17β-estradiol-3-glucuronide, 17α-ethinylestradiol-3-glucuronide, estrone-3-sulfate, 17α-estradiol-3-sulfate, 17β-estradiol-3-sulfate, and 17β-estradiol-17-sulfate. In the poultry litter stock solution, only the sulfate-conjugates as well as 17β-estradiol and estrone were detected (Figure 15 and Figure ***16***). In both the high and low PLAC exposures, only 17β-estradiol-17-sulfate, 17β-estradiol, and estrone were detected (Figure 16). A comparison of maximum hormone content in the high PLAC, low PLAC, and PLAC stock is shown in Figure 17. Finally, only 17β-estradiol and estrone were detected in the positive control.

Additionally, the presence of sulfadimethoxine (SDM) (glossary) was determined. Both the poultry litter stock solution, and the high and low PLAC exposures contained high amounts of SDM. The concentrations were so high that only an estimation of SDM concentration could be given without dilution. The positive control also contained SDM, but this was below quantification limits.

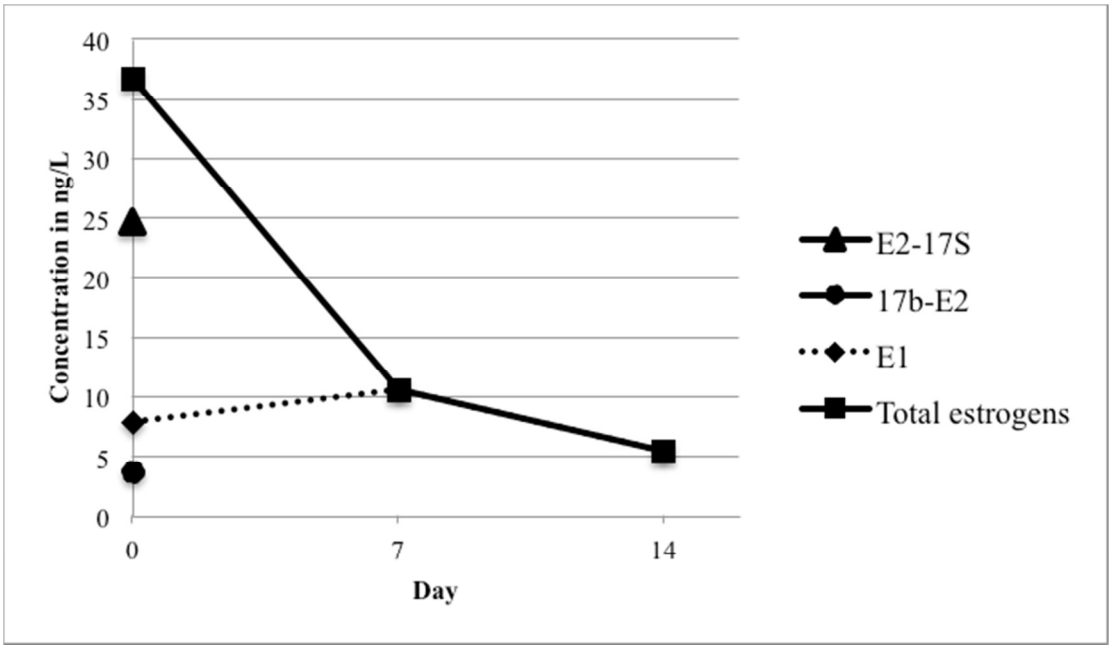

*Figure **15** :* Trends in Estrogens in High PLAC, measured with GC/MS. Copyright 2013 by Team KERMIT

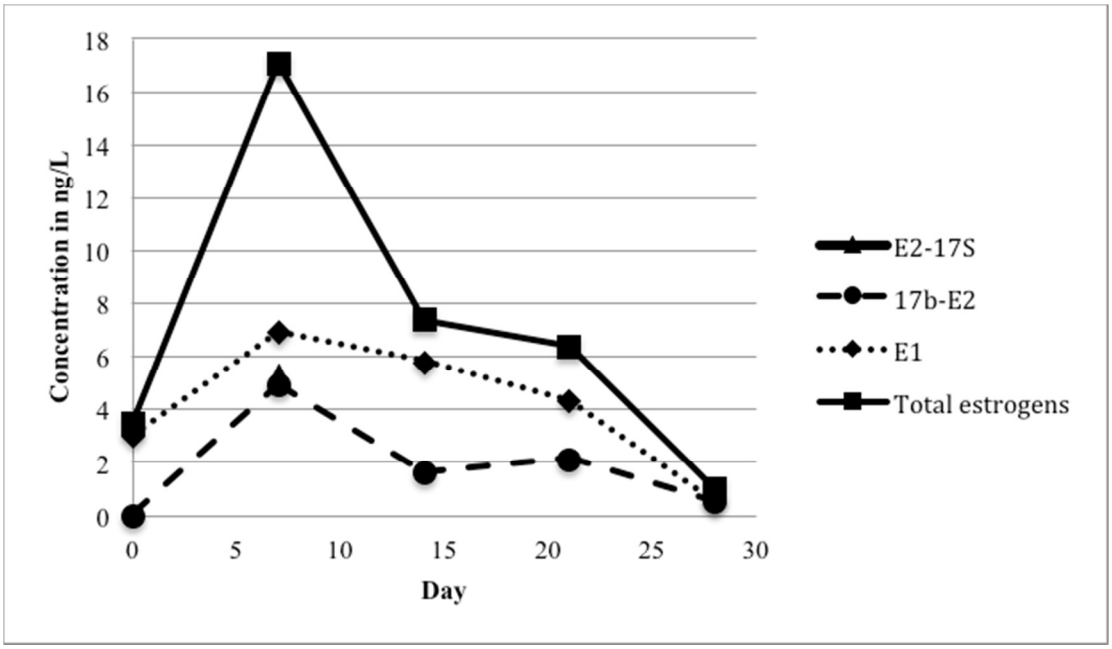

*Figure **16**:* Trends in Estrogens in Low PLAC, measured with GC/MS. Copyright 2013 by Team KERMIT

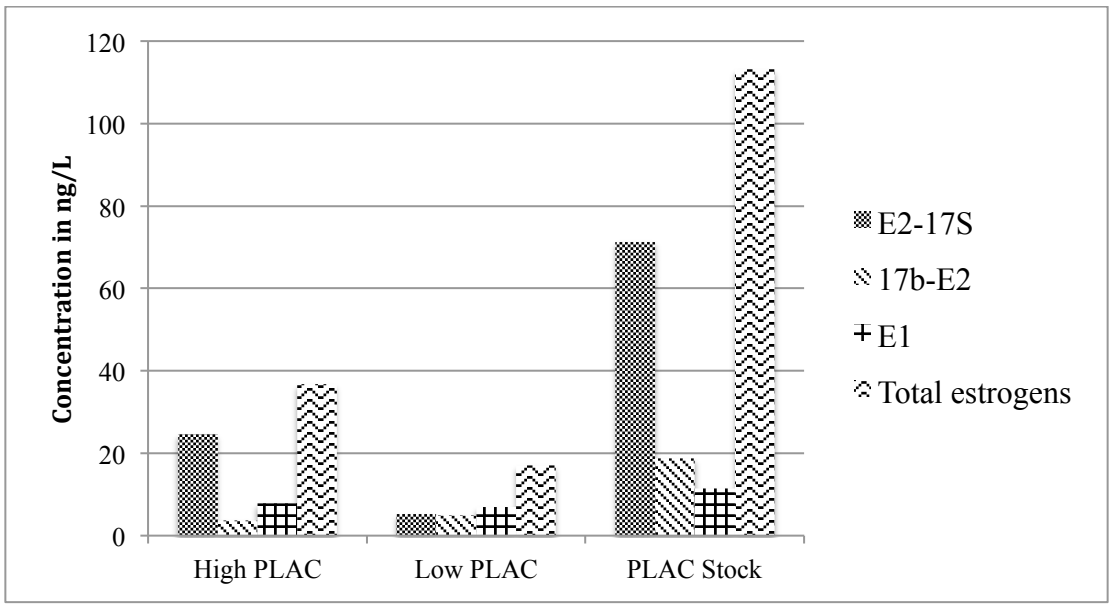

Figure 17: Comparison of Maximum Hormone Content. Copyright 2013 by Team KERMIT

4.2.2 Nitrogen containing compounds and heavy metals

The poultry litter solutions were also tested for the presence of nitrogen-containing compounds (ammonia, nitrite, and nitrate) and heavy metals (arsenic, chromium, copper, lead, and zinc). Nitrate was the only nitrogen-containing compound detected in the high PLAC solution, at a concentration of 11.1 mg/L (Figure 18). Ammonia, nitrate, and nitrite were all detected in the low PLAC solution at concentrations of 0.161 mg/L, 1 mg/L, and 15.5 mg/L, respectively. Chromium and lead were not detected in either the high or low PLAC exposure (Figure 19). Arsenic, copper and zinc were detected in the poultry litter samples; zinc was present in the highest concentration. In the UMD stock solution, the concentrations of arsenic, copper, and zinc were 0.062 mg/L, 0.15 mg/L, and 0.23 mg/L, respectively. The high carbon content in poultry litter likely bound to the heavy metals and decreased their bioavailability (

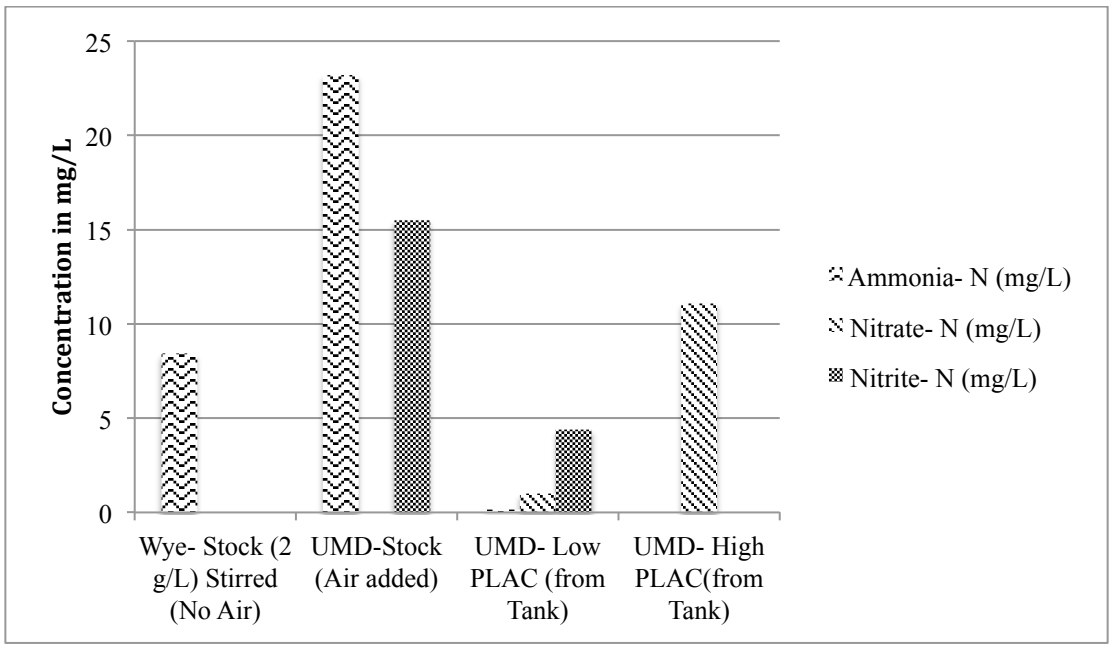

*Figure **18**:* Nitrogen Containing Contaminants in Poultry Litter. Copyright 2013 by Team KERMIT

