

## ABSTRACT

Title of Thesis: STEROID HORMONES IN BIOSOLIDS AND  
POULTRY LITTER: A COMPARISON OF  
POTENTIAL ENVIRONMENTAL INPUTS

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Steroid hormones can act as potent endocrine disruptors when released into the environment. The main sources of these chemicals are thought to be wastewater treatment plant discharges and waste from animal feeding operations. While these compounds have frequently been found in wastewater effluents, few studies have investigated biosolids or manure, which are routinely land applied, as potential sources. This study assessed the relative environmental contribution of steroid hormones from biosolids and chicken litter. Samples of limed biosolids collected over a four year period and chicken litter from 12 mid-Atlantic farms were analyzed for 17 $\beta$ -estradiol (E2), estrone (E1), estriol (E3), 17 $\alpha$ -ethinylestradiol (EE2), progesterone, and testosterone, and the conjugated hormones E1-sulfate (E1-S), E2-3-sulfate (E2-3-S), and E2-17-sulfate (E2-17-3). Results showed that E1 and progesterone were the most prevalent compounds in both of these materials, with E1-S also present in chicken litter.

STEROID HORMONES IN BIOSOLIDS AND POULTRY LITTER:  
A COMPARISON OF POTENTIAL ENVIRONMENTAL INPUTS

By

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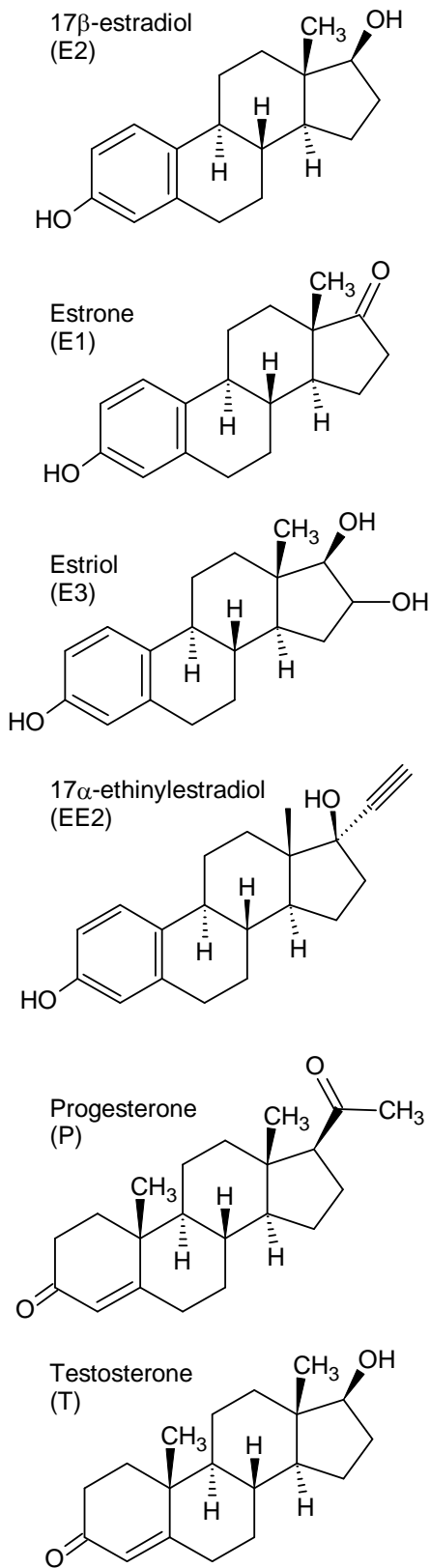
# Chapter 1: Introduction

## 1.1 Steroid Hormones Structure and Function

Steroid hormones are a class of biological chemicals derived from cholesterol that play a variety of roles in vertebrate systems. Within this classification are estrogens, gestagens, and androgens. Estrogens and androgens are generally considered the “female” and “male” sex hormones, respectively, while gestagens are largely responsible for initiation and maintenance of pregnancy.

All steroids are derived from cholesterol. This compound is composed of three cyclohexanes (labeled A, B, and C) and a cyclopentane (D), with a carbon side chain attached at the 17 position of the D ring. Partial loss of the side chain results in a series of C-21 compounds which includes progesterone, other gestagens, and corticosteroids. Complete loss of the chain results in the series which includes the androgens, of which testosterone is the primary compound. The next step of transformation includes the aromatization of the A ring and results in the estrane series, known as estrogens. Different compounds of all series are characterized by various functional groups at different positions of the carbon backbone. Main variations in the estrogen structures occur at C-16 and C-17 positions of the D-ring. The primary estrogen, estradiol, is characterized by the hydroxyl group in the C-17 position which can be in either the  $\alpha$  or the  $\beta$  conformation.  $17\beta$ -estradiol (E2) is the more common compound; a few species, most notably cattle, produce mainly  $17\alpha$ -estradiol. Estrone (E1) differs from this structure by containing a carbonyl group at the 17 position instead of the hydroxyl, while estriol (E3) has a hydroxyl at both the





**Figure 1. Structures of studied steroid hormones.**

17 and 16 positions. Synthetic steroids contain structural features that allow them to be more stable, and therefore able to be taken orally and survive to reach the active sites. For example, 17 $\alpha$ -ethinylestradiol (EE2) has an ethene group at the 17 position in addition to the hydroxyl present in natural 17 $\beta$ -estradiol. These structures are illustrated in Figure 1.

The primary estrogens are E2, E1, and E3. E2 is the most prevalent and potent of these forms, and is responsible for sexual differentiation in developing fetuses, for development of secondary female characteristics such as breasts, and for regulating the menstrual cycle together with progesterone and non-steroidal hormones Follicle Stimulate Hormone (FSH) and Luteinizing Hormone (LH). While an estrogen's dominant roles are in the reproductive tract, there are estrogen receptors in other organs as well, from the brain and hypothalamus to visceral organs such as the liver. E2 is also present in males, although in

much lower concentrations. E1 is the main degradation product of E2, and transformations between these two forms can readily occur during hormonal cycles. However, the estrogenic potency of E1 is half that of E2 (Johnson and Sumpter 2001). The third of these main forms of estrogen, E3, is mainly produced during pregnancy by the placenta and the developing fetus. It is the least potent compound, with potency between 0.005 and 0.04 that of E2 (Johnson and Sumpter 2001).

The male equivalents to the estrogens are the androgens, with the most potent of these being testosterone. They are responsible for growth and development of the male reproductive system and secondary sex characteristics such as increased facial and body hair. Testosterone is present in much lower amounts in females and influences female sexual behavior. It is also known to have anabolic effects in both sexes such as increasing muscle mass.

Progesterone is the most potent gestagen and can also be considered the only natural chemical in this group (Besse and Garric 2009). It is responsible for preparing the uterus for a fertilized egg and for maintaining pregnancy. While it is present in higher concentrations in females, progesterone is produced in both sexes and is an early precursor in the formation of other steroid hormones, including the estrogens and androgens. In addition to its direct reproductive role, progesterone acts on the nervous system and is involved in mating and parental care behaviors. It acts in both sexes; its role in males is now thought to be more prominent than originally believed (Schumacher and Robert 2002).

Certain synthetic forms of these chemicals have also been developed for pharmaceutical uses. Synthetic estrogens are used in birth control, hormone

replacement therapies, and in certain cancer drugs, either alone or in combinations with synthetic gestagens. As they need to remain in an active form in the body long enough to produce the desired effect, synthetic hormones are generally more potent and persistent than their natural counterparts. The most common synthetic estrogen, 17 $\alpha$ -ethynylestradiol (EE2), has an in vitro potency of 1-2x that of E2 (Johnson and Sumpter 2001)

### 1.2 Endocrine Disrupting Potential

Steroid hormones function by binding to a hormone receptor within the cytoplasm of the cell or on the cell membrane, which triggers a series of reactions associated with that hormone. The binding occurs mainly on the basis of shape. If another molecule is present that is similar in shape to the hormone associated with the receptor, it can bind in its place, thus triggering the reactions normally associated with that hormone. Similarly, an exogenous molecule could bind and therefore prevent the binding of the appropriate hormone and prevent the associated actions from occurring. Both of these events are classified as endocrine disruption. With an ever increasing amount of pharmaceuticals, personal care products, pesticides, and other organic chemicals on the market, concern has risen in recent years over the potential for endocrine disruption in humans and other animals when these chemicals are released into the environment.

While the natural steroid hormones have always been present in the environment, they are nevertheless a subject of concern. Increasing human population densities and intensive livestock production can result in concentrated

releases of hormones, resulting in potential endocrine disruption. As these chemicals are the ones designed to attach at receptor sites, they are active at much lower concentrations than exogenous chemicals. Metcalfe et al. (2001) observed males with a condition known as “intersex”, in which oocytes are found in the testes, when groups of Japanese medaka, (*Oryzias latipes*), were exposed to 10 ng/L of E2 or E1 from hatch to 100 days after hatch. In comparison, the industrial chemicals 4-nonylphenol and bisphenol A were less potent by a factor of  $10^6$ . While E3 was shown to be 100x less potent than the other estrogens, this is still 1000x more potent than those industrial chemicals (Metcalfe et al. 2001). There is particular concern with regard to exposure of aquatic or soil organisms to natural or synthetic hormones, as these populations are directly and continuously affected by any increasing environmental concentrations of these chemicals. This class of compounds can be considered pseudo-persistent, since they may degrade readily, but are constantly introduced to the soil or water environment (Besse and Garric 2009).

One useful marker of estrogen exposure is vitellogenin production. Vitellogenin is a precursor protein to egg yolk production that is produced in response to E2. It is found in the blood of developed females of oviparous (egg-laying) species, while concentration in juveniles or males is very low. Species in this category include fish, reptiles, and birds. Detection of increased levels of vitellogenin in the blood of these types of animals, especially males or juveniles, can be used to indicate exposure to estrogens or estrogen mimics. This technique is frequently used in fish species and a number of studies have demonstrated elevated vitellogenin levels in male or juvenile fish exposed to estrogenic chemicals (Routledge et al. 1998,

Jobling et al. 1998, Larsson et al. 1999, Yonkos 2005). Similarly, in a study of painted turtles living in ponds on cattle farms, Irwin et al. (2001) found that while E2 levels in the ponds were not sufficient to induce vitellogenin in male turtles, females had significantly higher levels of the protein than the females living in a nearby control pond. While the type of induction may vary between species, vitellogenin levels in blood are a useful indicator of exposure to estrogenic compounds and allow for long-term monitoring of a population, as the same individuals can be repeatedly tested over time.

The presence of vitellogenin itself may not have a deleterious effect on the organism; it is mainly useful as a biomarker of exposure. However, disruption in the system of steroid hormones can affect development, reproduction, or sex ratios in species. The development of intersex fish has been induced in the laboratory and has also been seen in fish in natural streams, especially those downstream of wastewater treatment facilities (Metcalf et al. 2001, Koger, Teh and Hinton 2000, Yonkos 2005, Jobling et al. 1998). In cases of more severe exposure, significantly more females than males are seen in some fish populations (Metcalf et al. 2001, Koger et al. 2000, Yonkos 2005). Whether or not the incidence of this condition will affect the overall population depends on how successfully the intersex individuals can reproduce. Kidd et al.(2007) observed near-complete reproductive failure and collapse of the fathead minnow population in an experimental lake in Ontario, Canada after exposure to 5-6 ng/L of EE2 over three years. During the exposure time, individuals in the population displayed impaired gonadal development and intersex conditions. A species can also be affected without these physically apparent effects, as demonstrated by a study in

which EE2 exposure reduced the chances of successful mating in a population of sand gobies (Saaristo et al. 2009).

