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## **Determination of 17 $\beta$ -estradiol and Estrone Concentrations in Runoff and Topsoil from Plots Receiving Dairy Manure**

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To the Graduate Council:

I am submitting herewith a thesis written by Angel Renea Peters Dyer entitled "Determination of  $17\beta$ -estradiol and Estrone Concentrations in Runoff and Topsoil from Plots Receiving Dairy Manure." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biosystems Engineering Technology.

D. Raj Raman, Major Professor

We have read this thesis and recommend its acceptance:

Mike Mullen, Robert Burns, Alice Layton

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council

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D. Raj Raman  
Major Professor

We have read this dissertation  
and recommend its acceptance:

Mike Mullen

Robert Burns

Alice Layton

Accepted for the Council:

Dr. Anne Mayhew  
Vice Provost and  
Dean of Graduate Studies

(Original signatures are on file in the Graduate Student Services Office.)

**DETERMINATION OF 17 $\beta$ -ESTRADIOL AND ESTRONE  
CONCENTRATIONS IN RUNOFF AND TOPSOIL FROM PLOTS RECEIVING  
DAIRY MANURE**

A Thesis  
Presented for the  
Master of Science  
Degree  
The University of Tennessee, Knoxville

Angel Renea Peters Dyer  
December 2001

## **DEDICATION**

This thesis is dedicated to my parents,

Steve and Angela Peters

who have always supported me in my educational endeavors

and given me endless encouragement, and to

Chad Dyer

my husband, who has believed in my abilities

and offered lots of support throughout my graduate studies..

## **ACKNOWLEDGMENTS**

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## ABSTRACT

Because of increasing concern about estrogenic compounds in animal wastes, the objective of this research was to measure 17 $\beta$ -estradiol and estrone concentrations in runoff and soil from plots fertilized with liquid dairy waste. Nine plots were established at The University of Tennessee Dairy Experiment Station located at Lewisburg, Tennessee, and dairy manure was applied to six of the nine plots in November, 2000. Three of the plots received manure at a rate sufficient to meet the nitrogen (N) requirement for winter wheat; three received manure at a rate sufficient to meet the phosphorus (P) requirement for winter wheat, and three received no manure. Runoff samples were collected after each of the first six runoff events producing natural rainfalls following manure application. A second manure application, based on the N and P requirements for sorghum/sudan grass, was made in March 2001. Soil samples were taken monthly after each manure application. Enzyme linked immunosorbant assays were used to determine the concentration of 17 $\beta$ -estradiol and estrone in the collected runoff samples, and soil samples. The results are reported herein, showing that 17 $\beta$ -estradiol concentrations in runoff from plots that historically received dairy manure, but which had not recently received manure, ranged from below detection threshold to 5.0 ng/L. In contrast, average 17 $\beta$ -estradiol concentrations in runoff from plots receiving manure at the N-rate were as high as 308 ng/L; plots receiving manure at the P-rate had average runoff 17 $\beta$ -estradiol concentrations as high as 29 ng/L. These values are above the 10 ng/L level that we have taken to be biologically significant. For estrone, runoff concentrations at the N-rate reached over 2000 ng/L; the runoff concentrations for the P-

rate reached 55 ng/L and the control plots reached 68 ng/L at one point. Runoff concentrations of both hormones generally decreased with time following manure application.

17 $\beta$ -estradiol soil concentrations for the N-based plots ranged from 2-600 ng/kg; P-based plot soil concentrations ranged from 6-160 ng/kg; and control plot soil concentrations ranged from 5.5-220 ng/kg. Estrone soil concentrations for N-based plots ranged from 67-1260 ng/kg; P-based plot concentrations ranged from 72-2580 ng/kg; and control plots ranged from 43-200 ng/kg.

In both winter and spring, biologically relevant concentrations of 17 $\beta$ -estradiol and estrone were observed in runoff from plots receiving dairy manure slurry at rates appropriate to crop N and P requirements. Masses of these compounds were positively correlated with estrogen mass application rates, and these compounds persisted in runoff water for several runoff events following manure application.

17 $\beta$ -estradiol masses in the soil positively correlated with estrogen mass application rates at statistically significant levels, but estrone masses did not. Both of these estrogens did persist in the soil for several months after manure applications in both winter and spring. Masses of 17 $\beta$ -estradiol in the soil did positively correlate with masses of this compound in the runoff, but concentrations of estrone in the soil did not correlate with runoff masses of estrone.



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## Chapter 1

### INTRODUCTION

#### Background

17 $\beta$ -estradiol (E2) is a highly potent, biologically active form of estrogen, and estrone (E1) is the ketone form of 17 $\beta$ -estradiol, which has a lower biological activity than 17 $\beta$ -estradiol (Arcand-Hoy et al., 1998). The structures of these compounds are shown in Figure 1. 17 $\beta$ -estradiol and estrone can be emitted into the environment from animal wastes (Monk et al., 1975; Knight, 1980) and from human wastes, via municipal wastewater treatment facilities (Desbrow et al., 1998; Routledge et al., 1998; Harries et al., 1997; Folmar et al., 1996; Purdom et al., 1994). A variety of environmental estrogens have been associated with human and wildlife health problems (Kaiser, 1996; McLachlan and Arnold, 1996; Pearce, 1996; Panter et al., 1998; Nakamura, 1984).

Sufficiently high concentrations of environmental estrogens can cause feminization of male fish. Fishermen first discovered hermaphroditic fish in the lagoons of a sewage treatment plant (Purdom et al., 1994). It is possible for a fish to have a complete sex reversal when exposed to environmental estrogens. Nakamura (1984) found that masu salmon (*Oncorhynchus masou*) had a sex distribution of 84-100% female when exposed to 17 $\beta$ -estradiol in concentrations of 250-5000 ng/L; control tanks in this experiment had only 49% females. Another biomarker for environmental estrogens is vitellogenin, a major component in fish egg yolk that is normally only found in female fish. 17 $\beta$ -estradiol at a concentration of 10 ng/L has been found to cause

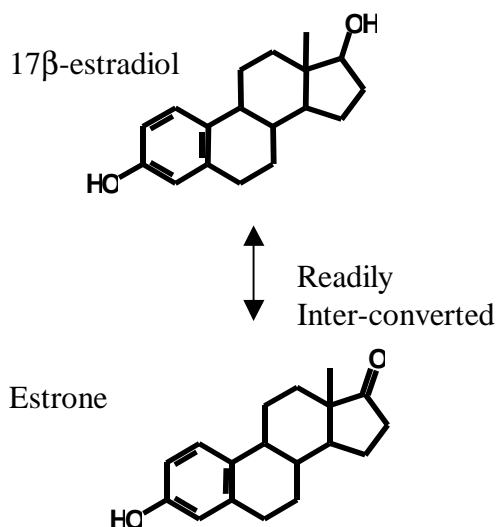


Figure 1. Structures of 17β-estradiol and estrone.

vitellogenin production in male rainbow trout and roach fish (*Oncorhynchus mykiss*, and *Rutilus rutilus*) (Routledge et al., 1998). The same investigators found that estrone at a concentration of 25 ng/L caused vitellogenin production in male rainbow trout (*O. mykiss*). Furthermore, there is evidence of synergism between 17β-estradiol and estrone: a combination of estrone at a concentration of 25 ng/L and 17β-estradiol at 25 ng/L caused more vitellogenin production than only 50 ng/L of estrone, or than 100 ng/L of 17β-estradiol (Routledge et al., 1998). In this thesis, 10 ng/L 17β-estradiol, and 25 ng/L estrone were considered threshold levels of biological relevance based on Routledge et al.'s study of vitellogenin production.

Environmental estrogens can not only feminize fish, but may cause problems in dairy heifers as well. Heifers fed a diet amended with poultry litter, which is high in 17β-estradiol and estrone (>300 µg/kg), have been diagnosed with premature udder development (Shore et al., 1993). The effect of environmental estrogens on humans is

unclear, but it has been suggested that declining male sperm counts and female breast cancer are linked to these compounds (Colborn, 1995; Bradlow et al., 1995). However, these investigations were primarily concerned with synthetic estrogens and estrogen mimicking compounds.

There is evidence that  $17\beta$ -estradiol is short-lived in the environment, with conversion to estrone likely the primary product.  $17\beta$ -estradiol may change forms into estrone over the course of hours or days (Routledge et al., 1998; Belfroid et al., 1999). Ternes et al. (1999) found that after a period of no more than 3 h, 95% of unconjugated  $17\beta$ -estradiol was gone, but estrone values had increased by 95% of the initial  $17\beta$ -estradiol concentration. In work with dairy manure solids, Raman et al. (2001) observed a similar rapid decrease of  $17\beta$ -estradiol concentrations and concomitant increase of estrone concentrations.

Poultry litter also contains elevated levels of environmental estrogens. Shore et al. (1993) reported that after six months of ensiling chicken manure, the concentration of total estrogen, including both estrone and estradiol, ( $533 \pm 40 \mu\text{g/kg}$ ) did not decrease. Nichols et al. (1997) reported significant levels of  $17\beta$ -estradiol (ca. 1000 ng/L) in runoff from plots receiving poultry litter, with the compound persisting for at least 7 d. In a later study, they determined that runoff concentrations of  $17\beta$ -estradiol are significantly reduced by fescue grass filter strips (Nichols et al., 1998). Since  $17\beta$ -estradiol concentrations did not decrease in chicken manure, and because grass filter strips significantly reduced these concentrations, it is possible that  $17\beta$ -estradiol could bind to

the soil, and cause soil contamination from application of animal waste to agricultural fields.

Measuring estrogen concentrations in samples is challenging because of the potential for significant storage losses and because of the extraction difficulty from the sample type. Acidification and low-temperature storage are crucial to sample preservation; Raman et al. (2001) determined that all samples should be acidified to a pH of 2 and kept at 4°C until they can be extracted with ether.

### **Problem Identification**

Little work has been reported on the potential of water contamination with 17 $\beta$ -estradiol and estrone from dairy manure. Since dairy manure is typically land applied to agricultural fields, and since pregnant animals excrete elevated levels of estrogen (Shore et al., 1993), the amount of 17 $\beta$ -estradiol and estrone in field runoff from land applied dairy waste may be environmentally relevant.

The objectives of this work were therefore to test the following hypotheses:

### **Runoff-Related Hypotheses**

#### *Hypothesis 1: Runoff estrogen concentrations*

Biologically relevant concentrations of 17 $\beta$ -estradiol and estrone are present in runoff from plots receiving dairy manure at rates appropriate to crop nitrogen and phosphorus requirements (nitrogen or phosphorus requirement is commonly used to determine manure application rates).



*Hypothesis 2: Correlation between estrogen application rate and runoff estrogen masses*

17 $\beta$ -estradiol and estrone masses in runoff positively correlate with estrogen application rates; that is, if more manure is applied, the off-field estrogen emissions will be higher.

*Hypothesis 3: Persistence of estrogen in runoff*

17 $\beta$ -estradiol and estrone concentrations persist in runoff water after several runoff events following manure application. Raman et al. (2001) found that 17 $\beta$ -estradiol quickly changes form to estrone, but that estrone persists for extended periods of time.

**Soil-Related Hypotheses**

*Hypothesis 4: Correlation between estrogen application rate and soil estrogen masses*

17 $\beta$ -estradiol and estrone masses in soil positively correlate with estrogen application rates.

*Hypothesis 5: Persistence of estrogens in soil*

17 $\beta$ -estradiol and estrone concentrations persist in the soil for several months following manure application.