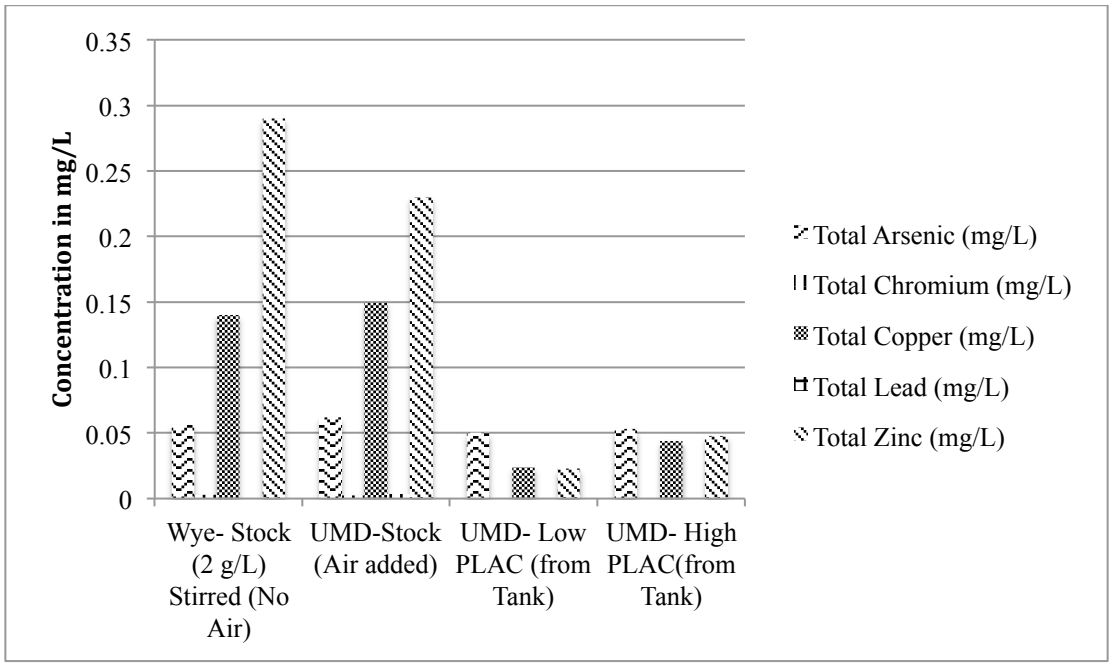

*Figure **19** :* Heavy Metal Contaminants in Poultry Litter. Copyright 2013 by Team KERMIT

Chapter 5: Discussion

5.1 Amphibian Discussion

5.1.1 Amphibian Survival

Negative control survival was lower than expected, and lower than observed in the pilot study under similar conditions. However, the surviving control juvenile frogs were all healthy, active and displayed normal gonadal development. There was no significant difference ($p>0.05$) in survival in the high and low PLAC treatments compared to the negative control. This study was designed to ensure survival in the low and high PLAC treatments so that adequate numbers of gonad samples could be examined. Ammonia, nitrate and nitrite exist in high and varying concentrations in poultry litter and are known to be toxic to amphibians at low concentrations. To decrease the potential toxicity of these nitrogenous compounds, we used very dilute poultry litter treatments.

The 96-hour LC50 (glossary) of total ammonia has been reported to range from 1.27 to 30 mg/L for *X. laevis* (Mann et al., 2009; Tietge, Ankley, DeFoe, Holcombe & Jensen 2009). Throughout the test, total ammonia levels of 1-3 mg/L total ammonia were measured colorimetrically (Hach kit). Tank pH levels in the PLAC treatments ranged from 7.60 to 8.10. At these pH levels, very little of the ammonia would be un-ionized ammonia, the most toxic form to aquatic organisms. Un-ionized ammonia levels of 0 to 0.161 mg/L were measured in the tanks (ALS Environmental, Middletown, PA). These levels are lower than the un-ionized

ammonia calculated 96-hour LC50s of 0.40 mg/L for *X. laevis* observed by Schuytema and Nebeker (1999).

Nitrate and nitrite are less toxic than ammonia to *Xenopus*, with 96-hour LC50s of 142-234 mg/L for nitrate and ~20 mg/L for nitrite (Mann et al., 2009). Sullivan and Spence (2003) determined a no-effects level of 66.0 mg/L nitrate in a 40-day study with *X. laevis* tadpoles. Nitrate concentrations as high as 11.1 mg/L and nitrite concentrations as high as 4.4 mg/L were measured in the PLAC treatments. These levels are far below nitrate and nitrite levels shown to cause mortality in *X. laevis*. However, the highest nitrate concentration measured in the low PLAC treatment, 11.1 mg/L, has been shown to be toxic to other more sensitive amphibian species, such as *Rana pipiens* (leopard frog) and *Bufo bufo* (common frog) (Camargo, Alonso & Salamanca 2004).

Heavy metals have also been shown to be toxic to amphibians at sufficiently high concentrations. In general, heavy metal concentrations measured in the low and high PLAC test solutions were well below those observed to cause amphibian mortality. Lead and chromium were found only in PLAC stock solutions, but were not detected in low or high PLAC treatments. Haywood, Alexander, Byrne & Cukrowska (2003) found significant *Xenopus* mortality results from zinc exposure at concentrations between 5 and 20 mg/L. Concentrations of zinc of 0.023 mg/L and 0.048 mg/L were measured in the low and high PLAC treatments, respectively. Bantle et al. (1996) determined a 96-hour LC50 of 0.980 mg/L of copper for *Xenopus* embryos. In a long-term exposure with *Rana sphenocephalus* (southern leopard frog), Lance et al. (2012) found that concentrations of 0.100 and 0.150 mg/L of copper

significantly decreased survival. Concentrations of 0.024 and 0.044 mg/L total copper were measured in the low and high PLAC tanks, respectively. Very few studies have been conducted on the effects of arsenic on amphibians. Khangarot, Sehgal, and Bhasin (1985) determined a 96-hour LC50 of 0.249 mg/L of arsenic for *Rana hexadactyla* (green pond frog). The USEPA (n.d.a) National Recommended Chronic Water Quality Criteria for arsenic is 0.150 mg/L arsenic. Total arsenic levels measured in low and high PLAC treatments were 0.050 and 0.053 mg/L, respectively; well below levels recommended to protect aquatic organisms. Dilution of the poultry litter stock solution into low and high PLAC treatments adequately reduced heavy metal concentrations to levels that likely reduced their toxicity to *Xenopus* embryos.

There was a significant reduction in survival in the positive control relative to the negative control (and the PLAC exposures). This could be because poultry litter contains a large amount of particulate matter compared to a pure estradiol dilution; it is possible that the frogs were able to feed on extra nutrients in the poultry litter. This extra source of nutrients could have kept frogs alive despite adverse environmental conditions. Additionally, little fecal material was noted in the positive control, indicating the frogs were not feeding well. The frogs in the positive control were significantly smaller than in other exposures. Smaller frogs have a greater ratio of surface area to body weight, resulting in faster uptake of estradiol through the skin (Tietge, et al., 2009). This increased estradiol uptake could result in more mortalities compared to the larger frogs in the experimental conditions.

5.1.2 Growth

The mean SVL and wet weight of low PLAC and positive control frogs were significantly lower compared to the SVL and wet weights of the negative control. The significant differences in size can be attributed to growth environment and nutrient availability. As expected, the specimens from the negative control group were among the largest and heaviest of all the frogs due to optimal tank conditions: clean water, adequate food sources, low ammonia levels, and high oxygen levels. Tietge et al. (2009) observed that amphibian growth is stunted when exposed to 15 mg/L of ammonia. Additionally, Haywood et al. (2003) have shown that increasing levels of zinc, copper, and lead decreases growth. However, the concentrations used in their study were significantly higher than the concentrations in our study. Thus, heavy metals likely contributed minimally to growth variations.

The mean SVL and wet weight of frogs in the high PLAC exposure were not significantly different from frogs in the negative control. This drastic size divergence from the low PLAC group may be ascribed to the high levels of particulate matter in poultry litter . The tadpole's capacity as a filter feeder allows it to uptake more carbon as a nutrient source from poultry litter in the water in addition to the scheduled feedings. Additionally, ammonia and nitrite levels were higher in the low PLAC solutions, while nitrate was higher in the high PLAC solution. Since ammonia and nitrite are more toxic than nitrate is to *X. laevis*, they may have contributed to the reduced growth in the low PLAC treatment. It is likely that concentrations of ammonia, nitrite, and nitrate varied throughout the test in the low and high PLAC treatments based on the levels of aerobic bacteria. These bacteria are known to

convert ammonia and nitrite to nitrate. Higher levels of bacteria in the high PLAC may have converted excess ammonia and nitrite into nitrate. We also found that there was a greater variation in size in the poultry litter exposures compared to the negative control.

The SVL and wet weight data in our experiment differed from similar studies because other studies conducted shorter exposures at higher estradiol concentrations. Therefore, comparisons that can be drawn from these studies are limited. Our specimens were sacrificed 152 DPF while comparable studies ran for just over half that time (Lutz, et al., 2008; Carr, et al., 2009). The study by Lutz et al. (2008), in which frogs were exposed to estradiol for no longer than 76 days, found that frog SVLs ranged from 16.3 mm in the 1.5 µg/L estradiol group to 23.0 mm in the 6.0 µg/L estradiol group. Lutz et al. found that the average SVL for frogs from the 0.2 µg/L estradiol group (our positive control concentration) was 22.2 mm. Carr et al. (2009) observed frog sizes ranging from 13.6 mm in the ethanol control to 14.5 mm in 100 µg/L of estradiol. Most existing research focuses on the consequences of isolated endocrine disruptor exposure rather than the effects of poultry litter as a whole.

5.1.3 External Abnormalities

None of the four exposures displayed significant external abnormalities. 4.8% of frogs in the high PLAC displayed limb deformities, which is within the natural abnormalities range. A baseline level of background abnormalities has been found to be between 0-2% (Ouellet, 2000) and 0-5% (Johnson et al., 2010). Malformations of the digits, and to a lesser extent limbs, appear to be a normal occurrences among wild

populations of frogs (Gardiner et al., 2003). It is estimated that <1% to 5% of frogs have observable malformations (Gardiner and Hoppe, 1999; Read, 1997; Read and Tyler, 1994; Stocum, 2000; Johnson et al., 2001b; Schoff et al., 2003; Eaton et al., 2004; Piha et al., 2006). In the present study, only 5% of the frogs in the high PLAC group had limb abnormalities, which falls into the normal range of abnormalities in frog populations. Therefore, these deformities were not considered to be significant.

Previous research shows that ammonia is a potential source of amphibian deformations. Surviving amphibians at a concentration of 30 mg/L of ammonia show "axial malformations of the tail and notochord, reductions in craniofacial and forebrain development, and cardiac edema" (Tietge et al. 2009). However, the ammonia in our exposures was quite low (0-0.161 mg/L) and may not have been concentrated enough to cause deformations. In a 100-day exposure, 10 mg/L of nitrate also produced edemas and head and digestive deformities (Hecnar, 1995). Our nitrate levels ranged from 1 mg/L in the low PLAC solution to 11.1 mg/L in the high PLAC solution. However, we did not observe any of these malformations in the surviving juvenile frogs from the PLAC test treatments.

Increased heavy metal concentrations have also been linked with increased malformations (Haywood et al., 2003). The study exposed tadpoles to varying solutions of zinc, copper, lead, or cadmium for seven days (Haywood et al., 2003). A maximum of 40% of the tadpole population exhibited malformations when exposed to 0.5-0.9 mg/L of copper; more than 50% exhibited deformations when exposed to 0.9 mg/L of lead; and around 50% exhibited deformations when exposed to 0.8-0.9 mg/L of cadmium (Haywood et al., 2003). Our 0.023 mg/L (low PLAC) and 0.048

mg/L (high PLAC) of zinc, 0.024 mg/L (low PLAC) and 0.044 mg/L (high PLAC) of copper, and 0 mg/L (low PLAC) and 0 mg/L (high PLAC) of lead levels may not have been high enough to induce malformations.

In this study, we observed deformed forelimbs in some of the high PLAC frogs. It is evident by understanding how *X. laevis* feeds that certain physical characteristics are essential for survival. After metamorphosis, *X. laevis* use both forelimbs for feeding. In the case of bilateral or unilateral forelimb abnormalities (glossary), the feeding motion becomes impossible or significantly more inefficient.

5.1.4 Sex Ratios

The low PLAC and high PLAC sex ratios were not significantly different from negative control ratios. The high PLAC ratios mirrored the negative control ratios; 44% of the frogs were female while 56% of the frogs were male. In the low PLAC, 59% of frogs were male while 41% of frogs were female. This bias toward males was unexpected but not significantly different from the negative control. The positive control ratios were significantly different from negative control ratios: 71% of the frogs were female while 29% were male. This supports the EC50 concentration through metamorphosis reported by Wolf et al. (2010). Since estradiol is a feminizing hormone, its EC50 concentration through metamorphosis is expected to alter the sex ratio from 50:50 to 75:25 female to male. The concentrations of estrogenic hormones in low and high PLAC were significantly lower than the concentrations in the positive control and were too weak to induce complete feminization.