As with male fish exposed to estrogens, female to male intersex has been induced in female fish by exposure to androgens in laboratory studies (Koger et al. 2000). However, the effects caused by androgens and estrogens are not entirely parallel, as testosterone and some of its metabolites can be aromatized to estrogens. For example, sexually undifferentiated medaka exposed to methyltestosterone displayed significantly higher levels of vitellogenin than controls, but in the presence of fadrozole, an aromatase inhibitor, fish displayed increased aggression and male secondary sexual characteristics. These results imply that when aromatization could occur, some of the androgen was converted to an estrogen (Zerulla et al. 2002). León et al. (2007), studied the response of medaka to 11-ketotestosterone, which can not be aromatized. In their study, they did not observe gonadal effects, but found differences in body weight and development, indicating the thyroid was affected (León et al. 2007). Androgenic activity and associated endocrine disruption has also been observed in environmental conditions in streams and ponds receiving effluent from cattle feedlots (Orlando et al. 2004). In general, however, androgens are less extensively studied than estrogens, and more studies on environmental occurrence of androgens focus on effects seen downstream of pulp and paper mills than on water or soil impacted by agricultural activities or domestic WTPs.

In addition to disrupting gonadal and egg development, changes in exogenous hormone levels can affect spawning behavior in some fish species. Androgens, estrogens, and especially progestins act as pheromones in fish and have been detected

in wastewater above the low ng/L levels at which fish will show an olfactory response (Kolodziej, Gray and Sedlak 2003). Aside from these pheromone studies, little research has been conducted on progesterone or synthetic progestins and their occurrence in or possible effects on environmental systems (Besse and Garric 2009).

### 1.3 Environmental Occurrence of Steroid Hormones

In order to be excreted from the body, steroid hormones are generally transformed into more soluble, inactive conjugated forms and excreted in urine. Most commonly, this conjugation involves the addition of a glucuronate or sulfate group at the 3 position of the A ring or the 17 position of the D ring. One or all of these conjugation possibilities could occur to a molecule. The majority of excreted estrogens are glucuronated; in humans, the ratio of sulfated to glucuronated estrogens in female urine is about 21% to 79% (D'Ascenzo et al. 2003). Once excreted, however, these conjugated compounds are susceptible to deconjugation, resulting in free and biologically active steroid hormones. Glucuronated compounds are less stable and are readily cleaved in wastewater processes, but sulfates will persist longer. This difference is likely due to the large amount of  $\beta$ -glucuronidase enzyme present in fecal bacteria which acts to cleave the conjugated compound. Arylsulfatase is more scarce (D'Ascenzo et al. 2003). This difference in stability results in free and sulfated hormones being the dominant species present in wastewater influent, with a ratio of free to conjugated species around 2 (D'Ascenzo et al. 2003).

Wastewater treatment plants (WTPs) and livestock operations, especially concentrated (or confined) animal feeding operations, are the two primary sources of

steroid hormones to water or soil. With  $\log K_{ow}$  (octanol-water partition coefficient) values between 2.6 and 4.0, free steroid estrogens are moderately hydrophobic and are likely to partition out of the water phase onto solids (Khanal et al. 2006).

Conjugated forms are more soluble and are more likely to be found in the water phase. However, their presence in sludge or sediment has been noted in a few studies (Nieto et al. 2008, Matejíček, Houserová and Kubán 2007, Isobe et al. 2006).

Both WTPs and animal feeding operations produce solid waste materials that are used as an amendment to agricultural fields. This is partially out of the necessity for an acceptable disposal route, but it can also be a very beneficial practice which recycles nutrients back into the soil and reduces dependence on chemical fertilizers, as well as providing additional organic carbon and aeration to the soil. Aside from benefits to the soil, reuse of waste materials is a sustainable practice which saves money for farmers and saves space in landfills. However, there is a risk of introducing contaminants such as steroid hormones into the soil, surface water, or groundwater through infiltration or runoff. Hormones have been detected in soil and runoff from both fields amended with biosolids and with poultry litter or other animal manure (Busheé, Edwards and Moore Jr 1998, Nichols et al. 1997, Finlay-Moore, Hartel and Cabrera 2000, Yonkos 2005, Jenkins et al. 2006, Beck et al. 2008). In order to make the best use of these materials in the safest way possible, it is important to understand the potential risks associated with the presence of these microconstituents



#### 1.4 Steroid Hormones in Biosolids

Of the approximately 8.2 million tons of biosolids that will be produced in the U.S. in 2010, approximately 70% is expected to be put to some beneficial use, including land application for agriculture or reclamation (USEPA 1999). This material consists of the wastewater sludge which has been separated and treated, primarily to reduce pathogens. The Environmental Protection Agency, in Part 503 of its Title 40 regulation, sets standards for the use or disposal of biosolids, including those that must be met before land application occurs. There are two classes, Class A and Class B, which are deemed safe for use, with Class A meeting higher standards and therefore being less restricted (potentially unrestricted) in usage (USEPA 1994). Standards include limits on the allowable concentrations of heavy metals and required treatments to reduce pathogens and vectors (flies, etc.). However, these regulations do not currently contain standards regarding concentrations of organic chemicals in biosolids, mainly due to the large number and wide variety potentially present (USEPA 1994). This gap makes land application of this material a potential source of environmental contamination.

Knowledge about the occurrence of steroid hormones in sludge and biosolids is still incomplete. Relatively few studies have investigated this issue, and those mainly focus on E2, E1 and EE2. Concentrations of hormones found in the literature are given in Table 1. Two studies also screened for conjugated hormones in sludge. Nieto et al. (2008), found E2-3S and E1-S in all of their samples with concentrations ranging from 0.64 to 7 ng/g. Muller et al. (2008) did not find any conjugated forms of hormones present.

**Table 1. Concentrations of steroid hormones found in biosolids**

Authors	Treatment	Concentration (ng/g dry wt)					
		E1	E2	E3	EE2	P	T
Ternes et al. (2002)	Digestion <sup>a</sup>	16, ND <sup>b,c</sup>	9, 49 <sup>c</sup>	NA <sup>b</sup>	17, 2 <sup>c</sup>	NA	NA
Andersen et al. (2003)	Digestion <sup>a</sup>	22.8-27.8	4.9-5.4	NA	ND	NA	NA
Muller et al. (2008)	Dewatered	2-8	1-10	ND	1-16	NA	NA
Nieto et al. (2008)	Unknown	ND-200 <sup>d</sup>	ND	ND-406	ND	NA	NA
Poithiou and Voutsas (2008)	Dried	ND	ND	ND	NA	NA	NA
US EPA <sup>e</sup> (2009)	Varied	26.7-965	22-355	7.56-232	ND	143-1290	30.8-2040

<sup>a</sup>Type (anaerobic vs. aerobic) not specified

<sup>b</sup>NA = Not analyzed, ND= Not detected

<sup>c</sup>Only two samples analyzed

<sup>d</sup>Exact number not given, value extracted from a graph

<sup>e</sup>Ranges given for samples where hormones were detected. E1 was detected in 60/84 samples, E2 in 11/84, E3 in 18/84, P in 19/84, and T in 17/84.

Of all these studies, the most comprehensive is the Targeted National Sewage Sludge Survey (TNSSS) carried out by the U.S. Environmental Protection Agency. This broad study was designed to obtain national estimates of the occurrence of a number of analytes of concern in sludge (USEPA 2009). Eighty-four samples were collected at 74 publicly-owned treatment works (POTWs) across 35 states, each treating more than 1 million gallons of wastewater per day. All free hormones which are part of the current study were included in the analyte list of the TNSSS. As shown in Table 1, values for these hormones varied widely, with the exception of EE2 which was not detected in any samples collected. Among the analytes found, E1 was detected in 71% of samples, while progesterone was found in 23%, E3 in 21%, testosterone in 20%, and E2 in 13%.

E1 was detected with similar constancy among the other studies. In addition to those in Table 1, Thomas et al. (2007) in a study of a wastewater treatment plant in Norway noted that E1 was present in all of their dewatered sludge samples, although they did not give exact numbers. E2 and EE2 were detected more frequently by other researchers than those involved in the TNSSS. This could be due to a higher reporting limit for the methods used by the EPA (EPA 2009), which could also help explain why other studies report mainly lower concentrations of E2; any samples having those levels of the hormone would not have been reported in the TNSSS. On the other hand, Nieto et al. (2008) also found E3 in two samples from one treatment plant at concentrations of 272 and 406 ng/g, which are larger values than those found in the TNSSS. Aside from the EPA study, no concentration values for testosterone or progesterone could be found in the literature.

Part of the wide variation in reported values is likely attributed to the variety of technologies used in treating sludge, resulting in a wide variety of final products. For example, the percent solids of the samples tested in the TNSSS ranged from 0.14% to 94.9%. Treatment options to produce biosolids for land application include aerobic or anaerobic digestions, composting, drying, and lime stabilization. These processes each subject any compounds in the sludge to a different set of conditions which may enhance or impede degradation or transformation.

In addition to these studies, which all utilize gas or liquid chromatography coupled with mass spectrometry, other researchers have used biological assays to detect hormonally active compounds in biosolids. Lorenzen et al. (2004) analyzed samples of biosolids from 19 sewage treatment plants across Ontario, Canada using a

recombinant yeast assay. In their anaerobically digested sludge, they found average receptor gene transcription activities of 1233 ng/g dry weight for estrogens, 543 ng/g dry weight for androgens, and no activity for progesterone. The drawback to this type of study is the lack of specificity, which does not allow for direct comparison with analytical methods. The values for estrogens and androgens initially look much higher than anything reported in Table 1, but they could include any compounds with hormonal activity, whether or not they are a hormone or even a contaminant. Additionally, assays use various types of cells which can give varying results. In the Lorenzen et al. (2004) study, some samples were analyzed using a human ovarian carcinoma cell assay instead of yeast and these results showed concentrations that followed the same pattern but had absolute values 25 times less than when the yeast was used.

### 1.5 Steroid Hormones in Chicken Litter

More than 13 million tons of poultry litter is produced across the U.S. per year and nearly all (>90%) is applied to agricultural land (Moore et al. 1995). This material is composed of manure, bedding material, and feathers and is a good source of nutrients (e.g. N, P, and K). In contrast to the regulations applied to biosolids, management of poultry litter in the U.S. is based solely on its nutrient content. The EPA's CAFO rule defines an Animal Feeding Operation (AFO) and a Confined Animal Feeding Operation (CAFO) and requires all CAFOs to apply and implement a nutrient management plan. AFOs, which include poultry houses, are facilities where animals are "stabled or confined and fed or maintained for 45 days or more in any 12

month period” (USEPA 2003). In the case of chickens, an AFO is considered a CAFO if it houses 37,500 or more chickens and uses a liquid manure handling system or 25,000 or more chickens and uses any other system (USEPA 2003). In Maryland, the Nutrient Management Law requires farmers who have more than \$2,500 in gross income and more than eight animal units (1,000 pounds of live animal weight) to have a management plan in place for nitrogen and phosphorus (MDA 2004). A nutrient management plan is defined as a plan to “manage the amount, placement, timing, and application of animal waste, commercial fertilizer, sludge, or other plant nutrients to prevent pollution by transport of bioavailable nutrients and to maintain productivity”(MDA 2004). Those farmers applying sludge or animal manure, which includes poultry litter, were required to have a plan for nitrogen prepared by a certified consultant by December 31, 2001 and for both nutrients by July 1, 2004. They were required to comply with the nitrogen plan by December 31, 2002 and the combined plan by July 1, 2005.