**Hypothesis on Soil-Runoff Relationship**

*Hypothesis 6: Correlation between runoff and soil estrogen masses*

Masses of 17 $\beta$ -estradiol and estrone in the soil positively correlate with the masses of these compounds in runoff.

**Relevance of Hypotheses**

If biologically relevant concentrations of 17 $\beta$ -estradiol and estrone are present in the runoff from plots receiving dairy manure and if 17 $\beta$ -estradiol and estrone

concentrations in runoff positively correlate with manure application rates, then manure application could be limited so that these estrogens will not be an environmental concern. If  $17\beta$ -estradiol and estrone concentrations persist in runoff water after several runoff events following manure application, then the timing of manure application may need careful consideration. If estrogens bind to the soil rather than accumulating in the runoff, there is less concern for the fish and wildlife in or around the stream the runoff water empties into unless the estrogens constantly wash out of the soil, thereby causing harmful concentrations of  $17\beta$ -estradiol and estrone in runoff water for extended periods of time.

## **Chapter 2**

### **MATERIALS AND METHODS**

#### **Overview**

Nine runoff plots (6 x 12 m each) were refurbished at The University of Tennessee Dairy Experiment Station in Lewisburg, Tennessee. Manure was applied to these plots twice throughout the experiment; once in the winter, and once in the spring. The first manure application was based on a winter wheat cropping system. Three plots received manure at a rate sufficient to meet the nitrogen (N) requirement; three received manure at a rate sufficient to meet the phosphorus (P) requirement, and three received no manure. The second manure application was based on a Sorghum/Sudan grass cropping system. The same three plots that had previously received manure at a rate sufficient to meet the N requirement received manure at the N requirement again. The P plots received manure at rates sufficient to meet crop P again, while the control plots remained unmanured.

#### **Dairy Description**

All field experiments were performed at the University of Tennessee Dairy Experiment Station in Lewisburg, TN. The experiment station maintains approximately 175 lactating Jersey cattle with free-stall housing. Manure is scraped into an agitated pit (sump), then passed through a solids separator. The liquid separator effluent (press liquor) is stored in a holding pond prior to land application. The solid portion of the separator effluent (press cake) is used as bedding for the dairy cattle.

## **Runoff Plots**

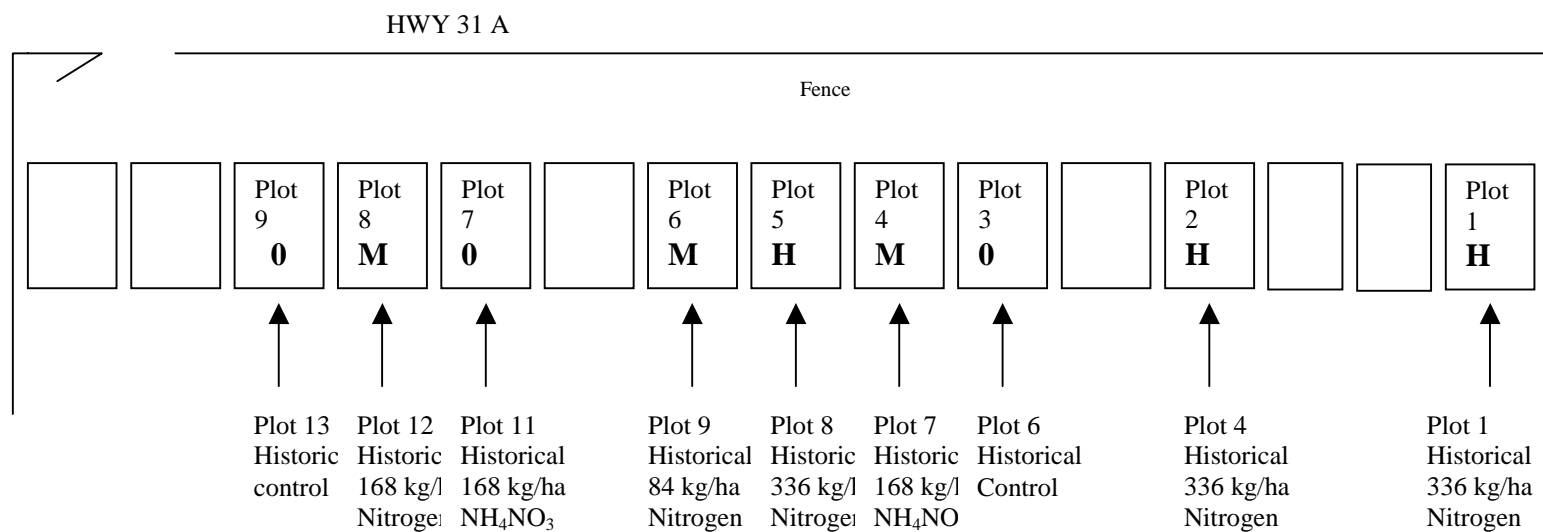
Nine plots were selected from 15 existing research plots at the University of Tennessee Dairy Experiment Station in Lewisburg, TN. Plot selection was based on current lysimeter quality, since lysimeter data was taken from these plots for a related project. Each of the nine 6 x 12 m plots was refurbished prior to the first manure application. Soil berms (15-cm tall) sown in bermuda grass were established around each plot to prevent excess runoff water from entering the plot area. Each plot had a slope of approximately 2%. Figure 2 shows the plot layout including the plots established prior to this experiment.

## **Plot History**

The soil at the site was an Armour silt loam (fine-silty, mixed, active, thermic Ultic Hapludalf). This soil had historically been manured and fertilized on a regular basis. In an earlier study (1991-1996), plots received manure ranging from 84 kg N/ha to 336 kg N/ha, or ammonium nitrate at 168 kg N/ha, in the spring. In the fall, the manured plots received half of the spring application, and the ammonium nitrate plots received 50 kg N/ha. Two of the three control plots in the current experiment were used as controls in the previous experiment. The remaining control was treated with ammonium nitrate (168 kg N/ha). A layout of the plots, including a key to the plot history, is provided in Figure 2.

## **Plot Installation**

Plots were marked off with wooden stakes (Figure 3). A triangular region was bermed off at the top of each plot to divert the runoff water coming from the land above.

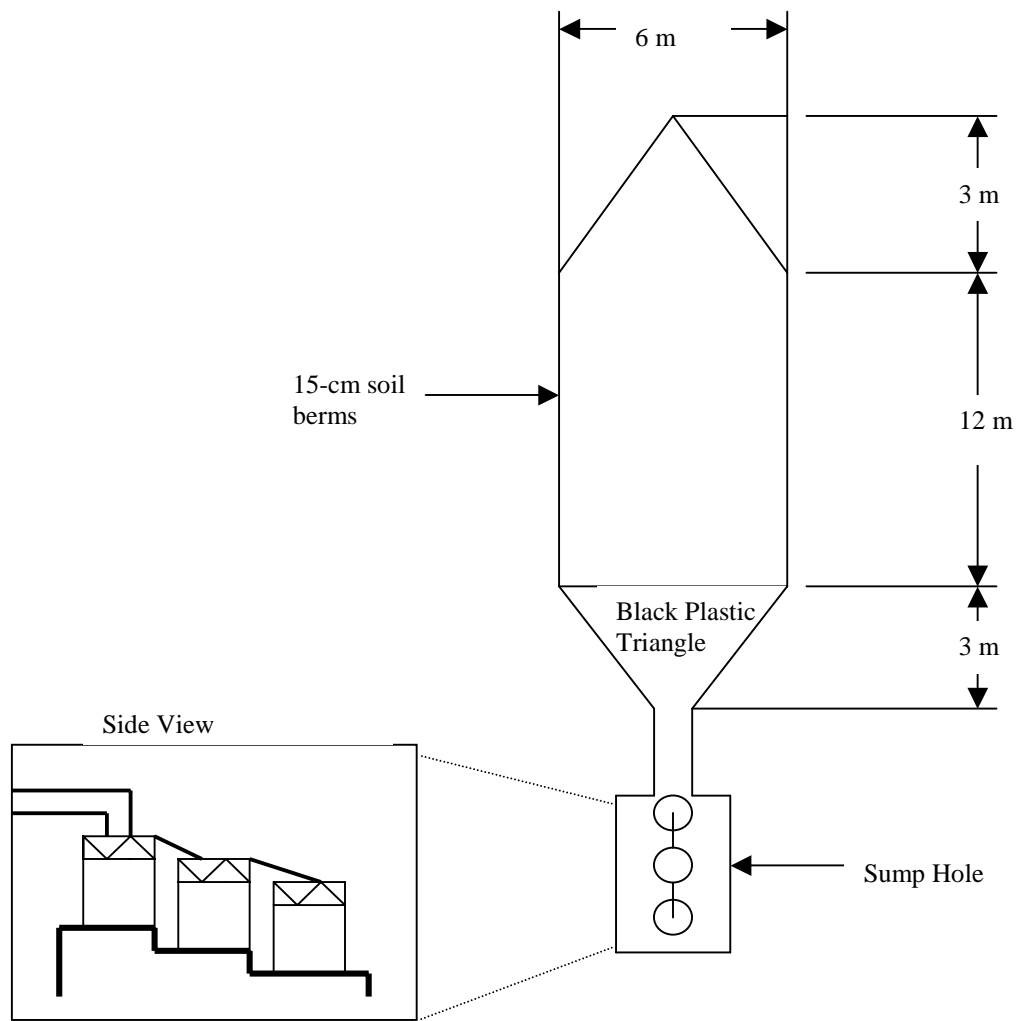


H = Plot receiving manure at the Nitrogen application rate.

M = Plot receiving manure at the Phosphorus application rate.

O = Plot receiving NO manure.

Figure 2. Layout of runoff plots, including previous plot history



NOT TO SCALE

Figure 3. Schematic of a runoff plot.

The outer edge of the plot was plowed with a tractor, and a rear tine tiller was used to further pulverize the soil, prior to hoeing into a 15-cm berm. Berms were tamped by walking over them, and then sown in bermuda grass to reduce erosion.

A backhoe was used to dig a sump hole approximately 6 m from the end of each plot (Figure 3). The sump holes were dug deep enough that the flow dividing bucket and PVC pipe used for runoff collection fit together. The sump holes were leveled and one wooden bucket stand was added to each hole. A 20-kg bag of pea gravel was added to each sump to aid in drainage. A metal triangle was placed on the wooden bucket frame for the first two flow dividing buckets that collected the runoff. These triangles were used to level the buckets after each rainfall event. A drainage pipe was installed to drain the rainwater from the sump hole.

A 1.2-m trench was dug at the bottom of each of the nine runoff plots. Black plastic sheeting was placed inside each trench and covered with soil to prevent the plastic from moving during the project. A triangular runoff collection area (Figure 3) was constructed to collect runoff from the plot and direct it into a PVC pipe. The two outer edges of the collection area were constructed of 3-m long pressure treated boards (5 x 10 cm cross section), which were also used to secure black plastic sheeting; the sheeting was rolled over the boards, stapled, then sealed with silicone caulk, to ensure the integrity of the collection area.

PVC pipe was placed at the bottom corner of the triangular collection area. The black plastic at the point was put into the PVC pipe and held in place with a 1-cm thick PVC ring. An elbow joint was added to the other end of the PVC pipe so that it would connect to the first runoff bucket in the sump hole.