5.1.5 Gonadal Abnormalities

As expected, gonadal abnormalities in both male and female negative control were infrequent; 5.2% of males, 0% of females, and 2.3% of the overall population displayed gonadal abnormalities. There are a number of studies that have observed similar frequencies of gonadal malformation in *X. laevis* exposed to negative control. In one study, approximately 2.2% of frogs developed abnormalities by NF (glossary) stage 66 (Hecker et al., 2003), closely echoing the 2.3% seen in this study. Comparable rates are found in nature; based on a survey of 233 male frogs, 13% had testicular oocytes, and 7% of male frogs that lived near agricultural areas developed intersex, higher than 5.2% observed in this study (Skelly, Bolden & Dion, 2010).

The frequency of gonadal abnormalities in males, females, and the overall population increased significantly ($p<0.05$) in positive control organisms. Our frogs were exposed to 202.0-236.5 ng/L of estrogenic hormones (138.7-160.1 ng/L of estradiol). A high frequency of gonadal abnormalities in the positive control treatments was expected. Wolf et al. (2010) determined that the EC50 through metamorphosis for estradiol was 200 ng/L; another study confirmed that significant numbers of frogs exposed to 200 ng/L of estradiol would develop gonadal abnormalities (Carr et al., 2003). Wolf et al. (2010) speculates that exposure to <200 ng/L of estradiol would induce gonadal abnormalities rather than complete feminization, which is confirmed by our data.

The frequency of gonadal abnormalities in males increased significantly ($p<0.05$) in low and high PLAC organisms. In the low PLAC exposure, four developed testicular dysgenesis and one developed testicular oocytes (19.2% total

deformation rate). In the high PLAC exposure, two developed testicular dysgenesis and two developed testicular oocytes (20% total deformation rate). McCoy et al. (2008) show that an increase in the number of gonadal abnormalities in *Bufo marinus* males is associated with an increase in proximity to agricultural lands. As agricultural proximity increased, the number of amphibians with gonadal tissue abnormalities such as multiple testes, abnormally shaped testes, small testes, and intersex also increased (McCoy et al., 2008). Similarly, agricultural land use and animal density correlate with intersex severity and frequency (Blazer et al., 2012). Furthermore, intersex prevalence also increased with the number of poultry houses in the vicinity (Blazer et al., 2012).

The frequency of gonadal abnormalities in females increased significantly ($p<0.05$) in low PLAC organisms. In the low PLAC exposure, one frog displayed ovarian dysgenesis, one frog developed both ovarian dysgenesis and a cyst, and one frog showed atresia and minor testicular tissue (16.7% total deformation rate). While not statistically significant, 12.0% of females in the high PLAC were affected by the increased levels of estrogenic hormones compared to 16.7% in the low PLAC, and 0% in the negative control.

5.2 Chemistry and Degradation

In this study, a novel approach was taken to examine the effects of a long-term exposure to PLAC, a natural source of EDCs in the environment. While many existing studies focus on the effect of pure hormone at high concentrations, only a few studies focus on low-level effects of complex compounds such as PLAC. Our

study found that even at dilute levels, the EDCs in poultry litter had an effect on *X. laevis*.

The exposure was conducted using environmentally relevant concentrations of EDCs. The GC-MS data show that the frogs were exposed to estrogenic substances at a maximum concentration of 36.8 ng/L in the high PLAC exposure or 17.1 ng/L in the low PLAC control. In the high PLAC exposure, the concentration of free and conjugated estradiol (glossary) was 28.4 ng/L and the concentration of free and conjugated estrone was 8.4 ng/L. In the low PLAC exposure, the concentration of free and conjugated estradiol was 10.2 ng/L and the concentration of free estrone was 6.9 ng/L. These concentrations are within the range previously observed in the environment (see Ecological Impact of EDCs section). In this way, our poultry litter exposure should simulate a single, natural runoff event.

Further, these hormones were allowed to degrade over time and the degradation was analyzed by GC-MS quantification. In the low PLAC exposure, all concentrations of estrogenic chemicals detected decreased to below quantification limits within 28 days. Most studies re-dose periodically with fresh exposure solution, creating an artificially high concentration of EDCs. In contrast, our study uses a large poultry litter stock solution, which was allowed to degrade over time. This degradation is a more realistic simulation to environmental conditions and allows the study of estradiol's degradation intermediates. Using poultry litter at an environmentally relevant concentration allows this study to be more relevant to normal conditions outside of the laboratory.

GC-MS quantification did not detect levels of any glucuronide-containing estrogen conjugates (glossary). This indicates that glucuronide conjugates are particularly unstable. This finding is supported in other studies that also examined conjugated estrogen degradation (Dutta et al., 2012). We also found that the poultry litter stock solution contained all the estrogen sulfate-conjugates that we tested for. In particular, high levels of 17β-estradiol-17-sulfate were detected in the low PLAC, high PLAC, and poultry litter stock solutions. It has been shown that sulfate conjugates are fairly stable and this is supported in our study (Dutta et al., 2012).

We wanted to test the persistence of estradiol in poultry litter runoff. The presence of 17β-estradiol-17-sulfate was only detected on one day (day 0 in the high PLAC and day 7 in the low PLAC) before the concentrations fell below detection limits. It would be expected that 17β-estradiol-17-sulfate would degrade into estradiol or estrone; however, our data do not show an increase in the concentration of either of these substances. Dutta et al. (2012) note that compared to free estrogens, the sulfate group-conjugated estrogens have a greater solubility in water. This may explain the high initial concentrations followed by rapid declines of conjugated estrogens in our study. Another explanation could be that the compound degraded into substances not tested for by the GC-MS. Alternatively, the degradation products, 17β-estradiol and estrone, could have rapidly degraded before the next quantification.

In the low PLAC degradation, the decrease in estrone concentration between days 7 and 21 was not substantial. Other studies have corroborated that estrone is a more persistent EDC in comparison to 17β-estradiol (Dutta et al., 2012). Between days 21 and 28, the estrone concentrations in our study decreased sharply. This

decrease could be the result of an increase in the number of estrone-degrading bacteria.

The positive control was also quantified by GC-MS for verification of the correct estradiol concentration. We were seeking a concentration between 200-220 ng/L of estradiol, the EC50 through metamorphosis determined by Wolf et al. (2010). While the concentration of estradiol alone was below the concentration we desired, the total estrogen concentration was between 202.0 and 236.5 ng/L when both estrone and estradiol were considered. This further supports that estradiol degrades quickly and that estrone is the primary degradation product, and confirms that our positive control was close to the EC50 through metamorphosis.

Because we wanted to look at estradiol degradation, using an ELISA based EDC quantification method was out of the question. ELISA and immunoassays are frequently overly sensitive, detecting other estrogenic compounds or organic matter. Because of this, they are unable to distinguish specific EDCs and report exaggerated values (Figure 20). Using GC-MS quantification allowed us to look at specific estrogens as well as estrogen conjugates, which was essential for studying the hormone composition of poultry litter as well as degradation.

In order to conduct a more thorough estradiol degradation study, it would have been beneficial to have more frequent sampling over a longer period of time for GC-MS quantification. However, this would have been prohibitively expensive for this study. Thus, we had to compromise between frequency of sampling and length of sampling time. Overall, all estrogenic substances degraded below quantification

limits by day 28, but it would have been beneficial to have similar data for the high PLAC exposure.

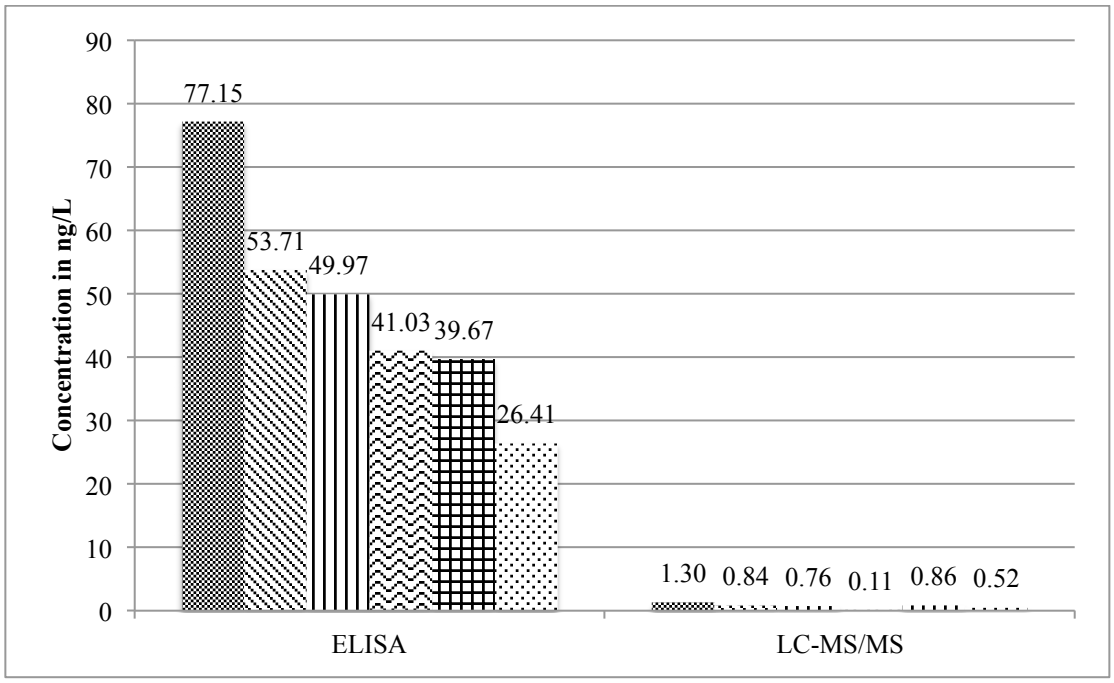

*Figure **20**:* Comparison of ELISA with LC-MS/MS Quantification Technique. Six samples were analyzed with ELISA (left) and the same six samples were analyzed with LC-MS/MS (right). This figure shows that ELISA reports falsely elevated concentrations.

5.3 Potential methods to decrease poultry litter pollution

Poultry litter is both an abundant and economical option for fertilization of large areas. However, its application leads to the disruption of natural nutrient and hormone balances. To address this issue, runoff and nutrient reduction should be considered. Several of these options include incorporating phytase (glossary) into chicken feed, using pelletized poultry litter (glossary), planting riparian buffer zones (glossary), and using cover crops.

5.3.1 Phosphorous, Poultry, and Phytase

Since phosphorous, often included in poultry diets and present in poultry litter, is a threat to water quality, a viable solution could focus on the presence of

phosphorous in poultry diets. Phosphorous is an essential element of poultry diets because it promotes bone growth, so limiting the intake of phosphorous is not a feasible solution. The main problem, however, in poultry phosphorous consumption is the presence of phytate (glossary), a molecule that prohibits phosphorous from being absorbed by poultry. According to Bergström et al. (2007), 80-90% of the phosphorus in cereal grain used for animal feed is stored as phytate, which is poorly digested by many animals. Chicken farmers use the enzyme phytase to increase the nutritional value of chickenfeed by breaking down phytate. When phytase is added to the feed, the chicken can retain greater amounts of calcium, phosphorous, carbohydrates and proteins, thereby enhancing its growth (Frias, Doblado, Antezana & Vidal-Valverde, 2003). Phosphorous levels in feed can be kept at 0.4% when phytase is supplementing the diet (Bergström et al., 2007). Additionally, phytase helps alleviate the negative effects of phytic acid (glossary) (Karimi et al., 2013). Phytic acid is prevalent in most cereals, which contributes to a high percentage of the food. Phytic acid inhibits the absorption of a variety of minerals during digestion, effectively making the food less nutritious (Karimi et al., 2013). Besides aiding digestion, phytase also acts as an antioxidant, making the chickens more resilient against disease (Frias et al., 2003). Farmers use phytase to increase the nourishing value of their chicken feed and decrease the amount of phosphorous in poultry litter and runoff.

Poultry that are fed high-availability phosphorous (HAP) corn have been experimentally shown to have comparable or even better performance than poultry that are fed corn with phytase (University of Delaware, 1999). Additionally,

preliminary results look promising in terms of reducing phosphorous levels. After feeding poultry flocks hybrid corn, phosphorous levels in poultry litter dropped 41 percent and water soluble phosphorous levels dropped 82 percent (University of Delaware, 1999).