Aside from these plans, treatment and management of poultry litter is largely at the discretion of the individual farmer or company. Ammonia build-up and moisture are deciding factors in when to remove and replace the litter in a house. Generally, farmers will “cake out”, or remove the top layer of litter, between flocks of birds, and about once a year scrape out the whole house. Most of the litter is either applied directly to land, or stockpiled until it is used. Other options include composting systems to cycle litter back into poultry houses, and pelletizing, which is a pasteurization process that condenses the nutrients and organic material into pellets that can be packaged and sold as organic fertilizer.

All studies found in the literature on steroid hormone concentrations in poultry litter used biological assay methods. As a result, they focus on E2 and testosterone; very little information could be found on any other compound. The concentrations reported are given in Table 2. As with the biosolids analyzed by these methods, there is a lack of specificity and a question as to which compounds exactly could produce the responses shown and are therefore represented in the total concentrations. Yonkos (2005) investigated this issue to some extent by attempting to separate the conjugated hormones from the free compounds. He found that about half of the E2 response was from the conjugated compounds, while most of the testosterone was in the free form. Likewise, Shore et al. (1993), report concentrations of “estrogen” in their study, acknowledging that the values are a combination of E2 and E1.

**Table 2. Concentrations of steroid hormones found in chicken litter. Concentrations given as ranges if all numbers were available, otherwise they are averages. NA = Not Analyzed, ND = Not Detected**

Authors	Litter Type	N	Method <sup>a</sup>	Concentration (ng/g dry wt)		
				E2	T	P
Finlay-Moore et al. (2000)	Broiler	8	EIA	20-35	15-55	NA
Jenkins et al. (2006)	Broiler	8	EIA	1.2-1.4	0.02-0.05	NA
Jenkins et al. (2008)	Broiler	2	EIA	1.88, 0.81	0.75, 0.13	NA
Jenkins et al. (2009)	Broiler	2	EIA	2.7, 0.99	0.34, 0.40	NA
Nichols et al. (1997)	Broiler	1	EIA	133	NA	NA
Nichols et al. (1998)	Broiler	1	EIA	904	NA	NA
Lorenzen et al. (2004)	Broiler	12	RYA	60	30 (2/12)	ND
	Breeder	5		70	30	67 (1/12)
	Layer	4		55	10	ND
Yonkos (2005)	Broiler	8	RIA	86-166 <sup>b</sup>	19-58 <sup>b</sup>	NA
Shore et al. (1993)	Broiler- Males	10	RIA	14 <sup>c</sup>	133	NA
	Broiler-Female	10		65 <sup>c</sup>	133	NA
	Layer	17		533 <sup>c</sup>	254	NA

<sup>a</sup>EIA = Enzyme Immoassay, RYA= Recombinant Yeast Assay, RIA = Radio Immoassay

<sup>b</sup>Wet weight

<sup>c</sup>Values given as concentration of E2 and E1

### 1.6 Biosolids and Poultry Litter Production and Use in Maryland

The State of Maryland has both a high human population density and a large poultry industry, making the issue of relative environmental inputs of steroid hormones from these sources relevant. More than 700,000 wet tons of sewage sludge is produced in Maryland each year and approximately 30% of this amount is applied to agricultural land in-state. An additional 42% is hauled out of state, likely for land application as well. The remaining material is used for land reclamation (3%),

composted or pelletized for commercial use (13%), or incinerated or disposed of in landfills (12%). The amount applied to land in-state has been generally decreasing since 2004, from 240,427 tons to 188,221 tons in 2008 (MDE 2009a). In order to use biosolids on farmland, the contractor must have a permit containing results of soil and sludge testing, and the application site is also inspected to ensure proper application. Currently, about 46,000 acres on more than 300 farms throughout Maryland are permitted to receive biosolids (MDE 2009b).

Nearly 300 million chickens were produced in Maryland in 2007 (USDA 2009). Broilers accounted for 99% of this total, or about 296 million. This is up from 287 million in 2002. Based on estimates of litter production per 1000 pounds of animal weight, these chickens produced approximately 415,000 tons of litter, most of which was (~85%) was applied to nearby farms (Penn State 2010). There is a pelletizing plant located in the region which takes in approximately 70,000 tons of poultry litter a year for processing into organic fertilizer.

### 1.7 Scope of Work

This study investigated the presence and concentrations of E1, E2, E3, EE2, progesterone, testosterone, E2-3-S, E2-17-S, and E1-S in biosolids and poultry litter produced in the mid-Atlantic region of the U.S. The primary objective was to compare these materials in terms of potential sources of steroid hormones to agricultural environments. The main hypotheses were as follows:

1. More of the studied steroid hormones were expected to be found in chicken litter, both in concentrations and in number of compounds present. This is



due to the less extensive processing that chicken litter is generally put through compared to biosolids.

2. Based on preliminary investigations and on a review of current literature, E1, progesterone, and potentially E2 were expected to be found in these materials.

Any conjugated hormones detected were expected to be in chicken litter.

To test these hypotheses and accomplish my objective, the following tasks were performed:

1. Biosolid samples collected every 2 months over 4 years from a large WTP servicing southern Maryland and the District of Columbia were extracted and analyzed.
2. Chicken litter collected from 12 poultry farms on the Eastern Shore of Maryland and Delaware was obtained, extracted, and processed.
3. To compare these materials in terms of potential environmental hormone inputs, production and use data for both materials in the state of Maryland were used to estimate yearly loads to this region.

## Chapter 2: Materials and Methods

### 2.1 Biosolid Sample Collection and Preparation

Biosolid samples were collected every 3 months over 4 years (July 2005 to August 2009) from a large municipal wastewater treatment plant in the mid-Atlantic region of the U.S. The plant has a raw wastewater treatment capacity of 1.4 million cubic meters (370 million gallons) per day. Treatment includes primary treatment, activated sludge secondary treatment, nitrification/denitrification, effluent filtration, chlorination/dechlorination and post aeration. Biosolids produced are a combination of sludge from primary, activated sludge, and nitrification treatments that are dewatered and treated with lime to approximately 15% based on dry weight to produce a Class B product. These biosolids are supplied as soil amendments to many mid-Atlantic farms. Samples were collected from the treatment line below the mixers, put in amber glass jars (60- 250 ml) and stored at -20°C until processing.

Prior to extraction, three-gram aliquots of each sample were freeze-dried to produce final weights of ~ 1 g. The fluffy, cotton-like texture of the freeze-dried biosolids made grinding impractical and therefore samples were cut up with scissors to reduce particle sizes.

### 2.2 Chicken Litter Sample Collection and Preparation

Chicken litter samples were collected at 12 broiler farms located in Maryland and Delaware on the Delmarva Peninsula. Each house had held a minimum of four flocks since the last complete clean-out of litter. For each house, 30 samples were

taken throughout the house, 12 in the brooding area and 18 in the rest of the house. Samples were collected with bulb planters to the depth where litter removal occurs. Once all samples were collected from the house, the litter was mixed well, spread out thinly and squared off into 16 quadrilles. Litter (250 g) was taken from the middle of each quadrille to compose the final sample.

Samples were dried in a forced-draft oven at 50°C and ground to pass through a 2 mm screen. They were then refrigerated until analysis.

### 2.3 Standards, Reagents and Materials

The following hormone standards were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA): E1 and E3 (VETRANAL<sup>®</sup> analytical standards), E2 (Sigma Reference Standard), and E1-S (sodium salt). Progesterone (99.0%), testosterone (99.5%), and EE2 (99.5%) were obtained from EQ Laboratories Inc. (Atlanta, GA, USA). E2-3-S and E2-17-S were kindly provided by Dr. Michael Meyer at the USGS Kansas Water Science Center (Lawrence, KS). Internal standards E1-2, 4, 16, 16-d<sub>4</sub> (95%), E1-3-sulfate-2,4,16,16-d<sub>4</sub> (95%), and E2-2,3,4-<sup>13</sup>C (99%) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA), and progesterone-3,4-<sup>13</sup>C (90%) from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

All solvents used were high purity pesticide grade. Ammonium hydroxide was acquired from Fisher Scientific (Pittsburg, PA, USA). The alumina used was neutral, 0.05-0.15 mm mesh, Brockmann Activity I obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Carbon free, deionized water (DI water) was produced with a NANOpure system (Barnstead International, Dubuque, IA, USA). Sand,

obtained from JT Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA), and all glassware used was baked at 400°C for 4 hours to drive off any organic materials.

#### 2.4 Pressurized Liquid Extraction

Extraction and clean-up methods for the biosolids and poultry litter were adapted from a method previously published by Nieto et al. (2008) and utilized an ASE 300 accelerated solvent extraction system ((Dionex Corp. Sunnyvale, CA, USA) and in-line alumina clean-up. The in-line clean-up step was incorporated into the extraction method by packing 5 g of neutral aluminum oxide, baked for 24 h at 120°C, on top of two glass fiber filters that were placed in the bottom of each 33 ml extraction cell. Sand was mixed with 1 gram of sample until the sample was evenly dispersed and this mixture was then poured into the extraction cell on top of the aluminum oxide plug. All internal standards were added to each sample in order to allow for internal standard quantitation and to gauge method recoveries. Selected samples were also spiked with non-labeled standard mixes to test method recovery. The remaining cell volume was topped off with sand. Details of the method development can be found in Appendix A.

Each cell was extracted twice, first with methanol and acetone (1:1) and then followed by methanol and deionized water (1:1). Operating conditions for both extractions were programmed into the ASE as follows: 75°C, 1500 psi, two static cycles of 3 min, 30% flush volume, and 200 sec of nitrogen purge (preheating period of 5 min for first cycle only). Final extract volumes were ~ 25 mL. The methanol/acetone fractions were concentrated under nitrogen to a volume < 5 mL,

combined with the corresponding methanol/water fractions, and concentrated again on a hotplate set to the lowest setting (~80 °C) until volumes reached < 5 mL.

Extracts were transferred to centrifuge tubes and diluted to 5 mL with deionized water and then to 10 mL with methanol. The final solutions were centrifuged for 7 min at 4500 rpm and the supernatant was decanted and analyzed by LC/MS/MS.

### 2.5 LC/MS/MS Analysis

Separation was achieved using a Waters 2695 LC (Waters Corp., Milford, MA) with a Waters XTerra C-18 column (5 µm MS C18 column - 150 x 2.1 mm) heated to 50°C at a flow rate of 200 µL/min. Injection volume was 20 µL. Solvent A was a 0.1% solution of ammonium hydroxide in water and Solvent B was a 0.1% solution of ammonium hydroxide in acetonitrile. The ammonium hydroxide was found to be necessary to aid in the ionization of analytes and to increase sensitivity in negative ionization mode (see Appendix C). Both solutions were made fresh before the start of each run. Initial conditions of 90% A:10% B were held static for 5 min, then shifted by linear gradient to 40% A:60% B by 20 min, and 20% A:80% B by 30 min. The column was then equilibrated to initial conditions for 10 minutes before the next run.