The flow dividing bucket system used for this thesis research was developed by Dr. Daniel Yoder (personal communication, Knoxville, TN, July 2000). Two 18.9-L flow dividing buckets (Figure 4), and one regular 18.9-L bucket were placed in each sump hole and strapped onto a wooden bucket stand. Each bucket had a top to prevent excess water from entering the bucket. One two-liter bottle filled with sand was placed in each bucket to increase its stability. Each flow-dividing bucket used a circular, multiple v-notch ring to divide any overflow into 24 equal subsections. A v-shaped channel reached from the flow divider of one bucket to the center of the next bucket so that 1/24 of the flow from bucket one could flow into bucket two and 1/24 of that runoff water flowed into bucket 3. The maximum runoff measurement capacity of the system was thus 11,400 L ( $18.9 + 454.2 + 10900.8$ ). The minimum amount needed to sample was 250 mL.

Initially, tarps were nailed over the sump holes to keep excess rain out of the holes, but this did not work. Therefore, black plastic tents framed with metal conduit and lumber were used to keep the rain out of the holes.

Problems also occurred with runoff water from around the plots draining into the sump holes, and the sump holes collapsed partially. Berms were built out from the plots to divert the runoff flow around each sump hole. To prevent the sides of the sump from collapsing, pressure-treated plywood sheets were installed around the inside of the sump holes and reinforced with additional lumber.

### **Manure Slurry Source**

Before manure was applied to the plots, tests were run to determine which manure source had the highest levels of 17 $\beta$ -estradiol. Manure was tested from the holding pond,





Figure 4. Placement of flow dividing buckets in the sump hole.

the sump, separated manure solids (press cake), and liquid separated manure (press liquor). Results of this test suggested that the press liquor had the highest levels of 17 $\beta$ -estradiol (ca. 15,000 ng/L), but there was not enough press liquor to readily apply to the plots. We chose to use the manure from the sump because the supply was plentiful and it had relatively high 17 $\beta$ -estradiol (ca. 10,000 ng/L).

In November 2000, manure containing 17 $\beta$ -estradiol at concentrations of  $3300 \pm 700$  ng/L was applied to the plots as indicated in Table 1. A winter wheat cropping system with an estimated yield of 40 bu/ac was assumed when determining the appropriate N and P application rates for the first application. The nitrogen requirement was 70 lbs/ac and the P<sub>2</sub>O<sub>5</sub> requirement was 30 lbs/ac (MWPS-18). Estrone concentrations were not available for the first manure application due to difficulties with the assay procedures.

A sorghum/sudan grass cropping system with an estimated yield of 8 tons/ac was assumed for the second manure application to determine appropriate N and P application rates. The nitrogen requirement was 319 lbs/ac and the P<sub>2</sub>O<sub>5</sub> requirement was 122 lbs/ac (MWPS-18). Manure application rates for the second application are shown in Table 2; the application was made in March 2001. The average 17 $\beta$ -estradiol concentration for manure application two was  $16,000 \pm 4,800$  ng/L. The average estrone concentration was  $1,069 \pm 160$  ng/L.

The second manure application was not all applied at the same time. Manure was applied to the N-based plots first, but before it could be applied to the P-based plots, a rainfall event occurred preventing the application to the P-based plots. The P-based plots

Table 1. Manure application rates for manure application 1.

Treatment	Volume Applied Per Plot	kg N/ha	kg P/ha
Control	0	0	0
P-based	450 L (5.4 mm)	65	24
N-based	980 L (11.7 mm)	142	53

Table 2. Manure application rates for manure application 2.

Treatment	Volume Applied Per Plot	kg N/ha	kg P/ha
Control	0	0	0
P-based	1094 L (15.2 mm)	158.9	59.9
N-based	2460 L (34.2 mm)	357.8	135

received manure two weeks after the N-based plots. For this reason, N and P-based plot data were only compared to the control plots, and not directly to one another.

## **Sample Collection Methods**

### *Runoff Water Samples*

After each rainfall event expected to generate runoff (>1.3 cm in 24 h), a 250-mL grab sample was taken from the first runoff collection bucket at each of the nine plots. Grab samples were collected in a 500-mL bottle; immediately after sampling, 1.0 mL of 4 N H<sub>2</sub>SO<sub>4</sub> was added to each sample because previous work in our lab demonstrated that acidifying samples is critical to preservation of 17 $\beta$ -estradiol and estrone (Raman et al., 2001). Samples were then stored on ice for transport back to the lab and were stored at 4°C in the lab until assay. Data from six significant rainfall events after each manure application were collected during the study.

### *Soil Samples*

Soil samples were taken with a 3/4" soil probe from the top 7.6 cm of soil. This depth was chosen because the soil at this site has deep topsoil and we could stay in the first horizon at this depth. Finlay-Moore et al. (2000) sampled the top 5 cm of soil. A representative sample was obtained from each plot by selecting five areas from each plot and mixing the soil together in a resealable bag. Shortly thereafter (ca. 2 hrs), 100 g of soil from each bag was weighed out, and 50 mL of acid was added to it. The acidified samples were shaken by hand, put in a cooler with ice to be transported back to the lab and were stored at 4°C in the lab until assayed. Samples were taken monthly starting one day before manure application and ending when the last water sample was collected.

## **Extraction Procedure**

### *Runoff Water Samples*

At the start of the assay procedure, 1 drop of 6 N NaOH and 5 mL of ether were added to a 30-mL aliquot of acidified sample. (NaOH was used to neutralize each sample for use with the assay kits.) Each sample was extracted in duplicate so there would be a sample for each assay kit. This mixture was shaken on a horizontal shaker for 2 h, and centrifuged for 10 min at 2300g to separate the ether layer. A 1.0-mL aliquot of ether was taken and evaporated to dryness under N<sub>2</sub> gas; the residue was then stored at –10 °C until analysis.

### *Soil Samples*

Soil samples were extracted by adding 3 drops of 6 N NaOH, to neutralize the mixture, and 5 mL of ether, to 5 g of soil acid mixture. This mixture was shaken on a horizontal shaker for 2 h and centrifuged for 10 minutes at 2300g to separate the ether layer. A 1.0 mL aliquot of ether was pulled and evaporated with N<sub>2</sub> gas; the residue was then stored at –10°C.

## **Assay Procedures**

Enzyme linked immunosorbent assay (ELISA) kits (Assay design Inc., Ann Arbor, MI; and Alpco Diagnostics, Windham, NH) were used to determine the concentration of 17 $\beta$ -estradiol and estrone in each sample. This method has been used in several studies involving estrogens because of its low-cost, sensitivity, specificity, and speed (Finlay-Moore et al., 2000; Bushee et al., 1998; Nchols et al., 1997; Vos, 1996). Separate kits were used to measure estrone and 17 $\beta$ -estradiol. A spiked matrix control

experiment was conducted for both 17 $\beta$ -estradiol and estrone by spiking distilled water with known amounts of the estrogens in the standard range. The 17 $\beta$ -estradiol ELISA kit works best between 220 and 27 800 ng/L (Figure 5). It over reported slightly for all concentrations tested except 3 470 ng/L, where it recovered 94 percent of the total 17 $\beta$ -estradiol available. The estrone ELISA kit works best between 54 and 27 800 ng/L (Figure 6). It under reported all values (88-97%) except 10 ng/L where it reported 215 percent of the total estrone was recovered.

Residues from the extraction were brought to room temperature, resuspended in 100  $\mu$ L of ethanol, vortexed for 30s, combined with 500  $\mu$ L of assay buffer provided with the 17 $\beta$ -estradiol ELISA kit, and vortexed for 30s more. Samples were then analyzed in triplicate for 17 $\beta$ -estradiol and estrone. ELISA standards were run using the same 1:5 ethanol : assay buffer ratio. Standard concentrations were 9.6 to 30,000 ng/L 17 $\beta$ -estradiol and estrone; however, since runoff water samples were concentrated by a factor of 6, the detection range of standards was 1.6 – 5000 ng/L. A standard curve for the 17 $\beta$ -estradiol ELISA is included in figure 7.

The extraction and assay procedures were the same for both estrone and 17 $\beta$ -estradiol. However, the estrone kit did not provide an assay buffer, so there was no way to resuspend the samples if they were extracted the same way as the 17 $\beta$ -estradiol samples. Several extraction techniques were tried. A 60% methanol solution was used to extract samples from the first manure application, but this was not successful. E. A. Vos (1996) reported success using a phosphate buffer (pH 7.2) for the estrone ELISA kits.

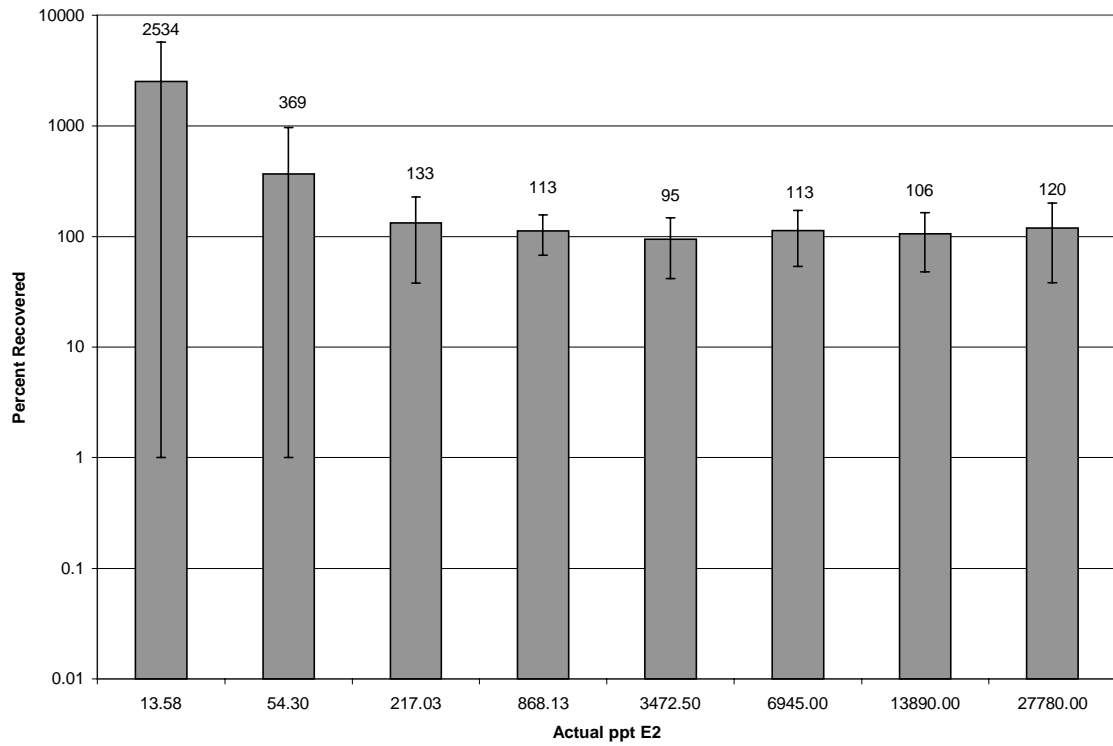


Figure 5. Spike-recovery test results for 17 $\beta$ -estradiol ELISA kit. The kit is highly accurate between concentrations of 220 to 27 800 ng/L, and over-reports significantly at 10 ppt.