Poultry companies are typically responsible for supplying the feed for poultry flocks. Therefore, if the recommendation of incorporating HAP corn is followed, the burden would fall on poultry companies to change the composition of their feed. Additionally, farmers will have to produce HAP corn at levels sufficient to satisfy poultry companies' needs. Because HAP corn depends on recessive genes, achieving adequate levels may be challenging without proper crossing techniques (University of Delaware, 1999).

5.3.2 Pelletized Poultry Litter

Using pelletized poultry litter instead of raw poultry litter presents additional economic and environmental advantages. Pelletized poultry litter has been shown to have a positive impact on crop biomass yields (Deksissa et al., 2008). From a practicality standpoint, pelletized poultry litter is odorless, easier to store and transport, and more easily applied to fields compared to non-pelletized poultry litter (Lopez-Mosquera, Cabaleiro, Sainz, Lopez-Fabal,& Carral, 2007). Additionally, pelletized poultry litter is processed at high heats such that it poses no risk of introducing fecal bacteria and coliforms into groundwater sources (Deksissa et al., 2008). This process also reduces the moisture content of the poultry litter and impacts the concentrations of nutrients and metals found in the poultry litter (Lopez-Mosquera et al., 2007).

Pellet size and composition vary by manufacturer (Wild et al., 2011), and there is no established recommended rate of application for specific crops (Lopez-Mosquera et al., 2007). Research has shown that there is uncertainty as to whether pelletizing poultry litter increases or decreases the variability of nutrient content among the batch of pellets (Lopez-Mosquera et al., 2007, Wild et al., 2011). Wild et al. (2011) found higher total and mineralizable nitrogen contents in pelletized poultry litter and lower nitrogen variability compared to non-pelletized poultry litter. However, Lopez-Mosquera et al. (2008) found that pelletized poultry litter had increased variability of total nitrogen and nitrate in comparison to fresh poultry litter. It was also found that pelletized poultry litter had comparatively a lower pH and lower concentrations of heavy metals such as chromium, copper, and cadmium (Lopez-Mosquera et al., 2007).

5.3.3 Guidance in the Chesapeake Bay Watershed

In May of 2009, President Obama signed Executive Order 13508, declaring the Chesapeake Bay a national treasure that needs to be protected by reducing sediment and nutrient runoff along coastal zones in the Mid-Atlantic area (CBF, 2012). As a result, the EPA published its *Guidance in the Chesapeake Bay Watershed* initiative in 2010, which was designed to highlight the most effective practices in reducing the impact of pollution on the bay. The guidelines addressed the impact of agriculture on the environment through non-point source pollution (EPA, 2010). The guidelines also suggest preferred methods for fertilizer application and land use assessment, as well as educational resources and lists of regional institutions that actively work to help farmers monitor their nutrient output. Specifically, for

agricultural practices, the EPA emphasizes the importance of creating sufficient riparian forest buffers (glossary) and vegetative strips, and using cover crops to reduce annual storm water runoff (U.S. EPA, 2012).

5.3.4 Buffer zones

Buffer zones are sediment traps that intercept nonpoint source pollutants prior to being deposited in the streams and ground water (Dillaha & Inamdar, 1996 as cited in Haycock et al., 1997). There are multiple types of buffer zones and no uniform standards or accepted methods for buffer zone creation (Dillaha & Inamdar, 1996 as cited in Haycock et al., 1997).

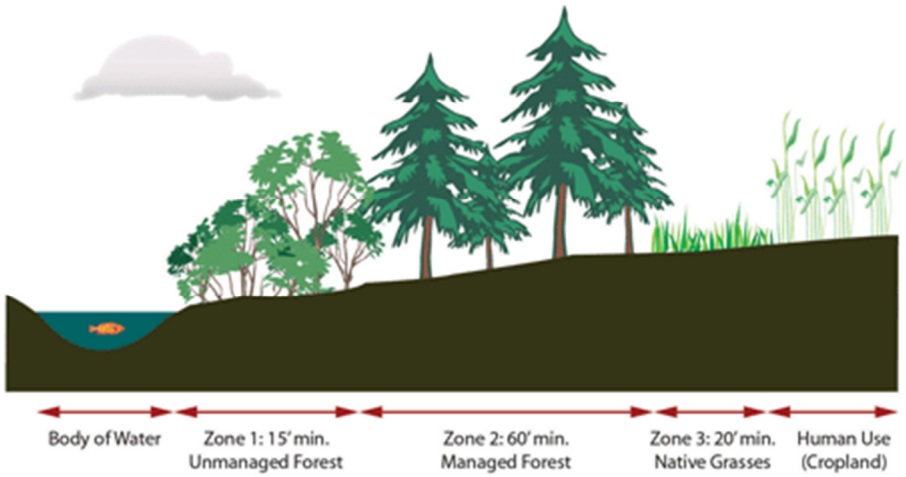

*Figure **21**:* Division of riparian buffer zones. From "Riparian Buffers," by A. Pierce.

Riparian buffer zones are the most complex of the three listed above because they consist of three distinct zones that are responsible for catching sediment, as shown in *Figure **21***. These zones are defined as vegetative areas that are of a sufficient width to intercept potential non-point source pollutants such as sediment, nutrients, pesticides and other runoff before they enter the watershed (USDA, 1997). Riparian buffer zones have been shown to reduce both nutrient and hormone

concentrations in surface runoff. Typically, buffer zones from 9 to 18 m in width can retain most nitrogen and phosphorus runoff, although nutrient saturation in the buffer zones reduces their effectiveness (Vought et al., 1994). Hormone concentrations have also been shown to decrease as runoff passes through riparian buffer zones. Dutta, Imander, Sims et al. (2010) found that the concentration of estrone was consistently lower through each stage of riparian forest buffer.

One of the main environmental issues in the United States involves the leaching of nitrate into ground water supplies. Nitrate levels greater than 10 mg/L are deemed unfit for human consumption, yet a study conducted in 1995 indicated that up to 26% of wells surrounding agricultural areas in the United States were contaminated with nitrate (Muller, Hamilton, Helsel, Hitt, & Ruddy, 1995). The level of nitrate in the water supplies may also be increased by the amount of nitrogen-related application to crops. An increase of nitrogen application rates from 100 to 134 kg/ha was found to nearly double the nitrates based on average flow-weighted tile drainage. Riparian buffers can help reduce the levels of nitrate contamination in water supplies. In the event of runoff, the bacteria that reside on riparian buffer plants help convert nitrates into gaseous forms of nitrogen (Mueller, Hamilton, Helsel, Hitt, & Ruddy, 1995).

The creation and conservation of riparian buffer zones along coastal wetlands has been addressed through both federal and state regulations regarding the setback limits and vegetative composition of these areas. The EPA specifies that a minimum width of 30 m of forest buffer is required for any active channel, and that additional setbacks may be required for areas depending on stream order, percent slope of the

channel bank, 100-year floodplain, and wetlands identified as critical areas (EPA, 2007). CAFOs require a setback of 76 m, but farms that use poultry litter or other manure as fertilizer but are not large enough to be considered CAFOs only require a setback of 30 m (EPA, 2007).

In 2008, the USDA Farm Service Agency created a voluntary program targeted at agricultural landowners in order to promote the conservation of wetlands by offering cost-share assistance and annual rental payments to owners who agreed to contribute to rebuilding wetlands and buffer zones along their property (USDA, 2012). Maryland has offered additional incentives for land owners who contribute land towards protecting the Chesapeake Bay watershed, and has set a goal to establish 31,000 hectares of additional riparian buffer habitat (Conservation Reserve Program, 2012). Farmers would receive benefits proportional to the amount of contribution they make towards achieving the program's goal.

In the Chesapeake Bay region, the Riparian Forest Buffer (RFB) Initiative (glossary) was created to encourage the establishment and preservation of riparian zones in the Delmarva area in 1996. The goal was to establish 3235 km of new buffer zones. In the same year, Maryland started Stream ReLeaf (glossary), a statewide initiative that brings together both state and federal agencies and nonprofit organizations to create and conserve buffers zones. Maryland committed to creating 966 of the 3235 km of buffer zones. This goal was met in 2001 largely due to the passing of the 1996 USDA Farm Bill which established the Conservation Reserve Enhancement Program (CREP) (glossary). The program helped increase incentives to help farmers and landowners convert land to buffer zones. In 1999 and 2000,

respectively, Virginia and Pennsylvania also initiated CREP and the 3235 km buffer zone goal was met in 2002. In 2003, a new goal was set to create 16,000 km of buffer zones by 2010, with Maryland establishing 3256 km (Maryland Department of Natural Resources 2012b).

Maryland has established statewide environmental regulations for the local watershed as well. The MDE originally defined a buffer as "a regulated area, 25 feet in width, surrounding a nontidal wetland, measured from the outer edge of the nontidal wetland" (Code of Maryland Regulations, 1974). In 1984 the General Assembly of Maryland passed its Critical Area Law, intended to "[foster] more sensitive development activity for certain shoreline areas so as to minimize damage to water quality and natural habitats" (Weschsler, 2010). Implementation of the law was to be done at a local level in accordance with established criteria by the state. The law was amended in 2002 to include a more comprehensive area of the coastal Chesapeake region. It declares that the critical area impacted by the law constitutes "roughly that area of land within [305 m] of the tidal waters of the State". Most of this area was required to have buffer widths of 30 m, and certain new subdivisions of development were subject to 61 and even 91 km buffer zones (Weschsler, 2010). The newest version of the law, which became effective in 2010, instated significantly stricter regulations and management procedures for coastal developments in the region. The regulations require the buffer strip to be replanted with native plants to ensure the maximum efficiency of the buffer zones (Weschsler, 2010). The property owners, after rebuilding the buffer zones, are required to submit a Buffer Management Plan that discusses how the area will be maintained, as well as

providing financial assurances to ensure that the plantings survive for a period of two to five years (Weschsler, 2010).

The newest version of the Critical Area Law (glossary) alters the definition of a buffer zone to include more specific identifiers. For example, the buffer is defined as the area from where the water reaches its highest level, as opposed to a "vegetated area established or managed to protect aquatic, wetland, shoreline and terrestrial environments from man-made disturbances" (Weschsler, 2010). The new definition also cites that land previously disturbed by human activity should be included in the buffer area. The term *disturbance*, which was previously undefined, is stated to be "any alteration or change to the land including any amount of clearing, grading, or construction activity" (Weschsler, 2010). The law seeks to limit the amount of disturbance of riparian buffer zones due to new construction by landowners. The regulations also specify new mitigation requirements that attempt to decrease the amount of harmful development within buffer zones.

The primary issue with this law is that it only affects new development projects, and does not have any direct impact on property owners who chose to change nothing on their land. The overall intentions of the law are for protecting the diminishing habitats in the Chesapeake watershed, but its strict and inflexible structure may serve as a deterrent for farm owners who want to make only minor adjustments to their land use.

5.3.5 Cover Crops

In addition to riparian buffer zones, cover crops have been shown to provide a number of environmental benefits including reducing soil erosion, minimizing

nitrogen leaching, increasing soil carbon storage, suppressing weed proliferation, and controlling pests. Cover crops are defined as vegetation planted to protect and improve soil, crop, and water quality. The crops are generally not harvested as cash crop. Instead, these crops are planted between cash crop seasons to help catch excess nutrients. They can be tilled into the soil as green manure or left untilled as surface mulch, which is beneficial to both conventional till and no-till practices (Dabney, 1998).

Cover crops increase water infiltration by reducing soil disturbance, increasing water storage capacity, and increasing soil porosity. Similarly, cover crops can also slow runoff rates by decreasing water flow velocity, erodibiliity, and sediment transport capacity. Soil erodibility is defined as the ease by which soil particles can be disturbed and transported by rain and wind. Cover crop roots have also been shown to hold soil in place and provide better soil stability to reduce erosion (Dabney, 1998).

Despite the benefits of using cover crops, there are numerous hurdles impeding farmers from adopting this practice. According to Claassen, Cattaneo, & Johansson (2008), 56% of surveyed farmers stated they would be more inclined to use cover crops if cost-sharing options were available.