Tandem mass spectrometry analysis was performed using a triple-quadrupole mass spectrometer (Quattro Ultima from Micromass Ltd., Manchester, U.K.) with an electrospray ionization source. Negative ionization mode was used for all compounds except testosterone and progesterone, which were analyzed in positive ionization mode. Acquisitions were done in Multiple Reaction Monitoring (MRM) mode and

peak integration and quantitation were performed using MassLynx v4.0 software (Micromass Ltd., Manchester, U.K.). The primary parent-daughter mass transitions and the mass spectrometer settings are listed in Table 3. Isotope dilution quantitation was used in order to account for matrix suppression of analytes. Isotope pairs used were E2/<sup>13</sup>C-E2, E1/ E1-d<sub>4</sub>, E1-S/E1-S-d<sub>4</sub>, and progesterone/<sup>13</sup>C-progesterone. These isotopes were also used as internal standards as follows: <sup>13</sup>C-Progesterone was used to quantitate testosterone, E1-S-d<sub>4</sub> was used for the conjugated hormones, and <sup>13</sup>C-E2 was used for E3 and EE2.

Compound	Parent (Da)	Daughter (Da)	Retention Time (min)	Cone (V)	Collision (eV)	Dwell (Sec)	Ion Mode
E2	271.0	183.0	21.7	100	46	0.2	ES-
E1	269.0	145.0	22.9	110	43	0.2	ES-
E3	287.4	171.4	16.5	112	50	0.2	ES-
EE2	295.0	145.2	22.7	105	44	0.2	ES-
Progesterone	315.0	108.9	26.2	65	31	0.2	ES+
Testosterone	289.0	108.9	22.4	62	31	0.2	ES+
E1-S	349.0	269.0	17.8	105	32	0.2	ES-
E2-3-S	351.0	271.0	16.7	105	35	0.2	ES-
E2-17-S	351.0	96.7	16.6	115	45	0.2	ES-
<sup>13</sup> C-E2	274.1	185.4	21.7	150	45	0.2	ES-
E1-d <sub>4</sub>	273.0	147.0	22.9	105	38	0.2	ES-
<sup>13</sup> C-Prog	317.0	98.9	26.2	75	31	0.2	ES+
E1-S-d <sub>4</sub>	352.0	272.0	17.8	105	32	0.2	ES-

**Table 3. MS conditions used for the detection of hormones and conjugated hormones**

### 2.6 Quality Control/Quality Assurance

Samples were run in groups of 5, with each sample being run in duplicate, and each group being run with a solvent blank to test for contamination and two samples spiked with a mixture of all free and conjugated hormones to test method recovery. Labeled E2, E1, progesterone, and E1-S were added as internal standards to all samples.

Limits of quantitation (LOQ) were defined as peaks with a signal-to-noise ratio of ~10:1; values less than the LOQ were not reported. Due to the variable nature of the samples and the interferences present, the 10:1 threshold varied but was approximately 5 ng/g for quantitated compounds. Duplicate sample values were averaged unless one value was below the LOQ, in which case only the quantitated value was used. For statistical purposes, concentrations in samples where both duplicates showed analyte peaks below the LOQ were set at half of the lowest standard (2.5 ng/g).

## Chapter 3: Results and Discussion

### 3.1 Results

#### 3.1.1 Method Performance

Average recoveries for the studied hormones in spiked samples using the isotope dilution internal standard method are given in Table 4. Average recoveries of

Hormone	Matrix	
	Biosolids	Chicken Litter
E2	90	108
E1	103	109
E3	99	103
EE2	87	103
Progesterone	85	88
Testosterone	139	78
E1-S	101	111
E2-3-S	98	104
E2-17-S	154	112
<sup>13</sup> C-E2	84	82
E1-d <sub>4</sub>	62	87
<sup>13</sup> C-Prog	44	43
E1-S-d <sub>4</sub>	29	60

**Table 4. Recoveries (%) of steroid hormones, conjugated hormones, and internal standards**

the internal standards are also shown.

Overall, this technique worked well to overcome matrix suppression in samples and losses during extraction and clean-up. In a few cases, namely testosterone and E2-17-S in biosolids, average recoveries indicate a tendency for the isotope used to overcorrect

the concentrations. However, these compounds were not detected above the

LOQ and therefore not quantitated in the samples so this issue was not investigated further. Isotope dilution was especially useful considering the highly variable nature of the samples, especially biosolids, which contain any number of interfering organic compounds that can vary the matrix even between duplicates of the same sample (matrix interferences are often a problem with MS electrospray analysis, see Appendix C). Using isotopes allowed for correction of response due to this variability and overall improved precision. Relative percent differences (RPDs) for duplicate samples are given in Appendix B along with concentration data.

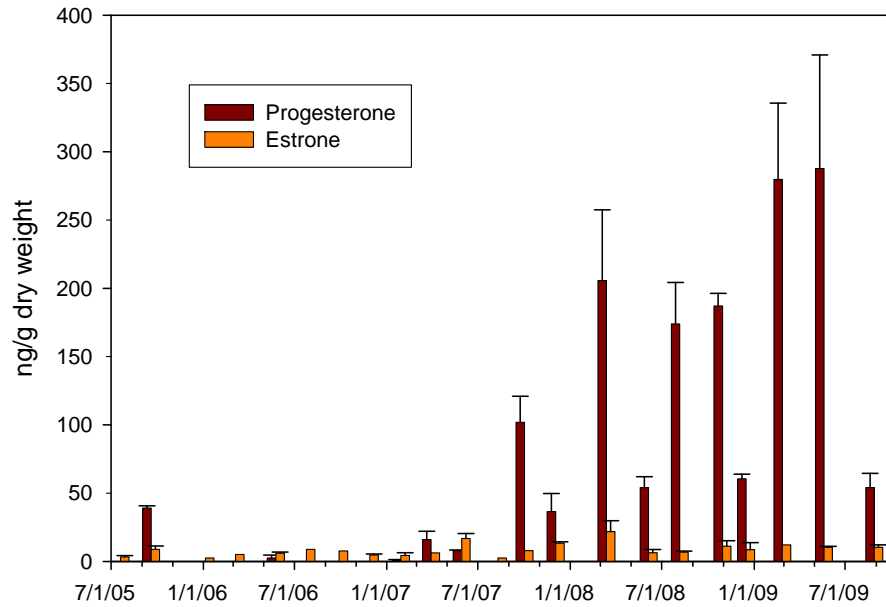


The poultry litter matrix was much cleaner and easier to work with than were the biosolids, as shown by the lower RPDs for these samples. This allowed for greater precision in the concentrations obtained. Nearly all RPDs for poultry litter samples were between 2% and 31%, with two outliers at 41% and 68%. Biosolid samples, on the other hand, were less consistent, with RPD values stretching into the 70% range and two values over 100%. These two extreme cases were both close to the LOQ. As would be expected, samples with concentrations closer to the LOQ showed less precision and therefore higher RPD values. Sample chromatograms illustrating differences in the two matrices are shown in Appendix C.

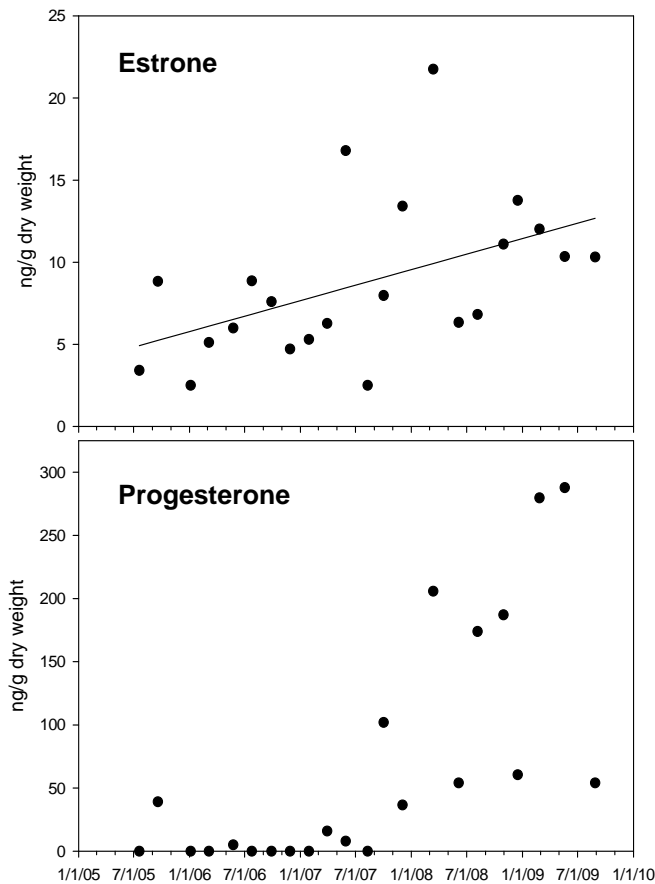
In addition to isotope dilution quantitation, dilution of sample extracts also aided in overcoming matrix effects. With the concentrations of background organic material lowered, peaks were better resolved and occasionally bigger than the more concentrated sample due to the decrease in matrix suppression. An example of this effect is shown in Appendix C. Therefore, sample extracts were concentrated from 25 ml to 10 ml in order to make peaks easier to quantitate, but left this dilute to avoid some of the matrix effects seen with more concentrated extracts.

### *3.1.2 Biosolids Analysis*

Figure 2 shows concentrations of hormones found in limed biosolids over the four years of collection. Progesterone and E1 were found in all samples, although not always above the LOQ. Traces of estriol and testosterone were seen in some samples, but not above the LOQ. Neither E2, EE2, nor any of the conjugated hormones were detected.



**Figure 2. Concentrations of estrone and progesterone in biosolids over 4 years. Error bars = Standard Error of Mean (SEM). No error bar indicates one value below LOQ, only one value used for the graph.**



A change in the concentration pattern is clearly seen in progesterone values in late 2007, while the shift in E1 is much more subtle. These differing trends are illustrated in Figure 3. Progesterone values show minimal fluctuation until August 2007, with an average concentration of 8.52 ng/g. After this point, values increase

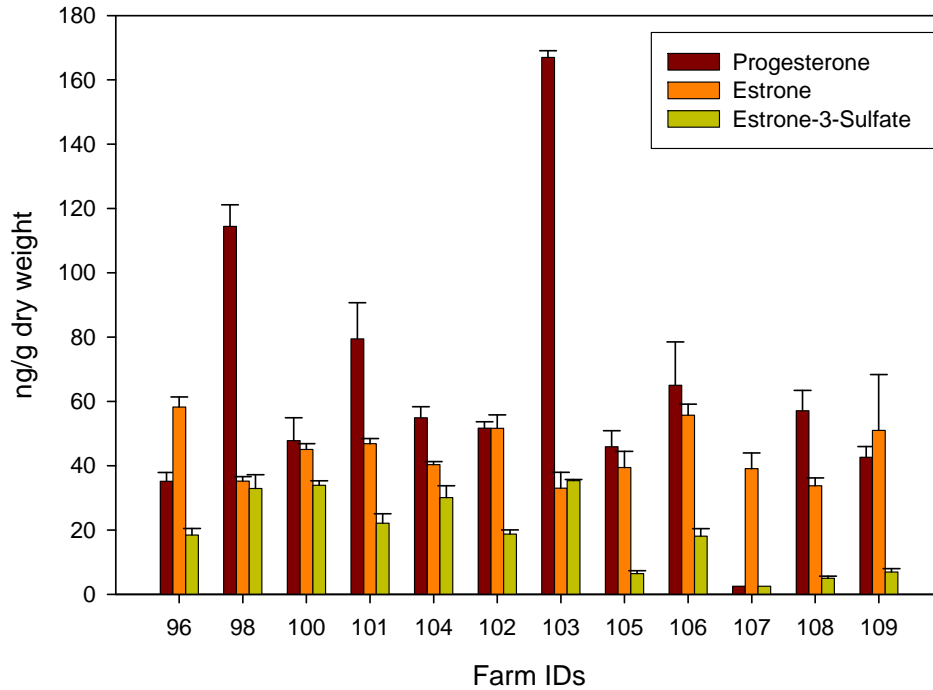
**Figure 3. Trends in estrone and progesterone values over 4 years. Note the difference in scales used.  $R^2$  for E1 regression is 0.83**

sharply and fluctuate widely, with an average concentration of 157 ng/g. Values of estrone are overall much lower, with an average over four years of 8.53 ng/g. Instead of an abrupt shift in 2007, the concentrations instead show a slightly increasing trend over the four years of sampling.