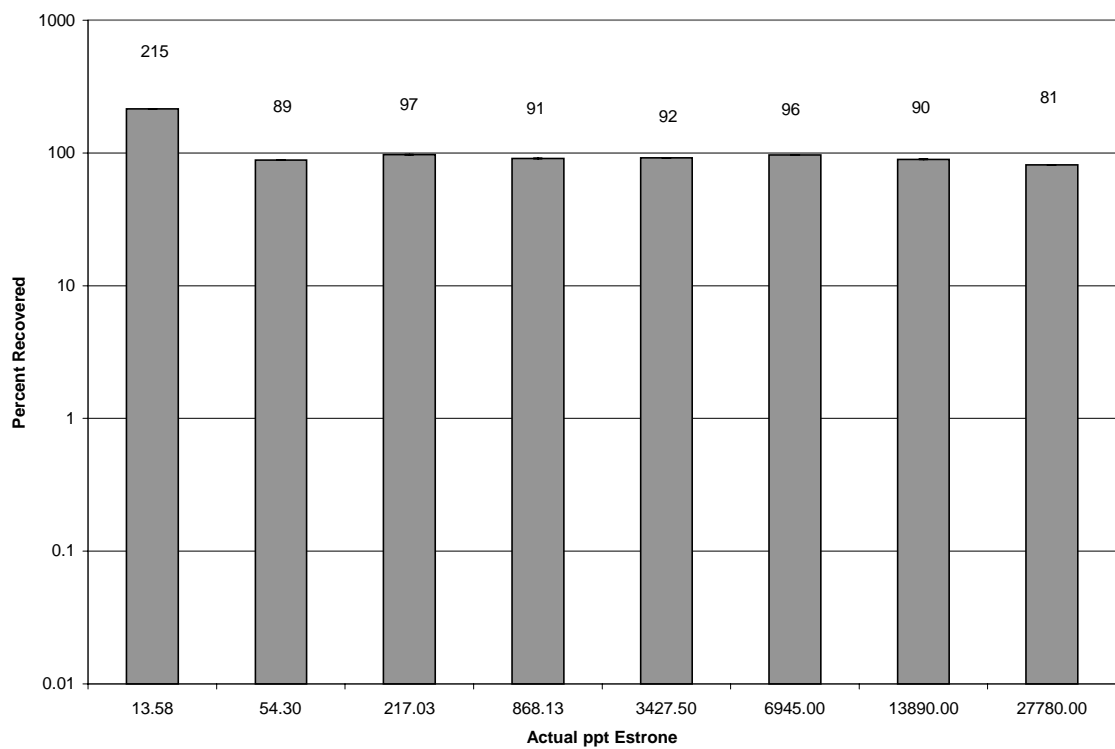


Figure 6. Spike-recovery test results for estrone ELISA kit. The kit is accurate between concentrations of 54 to 27 800 ng/L.



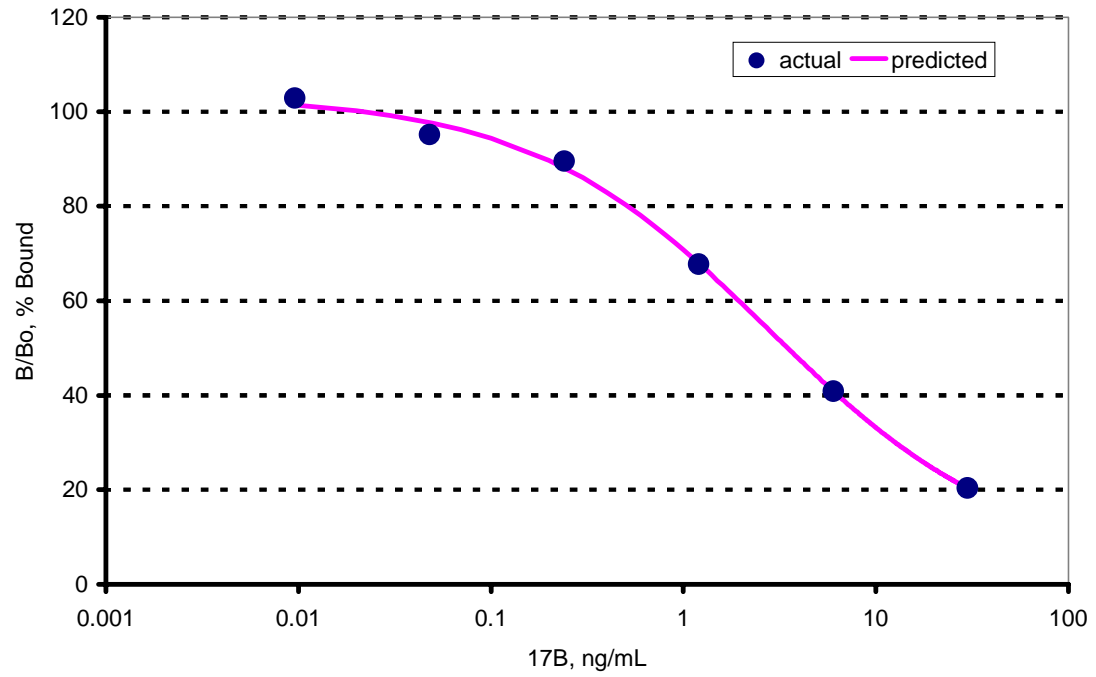


Figure 7. Representative standard curve for 17 $\beta$ -estradiol ELISA.

This method was used on samples from manure application 2, and was successful. A sample standard curve for the estrone ELISA can be seen in Figure 8.

### **Data Analysis**

Data analysis was performed on Excel (Microsoft, 1997). Each set of standards run were fit to a four parameter curve,  $y = \frac{y_0 + a}{1 + e^{\frac{-(x-x_0)}{b}}}$  and the  $r^2$  value was recorded ( $r^2$  values typically exceeded 0.98). Data outside the standard curve was not used. The binding values of the samples along with sample mass, ether volume, and extraction buffer volume were used to find the amount of  $17\beta$ -estradiol and estrone in each sample. The average of each triplicate sample was calculated along with the standard deviation and the coefficient of variation.

The mass of  $17\beta$ -estradiol and estrone in the soil (used in later calculations) was estimated by multiplying the concentration of estrogen (ng/kg) in the soil by a conversion factor of  $1.4 \text{ kg soil m}^{-3}$ , and multiplying that by the volume of soil ( $\text{m}^3$ ) in the sample area per plot assuming a depth of 7.6 cm.

Statistical analysis was performed with SAS version 8.12 (1999, SAS institute, Carey, NC). A statistical significance of  $\alpha=0.05$  was used. Analysis of variance for a completely randomized design with missing values was performed. Mean separation was done using Duncan's test.

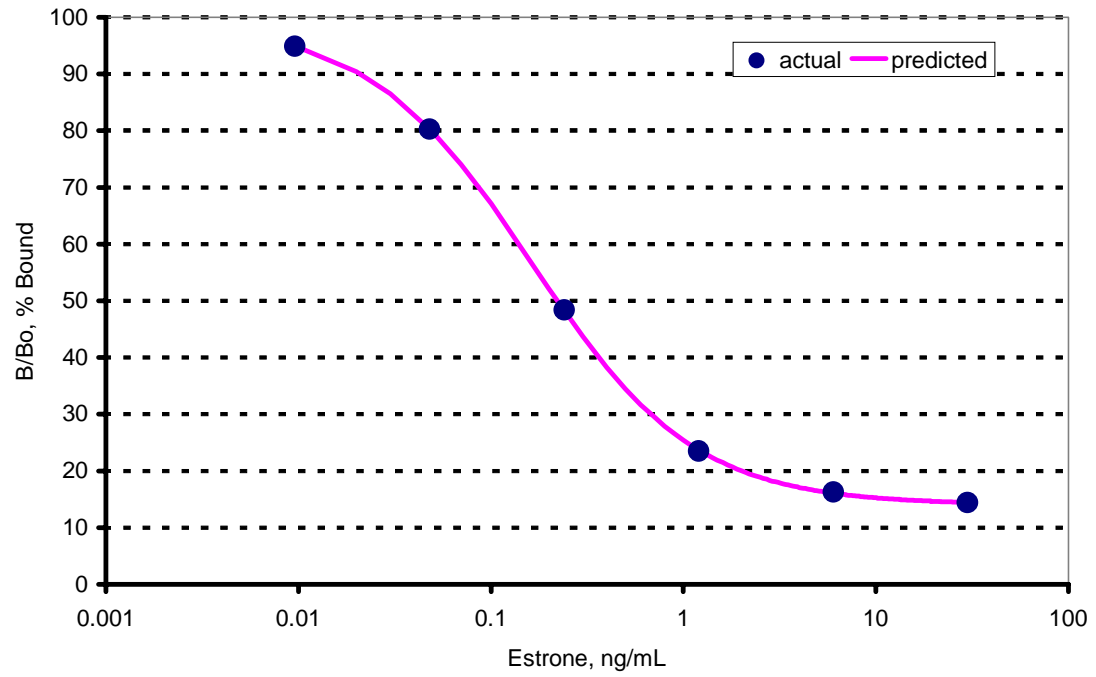


Figure 8. Representative standard curve for estrone ELISA.

## Chapter 3

### RESULTS AND DISCUSSION

#### Control Plot Check

Since the control plots had not all received the same treatment in the previous study, statistics were run on runoff concentrations of 17 $\beta$ -estradiol and estrone, as well as soil concentrations of 17 $\beta$ -estradiol and estrone to determine if there were significant differences between concentrations in the control plots ( $\alpha=0.05$ ). There was none.

#### Runoff Related Hypotheses

*Hypothesis 1: Biologically relevant concentrations of 17 $\beta$ -estradiol and estrone are present in runoff from plots receiving dairy manure at rates appropriate to crop N and P requirements.*

For both manure applications, and for both manure application rates, this hypothesis was accepted. Following manure Application 1, the concentration of 17 $\beta$ -estradiol in the runoff from the N and P based plots were above 10 ng/L for runoff events 22 and 24 days after manure application. Furthermore, this level was statistically significantly above the background level observed from the control plots ( $\alpha=0.05$ ). However, for subsequent runoff events, levels of 17 $\beta$ -estradiol were not biologically relevant (Table 3). 17 $\beta$ -estradiol concentrations in runoff from control plots were never above biologically relevant levels. Estrone was not tested for manure application 1.

After manure application 2, 17 $\beta$ -estradiol concentrations were above biologically relevant levels for runoff events occurring 1, 26 and 82 days after manure application on

Table 3. Runoff 17 $\beta$ -estradiol concentrations (ng/L) following manure application 1.

Bold indicates concentrations above 10 ng/L, and lowercase letters indicate significant differences between treatments down the columns; they are not applicable across rows.

Note: BDL (Below Detection Limits of 1.6 ng/L)

Treatment	4 days	22 days	24 days	54 days	60 days	69 days
Control	1.77 $\pm$ 1.0 b	BDL b	BDL b	BDL a	BDL a	BDL a
P-based	2.2 $\pm$ 1.3 b	<b>28.9 <math>\pm</math> 24.3 a</b>	<b>29.1 <math>\pm</math> 25.7 a</b>	1.62 $\pm$ .69 a	BDL a	BDL a
N-based	9.3 $\pm$ 7.1 a	<b>34.3 <math>\pm</math> 28.1 a</b>	<b>25.7 <math>\pm</math> 38 a</b>	1.9 $\pm$ 1.1 a	1.5 $\pm$ 0.7 a	BDL a
Rainfall Amt.	2.6 cm	5.0 cm	2.5 cm	1.3 cm	7.5 cm	2.5 cm

the N-based plots (Table 4). Interestingly, the concentration of 17 $\beta$ -estradiol in the runoff from the N-based plots was also biologically relevant for the runoff event that occurred 26 days after manure application (Table 4). This could possibly be caused by migrating birds in the area such as geese, which are frequently seen on the farm during this season.