Maryland is one of the states that provides cover crop cost sharing incentives. Under the Cover Crop Cost Program (glossary), farmers can get subsidies for planting various types of cover crops depending on crop type and planting convention (conventional till versus no-till) (Maryland Department of Agriculture, 2012). As of fall 2010, 1,567 farmers had planted 162,000 hectares of cover crops,

exceeding Maryland's first two-year milestone goal of 132,000 hectares. In 2011, 1,585 farmers planted 174,000 hecatres of cover crops, again exceeding the State's second two-year milestone goal of 144,000 hectares (Maryland Department of Natural Resources, 2012a).

5.4 Other potential source of hormones – wastewater treatment plants

In addition to agricultural sources, wastewater treatment plants (WWTPs) (glossary) also release EDCs into the environment. WWTPs are established to remove pollutants from wastewater so that when it enters larger aquatic systems, it has minimal effects on wildlife. However, research has indicated that effluents from WWTPs in the United States and United Kingdom contain estrogens at sufficiently high concentrations to affect fish (Auriol, Filali-Meknassi, Tyagi, Adams, & Surampalli, 2006). Estrogenic steroids were reported from sewage treatment plants in concentrations ranging up to 64 ng/L for estradiol (Ying et al., 2002). Experiments that have been conducted to measure hormone levels in biosolids applied to farmlands show estradiol concentrations ranging from as low as 1-10 ng/g (Muller et al., 2008 as cited in Bevacqua et al., 2011) and as high as 22-355 ng/g (USEPA, 2009 as cited in Bevacqua et al., 2011). However, it appears that different wastewater treatment techniques applied to the sludge greatly affect the estrogenic steroid content of the biosolids produced. Bevacqua et al. (2011) measured steroid hormone concentrations in human biosolids treated with lime from wastewater plants. In this study, of all estrogenic compounds, only progesterone (glossary), estrone, and estrone-3-sulfate were detected. The absence of estradiol was attributed to both the high pH levels detected as a result of added lime to the sludge and to the degradation of estradiol to

estrone (Bevacqua et al., 2011).

Additionally, EDC's such as estradiol are common chemicals in human waste, Several studies have determined that human waste contain partially metabolized and un-metabolized pharmaceuticals, natural estrogens, and synthetic estrogens, such as 17α-ethinylestradiol, and mestranol (glossary) from birth control (Jones, Voulvoulis, & Lester, 2005; Limpiyakorn, Homkin, & Ong, 2011).

Other research has also examined how chemicals disperse from these plants into streams, rivers, and other bodies of water from WWTPs. Ferrey et al. (2011) examined the influents and effluents from 25 WWTPs in Minnesota and tested for 78 chemicals. Ferrey et al. found that many chemicals were located both upstream and downstream of the WWTPs. In general, more chemicals were detected downstream than upstream. However, at four of the 25 WWTPs, equal or greater amounts of chemicals were detected downstream than upstream. Additionally, estradiol was detected in 13% of upstream water samples and 42% of downstream water samples at a maximum concentration of 3 ng/L. Interestingly estradiol was detected in 40% of sediment samples upstream and 100% of sediment samples downstream. The Ferrey et al. study shows that chemicals, including EDCs, are not removed by WWTPs and can be detected in nearby surface water and sediment.

Water is treated primarily to remove organic solids whose decay can deplete oxygen from the ecosystem (United States Geographical Survey, 2012). In 2005, a study of 18 municipal WWTPs in Canada found that treatment was 75-98% effective in the removal of estradiol when comparing composite influent and effluent samples (Servos et al., 2005). Plants that achieved de-nitrification also had the highest

estradiol removal rates. While comprehensive treatment systems are best for removal of nutrients, organic matter, hormones, and other pharmaceutical pollutants, they are not always cost effective solutions that can be implemented (Koh et al., 2009).

A review by Limpiyakorn et al. (2012) found that while sewage treatment plants were able to appreciably decrease the amount of estrogens in water, they persisted at levels which could affect aquatic organisms. In some sewage treatment plants, the concentration of estrone in effluent was higher than in influent. This is likely because estrone is a degradation product of estradiol. Sewage treatment plants using only mechanical separation to remove particulates are nearly completely ineffective at removing estrogens. Other studies have noted that WWTPs can actually increase estrogenicity by deconjugating estrogens, thus creating an even greater hazard for aquatic ecosystems (Liu, Kanjo & Mizutani, 2010).

Locally, Maryland Senate Bill 320 set up the Bay Restoration Fund (glossary) in 2004 to improve water quality of effluent from WWTPs. The mission is to enhance nutrient removal before treated water reaches the Bay. Specifically, the bill seeks to reduce nitrogen and phosphorus loading into the Bay by 3.4 million kilograms and 118,000 kilograms, respectively (Bay Restoration Advisory Committtee, 2012). In 2012, Maryland House Bill 446 requires septic systems users pay $5/month per household that goes toward upgrading sewage treatment technology. As of late 2011, the state of Maryland has set aside $352 million for the Wastewater Treatment Plant fund, $42 million for the Septic Systems Upgrade fund, and $37 million to plant cover crops.

Thus far, in the state of Maryland, 22 WWTPs have been updated in an effort to enhance nutrient removal systems (Bay Restoration Fund Committee, 2012). There have been some funding challenges in upgrading all 67 plants, resulting in a shortfall of $385 million. Farmers planted 162,000 hectares of cover crops in 2011, reducing nitrogen by an estimated 1.1 million kilograms. In 2012, about 231,000 hectares of cover crops are scheduled to be planted, which exceeds the goal set out by the state of Maryland to plant 144,000 hectares (Bay Restoration Fund Committee, 2012).

While the state of Maryland is working hard to update WWTPs, there is still an issue of directing wastewater into these facilities. Industrial establishments are allowed to dispose of wastewater through a pipe directly to surface waters, but are required to have a Surface Water Discharge Permit per the NPDES as part of the CWA (USEPA, 2010b). The USEPA is responsible for conducting periodic inspections of facilities with this permit. Factories and other industrial complexes are point sources of pollution, and wastewater treatment facilities can be strategically positioned to be downstream from waste pipelines. Runoff from fields fertilized with poultry litter is more difficult to treat since this is a nonpoint-source pollutant. Although WWTPs can be effective at removing hormones and other pollutants, more research needs to be conducted to determine where plants should be placed to reduce nonpoint-source pollution and increase cost-effectiveness.

Chapter 6: Future Directions and Conclusion

6.1 Future Directions

It remains to be seen whether the types of abnormalities found in this study could lead to fertility issues in the exposed population. Future studies should address whether certain gonadal deformities could lead to a change in reproductive ability or a lack of development of secondary sex characteristics. Furthermore, it would be important to assess if offspring of the affected frogs would be able to reproduce. Du Preez et al. (2008) conducted studies on *X. laevis* examining trans-generational effects of atrazine exposure. F1 generation *X. laevis* frogs were exposed to atrazine and their offspring, the F2 generation, were studied to determine any detrimental effects on fecundity. Du Preez et al. tested for clutch size and survival of offspring as their main endpoints. Clutch size, time to metamorphosis, hatching success, and sex ratios of the F2 generation were all found to be consistent with the control.

Although many studies have been conducted to observe the developmental effects of EDCs on wildlife, there has not been as much research that specifically examines the effects of EDCs under environmentally relevant conditions. More research should be conducted using natural sources of EDCs, such as poultry litter, to examine the true environmental impact of nonpoint-source pollution. Additionally, tests should be conducted over longer durations of exposure to more accurately simulate environmental runoff conditions.

To create a more complete picture of the effects of poultry litter runoff and its impact on Chesapeake Bay ecology, native organisms should be studied using a

similar methodology to our study. Although we chose to study the effects of PLAC on *X. laevis,* this does not exactly replicate what may be happening to Chesapeake Bay organisms. Thus, such a study would be helpful in making decisions about what types of rules and regulations would best help improve the state of the Bay.

Additionally, it would be beneficial to quantify the hormone levels in streams in the Delmarva region. Kolpin et al. (2002) tested 139 streams impacted by animal wastes to measure estradiol and estrone concentrations. Because WWTPs are a major point source of EDCs in the environment, a similar study correlating hormone concentration and WWTP location in the Delmarva area would be highly illuminating. Such a study would also help track the movement of hormones in the effluent from WWTPs. Following this, further study would be necessary to determine the most effective type of water treatment for removal of hormones in wastewater.

Finally, the hormone quantification methodology used in this study was GC-MS. GC-MS and LC/MS are the best techniques for quantifying contaminants in water, but GC-MS is advantageous over LC/MS for analysis of non-polar, volatile compounds. GC-MS is also less prone to errors due to water matrix effects and has a lower limit of detection compared to LC/MS. However, many prior studies which measure hormone levels use methods such as ELISA, which may produce measurements that are artificially high. Due to the existence of so many discrepancies, comparative studies should be done on hormone measurement techniques. This would create a definitive methodology that is suitable for detection of low concentrations of hormones. It would also aid in reconciling the different levels of hormones measured in different studies.

While this study focused on the effects of EDCs on gonadal development, EDCs are also known to have effects on the thyroid axis (Helbing, Gallimore, & Atkinson, 1996). Future studies could utilize our water renewal methodology to examine thyroid axis, particularly at the cellular level and at specific genes. Such a study could focus on endpoints such as tail resorption, and gene regulation to determine the extent of thyroid disruption (Helbing, Gallimore, & Atkinson, 1996; Helbing et al., 2003).

It is known that bacteria play an important role in hormone and nutrient degradation. Future studies could identify and quantify bacteria species present in poultry litter, soil, and runoff. Adding a bacterial component would allow for the identification of bacterial species that play a key role in the decomposition of hormones in poultry litter runoff.

6.2 Conclusion

The present study has successfully established that poultry litter runoff does indeed have an effect on the sexual differentiation and development of *X. laevis* frogs. It is clear from the histology performed in this study that abnormalities occur in gonadal tissue after a poultry litter runoff exposure starting at 12 DPF. Additionally, we have been able to correlate some of these abnormalities with changes in water chemistry.

In this study, the poultry litter exposure solution was diluted to focus on the effects of estrogenic hormones. Higher concentrations of nitrogen containing compounds and heavy metals would have resulted in inhibited survival, slower growth and a higher proportion of frogs with external abnormalities and deformities.

This is consistent with what was seen in our study; although the rate of external abnormalities was not statistically significant, instances of external abnormalities were only found in the high PLAC treatment. It is important to note that at such low concentrations hormones in the poultry litter still led to increased gonadal abnormalities. Even though hormones typically degrade quickly in nature, their presence during critical stages of development (glossary) can cause gonadal abnormalities.

Estrogenic hormones from runoff clearly pose a problem for the ecosystem; however, the use of poultry litter as crop fertilizer cannot be done away with because it is too prevalent in the Delmarva region. However, steps are being taken to address this problem, in particular to nutrient runoff. Progress has been made to reduce heavy metal levels found in runoff with the removal of Roxarsone from feed. High phosphorous levels in runoff have been addressed with the addition of phytase to animal feed, the use of hybrid corn diets, and changes in the application of fertilizer. The addition of inorganic nitrogen at specific times during planting season also improves efficiency, reducing nitrogen runoff. Unlike these other runoff contaminants, hormones occur naturally in poultry litter, and thus cannot be removed in a similar way. Riparian buffer zones, cover crops, and no-till, injection farming can help to reduce hormone runoff. With the legal measures being taken and research being performed on these topics, progress is both likely and possible in addressing issues with poultry litter use in the Chesapeake Bay.