### *3.1.3 Chicken Litter Analysis*

Figure 4 shows concentrations of hormones found in chicken litter samples. Progesterone and E1 were again found in all samples and above the LOQ in all but the progesterone and E1-S peaks for Farm 107. These extracts showed an unusually large amount of matrix interference compared to other chicken litter samples, and this likely affected the quantitation of those peaks. The average concentrations for progesterone and E1 were 63.4 ng/g and 44.1 ng/g, respectively. These samples also contained E1-S, with an average concentration of 19.2 ng/g. Traces of E3 and testosterone were again seen in some samples, but all below the LOQ. No E2 was seen. EE2 was also not seen, however this would not be expected in these samples, as chickens should not be exposed to this synthetic hormone.

**Figure 4. Concentrations of steroid hormones in chicken litter. Error bars = SEM. No error bar (Farm 107) indicates peaks seen below LOQ. Nominal bar shown for this farm at half the lowest standard, 2.5 ng/g.**



### 3.2 Discussion

#### *3.2.1 Biosolids Analysis*

Biosolids are a very heterogeneous material; among the factors that can cause variation in the compounds found are the sources of wastewater feeding the WTP, the attributes of the population that the plant serves, and the processes the plant uses for treatment of biosolids. When comparing the hormone levels detected in this study to those found in previous studies, the potential for inherent variability in the studied material needs to be taken into account. Of the studies listed in Table 1, only the TNSSS is known to contain treatment plants which produce limed biosolids, however

the number of plants employing this method and the hormone levels associated with their biosolids are not disclosed.

With these considerations in mind, the concentrations detected here generally agree well with those found in other studies. The E1 concentrations I quantitated, ranging from 2.73-21.7 ng/g, fall in the same range (2-27.8 ng/g) as those reported in digested and dewatered biosolids (Andersen et al. 2003, Ternes et al. 2002, Muller et al. 2008). The non-detectable levels of E3 in limed biosolids were also in agreement with these results (Muller et al. 2008, Andersen et al. 2003). On the other hand, E2 was detected in all of these previous studies, but not in the samples analyzed here. This could partly be a result of detection limits. Many of the reported values are between 1 and 10 ng/g, which is below or at the limit of sensitivity in this analysis. While method detection limits were not formally tested in this study, it was frequently difficult to get a clean peak for this compound in the standard equivalent to 50 ng/g dry weight in a sample. Another possibility could be that liming biosolids more effectively eliminates E2 in comparison to digestion or dewatering. None of these compounds were detected in biosolids which were treated by dewatering and transferring to drying beds, with reporting limits at 5 ng/g for E1 and E2, and 3 ng/g for E3 (Pothitou and Voutsas 2008). This agrees with the results of my study for E2 and E3, but indicates a more effective removal of E1. In contrast is the study by Nieto et al. (2008), which also did not detect E2, but found E1 at levels up to 200 ng/g and E3 as high as 406 ng/g. They do not specify how this sludge was treated. Combined, these results illustrate the variability in the levels of these hormones that can be found in biosolids following different treatments.

The TNSSS is a broader survey of many types of WTPs, making the data useful for comparing the frequency with which various hormones are found in sludge in addition to comparing the levels reported (USEPA 2009). The findings of my study indicate that E1 is the most consistently detected estrogen in biosolids. This is supported by a higher frequency of detection in the TNSSS (71%) for E1 than for any of the other hormones (USEPA 2009). The levels reported in the TNSSS are higher than those reported in my study, but this could again be partly due to a higher reporting limit (21 ng/g), which would exclude samples with the concentrations found here (USEPA 2009). The TNSSS also provides the only data on progesterone and testosterone to compare with the results reported here for those compounds. The average progesterone concentration of 157 ng/g for the second two sampling years puts the concentration within the lower range of the TNSSS, but their reporting limits for this compound are above all of the levels detected for the first two years. Testosterone was detected in the TNSSS, although only in 20% of samples (USEPA 2009).

Characteristics of the populations served by the WTP are more likely to affect the concentrations of EE2 found in biosolids than those found for the other compounds. Whereas the others are naturally occurring and always being excreted, EE2 is a pharmaceutical and its rate of detection will be affected by how frequently it is used. The fact that no EE2 was seen in the limed biosolids is consistent with the results of the TNSSS, which did not detect the compound in any of their samples, all of which were collected in the U.S. like the ones in my study (USEPA 2009). EE2 was also not detected in two of the four European studies (Andersen et al. 2003, Nieto

et al. 2008). As these results are not consistent among plants with similar treatment processes, they could instead correlate with the frequency of usage in the various locations served by the treatment plants studied.

Non-detectable levels of conjugated hormones was an expected result as the biosolids are highly processed and exposed to harsh conditions of high pH and high temperature prior to our sampling point. These findings are in agreement with those of Muller et al. (2008). Nieto et al. (2008) did find conjugated hormones up to 7 ng/g but they do not specify what prior treatment the sludge had gone through. Again, differing processes may allow for some sulfates to persist.

The advantage to this study compared to the others discussed is that samples were collected over a period of years and therefore provides both more samples (from one WTP) and also a broader picture of what is occurring in the WTP in terms of concentrations of steroid hormones in the biosolids. This analysis over time brought up the question of what occurred in 2007 to raise the values of progesterone so abruptly and cause the larger variation in the levels. One possibility was that refinements made in the way the extraction and analytical method was performed over the course of the study caused the results to appear different due to variable recoveries. To test this, one sample from each year was randomly chosen to be re-run from the beginning of the method (including a duplicate of each). Another sample (5-20-09) was repeated because a large discrepancy was seen between the two duplicates of the first run. When these extractions were complete, all originals and duplicates were run together and the results were compared. This comparison is shown in Table 5. Both calculated RPDs for the random duplicated samples are

<50%, which is generally considered acceptable given the inherent variability in biosolid samples (USEPA 2009). The RPD for the duplicated run of the 5-20-09 sample is 14% higher than the 50% value, which is not completely unreasonable and more importantly, stayed within the trend seen in the data. Based on these results, I concluded that method variations could not account for the abrupt shift or the high variability seen in the progesterone concentrations after August 2007. For consistency, these same analyses were performed for E1, with similar results to those observed for progesterone (RPD values ranged from 6 to 54%).

Sample Date	Concentration (ng/g dry wt)		RPD
	First Extraction	Second Extraction	
5-25-06	<LOQ	<LOQ	ND
1-29-07	<LOQ	<LOQ	ND
8-6-08	205	146	33
8-28-09	49.8	35.3	34
5-20-09	338	175	64

**Table 5. Averages and Relative Percent Difference (RPD) from two extraction runs of the same sample (n=2). ND = Not Determined**

With the method eliminated as the cause of the sudden shift in levels of progesterone, the most likely cause for this increase in concentrations was a change in the treatment process at the studied WTP. After consultation with staff at the plant, I learned that around the time when the shift occurred, the plant changed its supply of lime from one with a fine texture to one with a courser texture. A previous study at this plant had shown that while no pH changes occur in biosolids based on lime texture, coarser lime resulted in an increase in the levels of *E. coli* detected (North et al. 2008). The authors of that study believed that the coarser lime may have left larger pockets of biosolids between lime particles where the pH did not get quite as



high and therefore more bacteria were able to survive (North et al 2008). While this is not a direct comparison, I suspect that lime texture has an effect on the progesterone concentrations as well and perhaps to a lesser extent, on the E1 concentrations. A supplementary experiment was performed to test this hypothesis and the results indicated that amending sludge with coarser lime vs. finer lime resulted in concentrations of both hormones that mirrored the differences seen in samples from pre-2007 and post-2007. However, the reasons behind this are still uncertain. See Appendix D for a more detailed description of these findings.

### *3.2.2 Chicken Litter Analysis*

This analysis of broiler litter showed very different results compared to previous studies. My findings did not indicate the presence of E2 or testosterone, the main compounds reported in chicken litter to date. Much of the difference is likely due to differences in detection methodologies. Whereas other studies used bioassays that quantify overall biological responses to hormonally active compounds present in the material, I used LC/MS/MS which allowed specific hormones to be identified. Average concentrations for E1 (44.1 ng/g) and for E1-S (19.2 ng/g), or both combined, fall within the range of values reported previously for E2 in broiler litter. It is possible, and even likely, that at least a part of the biological activity these reported values represent can be attributed to the presence of E1. Shore et al. (1993) acknowledged this in his reported concentrations by ascribing them to the concentration of “estrogen,” a combination of E2 and E1. Similarly, the lack of testosterone in my samples could be an indication that there are other active androgenic compounds in the litter which contribute to the testosterone response.

Analysis was performed for three possible conjugated hormones and found that E1-S was present in the chicken litter samples at concentrations as high as 35.4 ng/g. This compound could contribute to the total estrogenicity of the chicken litter if conditions favor deconjugation. These results support the findings of Yonkos (2005) that approximately half of the E2 response came from conjugated forms of estrogens, especially as many other conjugation possibilities exist other than those studied here.

Variability was seen in measured concentrations of hormones in chicken litter, but no distinct trends were apparent. Factors potentially contributing to variability include the number of flocks that have been in the house since the last complete clean-out, the age of the current flock, the type of chickens raised, and litter treatment or prolonged storage of litter. Of these factors, data was available for the age of the current flock and the number of flocks raised in the houses since the last clean-out. When the samples were divided based on the current flock age, no significant difference could be seen between houses with flocks <10 days old (n=4) and those with flocks >25 days old (n=6). All houses but two had the same number of flocks in them since the last clean-out, making that comparison less meaningful. Samples were chosen based on location in the studied region and not for any particular features, so only a limited analysis of this type is possible. See Appendix B for complete data.