After manure application 2, runoff 17 $\beta$ -estradiol concentrations from the P-based plots were only above biologically relevant levels for the runoff event that occurred 12 days after manure application (Table 5). Interestingly, the control plots were also above biologically relevant levels for this runoff event and there was no statistically significant difference between the control and P-based runoff concentrations. After the first runoff event, both P and control plots were below biologically relevant levels.

For N-based plots, runoff estrone concentrations reached biologically relevant levels (25 ng/L) for runoff events occurring 1, 52, 82, and 98 days after manure application (Table 6). Surprisingly, control plot runoff from events occurring on 82 and 98 days, were also above biologically relevant levels (Table 6).

Estrone concentrations in runoff from the P-based plots exceeded biologically relevant levels (25 ng/L) for all but two runoff events (Table 7). For the last 3 runoff events, the control plots were also above biologically relevant levels.

The concentrations of 17 $\beta$ -estradiol for this experiment are much lower than those observed by Nichols et al. (1998), who reported a mean runoff water concentration of 3500 ng/L 17 $\beta$ -estradiol with the use of grass filter strips. The highest runoff

Table 4. Runoff 17 $\beta$ -estradiol concentrations (ng/L) for Control and N-based plots following manure application 2. Bold indicates concentrations above 10 ng/L. Lower case letters indicate significant differences between treatments. Statistical data can only be compared down the columns. They are not applicable across rows. BDL indicates below detection limits of 1.6 ng/L.

Treatment	1 day	26 days	52 days	64 days	75 days	82 days	96 days	98 days
Control	BDL a	<b>188 <math>\pm</math> 371 a</b>	1.8 $\pm$ 0.7 a	7.8 $\pm$ 6.7 a	4.0 $\pm$ 4.0 a	5.0 $\pm$ 5.5 a	4.2 $\pm$ 3.0 a	2.4 $\pm$ 2.1 a
N-based	<b>308 <math>\pm</math> 166 b</b>	<b>108 <math>\pm</math> 68 a</b>	2.9 $\pm$ 2.9 a	8.0 $\pm$ 5.5 a	2.6 $\pm$ 3.1 a	<b>16.4 <math>\pm</math> 11.5 b</b>	3.7 $\pm$ 3.9 a	4.4 $\pm$ 4.3 a
Rainfall Amt.	2.76 cm	6.1 cm	1.73 cm	6.45 cm	2.24 cm	7.42 cm	2.36 cm	4.27 cm

Table 5. Runoff 17 $\beta$ -estradiol concentrations (ng/L) for control and P-based plots following manure application 2. Bold indicates concentrations above 10 ng/L. Lowercase letters indicate significant differences among treatments. Statistical data can only be compared down the columns. They are not applicable across rows.

Treatment	12 days	38 days	60 days	71 days	78 days	92 days	94 days
Control	<b>188 <math>\pm</math> 371 a</b>	1.8 $\pm$ 0.7 a	7.8 $\pm$ 6.7 a	4.0 $\pm$ 4.0 a	5.0 $\pm$ 5.5 a	4.2 $\pm$ 3.0 a	2.4 $\pm$ 2.1 a
P-based	<b>142 <math>\pm</math> 103 a</b>	2.8 $\pm$ 3.1 a	6.0 $\pm$ 5.4 a	4.4 $\pm$ 1.8 a	4.4 $\pm$ 3.9 a	7.7 $\pm$ 4.4 a	5.4 $\pm$ 5.0 a
Rainfall Amt.	6.1 cm	1.73 cm	6.45 cm	2.24 cm	7.42 cm	2.36 cm	4.27 cm

Table 6. Runoff estrone concentrations (ng/L) for control and N-based plots following manure application 2. Bold indicates concentrations above 25 ng/L. Lowercase letters indicate significant differences among treatments. Statistical data can only be compared down the columns. They are not applicable across rows.

Treatment	1 day	26 days	52 days	64 days	75 days	82 days	96 days	98 days
Control	1.9 $\pm$ 24 b	4.8 $\pm$ 4.8 b	19.0 $\pm$ 11 b	<b>27 <math>\pm</math> 34 a</b>	16 $\pm$ 4.1 b	<b>25 <math>\pm</math> 5.4 b</b>	<b>41 <math>\pm</math> 20 a</b>	<b>68 <math>\pm</math> 15 a</b>
N-based	<b>2524 <math>\pm</math> 1614 a</b>	18.1 $\pm$ 8.4 a	<b>32.2 <math>\pm</math> 2.2 a</b>	10.6 $\pm$ 9.9 a	21 $\pm$ 1.7 a	<b>32 <math>\pm</math> 7.0 a</b>	23 $\pm$ 5.8 a	<b>72 <math>\pm</math> 11 a</b>
Rainfall Amt.	2.76 cm	6.1 cm	1.73 cm	6.45 cm	2.24 cm	7.42 cm	2.36 cm	4.27 cm

Table 7. Runoff estrone concentrations (ng/L) for control and P-based plots following manure application 2. Bold indicates concentrations above 25 ng/L. Lowercase letters indicates significant differences among treatments. Statistical data can only be compared down the columns. They are not applicable across rows.

Treatment	12 days	38 days	60 days	71 days	78 days	92 days	94 days
Control	4.8 ± 4.8 b	19.0 ± 11 b	<b>27 ± 34 a</b>	16 ± 4.1 a	<b>25 ± 5.4 a</b>	<b>41 ± 20 a</b>	<b>68 ± 15 a</b>
P-based	<b>39 ± 10 a</b>	<b>32.1 ± 12 a</b>	7.0 ± 2.1 a	15 ± 0.4 a	<b>25 ± 4.1 a</b>	<b>25 ± 2.7 a</b>	<b>55 ± 28.9 a</b>
Rainfall Amt.	6.1 cm	1.73 cm	6.45 cm	2.24 cm	7.42 cm	2.36 cm	4.27 cm



concentration of 17 $\beta$ -estradiol recorded in this work is only 308 ng/L, or ten percent of that observed by Nichols et al. This difference could possibly be explained by the amount of 17 $\beta$ -estradiol in the manure applied. Nichols et al. applied 17 $\beta$ -estradiol at 450  $\mu\text{g}/\text{m}^2$ ; the highest application rate used in this thesis was 220  $\mu\text{g}/\text{m}^2$ , one half of the amount applied by Nichols et al..

*Hypothesis 2: 17 $\beta$ -estradiol and estrone masses in runoff positively correlate with estrogen application rates.*

This hypothesis was accepted for 17 $\beta$ -estradiol for the first runoff event and was rejected for estrone for all runoff events. Five separate application rates were used to test this hypothesis for 17 $\beta$ -estradiol, and three application rates were used for estrone. 17 $\beta$ -estradiol mass in runoff is positively correlated with estrogen application rates for the first runoff event ( $r=0.65$ ) ( $p=0.0029$ ) (Figure 9). After the first runoff event following manure application, 17 $\beta$ -estradiol concentrations become negligible and there is no significant difference between runoff concentrations and estrogen application rates.

Estrone masses in runoff were not statistically significantly correlated with estrogen application rates (Figure 10). Estrone concentrations were generally higher than 17 $\beta$ -estradiol concentrations, and persisted for longer amounts of time. Nichols et al. (1997) reported that the first rainfall event after manure application produced the greatest amount of 17 $\beta$ -estradiol in runoff. This was also evident with this thesis data, and could be a contributing factor to why 17 $\beta$ -estradiol was only significantly correlated with manure application rates for one rainfall event.

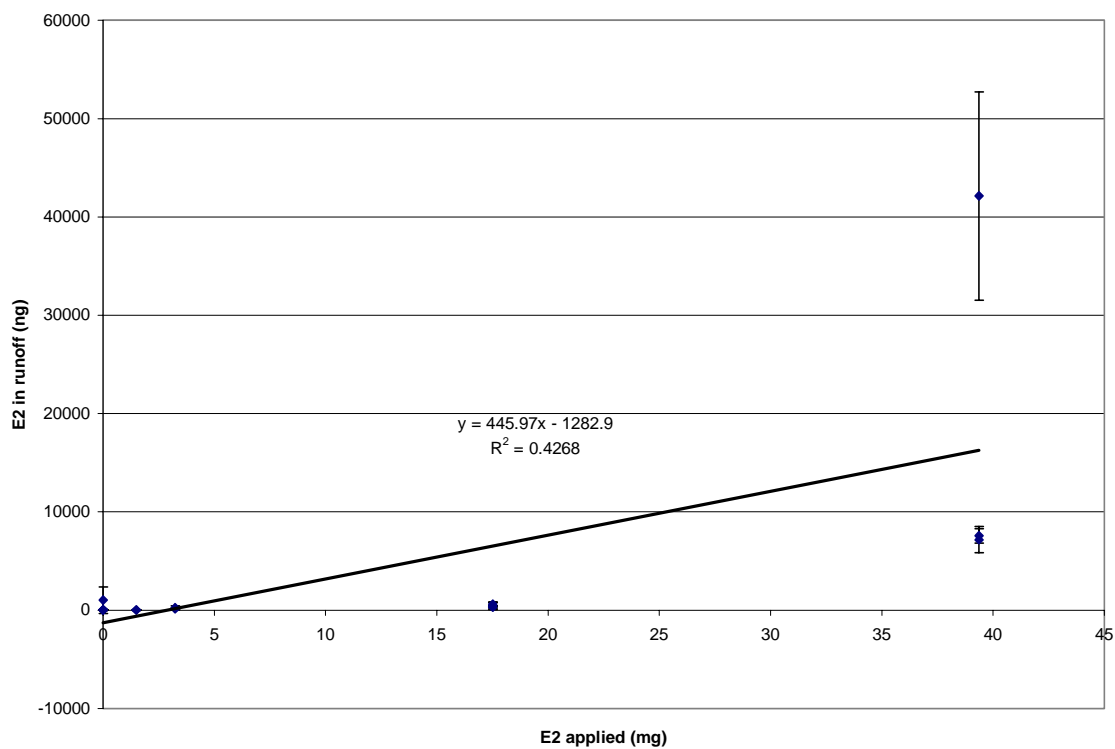


Figure 9. Mass of  $17\beta$ -estradiol applied vs. mass of  $17\beta$ -estradiol in runoff for the first runoff event from both manure applications.