Appendices

Appendix A – Exposure volume increases and feeding rates

Day (M/W/F)	Exposure Volume	Volume Removed	Volume Added	Amount Fed
Day 0 (W)	5L	N/A	N/A	5mL
Day 2 (F)	5L	1L	1.25L	5mL
Day 5 (M)	5L	1L	1.25L	5mL
Day 7 (W)	5L	1L	1.25L	6mL
Day 9 (F)	5L	1L	1.25L	6mL
Day 12 (M)	5L	1L	1.25L	6mL
Day 14 (W)	6L	1.5L	2.5L	8mL
Day 16 (F)	6L	1.25L	1.5L	8mL
Day 19 (M)	6L	1.25L	1.5L	8mL
Day 21 (W)	7L	1.5L	2.75L	10mL
Day 23 (F)	7L	1.5L	1.75L	10mL
Day 26 (M)	7L	1.5L	1.75L	10mL
Day 28 (W)	8L	2L	3L	12mL
Day 30 (F)	8L	1.75L	2L	12mL
Day 33 (M)	8L	1.75L	2L	12mL
Day 35 (W)	9L	2.25L	3.25L	14mL
Day 37 (F)	9L	2L	2.25L	14mL
Day 40 (M)	9L	2L	2.25L	14mL
Day 42 (W)	10L	2.5L	3.5L	20mL
Day 44 (F)	10L	2.25L	2.5L	20mL
Day 47 (M)	10L	2.25L	2.5L	20mL
Day 49 (W)	10L	2.25L	2.5L	30mL
Day 51 (F)	10L	2.25L	2.5L	30mL
Day 54 (M)	10L	2.25L	2.5L	30mL
Day 56 (W)	10L	2.25L	2.5L	32mL
Day 58 (F)	10L	2.25L	2.5L	32mL
Day 61 (M)	10L	2.25L	2.5L	32mL

Appendix A Exposure volume increases and feeding rates (Continued)

Day 63 (W)	10L	2.25L	2.5L	32mL
Day 65 (F)	10L	2.25L	2.5L	32mL
Day 68 (M)	10L	2.25L	2.5L	32mL
Day 70 (W)	10L	2.25L	2.5L	40mL
Day 72 (F)	10L	2.25L	2.5L	40mL
Day 74 (M)	10L	2.25L	2.5L	40mL
Day 77 (W)	10L	2.25L	2.5L	60mL
Day 79 (F)	10L	2.25L	2.5L	60mL
Day 82 (M)	10L	2.25L	2.5L	60mL
Day 84 (W)	10L	2.25L	2.5L	60mL
Day 86 (F)	10L	2.25L	2.5L	60mL
Day 89 (M)	10L	2.25L	2.5L	60mL
Day 91 (W)	10L	2.25L	2.5L	60mL
Day 93 (F)	10L	2.25L	2.5L	60mL
Day 96 (M)	10L	2.25L	2.5L	60mL
Day 98 (W)	10L	2.25L	2.5L	100mL
Day 100 (F)	10L	2.25L	2.5L	100mL
Day 103 (M)	10L	2.25L	2.5L	100mL
Day 105 (W)	10L	2.25L	2.5L	120mL
Day 107 (F)	10L	2.25L	2.5L	120mL
Day 110 (M)	10L	2.25L	2.5L	120mL
Day 112 (W)	10L	2.25L	2.5L	160mL
Day 114 (F)	10L	2.25L	2.5L	160mL
Day 117 (M)	10L	2.25L	2.5L	160mL

Note. feeding rates do not change within an exposure week, defined from Wednesday to Tuesday. Thus, even though only Mondays, Wednesdays, and Fridays are shown in the table, the feeding rate remained constant throughout the week. Volume increase days are in bold. Last point: Feeding amounts were decreased by 5% per death

Appendix B – Growth and Abnormalities Data of Frogs Post Exposures

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T2-E2-3	E2	F	25	2.5	Normal	Mixed sex, ovary with testicular tissue
T2-E2-5	E2	F	24	2.4	Normal	--
T2-E2-6	E2	F	22	1.7	Normal	Mixed sex, ovary with testicular tissue
T2-E2-7	E2	F	24	2	Normal	Mixed sex, ovary with testicular tissue
T2-E2-8	E2	F	26	2.7	Normal	Mixed sex, ovary with testicular tissue, some atretic oocytes
T2-E2-9	E2	F	23	1.6	Normal	Mixed sex, ovary with testicular tissue, dysgenesis
T2-E2-10	E2	F	21	1.3	Normal, small	Some atretic oocyets
T2-E2-12	E2	F	27	2.7	Normal	Mixed sex, female with testicular tissue
T2-E2-13	E2	F	23	1.6	Normal, small	--
T2-E2-15	E2	F	27	2.5	Normal	Atresia

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet Weight (g)	External observations	Microscopic observations
T2-E2-16	E2	F	25	1.6	Normal	--
T2-E2-17	E2	F	26	2.3	Normal	--
T2-E2-2	E2	M	25	2.6	Normal	--
T2-E2-4	E2	M	25	2.1	Normal	1 testicular oocyte
T2-E2-14	E2	M	21	1.6	Normal, small	--
T2-E2-18	E2	M	21	1.1	Normal, small	2 testicular oocyte
T2-E2-19	E2	M	26	2	Normal	--
T2-E2-1	E2	-	SVL-27/ Tail-79	2.8	Non-morph	No slide
T2-E2-11	E2	-	22	1.6	Stunted arms	No gonads observed

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-CON-1	CON	F	32	4.7	Normal, large, plump	--
T3-CON-3	CON	F	31	3.7	Normal, plump	--
T3-CON-6	CON	F	36	8.5	Normal, large, plump	--
T3-CON-8	CON	F	39	8.6	Normal, large, plump	--
T3-CON-9	CON	F	33	6	Normal, plump	--
T3-CON-11	CON	F	33	5.9	Normal, plump	--
T3-CON-13	CON	F	37	4.6	Normal	--
T3-CON-14	CON	F	25	2.3	Normal	--
T3-CON-15	CON	F	26	2.5	Normal	--
T3-CON-18	CON	F	33	5.3	Normal	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-CON-19	CON	F	30	3.8	Normal	--
T3-CON-22	CON	F	29	2.8	Normal	--
T3-CON-25	CON	F	25	2.2	Normal	--
T3-CON-28	CON	F	28	2.8	Normal	--
T3-CON-30	CON	F	30	3.9	Normal	--
T3-CON-32	CON	F	35	7.6	Normal, plump, large	--
T3-CON-35	CON	F	32	5.1	Normal, plump	--
T3-CON-37	CON	F	25	2.2	Normal	--
T3-CON-38	CON	F	30	3.6	Normal	--
T3-CON-39	CON	F	26	24	Normal	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-CON-41	CON	F	31	4.1	Normal	--
T3-CON-42	CON	F	27	2.2	Normal	--
T3-CON-43	CON	F	21	1.3	Normal, small	--
T3-CON-46	CON	F	20	0.9	Normal, small	--
T3-CON-2	CON	M	28	4	Normal, plump	1 testicular oocyte
T3-CON-5	CON	M	27	2.7	Normal	--
T3-CON-7	CON	M	36	7.2	Normal, large, plump	--
T3-CON-12	CON	M	45	6.4	Normal, plump	--
T3-CON-16	CON	M	27	2.6	Normal	--
T3-CON-17	CON	M	31	3.8	Normal	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-CON-20	CON	M	35	5.9	Normal, plump	--
T3-CON-21	CON	M	29	3.1	Normal	--
T3-CON-23	CON	M	29	3.8	Normal, plump	--
T3-CON-24	CON	M	37	7.5	Normal, plump, large	--
T3-CON-26	CON	M	26	2.5	Normal	--
T3-CON-27	CON	M	34	6.1	Normal, large, plump	--
T3-CON-29	CON	M	29	3.9	Normal	--
T3-CON-31	CON	M	32	4.8	Normal	--
T3-CON-34	CON	M	28	3.1	Normal	--
T3-CON-36	CON	M	25	2.3	Normal	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-CON-44	CON	M	27	2.9	Normal	--
T3-CON-45	CON	M	21	1.1	Normal, small	--
T3-CON-47	CON	M	24	2.2	Normal	--
T3-CON-4	CON	-	28	3.2	Normal	No gonads
T3-CON-10	CON	-	36	7.5	Normal, plump	No gonads
T3-CON-33	CON	-	27	3.2	Normal	No gonads
T3-CON-40	CON	-	22	1.3	Normal, small	No gonads

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-LP-A-2	LP	F	31	3.9	Normal	
T3-LP-A-7	LP	F	26	2.3	Normal	
T3-LP-A-12	LP	F	22	1.5	Normal, small	
T3-LP-B-1	LP	F	28	3.1	Normal, light color	
T3-LP-B-5	LP	F	25	1.8	Normal	Ovarian dysgenesis
T3-LP-B-6	LP	F	24	2.1	Normal	
T3-LP-C-2	LP	F	25	2.5	Normal	
T3-LP-C-3	LP	F	23	1.4	Normal, small	
T3-LP-C-8	LP	F	25	1.8	Normal	
T3-LP-C-9	LP	F	22	1.2	Normal, small	Testicular tissue in ovary; some atresia (mixed sex)

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-LP-D-7	LP	F	23	1.4	Normal	--
T3-LP-D-8	LP	F	23	1.7	Normal	--
T3-LP-E1	LP	F	29	3.2	Normal	--
T3-LP-E2	LP	F	27	2.5	Normal	--
T3-LP-E6	LP	F	27	2.6	Normal	--
T3-LP-E7	LP	F	27	2.4	Normal	--
T3-LP-E8	LP	F	33	4.7	Normal, large	--
T3-LP-A-3	LP	M	21	1.3	Normal, small	--
T3-LP-A-4	LP	M	22	1.1	Normal, small	--
T3-LP-A-5	LP	M	31	4.4	Normal	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-LP-A-8	LP	M	24	2	Normal	--
T3-LP-A-10	LP	M	23	1.6	Normal, small	--
T3-LP-B-3	LP	M	30	3.6	Normal	--
T3-LP-B-4	LP	M	27	2.3	Normal	--
T3-LP-B-7	LP	M	23	1.8	Normal	--
T3-LP-B-8	LP	M	21	1	Normal, small	Some testicular dysgenesis
T3-LP-B-9	LP	M	22	1.4	Normal, small	--
T3-LP-B-10	LP	M	17	0.7	Normal, tiny	Testicular dysgenesis
T3-LP-C-1	LP	M	30	3.1	Normal, light color	
T3-LP-C-5	LP	M	32	4.4	Normal, plump	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-LP-C-6	LP	M	23	1.9	Normal	--
T3-LP-C-7	LP	M	22	1.4	Normal, small	--
T3-LP-D-1	LP	M	25	2.2	Normal	1 testicular oocyte
T3-LP-D-2	LP	M	24	1.9	Normal	Testicular dysgenesis
T3-LP-D-3	LP	M	23	1.7	Normal	--
T3-LP-D-4	LP	M	29	3.1	Normal	--
T3-LP-D-5	LP	M	20	1.1	Normal, small	--
T3-LP-D-6	LP	M	23	1.6	Normal	--
T3-LP-D-9	LP	M	21	1.3	Normal	Testicular dysgenesis
T3-LP-D-10	LP	M	18	0.7	Normal, tiny	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-LP-E3	LP	M	33	4.9	Normal, large	--
T3-LP-E4	LP	M	29	3.4	Normal	--
T3-LP-E5	LP	M	26	1.9	Normal	--
T3-LP-A-6	LP	-	25	1.6	Normal, small	No gonads
T3-LP-A-9	LP	-	24	1.5	Normal	No gonads
T3-LP-A-11	LP	-	22	1.5	Normal, small	Indeterminate
T3-LP-B-2	LP	-	29	3.5	Normal, light color	No gonads
T3-LP-C-4	LP	-	25	2.2	Normal	Bad slide
T3-LP-C-10	LP	-	SVL-23, Tail-72	2.1	Morph with tail remaining	No slide
T3-LP-D-11	LP	-	16	0.5	Normal, tiny	No slide
T3-LP-D-12	LP	-	15	0.2	Normal, tiny, thin	No slide