### *3.2.3 Comparison of Environmental Inputs by Biosolids and Poultry Litter*

In order to give some perspective on environmental inputs, average hormone concentrations were used to estimate the annual load by both chicken litter and biosolids in the state of Maryland. This state was chosen both because it is the region from which the samples were obtained and because the large population density and

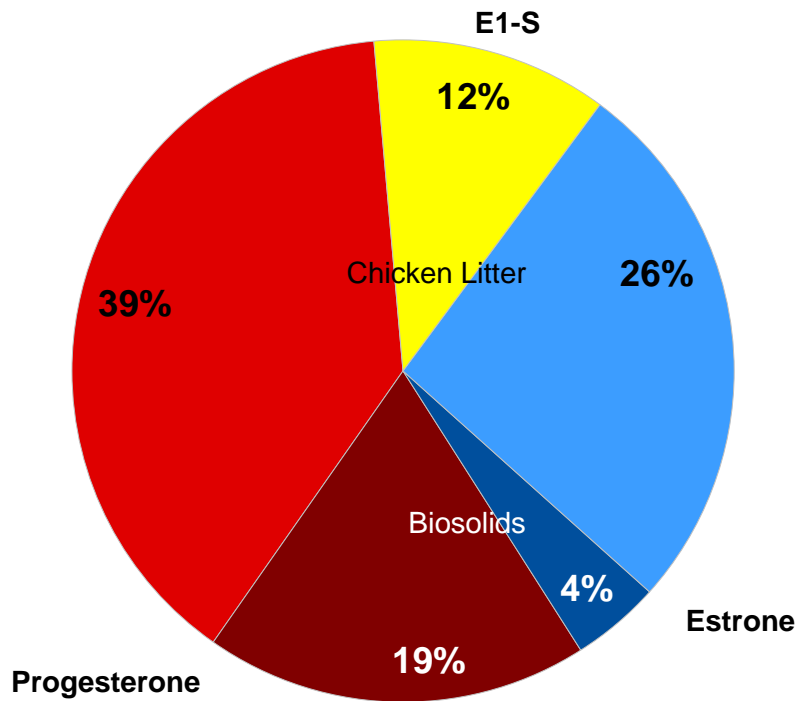
large poultry farm density, especially on the Eastern Shore, make the issue particularly relevant.

Based on reported yearly land application data for biosolids and estimated production and application of poultry litter (See Section 1.6), potential yearly loads were calculated (See Appendix E for more details on the calculations). These values are reported in Table 6. For this analysis, the average biosolid concentrations over the last two years were used.

Source	Hormone Inputs (kg/yr)		
	Progesterone	Estrone	Estrone Sulfate
Biosolids (2 yr avg)	8.0	1.9	ND
Chicken Litter	16	11	5.0

**Table 6. Estimated yearly inputs of steroid hormones from biosolids and chicken litter. ND = Not detected**

Chicken litter has a higher potential to be a source of steroid hormones when applied to land. A graph of relative inputs is shown in Figure 5. This estimate is reasonable considering the more extensive processing that takes place in a WTP and the smaller total dry weight of biosolids that are land applied. However, the distribution is a statewide average; the actual ratio of inputs by biosolids to inputs by chicken litter will vary by location. The majority of poultry farming takes places on Maryland’s Eastern Shore, and due to high costs of shipping, most of the litter is applied to the farm where it was produced or very nearby. This makes inputs from chicken litter much more likely in that area compared to farms elsewhere in the state that are more likely to receive biosolids.



**Figure 5. Relative environmental inputs of steroid hormones from biosolids and chicken litter. Red/Maroon = Progesterone, Blue = Estrone, Yellow = E1-S**

The fate of steroid hormones after land application depends on the rate and pathways of degradation as well as processes such as diffusion into soil and leaching or runoff into water bodies after rain events. An estimate of the initial Predicted Environmental Concentration ( $PEC_{ini}$ ) in soil was calculated for progesterone, E1, and E1-S using Equation 1 (Jackson and Eduljee 1994).

$$PEC_{ini} = C_{soil(0)} + \frac{C_{amendment} \times AR_y}{D \times S_z \times CF} \quad (1)$$

$C_{soil(0)}$  is the background concentration of the specific hormone in soil,  $C_{amendment}$  is the mean concentration of the hormone in chicken litter or biosolids (average of the last two years),  $AR_y$  is the application rate of the soil amendment,  $D$  is the soil density ( $1.4 \times 10^{-3} \text{ kg/cm}^3$ ),  $S_z$  is soil depth (taken to be 10 cm), and  $CF$  is the

conversion factor ( $1 \times 10^8 \text{ cm}^2/\text{ha}$ ). For these calculations, the average concentrations determined for each hormone were converted into wet weight based on 70% moisture in biosolids and 20% moisture in chicken litter. Given the very limited data on hormone concentrations in soil,  $C_{\text{soil}(0)}$  was assumed to be 0 for these compounds. This is in agreement with the findings of Beck et al. (2008) that no estrogens were detected in soils with no history of manure amendment. An average for  $AR_y$  of 9200 kg/ha was determined from the total amount of biosolids applied in Maryland 2008 (188,220 tons) and the number of acres (46,000) that received biosolid application in 2008 (see section 1.6) (MDE 2009a, MDE 2009b). As the nitrogen content of chicken litter is approximately the same as that of biosolids, the same application rate was used. This amendment rate is in agreement with other studies involving chicken litter or biosolids application to agricultural land in this region (Jenkins et al. 2008, Jenkins et al. 2009, Lozano et al. 2010).

The values for  $PEC_{\text{ini}}$  of each hormone after land application of biosolids or chicken litter are given in Table 7. How quickly these hormones dissipate after application, how much will remain in the soil, and how much will end up in ground or surface water will depend on the processes listed above. Degradation pathways and rates are especially important for compounds like E1-S, which will degrade to form an active hormone whereas the parent was inactive. When poultry litter is applied, E1 concentrations could increase nearly 50% as E1-S is deconjugated.

Source	$PEC_{\text{ini}}$ (ng/kg)		
	Progesterone	Estrone	Estrone Sulfate
Biosolids (2 yr avg)	310	21	ND
Chicken Litter	340	230	100

**Table 7. Initial Predicted Environmental Concentrations ( $PEC_{\text{ini}}$ ) of steroid hormones in soil after amendment with biosolids or chicken litter.**

## Chapter 4: Conclusions

This study presents a profile and comparison of steroid hormones seen in biosolids and chicken litter, two materials which are both applied to agricultural land. Results show frequent detection of progesterone and E1 in both materials and E1-S also detected in chicken litter. Concentrations of E1 are higher in chicken litter while progesterone concentrations are higher in biosolids; however levels of progesterone are higher than E1 in both materials.

In terms of biosolids data, this study is unique in that it documents levels of hormones over a period of years, allowing for investigation of concentration trends and also a larger number of samples. Additionally, progesterone and testosterone, compounds for which little data is available, were included in the analysis. This proved to be especially important, and progesterone was one of two compounds detected in the majority of samples and the one present at the higher concentrations. While potential effects of steroid estrogens are well documented, little is known about progesterone, its potential for transport, its fate, or its possible effects when released into the environment at high concentrations. These results indicate that more work is needed in this area.

The concentrations of steroid hormones in the biosolids agree well with other studies considering the variations that are possible due to the variety of treatments available for sludge. The treatment process will have an effect on hormone levels, as was seen here in the change in concentration and variability in progesterone levels (and to a lesser extent, E1) that occurred with the switch to a coarser lime. The mechanism of the effect caused by the grain size of the lime is not well understood.

Future work should include a more detailed study of how liming affects these hormones, and if this effect is seen in other compounds as well (See Appendix D for more details). Continued work on biosolids from all types of treatment will aid in the understanding of these compounds and how processing affects their concentrations. More studies involving larger sample sets would be especially useful considering the very heterogeneous nature of biosolids.

Levels of steroid hormones in chicken litter are even less well documented. While bioassay techniques provide a general measure of estrogenic or androgenic activity in the litter, this study contributes more concrete analytical measurements of the concentrations of steroid hormones present. Results indicate that E1 is the primary estrogen seen, and that conjugated estrogens are present as well. Three conjugated forms were included here. While E1-S was the only one detected, there are other combinations possible that were not studied and may be present. For instance, while glucuronated hormones are less stable, there is a chance they will exist in litter as it is less extensively processed than sludge. These conjugated compounds have the potential to contribute to the overall estrogenicity of the litter as conditions allow for deconjugation and reformation of the active hormone.

Progesterone was also present at high levels in the chicken litter. Until now, very little work has been done with this compound and almost no data on levels in chicken litter are available. Its consistent presence in these samples suggest that more work be done in this area both on concentrations present in the litter and also on potential effects when released into the environment in this way. On the other hand, testosterone was only detected at trace levels in a few samples. This is in contrast to

other studies on this topic, all of which report some level of this hormone. As these studies used non-specific bioassay techniques, this could indicate the presence of other androgenic compounds in the litter capable of producing a biological response. There are other androgens and testosterone metabolites which are potentially present and future analytical work should investigate these possibilities. Overall, much more analytical work is needed in this area to better understand which hormones or hormonally active compounds are present in chicken litter and at what concentrations.

As with biosolids, treatment of chicken litter can effect hormone concentrations. Levels of hormones in the chicken litter varied, especially progesterone concentrations. However, a detailed analysis of possible reasons for variability was beyond the scope of this study. Future work on litter from houses chosen based on variations in flock characteristics and litter treatment and storage could improve understanding of the way these differences affect the concentrations of hormones in chicken litter.

The next step in understanding the potential impacts of steroid hormones released into the environment with biosolid or poultry litter land application is to study the processes that will affect their distribution and fate. Very few studies have investigated hormone levels in soils amended with these materials or what effects these concentrations may have on soil organisms (Finlay-Moore et al. 2000, Jenkins et al. 2006, Beck 2008, Jenkins et al 2008). Considering the  $PEC_{ini}$  calculated for progesterone, E1, and E1-S, more investigations of this type are warranted. Similarly, while concentrations of hormones in runoff from biosolid- or poultry litter-amended fields have been investigated, these studies were done with bioassay



techniques (Nichols et al. 1997, Busheé et al. 1998, Nichols et al. 1998, Finlay-Moore et al. 2000, Yonkos 2005, Jenkins et al. 2006, Jenkins et al. 2008, Jenkins et al. 2009). This work should be expanded to include analytical measurement of the various estrogens and other hormones.

Application of biosolids and poultry litter to agricultural land is both necessary and beneficial. While there are risks associated with compounds such as steroid hormones present in these materials, a greater understanding of the magnitude of these risks and ways they may be mitigated can make recycling of these waste materials a truly sustainable practice.

## Appendix A: Method Development

The first method tried was an adaptation of a method developed by Burkhardt et al. (2008), which also used ASE extraction, followed by SPE (solid phase extraction) with Oasis<sup>®</sup>HLB cartridges (Waters Corporation, Milford, MA, USA) and Florisil. However, after the conjugated hormones were added to the analyte list, I found that I was unable to achieve adequate recoveries of these compounds with that method. Trials were carried out with the method by Nieto et al. (2008), which also included conjugated hormones, and sufficient improvements were seen in the results to continue with this method for the analysis of biosolid and chicken litter samples. In addition to improvements in detection of the conjugated hormones, there was also an improvement in the recovery of the <sup>13</sup>C-E2 internal standard (42% vs. 84% reported in this study).

The original method described by Nieto et al. (2008) called for freeze-dried biosolids to be mixed with the alumina as both a dispersant and clean-up material and packed in an 11 ml extraction cell. The cells used in this experiment were 33 ml, and therefore some adaptation was needed. To optimize the amount of alumina used, six replicates of both a chicken litter sample and a biosolid sample were run through the method using different amounts of alumina. Two samples were run using 5 g, 2 with 10 g, and 2 with 15 g. As there is extra space in the 33 ml cell compared to the 11 ml cell originally specified, the alumina was packed on the bottom of the cell and sand was used as the dispersant in order to optimize the contact time of the sample extract

with the alumina. Each sample was spiked with standards of all hormones studied.