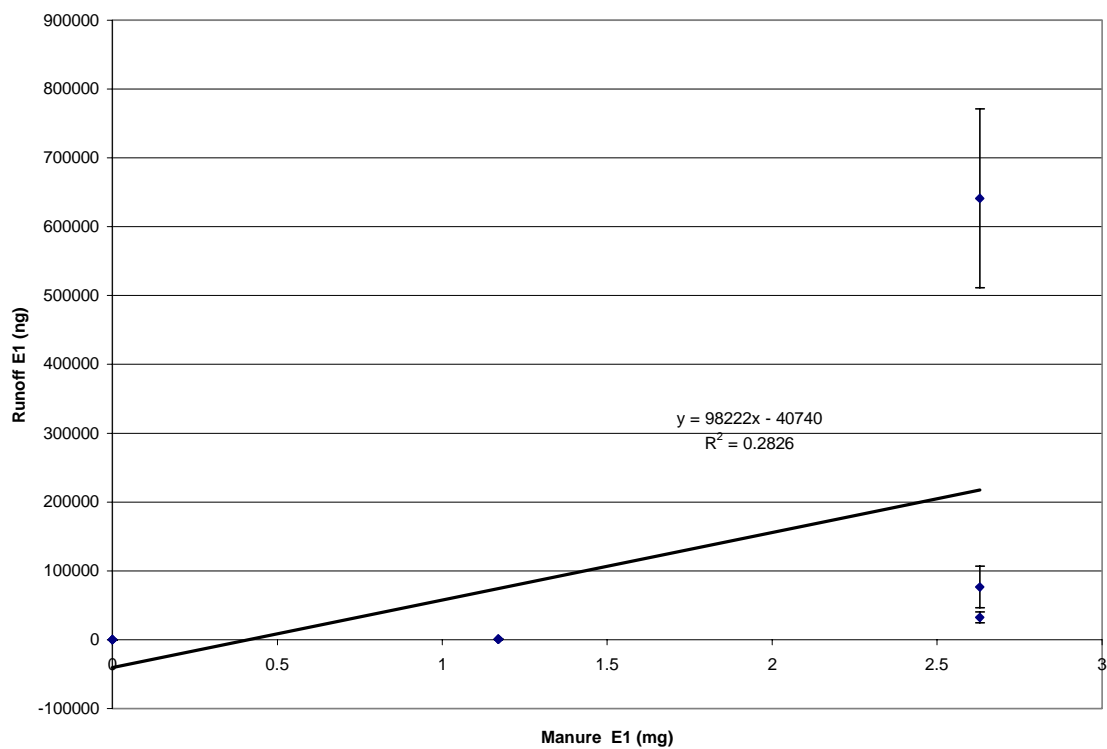


Figure 10. Mass of estrone applied in manure (mg) vs. mass of estrone in runoff (ng) for the first runoff event after manure application.

*Hypothesis 3: 17 $\beta$ -estradiol and estrone concentrations persist in runoff water for one month following manure application.*

For manure application 1, N and P-based plots, this hypothesis was accepted. 17 $\beta$ -estradiol concentrations on the N and P based plots were statistically significantly different ( $\alpha=0.05$ ) from control plots for the first 3 runoff events (Table 3). Runoff concentrations of 17 $\beta$ -estradiol from both the N and P-based plots were only above biologically relevant levels for runoff events occurring 22 and 24 days after manure application.

For manure application 2, 17 $\beta$ -estradiol concentrations from the N-based plots, this hypothesis was also accepted. For runoff events occurring 1 and 82 days after manure application, N-based plots were statistically significantly different from the control plots (Table 4). Runoff 17 $\beta$ -estradiol concentrations from N-based plots were only above biologically relevant levels for runoff events occurring 1, 26, and 82 days after manure application. However for 17 $\beta$ -estradiol concentrations in runoff from the P-based plots, this hypothesis was rejected because the runoff concentrations of 17 $\beta$ -estradiol from the P-based plots were never statistically significantly different from the control plots in this work (Table 5). Therefore, we cannot conclude that the runoff from the P-based plots was any more harmful than the unmanured plots. Runoff concentrations from P-based and control plots were only above biologically relevant levels for 12 days after manure application.

For the estrone concentrations in runoff from the N-based plots, this hypothesis was accepted. The N-based plots were statistically significantly different from the

control plots for runoff events occurring 1, 26, 52, 75, and 82 days after manure application (Table 6). This hypothesis was also accepted for the estrone concentrations in runoff from the P-based plots. Estrone concentrations in runoff from the P-based plots were statistically significantly different from the control plots for runoff events occurring 12 and 38 days after manure application (Table 7). Estrone however, persisted at biologically relevant levels for as many as 8 runoff events after manure application 2, from both the N and P-based plots. Estrone probably persists for longer periods of time because it is less likely to change forms. However,  $17\beta$ -estradiol can easily be converted to estrone in the environment (Routledge et al. 1998; Belfroid et al; 1999). If  $17\beta$ -estradiol is converted to estrone, this could cause elevated levels of estrone in the runoff, and less  $17\beta$ -estradiol.

### **Soil Related Hypotheses**

*Hypothesis 4:  $17\beta$ -estradiol and estrone masses in soil positively correlate with estrogen application rates.*

Based on five different estrogen application rates, this hypothesis is accepted for  $17\beta$ -estradiol concentrations in the soil for three months after manure application (Figure 11). During this time,  $17\beta$ -estradiol masses in the soil do positively correlate with estrogen application rates. They are positively correlated for 3 sampling events. Month one was a strong positive correlation ( $p=0.0001$ ). The strength of the correlation decreased with time, and month 4 was weakly negatively correlated with estrogen application rates ( $p=0.09$ ).

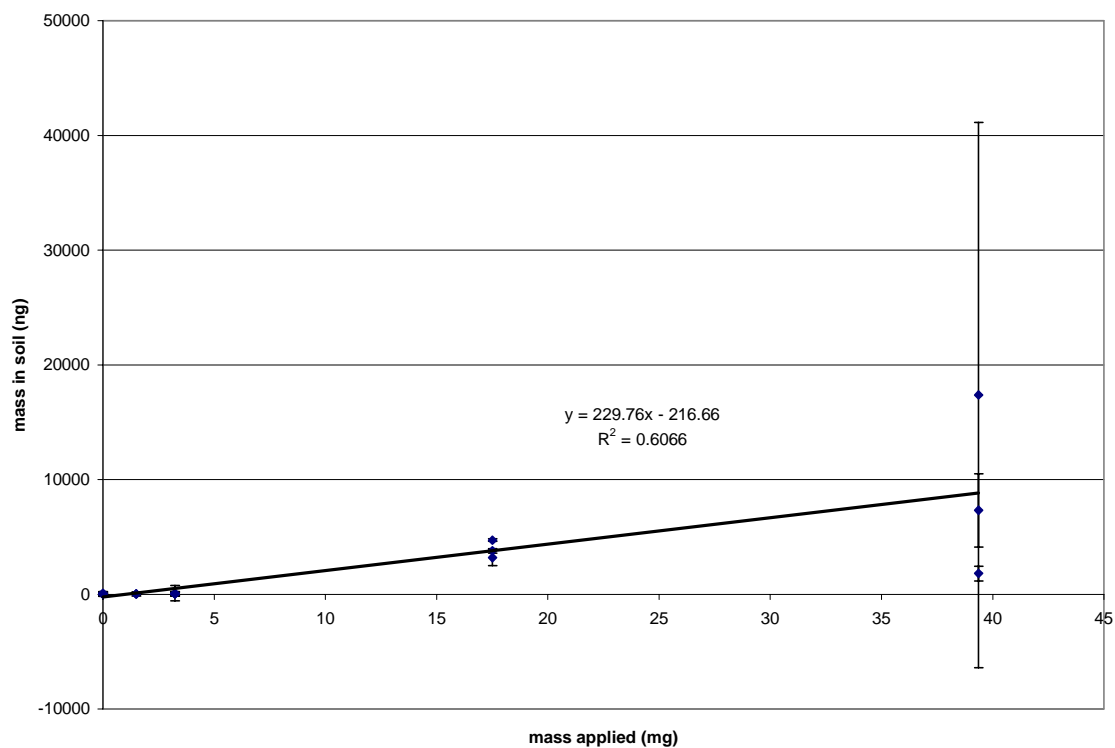


Figure 11. Mass of  $17\beta$ -estradiol applied to plots vs. mass of  $17\beta$ -estradiol in soil. This figure only shows the first month of soil samples after manure application.

This hypothesis was rejected for the estrone soil samples. Estrone masses in the soil were not statistically significantly correlated with the estrogen application rate. Only three application rates were available for estrone because estrone was not measured for the first manure application due to difficulties with the estrone ELISA kit.

If  $17\beta$ -estradiol is readily converted to estrone, this would explain why for the first month of soil sampling,  $17\beta$ -estradiol masses are positively correlated with estrogen application and after that, they get weaker and are not positively correlated by month 3. It would also explain why estrone is not positively correlated with estrogen application rates, because when  $17\beta$ -estradiol converts to estrone, it may not all convert at once which would cause the estrone masses to be higher than the  $17\beta$ -estradiol masses in the latter runoff events, or latter soil samples. Fluctuations in estradiol levels were also observed by Finlay-Moore (1999).

*Hypothesis 5:  $17\beta$ -estradiol and estrone concentrations persist in the soil for several months following manure application.*

This hypothesis was accepted for both  $17\beta$ -estradiol and estrone for manure applications 1 and 2, except for the P based plots for  $17\beta$ -estradiol for manure application 2. Manure application 1 data (Table 8) shows that  $17\beta$ -estradiol is present in the soil for 2 months after manure application. Soil  $17\beta$ -estradiol concentrations from the N-based plots for manure application 1 were statistically significantly different from the control plots for both December and January after manure application. In February, there was no

Table 8. Soil concentrations 17 $\beta$ -estradiol (ng/kg) manure application 1. November samples were taken before manure application. Lowercase letters indicate significant differences among treatments. Statistical data can only be compared down the columns. They are not applicable across rows.

Treatment	November	December	January	February
Control	217 $\pm$ 122 a b	11.2 $\pm$ 12 b	26.6 $\pm$ 32 c	24.3 $\pm$ 17 a
P-based	162 $\pm$ 78 b	6.49 $\pm$ 7.9 b	61.8 $\pm$ 35.6 b	41.7 $\pm$ 32 a
N-based	340 $\pm$ 181 a	28.9 $\pm$ 26 a	99.0 $\pm$ 37 a	29.3 $\pm$ 21 a



significant differences among treatments. In January, the concentration of  $17\beta$ -estradiol in the soil from the P-based plots were also statistically different from the control plots.

The November soil samples were taken before manure application and surprisingly, they have the highest concentrations of  $17\beta$ -estradiol. The reason for this is unclear, though one possible explanation is that manure deposited by flocks of geese on the plots.

As for manure application 2,  $17\beta$ -estradiol concentrations in soil from the N-based plots were statistically significantly different from the control plots shortly after manure application (Table 9). The P-based plots for  $17\beta$ -estradiol were never statistically different from the control plots, therefore, we cannot conclude that this soil contains more  $17\beta$ -estradiol than it would if the plots had not been manured.

Soil estrone concentrations in the N-based plots were statistically significantly different from the control plots in March, immediately after manure application, and in May. The rest of the time, there was no significant difference between the N-based plots and the control plots. Immediately after manure application, all three treatment types were statistically significantly different. However, the P-based plots were statistically significantly different from the control plots for three months after manure application (Table 10).

Table 9. Soil concentrations 17 $\beta$ -estradiol (ng/kg) manure application 2. Lowercase letters indicate statistically significant differences among treatments. Statistical data can only be compared down the columns. They are not applicable across rows.

Treatment	Before manure (March)	After Manure (March)	April	May	June	July
Control	49 $\pm$ 47 a	5.5 $\pm$ 5.9 b	67.7 $\pm$ 64 a	33.6 $\pm$ 13 a	45.8 $\pm$ 90 a	38.0 $\pm$ 35 a
P-based	92 $\pm$ 84 a	51.6 $\pm$ 21 b	124 $\pm$ 114 a	45.8 $\pm$ 34 a	20.3 $\pm$ 44 a	32.9 $\pm$ 40 a
N-based	49.6 $\pm$ 77 a	600 $\pm$ 939 a	97.5 $\pm$ 93 a	56.6 $\pm$ 35 a	2.1 $\pm$ 1.0 a	70.6 $\pm$ 54 a

Table 10. Soil estrone concentrations (ng/kg) manure application 2. Lowercase letters represent significant statistical differences among treatments. Statistical data can only be compared down the columns. They are not applicable across rows.