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-HP-A-1	HP	F	42	14.9	Normal, huge	--
T3-HP-A-2	HP	F	37	7.1	Normal, large	--
T3-HP-A-4	HP	F	39	7.3	Normal, large	--
T3-HP-A-5	HP	F	39	7.1	Abnormal right hand	--
T3-HP-B-1	HP	F	36	5.6	Normal	--
T3-HP-B-4	HP	F	43	11.2	Normal, huge	--
T3-HP-C-1	HP	F	32	4.2	Normal	--
T3-HP-C-2	HP	F	29	2.9	Normal	--
T3-HP-C-4	HP	F	28	2.7	Normal	--
T3-HP-C-5	HP	F	27	2.5	Normal	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-HP-C-6	HP	F	30	3.6	Short left arm	--
T3-HP-C-7	HP	F	27	2.5	Normal	--
T3-HP-D-6	HP	F	26	2.3	Normal	--
T3-HP-D-9	HP	F	26	2	Normal	--
T3-HP-D-10	HP	F	31	4	Normal	--
T3-HP-D-12	HP	F	23	1.6	Normal, small	Ovarian dysgenesis; spermatocytes (mixed sex)
T3-HP-D-13	HP	F	28	2.4	Normal	--
T3-HP-D-16	HP	F	26	3.1	Normal	--
T3-HP-E-1	HP	F	29	2.8	Normal	--
T3-HP-E-2	HP	F	29	3.9	Normal	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-HP-E-3	HP	F	32	5	Normal	--
T3-HP-E-4	HP	F	32	4.7	Normal	--
T3-HP-E-10	HP	F	31	4.3	Normal	--
T3-HP-E-12	HP	F	29	3.8	Normal	Ovarian spermatocytes (mixed sex)
T3-HP-E-13	HP	F	26	2.6	Normal	Female with male tissue (mixed sex)
T3-HP-A-3	HP	M	35	5.3	Normal	--
T3-HP-A-6	HP	M	33	4.5	Normal	--
T3-HP-B-2	HP	M	39	9.3	Normal, large	--
T3-HP-B-3	HP	M	33	4.5	Normal	--
T3-HP-C-8	HP	M	16	0.5	Normal, tiny	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-HP-C-9	HP	M	19	0.7	Normal tiny	--
T3-HP-D-1	HP	M	32	5	Normal	--
T3-HP-D-2	HP	M	29	2.9	Normal	5 Testicular oocytes
T3-HP-D-3	HP	M	30	3.4	Normal	--
T3-HP-D-4	HP	M	43	11.3	Normal, very large	Testicular dysgenesis
T3-HP-D-5	HP	M	30	3.6	Normal	2 testicular oocytes
T3-HP-D-7	HP	M	28	3	Normal	--
T3-HP-D-8	HP	M	20	0.9	Normal, small	--
T3-HP-D-15	HP	M	28	4.1	Normal	--
T3-HP-D-17	HP	M	31	3.8	Normal	--

Appendix B—Growth and Abnormalities Data of Frogs Post Exposures (continued)

Frog ID	Treatment	Sex	SVL (mm)	Wet weight (g)	External observations	Microscopic observations
T3-HP-E-5	HP	M	25	2.5	Normal	--
T3-HP-E-6	HP	M	31	4.7	Normal	Testicular dysgenesis
T3-HP-E-7	HP	M	26	3	Normal	--
T3-HP-E-8	HP	M	23	1.5	Normal, small	--
T3-HP-E-11	HP	M	30	4.2	Normal	--
T3-HP-C-3	HP	-	36	6.5	Normal	No gonads
T3-HP-D-11	HP	-	33	4.9	Normal	No gonads
T3-HP-D-14	HP	-	27	2.4	Normal	No gonads
T3-HP-E-9	HP	-	23	2.3	Normal	No slide

Appendix C – GC-MS Hormone Quantitation

Positive Control

Day	SDM	E2-17S	17b-E2-3S	17a-E2-3S	E1-3S	17b-E2	E1	Total estrogens
7	trace	ND	ND	ND	ND	138.7	63.3	202
14	trace	ND	ND	ND	ND	145.3	68.2	213.5
21	trace	ND	ND	ND	ND	160.1	76.4	236.5

High PLAC

Day	SDM	E2-17S	17b-E2-3S	17a-E2-3S	E1-3S	17b-E2	E1	Total estrogens
0	757	24.7	ND	ND	trace	3.7	7.9	36.3
7	1855	ND	ND	ND	ND	ND	10.6	10.6
14	92	ND	ND	ND	ND	ND	5.5	5.5

Low PLAC

Day	SDM	E2-17S	17b-E2-3S	17a-E2-3S	E1-3S	17b-E2	E1	Total estrogens
0	71	ND	ND	ND	trace	ND	3.0	3
7	745	5.3	ND	ND	ND	4.9	6.9	17.1
14	206	ND	ND	ND	ND	1.6	5.8	7.4
21	64	ND	ND	ND	ND	2.1	4.3	6.4
28	21	ND	ND	ND	ND	trace	trace	1

PLAC Stock

Day	SDM	E2-17S	17b-E2-3S	17a-E2-3S	E1-3S	17b-E2	E1	Total estrogens
0	3405	71.3	6.2	1.1	4.4	18.7	11.5	113.2

Note. Trace indicates concentration was below method limit of quantitation. For any calculations, this was approximated at half the method limit of quantitation.
E2-17S: 17β-estradiol-17-sulfate
17b-E2-3S: 17β-estradiol-3-sulfate
17a-E2-3S: 17α-estradiol-3-sulfate
E1-3S: estrone-3-sulfate
17b-E2: 17β-estradiol
E1: estrone

Appendix D – Water Chemistry Analysis

	Wye Stock (2 g/L) (Stirred, No Air)	UMD Stock (Air added)	UMD Low PLAC (from Tank)	UMD High PLAC (from Tank)
Ammonia-N (mg/L)	8.42	23.2	0.161	0
Nitrate- N (mg/L)	0	0	1	11.1
Nitrite- N (mg/L)	0	15.5	4.4	0
Total Arsenic (mg/L)	0.056	0.062	0.05	0.053
Total Chromium (mg/L)	0.0031	0.0026	0	0
Total Copper (mg/L)	0.14	0.15	0.024	0.044
Total Lead (mg/L)	0	0.0035	0	0
Total Zinc (mg/L)	0.29	0.23	0.023	0.048

Appendix E – State of the Bay Report Analysis Summary

The average summer volume of dead zone dropped from 27% in 2011 to 18% in 2012, (percentages being a measure of volume of the Chesapeake Bay). While rates dropped in the last year, there is no definite downward trend in deadzones over the last 27 years. The overall health score for the Bay was a D+ in 2011. This testing was done using six indicators separated into three more general categories: water quality index (which took into account water clarity, cholorophyll a presence, and dissolved oxygen), biotic index (which took into account the benthic community, aquatic grasses, and phytoplankton community), and the overall bay health index. Sampling of scores from fifteen different areas of the Bay were averaged to find overall bay scores (University of Maryland Center for Environmental Science, 2011).

State of the Bay

State of the Bay is an annual report compiled by the CBF (CBF), an environmental group that aims to initiate citizen action against pollution in the Chesapeake Bay. To accomplish its goals, the CBF uses four methods: educate, advocate, litigate, and restore. According to CBF, the biggest problem facing the Chesapeake Bay is nitrogen pollution from agricultural runoff (CBF).

Although the CBF uses 13 metrics to assess pollution in the Chesapeake Bay, we have selected seven to analyze longitudinally for the purposes of our study. The trends of each of these metrics are described below and shown on a year-over-year basis.

Metric Analysis

The CBF has conducted the State of the Bay report annually, with the exception of 2009. Although data on 2011 and 2012 is not available, an analysis from 2004 to 2010 was conducted with data reported on the CBF's State of the Bay reports. On each report the CBF reports a numerical score for 13 environmental metrics: nitrogen, phosphorous, water clarity, toxicity, dissolved oxygen, forested buffers, wetlands, underwater grasses, resource lands, crabs, rockfish, oysters, and shad. Because of the scope of the study, we have selected to examine and analyze seven metrics: nitrogen, phosphorous, water clarity, dissolved oxygen, toxicity, forested buffers, and wetlands. CBF scores for all metrics — except for wetlands, which has had a constant score year-over-year — have been trending positively over the six year period. Although only the numerical scores are reported in the graphs located in the [appendices], the numerical score can be translated into a letter grade based on the CBF's scoring scale, listed below:

A+: 70 and above

A: 60 to 69

B+: 50 to 59

B: 45 to 49

C+: 40 to 44

C: 35 to 39

D+: 30 to 34

D: 25 to 29

D-: 20 to 24

F: 19 and below

Nitrogen and Phosphorous

Although reported as two different categories, CBF analyzes both metrics together when presenting its annual State of the Bay report. According to the CBF, nitrogen and phosphorous loads must be no more than 79 million kilograms and 58.4 million kilograms, respectively, to restore the Bay's clean water and health. Positive drivers for nitrogen and phosphorous include the upgrades of sewage plants, no-till farming, cover crops, and streamside fencing.

Water Clarity

Annual average visibility in the Bay's mainstem is 1 to 1.2 m. Adequate water quality requires visibility to be several times greater than what is currently the norm. Algal blooms, a byproduct of nitrogen and phosphorous, are responsible for the current state of water quality in the Bay. To improve water quality, appropriate controls on industrial and agricultural runoff must be imposed, along with strong riparian forest buffers and adequate erosion control methods.

Dissolved Oxygen

A lack of oxygen, commonly known as a dead zone, has become commonplace across the Bay. Dead zones are caused by algal blooms, which consume the oxygen as they die and decompose. The impact of dead zones, however, may be counteracted by weather and wind patterns. Scores from crab and rockfish can be a leading indicator of water quality and dead zones.

Toxics

Bay scientists have estimated that approximately 67% of the Bay's tidal segments have been impaired due to chemical contaminants. Additionally, a study by

NOAA detected PCBs, herbicides, oil and its byproducts in every sediment sample collected in the Chesapeake. Although most chemicals found in the Bay have been banned since the 1970s, such chemicals are slow to degrade, explaining their longstanding presence.

The implementation of forested buffers has fallen in the mid-2000s. This can be explained due to a lack of government assistance and growing economic incentive. Because state governments are cutting their budgets, farmers have no technical expertise on hand to implement riparian buffers. At the same time, farmers have a strong incentive to keep the greatest amount of land in production. Coupled together, these two factors have decreased the amount of forested buffers maintained by farms near the Chesapeake Bay.

Wetland

According to the CBF, wetland restoration is crucial to restoring the Bay's overall health. To restore the Bay's health, 809,000 hectares of wetlands must be restored. Wetlands are natural filters that trap and treat polluted runoff and protect shorelines from flooding. States in the Chesapeake Bay watershed have succeeded in creating 405 hectares of wetlands per year, but gains have been offset by illegal filling of wetlands for water supply and development. Additionally, the onset of rising global temperatures and sea levels has led to wetland disturbance in certain areas.

Glossary

Adipocyte development – development of cells that store fat.

Alfalfa – purple green perennial legume in the pea family *Fabaceae*.

Alkyl-phenols – xenoestrogens, which is an estrogen imitation hormone, formed by the alkylation of phenols.

Ammonia volatilization – vaporization of volatile ammonia which can result in damage to crops.

Amplexus – pseudocopulation where the male fertilizes the eggs while grasping the female with his front legs. Fertilization of the eggs is external.

Anaerobic digestion – process during poultry litter disposal by which microorganisms break down biodegradable materials in the absence of oxygen.

Androgens – steroid hormones responsible for the development and activity of the sexual characteristics of male vertebrate.

Antimicrobials – chemicals that kill or hamper the growth of microorganisms such as bacteria.

Antimicrobial growth promoters – medicine that destroys or inhibits bacteria. Typically administered at a low, subtherapeutic dose.

Aortic ruptures – tearing of the aorta, the largest artery in the body. Usually fatal because the aorta is the artery responsible for transporting blood from the heart to the rest of the body.

Aromatase – enzyme that synthesizes estrogen from androgens and transforms testosterone into estradiol.

Atrazine – popular organic herbicide that protects crop fields from weeds. Controversial due to potential contamination of drinking water and alleged induction of birth defects.

Atresia – absence or abnormal closure of body opening.

Benthic organisms – organisms that live near bodies of seawater.

Bilateral deformations – internal or external body deformations that occur on both sides of the body.

Binding – process by which poultry litter amino acids bind together resulting in proteins that cannot be broken down by digestion. Occurs when poultry litter is stacked too high.

Bisphenol A – organic compound used to create plastic which displays hormonal properties. It is not very soluble in water.

Broiler chicken – chicken bred for meat production.

Caged layer manure – poultry waste that is mostly free of chicken litter and contains a higher concentration of water than of other forms of poultry waste.

Cardiovascular lesion – trauma to the tissue in the heart or to the blood vessels of an organism.

Chesapeake Bay – largest U.S estuary, extending over 322 km and bordered by Maryland, Virginia, and the Atlantic Ocean.

Chesapeake Bay watershed – all rivers, streams, and creeks that flow into the Chesapeake Bay; the Chesapeake drainage basin.

Clean Water Act (CWA) – 1972 federal law that enforced stricter regulations in an attempt to decrease water pollution from agricultural and industrial facilities.

Coccidiosis – intestinal disease in many animal species caused by the parasite Coccidia.

Concentrated animal feeding operations (CAFOs) – agricultural facility that houses a large number of livestock for extended periods of time during the growing season.

Conjugated estradiol – estradiol that has ions bonded to it.

Conservation Reserve Enhancement Program (CREP) – government program that provides incentives for farmers to retire sensitive or erodible agricultural lands into buffer zones.