Recoveries of each standard are shown in Table A1.

**Table A 1. Recoveries (%) of standards after extraction and clean-up with three different amounts of alumina**

<b>Sample</b>	<b>E2</b>	<b>E1</b>	<b>E3</b>	<b>EE2</b>	<b>P</b>	<b>T</b>	<b>E1-S</b>	<b>E2-3-S</b>	<b>E2-17-S</b>
<i>Biosolids</i>									
5 g	117	59	120	62	107	117	65	54	8
10 g	105	81	118	75	98	113	41	30	0
15 g	98	72	109	68	79	136	4	3	2
<i>Litter</i>									
5 g	98	82	124	78	104	117	66	66	61
10 g	154	94	133	86	96	113	60	56	25
15 g	112	94	95	74	102	136	40	29	6

Free hormones show similar recoveries in all extracts. The recoveries of the conjugated hormones, however, dropped off when run through increasing amounts of alumina. This was especially true in the biosolid samples. As there was not a large decrease in the matrix interferences seen as more alumina was added, 5 grams was used in order to maximize recoveries of the conjugated hormones.

## Appendix B: Sample Data

**Table A 2. Concentrations of progesterone and estrone detected in each biosolid sample based on dry weight. Averages are reported when both duplicates were quantitated, otherwise the value is the concentration in the quantitated sample or labeled as <LOQ if neither were quantitated. RPD= Relative Percent Difference, lbs = limed biosolids, ND= Not Determined.**

Sample	Progesterone			Estrone		
	ng/g	Averages	RPD	ng/g	Averages	RPD
lbs 8-28-09	87.7	94.6	15	18.0	15.1	38
lbs 8-28-09 dup	102			12.2		
lbs 5-20-09	629	470	67	11.7	11.0	12
lbs 5-20-09 dup	312			10.3		
lbs2-26-09	224	280	40	<LOQ	12.0	ND
lbs2-26-09 dup	336			12.0		
lbs 12-16-08	57.1	60.5	11	3.46	8.61	120
lbs 12-16-08 dup	63.9			13.8		
lbs 10-30-08	178	187	10	7.05	11.1	73
lbs 10-30-08 dup	196			15.1		
lbs 8-6-08	268	233	30	10.0	8.18	44
lbs 8-6-08 dup	197			6.36		
lbs 6-5-08	62.0	54.0	30	3.91	6.33	76
lbs 6-5-08 dup	46.0			8.75		
lbs 3-13-08	257	206	50	29.8	21.7	74
lbs 3-13-08 dup	154			13.7		
lbs 12-3-07	23.3	36.5	73	12.5	13.4	14
lbs 12-3-07 dup	49.8			14.4		
lbs 10-2-07	82.9	102	37	7.97	7.97	ND
lbs 10-2-07 dup	121			<LOQ		
lbs 8-10-07	6.24	6.24	ND	<LOQ	<LOQ	ND
lbs 8-10-07 dup	<LOQ			<LOQ		
lbs 5-30-07	7.53	7.95	11	13.1	16.8	44
lbs 5-30-07 dup	8.38			20.5		
lbs 3-30-07	9.74	15.9	78	<LOQ	6.27	ND
lbs 3-30-07 dup	22.1			6.27		
lbs 1-29-07	<LOQ	<LOQ	ND	<LOQ	2.73	ND
lbs 1-29-07 dup	<LOQ			2.73		
lbs 11-28-06	<LOQ	<LOQ	ND	3.96	4.71	32
lbs 11-28-06 dup	<LOQ			5.45		
lbs 9-28-06	<LOQ	<LOQ	ND	7.60	7.60	ND
lbs 9-28-06 dup	<LOQ			<LOQ		
lbs 7-25-06	<LOQ	<LOQ	ND	<LOQ	8.86	ND
lbs 7-25-06 dup	<LOQ			8.86		
lbs 5-25-06	0.95	4.96	162	2.91	3.03	8
lbs 5-25-06 dup	8.98			3.15		
lbs 3-6-06	12.7	12.7	ND	5.11	5.11	ND
lbs 3-6-06 dup	<LOQ			<LOQ		
lbs 1-5-06	2.90	2.90	ND	<LOQ	<LOQ	ND
lbs 1-5-06 dup	<LOQ			<LOQ		
lbs 9-19-05	40.7	39.1	8	6.38	8.83	56
lbs 9-19-05 dup	37.4			11.3		
lbs 7-20-05	<LOQ	<LOQ	ND	2.50	3.41	53
lbs 7-20-05 dup	<LOQ			4.32		

**Table A 3. Concentrations of progesterone, estrone, and estrone sulfate in poultry litter samples based on dry weight. <LOQ = peaks below the limit of quantitation.**

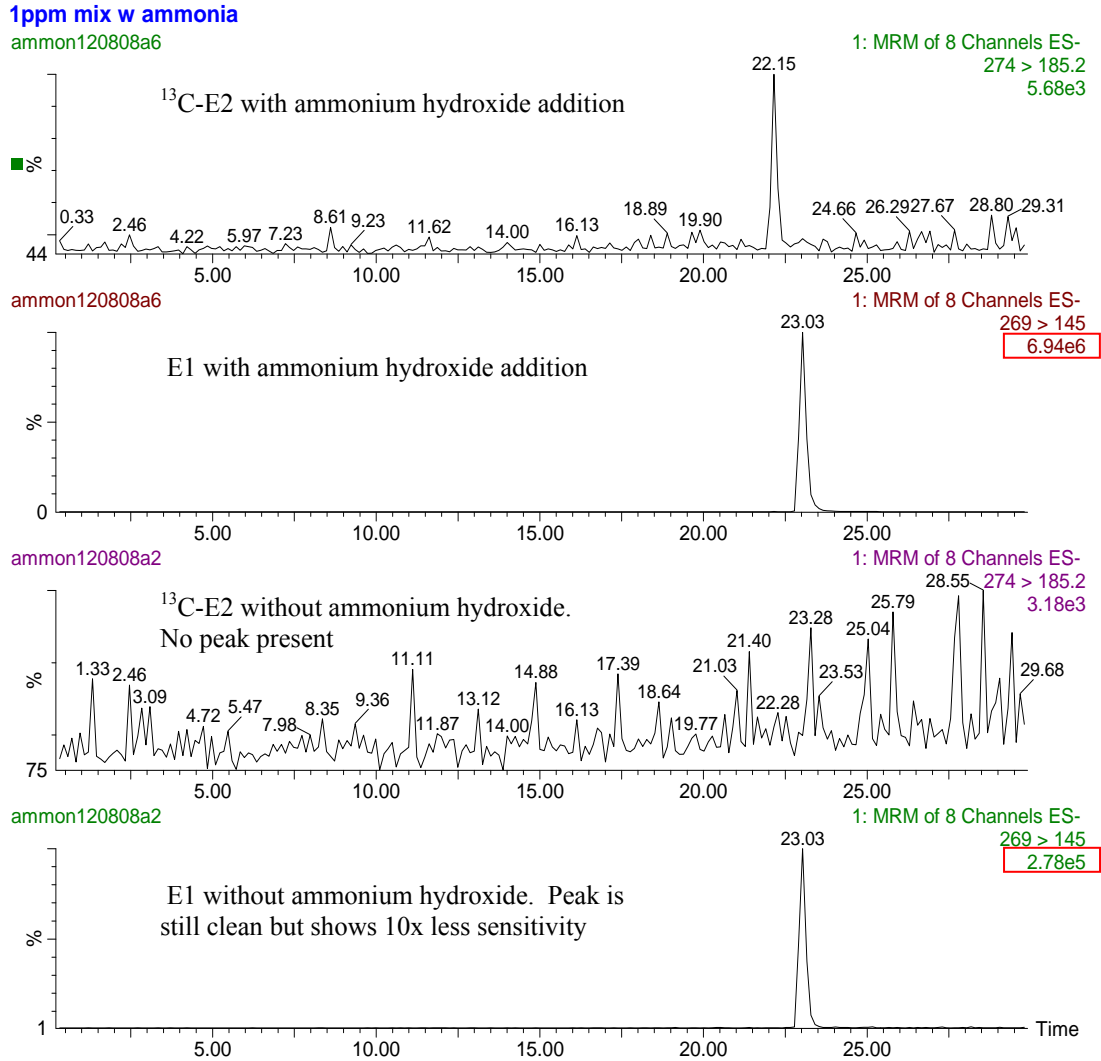
Sample	Progesterone			Estrone			Estrone Sulfate		
	ng/g	Averages	RPD	ng/g	Averages	RPD	ng/g	Averages	RPD
96	37.9	35.1	16	55.1	58.2	11	16.4	18.5	22
96 dup	32.4			61.4			20.5		
98	121	114	12	36.6	35.2	8	37.2	32.9	26
98 dup	108			33.8			28.5		
100	54.9	47.8	30	46.8	45.1	8	35.3	33.9	8
100 dup	40.7			43.4			32.6		
101	90.7	79.5	28	48.4	46.8	7	19.1	22.1	27
101 dup	68.2			45.3			25.1		
104	51.5	54.9	12	39.4	40.4	5	33.8	30.1	25
104 dup	58.3			41.3			26.4		
102	53.7	51.7	8	47.4	51.6	16	20.0	18.8	13
102 dup	49.6			55.8			17.5		
103	165	167	3	28.0	33.0	30	35.0	35.4	2
103 dup	169			37.9			35.7		
105	50.9	45.9	22	34.4	39.4	26	7.31	6.46	26
105 dup	41.0			44.5			5.61		
106	78.5	65.1	41	52.3	55.7	12	20.4	18.1	26
106 dup	51.6			59.1			15.7		
107	<LOQ	<LOQ	ND	34.2	39.1	25	<LOQ	<LOQ	ND
107 dup	<LOQ			44.0			<LOQ		
108	50.7	57.1	22	36.2	33.8	14	5.64	5.01	25
108 dup	63.4			31.4			4.38		
109	45.9	42.6	16	68.3	51.0	68	7.98	6.92	31
109 dup	39.2			33.7			5.86		

**Table A 4. Chicken litter samples separated by age of flocks at the time of sampling. T-tests on averages showed no significant differences. For statistical purposes, values <LOQ were estimated at half the concentration of the lowest standard, or 2.5 ng/g. Empty houses were not considered as the time since the last flock was unknown.**