Treatment	Before manure (March)	After Manure (March)	April	May	June	July
Control	63 $\pm$ 39 a	204 $\pm$ 140 c	112 $\pm$ 68 b	43 $\pm$ 40 b	119 $\pm$ 18 a	59 $\pm$ 12 b
P-based	245 $\pm$ 83 a	2579 $\pm$ 855 b	188 $\pm$ 16 a	130 $\pm$ 66 a	106 $\pm$ 35 a	72 $\pm$ 12 a
N-based	463 $\pm$ 1076 a	1262 $\pm$ 538 a	138 $\pm$ 18 b	182 $\pm$ 61 a	124 $\pm$ 45 a	67 $\pm$ 9.0 ab

## **Hypothesis on Soil-Runoff Relationship**

*Hypothesis 6: Masses of 17 $\beta$ -estradiol and estrone in the soil positively correlate with masses of these compounds in the runoff.*

This hypothesis is accepted for 17 $\beta$ -estradiol, and rejected for estrone. 17 $\beta$ -estradiol masses in soil are highly positively correlated with masses of this substance in the runoff ( $p=0.0001$ ) (Figure 12). Estrone masses in the soil were not correlated with estrone masses in the runoff. This could be because most of the labile estrone and 17 $\beta$ -estradiol comes off the plots in runoff during the first few runoff events. After that, the remaining estrogens tend to stay in the soil. The amount of 17 $\beta$ -estradiol and estrone fluctuate somewhat. This could be caused by the conversion of 17 $\beta$ -estradiol to estrone and the degradation rates of these two estrogens. These estrogens might move vertically or horizontally in the soil profile, which would cause fluctuating concentrations of soil estrogens. Temperature and microbial activity could also have an effect on the persistence of these estrogens in the soil. In an earlier study, Finlay-Moore et al. (2000) also observed fluctuations in soil concentrations of 17 $\beta$ -estradiol.

## **Mass Lost to Runoff**

For manure application 1, less than 1% of the mass fraction of 17 $\beta$ -estradiol applied left the plots through runoff (Figure 13 and 14). For manure application 2, less than 10% of the total mass of 17 $\beta$ -estradiol and estrone applied to the plots was removed in the runoff water (Figure 15-18). This would suggest that the estrogens are remaining in the soil until they degrade, or move further down the soil profile.

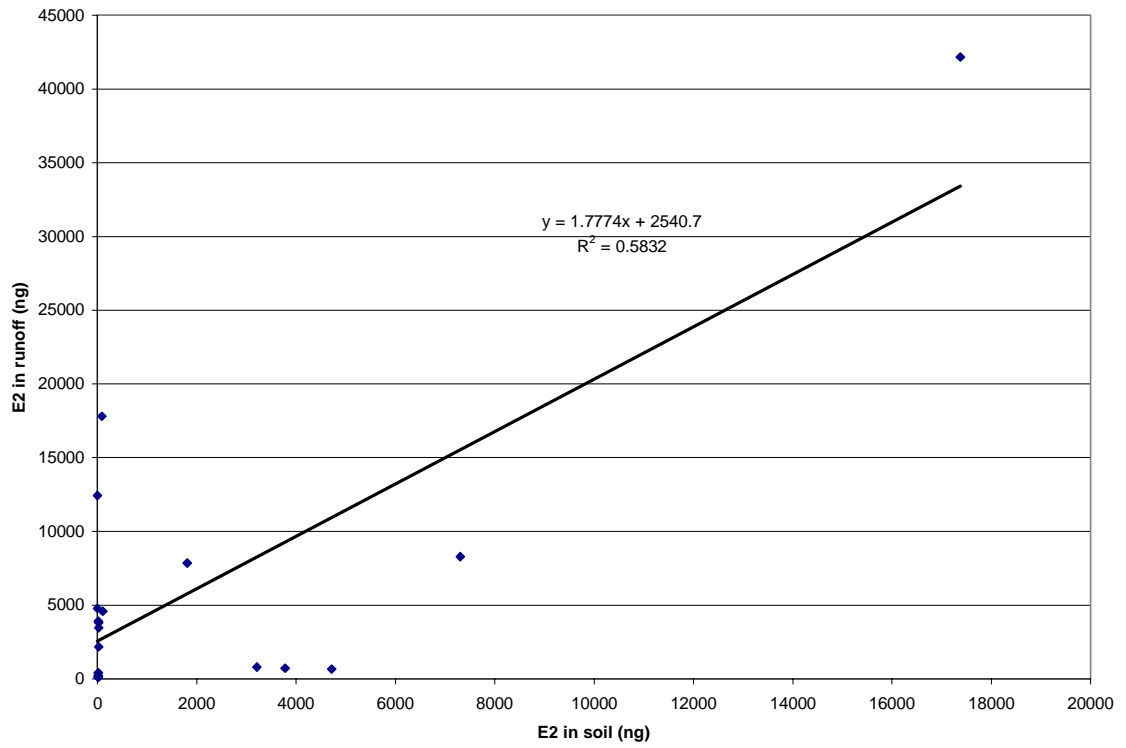


Figure 12. Mass of 17β-estradiol in soil (ng) vs. total mass of 17β-estradiol lost in subsequent runoff events (ng).

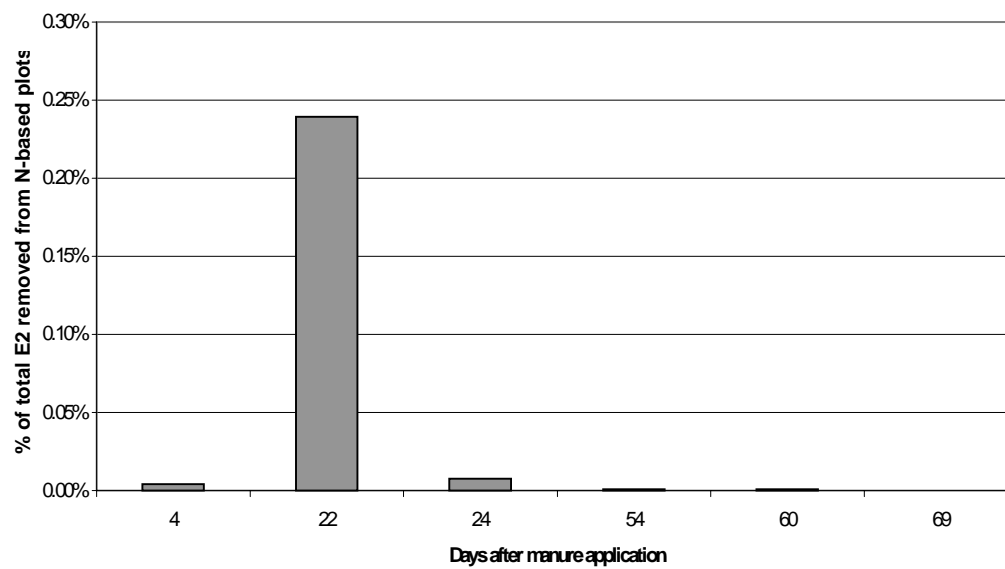


Figure 13. Percentage of 17β-estradiol removed from N-based plots after manure application 1 during rainfall events.

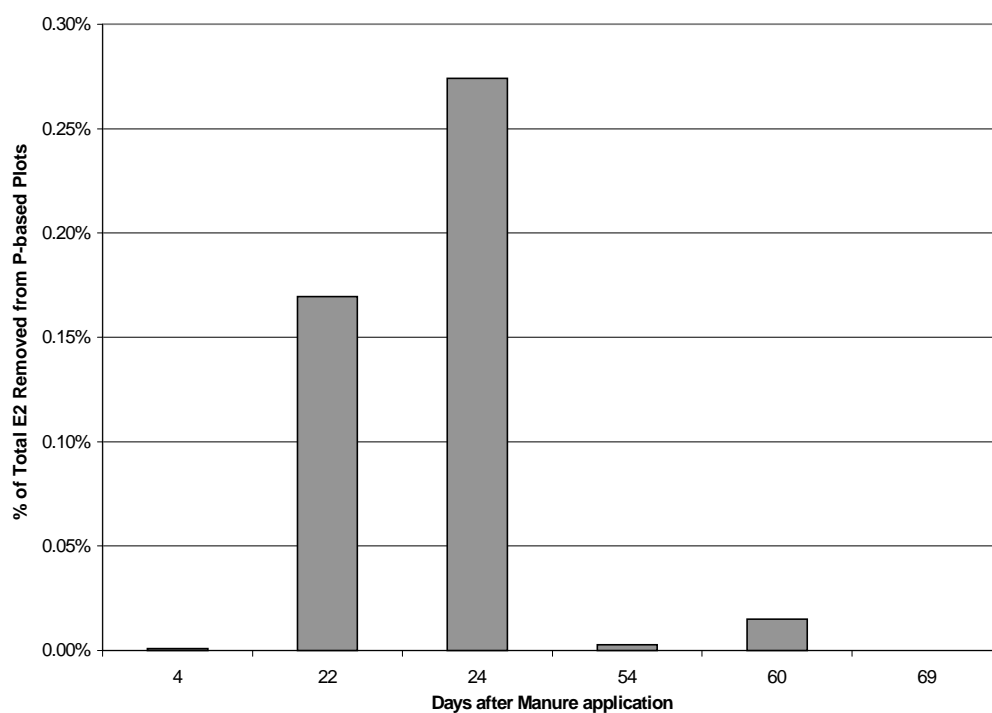


Figure 14. Percentage of 17β-estradiol removed from P-based plots after manure application 1 during rainfall events.

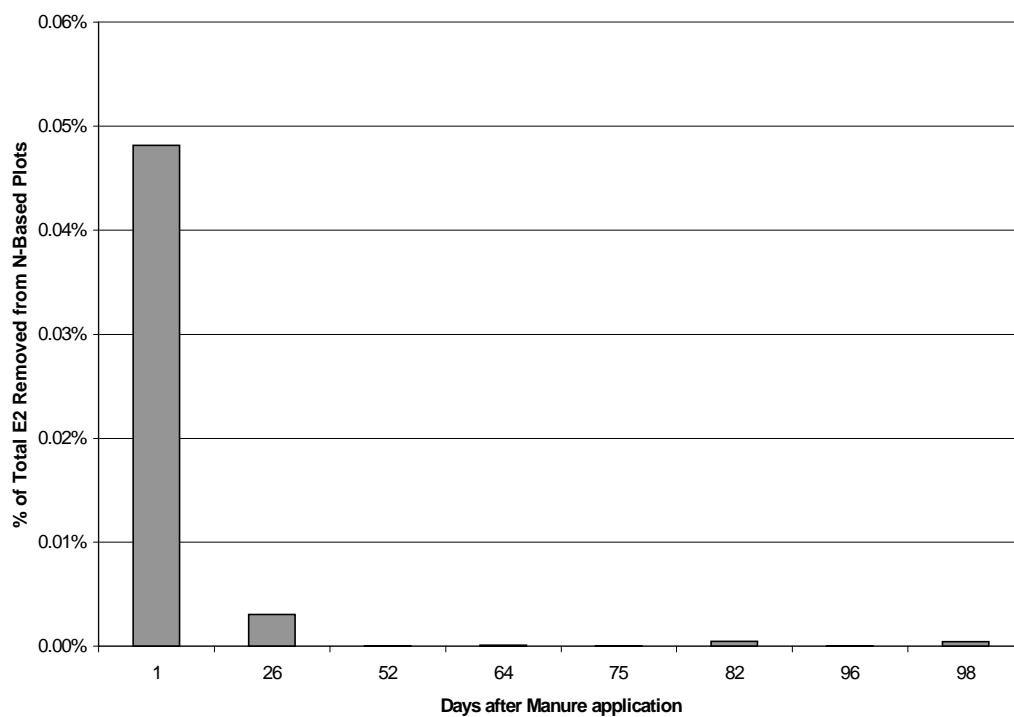


Figure 15. . Percentage of 17β-estradiol removed from N-based plots after manure application 2 during rainfall events.