Conventional till – act of preparing agricultural land for the planting of new crops by loosening up the soil and removing old crop residue.

Copper loading – ability of enzymes to acquire and utilize copper.

Cover crops – specific crops planted for the purpose of improving soil sustainability, fertility, and quality.

Critical period of reproductive development – time period in which the reproductive organs are developing and the organism is particularly sensitive to environmental factors.

Delmarva – region within the United States consisting of the states Delaware, Maryland, and Virginia.

Demasculinization – anomaly in the male gonads of fish, amphibians, reptiles and mammals including but not limited to oocytes in the testes, testicular lesions and lagging gonadal growth.

Denitrification – microbial process of nitrate reduction.

Diethylstilbestrol – synthetic estrogen that was used in cattle feed and has correlated with breast and prostate cancer.

Dinoflagellate – a large group of eukaryote microorganisms that have one or more whip-like organelles called flagella.

Dioxins – a diverse group of toxic chemical compounds that share certain biological characteristics and chemical structures.

Direct combustion (poultry litter disposal) - the burning of poultry litter which has been considered as a possible method of producing electricity.

Discontinuous gonad – multiple gonad or segmented gonad, discrete subunits with obvious gonadal tissue separated by thin pieces of connective or non-gonadal tissue.

Dividing gonocytes – a state of growth split into the primary and secondary gonocyte stage. The primary stage includes primary oocytes and primary spermatocytes and is where the homologous chromosomes separate. The secondary stage includes secondary oocytes and secondary spermatocytes where chromatids of each chromosome separate from each other.

DNA methylation – a biochemical process where a methyl group is added to the cytosine or adenine DNA nucleotides.

Dorsal lymph sac – area in the upper dorsal surface of the frog's back, superior to the scapula injected with hCG in the frogs to induce ovulation prior to fertilization.

DPF – days post fertilization.

EC50 – concentration of a compound that elicits a response in 50% of the population

Ectoderm – most external layer of three primary germ cell layers present in early embryos. The ectoderm eventually differentiates to form the nervous system and the epidermis.

Elastin – elastic protein in connective tissue that enables flexibility

Emulsifiers – substance that stabilizes the kinetic energy in a mixture of two immiscible liquids.

Endocrine disrupting chemical (EDC) – chemicals that interfere with the endocrine systems in organisms, sometimes with negative effects.

Endoderm – most internal layer of three primary germ cell layers present in early embryos. The endoderm differentiates in the gastrointestinal and respiratory tracts, endocrine glands, and the auditory and urinary systems.

Estradiol – cholesterol based sex hormone present in both males and females. In males it is responsible for early brain development and is present at low levels thereafter. In females, it is involved in sexual development and the reproductive cycle.

Estradiol degradates – estradiol naturally degrades into products such as estrone and 17α-estradiol .

Estriol – estrogen derivative produced in significant amounts during pregnancy in the placenta.

Estrogens – primary female sex hormones that are also used in oral contraceptives.

Estrone – estrogen secreted in the ovaries that is known to have adverse effects in men and women in high quantities.

Ethinylestradiol – bioactive estradiol derivative used in some medications that could carry risks of blood clot formation if taken in high quantities.

Fadrozole – aromatase inhibitor currently used in breast cancer treatment.

Feminization (morphological) – partial or complete conversion from the male to the female phenotype.

Fluoroquinolones – broad-spectrum antibiotics used to treat bacterial infections such as urinary tract infections. They function by inhibiting topoisomerase ligase, thereby fragmenting bacterial DNA.

Formalin – diluted formaldehyde used commercially to preserve tissue specimens.

Forest buffer – natural or manmade strips of trees or bushes adjacent to bodies of water that serve to capture and filter runoff.

Free estradiol – biologically active estradiol that constantly circulates at a constant rate in the body.

Gas chromatography-mass spectrometry (GC-MS) – method of substance indentification that uses he specific phase properties of a substance along with a specific column dimension to separate molecules on a column. The mass spectrometer portion of the device them captures and detects the molecules by their ionized fragments. The results are very specific to the compound, and allow for very fine and accurate identification.

Gentamicin – antibiotic used primarily on Gram negative organisms.

Gestagens – class of steroid hormones that are secreted by the ovaries and prepare the uterus for fertilization and pregnancy.

Glucuronide conjugates – common soluble conjugates that often combines with toxic organic compounds and are excreted.

Hermaphroditism – form of intersexuality in which both male and female gonadal tissues are present in the same individual

Histopathological – having to do with the study of the miscroscopic structure of diseased tissue.

Human chorionic gonadotropin – hormone that induces pregnancy.

Injection technique – fertilizer application method that injects the poultry litter into the ground and is used to avoid tilling while minimizing runoff.

Inorganic nitrogen – nitrogen that is derived from nonliving material.

Internal melanophores – black/brown cells that contain pigment and light-reflecting organelles.

International Unit (I.U.) – unit of measurement for an amount of substance which is based on biological activity or effect.

Intersex – assessed as ovarian and testicular tissue in the same individual as separate gonads (left/right).

Ionophores – liquid-soluble molecule that is usually synthesized by microorganism to transport ions across the lipid bilayer of the cell membrane.

Juvenile frog stages – juvenile frog states are the stages of a frog's development that are in-between tadpole and adult frog.

LC50 – lethal concentration of toxic material that will cause 50% death for the animal test group.

Liquid chromatography-mass spectrometry (LC-MS) – chemistry technique that is usually used for general detection and potential identification of chemicals in the presence of other chemicals. This technique combines the physical separation capabilities of liquid chromatography and the mass analysis capabilities of mass spectrometry.

Maryland Bay Restoration Fund – dedicated fund, financed by wastewater treatment plant users, with the goal of upgrading wastewater treatment plants with enhanced nutrient removal technology so that waste water will have an effluent quality of 3 mg/L total nitrogen and 0.3 mg/L total phosphorus.

Maryland Cover Crop Cost Program – program that rewards the use of cover crops in order to control soil erosion, reduce nutrient runoff and protect the water quality of streams, rivers and the Chesapeake Bay.

Maryland Critical Area Law (1984) – law that minimizes the environmental impact in land within 305 m of the Mean High Water Line of tidal waters or the landward edge of tidal wetlands and all waters of and lands under the Chesapeake Bay and its' tributaries.

Maryland Riparian Forest Buffer (RFB) Initiative (1996) – adoption of goals that would protect, maintain and increase riparian buffers on 3235 km of stream and shorelines by 2010.

Maryland Stream ReLeaf – formation of a committee to implement the RFB Initiative; this committee addressed tracking and reporting buffer restoration and conservation, technical and financial resource management, outreach and networking, Stream Releaf awards, and goal setting.

Mayer's hematoxylin and eosin (MHE) – staining protocol for hemotoxylin and eosin.

Medulla – the middle.

Mesoderm – one of three primary germ layers in an early embryo of all bilaterian animals.

Mestranol – synthetic estrogen that is the 3-methyl ether of ethinylestradiol.

Methemoglobinemia – blood disorder where hemoglobin is unable to release oxygen effectively to body tissue and is characterized by abnormally high methemoglobin.

Microcytic hypochromic anemia – type of anemia where blood has a low mean corpuscular volume (MCV), a low mean corpuscular hemoglobin (MCH) content, and a low mean corpuscular hemoglobin concentration (MCVC).

Mixed sex – defined as the co-occurrence of both ovarian and testicular tissue in a single gonad.

MS-222 (tricane mesylate) – white powder commonly used for sedation or euthanasia.

National Pollution Discharge Elimination System (NPDES) – permit program controls water pollution by regulating point sources that discharge pollutants into waters of the United States.

Natural Hormone – hormones released from animals.

Neurula – embryo at the early stage of development when the development of the nervous system and vertebrate occurs.

Nieuwkoop and Faber (NF) stages – developmental stages of Xenopus.

Nitrogen mineralization – process by which organic nitrogen is converted into inorganic forms.

No-till farming – method of farming where the soil is not disturbed.

Nonylphenol - organic compounds that are part of a larger group of alkylphenols and are suspected to be endocrine disruptors.

Nutrient Management Plan (NMP) – set of conservation practices designed to use fertilizer and/or manure to effectively provide needed crop nutrients while protecting against the potential adverse impacts of manure, erosion and organic byproducts on water quality.

Octylphenol – isomeric compounds that have been proven to be harmful to the environment and are used in rubber, pesticides and paints.

Oogonia – immature female germ cells that are forming oocytes by repeated divisions.

Organic nitrogen – nitrogen that originated from living material.

Organogenesis – process by which the three primary germ cells ectoderm, endoderm and mesoderm develop into internal organs.

Ovarian cyst – a collection of fluid that is surrounded by a very thin wall and is within an ovary.

Ovarian dysgenesis – defective development of the ovaries and can be accompanied by abnormalities of sex chromosomes.

Ovariectomized – removed ovaries.

Ovotestes – gonadal tissue that is more than 30% female.

Pathogenic microorganisms – bacteria, viruses, protozoa and fungi that are inside and outside, skin and mucosal surface of an organism's body.

PCBs – an environmentally harmful and persistent organic compound that is widely use in dielectric and coolant fluids.

Pelletized poultry litter – poultry litter that has been dried and transformed into pellet form.

Perinucleolar phase oocytes – oocytes that have developed in the perinucleolar stage where the ovaries is composed of nests of oocytes that are mostly polygonal.

Perosis – a disease in birds characterized by enlargement of the hook, twisted metatarsi and slipped tendons. It is usually caused a lack of manganese or choline.

Phthalates – esters that are used as plasticizers in variety of consumer products. Exposures via air, water, food, plastic or vinyl products of certain forms of phthalates have been anticipated as carcinogen and concluded to negatively affect hormone levels, reproduction and development of reproduction system.

Phytase – a phosphatase that hydrolyzes phosphate ion from phytate.

Phytate – insoluble solid formed from phytic acid in presence of excess metal ions.

Phytic acid – most common form of phosphorus in plants.

Poultry litter – mixture of poultry manure and bedding. Economic and nutrient-rich alternative for synthetic fertilizers. Source of estradiol and other PLAC that end up in large bodies of water.

Poultry litter associated contaminants (PLAC) – estrogenic hormones, heavy metals, antibiotics, pesticides.

Primordial germ cells – lead to spermatozoa and oocytes.

Progesterone – naturally occurring steroid hormone that has a central role in reproduction.

Prostatic intraepithelial neoplasia (PIN) – condition when abnormalities are seen on prostate epithelial cell.

Radioimmunoassay – assay technique that allows accurate measurement of substances such as hormones from ratio of radioactively labeled and bound antigens to unbound antigens.

Retinoids – vitamin A like compound that can induce limb malformation.

Riparian buffer zones – nutritionally rich area on the edge of streams.

Roxarsone – arsenic-based compound that is added to chicken feed to prevent parasitic disease.

Segmental hypospadias – defect in which the opening of the urethra is on the underside, rather than at the end, of the penis.

Sex ratios – ratio of males to females in a specific population.

Sex reversal – change of sex to another.

Spermatogonia – germ cell that regulates initiation of spermatogenesis.

Sulfadimethoxine (SDM) – chemical compound that kills infectious agents and used as anti-infection drug.

Surfactants – alkyphenol compounds containing detergent that can be used as plasticizer or carrier for pesticides.

Snout-vent length (SVL) – distance from the tip of the snout to the anus.

Tamoxifen – anti-estrogen medication prescribed to reduce activity of estrogen and reduce growth of breast tumors.

Testicular dysgenesis - failure of the testes to completely develop.

Testicular oocytes – ovarian tissue present in the testes of a male frog.

Unilateral deformities – deformity that occurs only on one side of a body structure.

Vitellogenin – protein used as a precursor in production of egg yolks; its presence in males is evidence of exposure to estrogenic EDCs.

Water matrix effects – consistently present factors such as water cohesion and surface tension that can cause increased false positive or false negative experimental results.

Waste Water Treatment Plant (WWTP) – facility in which polluted wastewater is cleaned and released into the environment.

Water Quality Improvement Act (WQIA) – legislation that gives the federal government punitive power over water polluters, as well as mandating various regulations for the reduction of water pollution.

Wet weight – frog weight measured after frog death.

Xenoestrogen – synthetic compounds that are chemically similar to naturally-occurring estrogens.

Xenopus laevis – African clawed frog, a model organism for amphibian biology.

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