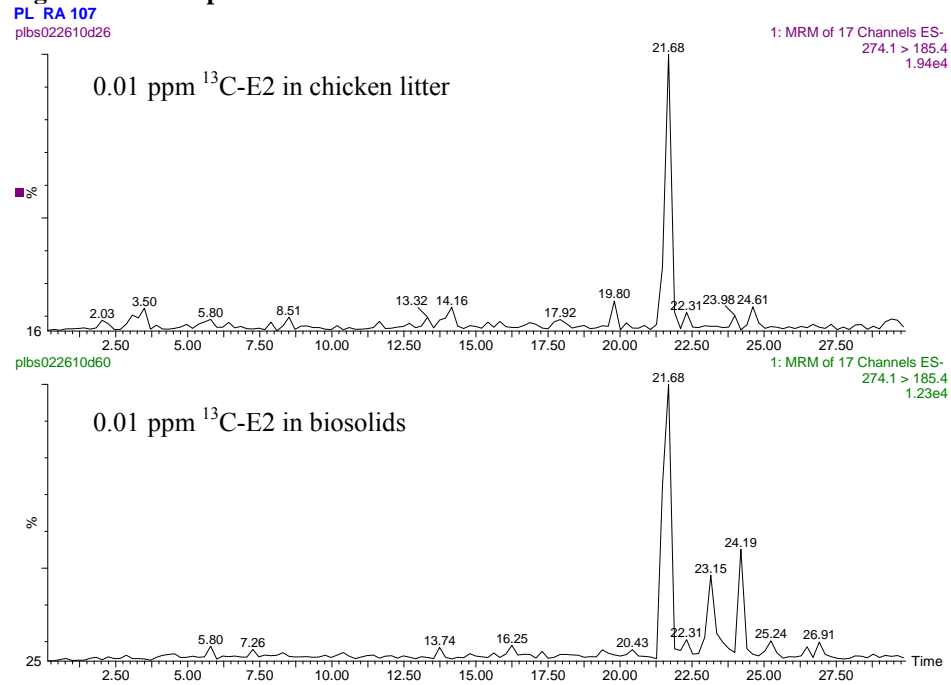
Flock age	Concentration (ng/g dry wt)			Concentration (ng/g dry wt)		
	Progesterone	Estrone	E1-S	Progesterone	Estrone	E1-S
<10 days				<i>Empty house</i>		
101	79.5	46.8	22.1	103	167	33.0
102	51.7	51.6	18.8	104	54.9	40.4
105	45.9	39.4	6.5	Average	111	36.7
107	16.7	39.1	2.7			
Average	48.4	44.2	12.5			
>25 days						
96	35.1	58.2	18.5			
98	114	35.2	32.9			
100	47.8	45.1	33.9			
106	65.1	55.7	18.1			
108	57.1	33.8	5.0			
109	42.6	51.0	6.9			
Average	60.3	46.5	19.2			

# Appendix C : Chromatogram Comparisons

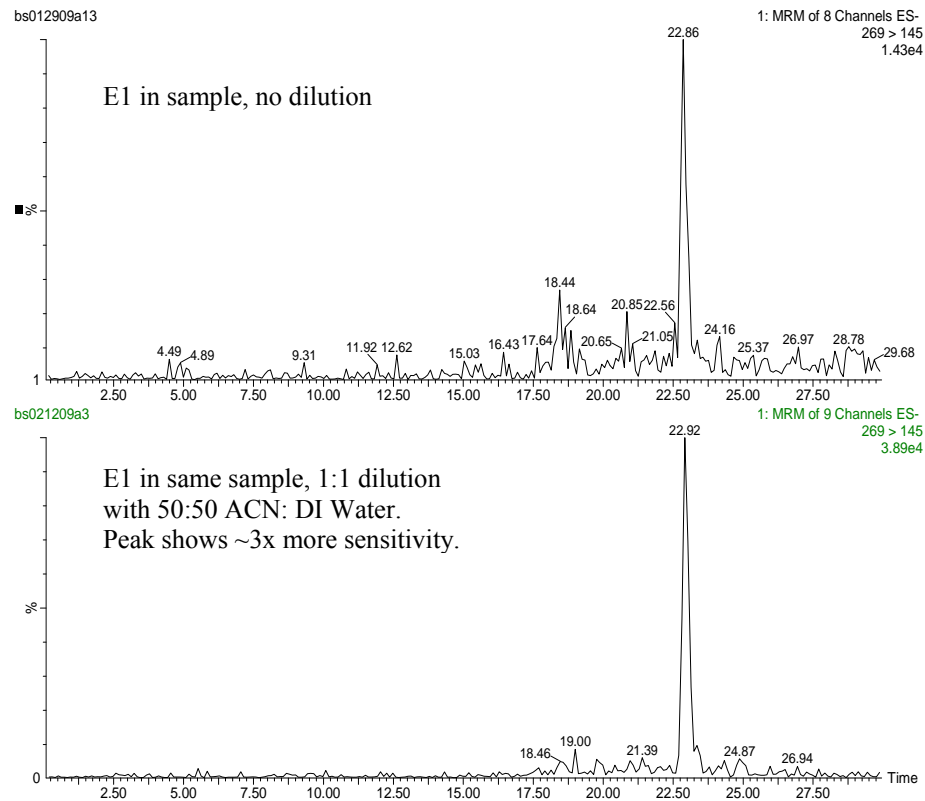
Figure A 1. Chromatograms of two hormone standards in the same solution run with and without ammonium hydroxide addition



**Figure A 2. Comparison of biosolid and chicken litter matrices**



**Figure A 3. Comparison of E1 peaks in an undiluted vs. a diluted sample.**



## Appendix D: Lime Experiment

In order to test the hypothesis that a change to coarser lime resulted in the increase of progesterone values observed, an experiment was run in which unlimed sludge was collected and then mixed with either coarse or fine lime. These samples were then extracted and analyzed according to the experimental method described.

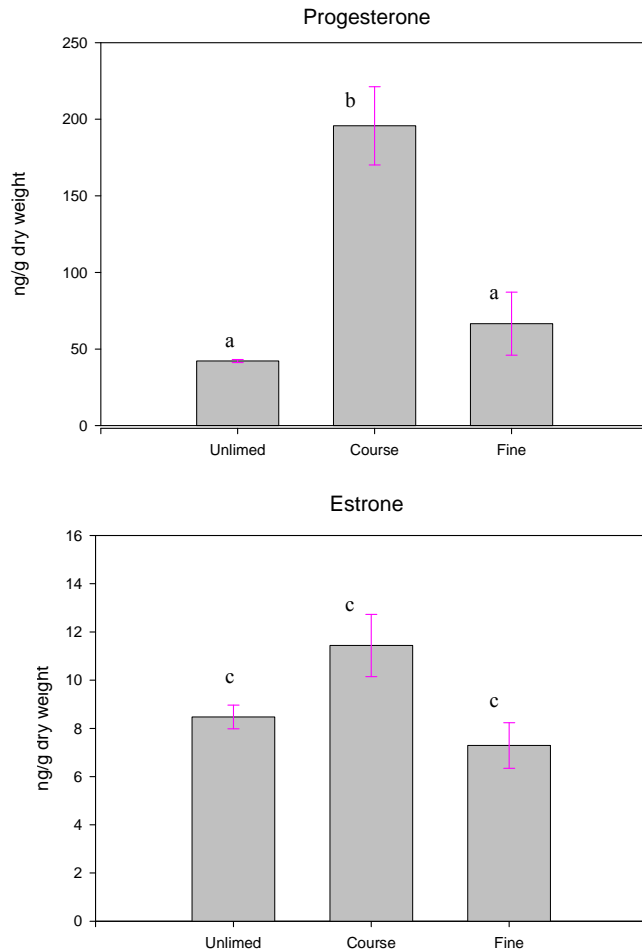
The method for this experiment was as follows. Lime was obtained from a standard daily shipment received by the studied WTP. In the previous study on lime incorporation conducted at this plant (North et al. 2008), lime was made finer by grinding, after which all lime could pass through 2 mm mesh. As equipment for grinding was not readily available for this study, it was decided that the finer lime would consist of all lime that passed through a 2 mm sieve. The coarser lime was a sample taken as it was received. To sieve, approximately 160 grams of lime was poured onto a series of sieves, ranging from 0.15 mm to 6.35 mm, stacked on a shaker and shaken for 2 minutes. All lime passed through 6.35 mm mesh, and approximately 46% was retained on the 2 mm sieve. Of the finer fraction, 98% was between 0.15 mm and 1.4 mm.

An unlimed sample of sludge was collected from the line after the centrifuges and before the lime mixers. It was divided into 4 aliquots of 100 g. Two of these samples were amended with coarser lime and two were amended with finer lime, all a rate of 15% by dry weight of sludge. All samples were mixed in a KitchenAid stand mixer for 40 seconds (the time estimated that sludge and lime are mixed before the limed biosolid collection point). Samples of each mixture were collected and run



through the procedure described previously. All samples were run in duplicate along with a spike and a blank.

The results of the analysis are shown in Figure A4. The pattern seen in the



**Figure A 4. Progesterone and estrone concentrations in sludge amended with coarse vs. fine lime. Different letters on the same graph indicate significant differences ( $p < 0.05$ ). Error bars = SEM**

original sample analysis was mirrored in these results, with coarser lime resulting in significantly higher levels of progesterone than finer lime. The smaller but still noticeable difference between levels of E1 in biosolids after amendment with the coarse vs. fine lime also indicated a strong trend ( $p < 0.1$ ).

However, these results are complicated by the hormone levels seen in the unlimed biosolid

samples analyzed. In both cases, amendment with coarse lime resulted in an increase in the hormone level. The difference in progesterone is significant, and the difference in E1 indicates a trend. The reason behind this increase is uncertain. It could be that the increase in temperature and pH that accompanies lime amendment causes

transformations in the hormones present. As progesterone is a precursor to other steroid hormones, it is possible that transformations could occur which would result in the re-formation of this chemical. Also, any conjugated hormones present are likely to be broken apart in this process which could result in an increase in free concentrations seen. However, this would not explain why the finer lime, which is believed to be better incorporated, does not result in this same effect. Finer lime is potentially deactivated to a greater degree as there is a greater surface area available to interact with air, which breaks down the CaO. It is therefore possible that, despite an increase in incorporation, the lime is less effective and does not result in as much transformation activity. The opposite effect is also possible, that the finer lime is better incorporated and creates conditions that are actually more effective at continuing transformations until the compounds are degraded back to original levels. The coarser lime may leave more space open for the transformed compounds to remain in their altered conformations.

In order to understand the mechanisms taking place, a more in depth study will have to be conducted. This experiment should be repeated to see if results are duplicated. Additionally, ground lime should be compared to the sieved lime used here. Grinding the lime would increase activity since fresh surface area is exposed to interact with the sludge. Experiments should also be extended to test the effects of mixing time and the amount of lime added to the sludge. All of these factors could result in changes in hormone concentrations.

## Appendix E: Calculation of Yearly Inputs

For biosolids calculations, the amount of biosolids applied to land in 2008 was used. This number, 188,220 wet tons, was converted to dry weight by multiplying by 0.3 (30%), which is the average percent solids in the biosolids collected. Based on this calculation, a dry weight of 56,466 tons of biosolids was applied. This number was multiplied by the average concentrations of progesterone and E1 and converted into kg/year to give the values reported in Table 6.

The amount of chicken litter produced per year was calculated using the formula laid out by the Penn State Agronomy Guide (Penn State 2010). The number of broilers produced in Maryland, as reported by the 2007 Census of Agriculture was 296,341,690 (USDA 2007). This number was multiplied by the Animal Equivalence Unit (AEU), 0.0026, and the manure production estimate per AEU per year (0.53805 tons) to give the estimate on litter production of 415,000 (Penn State 2010). The AEU is based on 1000 lbs of animal weight and takes into account the average weight of the animals.

For the purpose of this estimate, it was assumed that all of the litter was land applied besides the ~70,000 which was pelletized. Subtracting this amount from the production estimate gives a yearly application rate of 345,000 tons. Poultry litter is approximately 20% water, so the final dry weight is 80% of 345,000, or 276,000. Once this number was obtained, the inputs were calculated in the same way as the biosolids inputs and reported in Table 6.

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