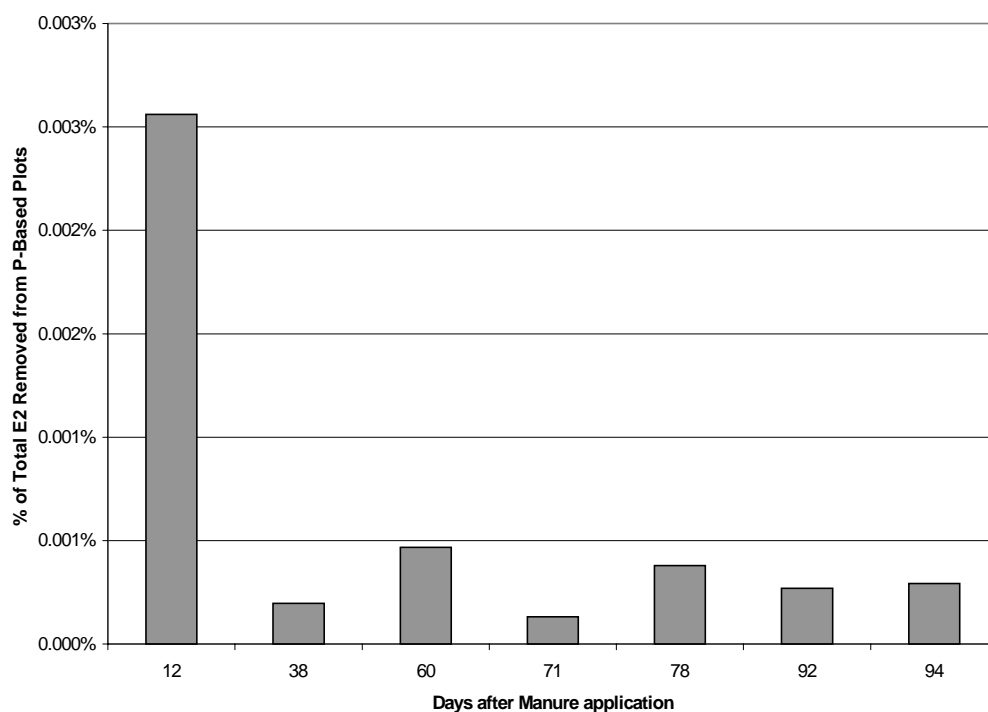


Figure 16. Percentage of  $17\beta$ -estradiol removed from P-based plots after manure application 2 during rainfall events.



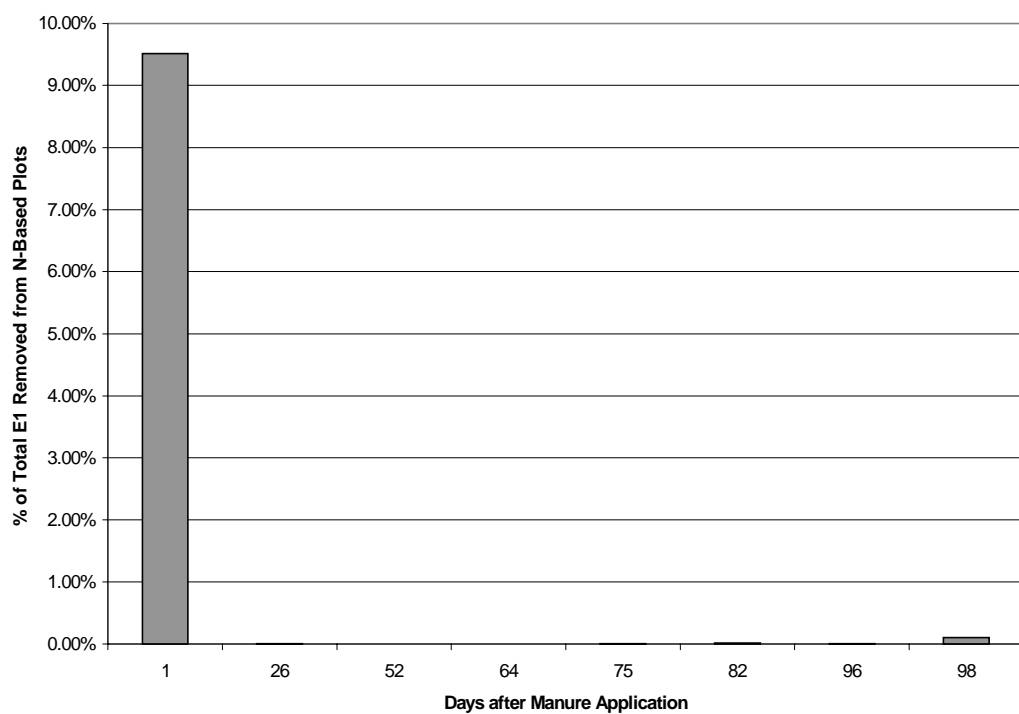


Figure 17. Percentage of estrone removed from N-based plots after manure application 2 during rainfall events.

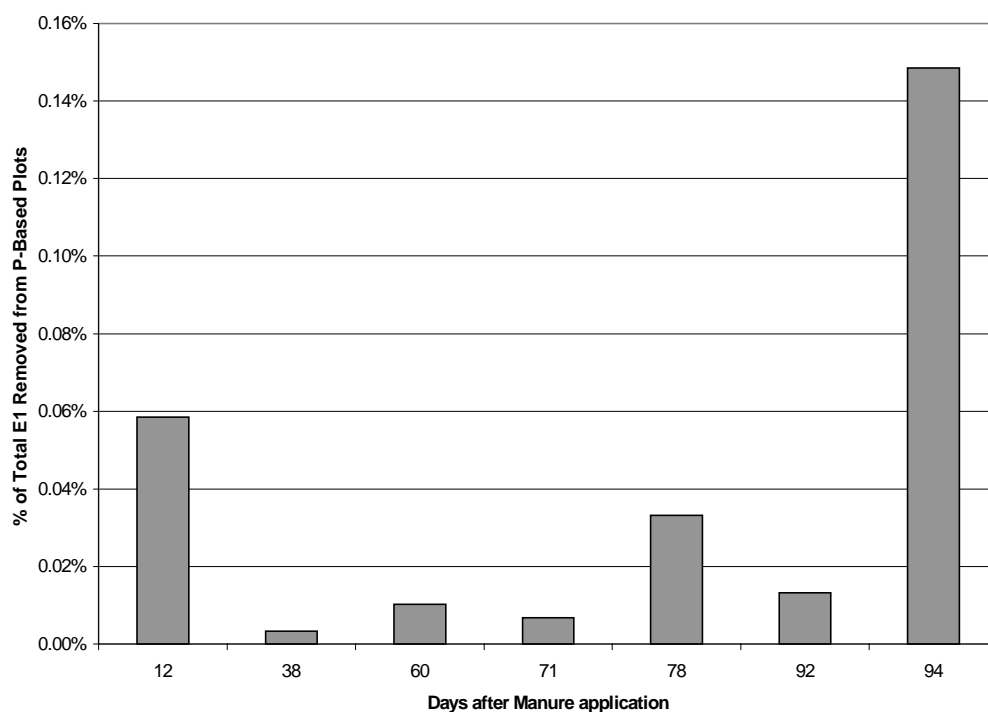


Figure 18. Percentage of estrone removed from P-based plots after manure application 2 during runoff events.

## Chapter 4

### CONCLUSIONS AND FUTURE WORK

#### Conclusions

In both winter and spring, biologically relevant concentrations of  $17\beta$ -estradiol and estrone were observed in runoff from plots receiving dairy manure slurry at rates appropriate to crop nitrogen (N) and phosphorus (P) requirements. Masses of these compounds in runoff were positively correlated with estrogen application rates, and these compounds persisted in runoff water for several runoff events following manure application.

$17\beta$ -estradiol concentrations in the soil positively correlated with estrogen mass application rates at statistically significant levels, but estrone concentrations did not. Both of these estrogens did persist in the soil for several months after manure applications in both winter and spring. Masses of  $17\beta$ -estradiol in the soil did positively correlate with masses of this compound in the runoff, but masses of estrone in the soil did not correlate with runoff masses of estrone.

Since biologically significant levels of both  $17\beta$ -estradiol and estrone were found in runoff, manure application may need to be limited so that lower amounts of these estrogens come off in the runoff. This would especially help with the first rainfall after manure application. If less estrogen containing manure were applied, less would be in the runoff and eventually reach receiving bodies of water. If manure were applied to agricultural fields based on crop P-requirement, rather than on crop N-requirements, the

impact from these estrogens may be minimized. The P-based plots were statistically significantly different from control for no more than 2 runoff events after manure application, where the N-based plots were statistically significantly different from control plots for up to 6 runoff events after manure application. The runoff water from fields receiving manure would flow into an entire watershed further diluting the estrogens. If the manure was applied at the P-rate, the impact of these estrogens could be minimized.

Much of the estrogens in the manure could also bind to the soil. This could prevent more estrogen from moving into the stream water. If the estrogens bind to the soil, they can degrade and not be of concern for the fish and wildlife. As long as manure application rates are monitored and controlled, and only trace amounts of 17 $\beta$ -estradiol and estrone are found in the runoff water, these estrogens should not be a problem for their surroundings.

### **Future Work**

Future areas of research on this topic should include an exploration of how 17 $\beta$ -estradiol and estrone behave at different depths in the soil. It is possible that these estrogens could move down through the profile of the soil and accumulate. Leachate water was collected from these runoff plots with lysimeters, for another project. It would be of interest to compare the leachate data with the soil and runoff water, for a better picture of what happens to the estrogens once they leave the top 7.6 cm of soil.

A large-scale study looking at estrogen concentrations in streams receiving runoff from heavily manured areas would provide more information on the dilution of estrogens in a watershed setting. That would be the true test to determine if these estrogens are

actually in receiving bodies of water at ecologically harmful levels. It is also unclear how estrogens behave in the soil. More studies should be conducted on the ability of estrogens to bind to soil organic matter, and on the impact of soil organic matter on different estrogen assay methods.

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## **APPENDIX**

Table A1. Runoff volumes for manure application 1. All volumes are reported in Liters.

	R1	R2	R3	R4	R5	R6
Plot 1 (H)	10.795	285.7	0	17.145	19	17.145
Plot 2 (H)	26.62	72.34	171.4	17.78	19	16.1925
Plot 3 (C)	475	179.02	475	18.7325	34.24	16.8275
Plot 4 (L)	0	125.68	79.96	15.875	415.24	17.4625
Plot 5 (H)	0	140.92	140.92	0	12.7	407.62
Plot 6 (L)	5.08	72.34	323.8	0	232.36	16.8275
Plot 7 (C)	19	19	19	64.72	19	17.145
Plot 8 (L)	8.89	83.77	19	57.1	17.145	17.145
Plot 9 (C)	0.635	19	49.48	0	19	0.3175

Table A2. Runoff volumes for manure application 2. All volumes are reported in Liters.

	R1	R2	R3	R4	R5	R6	R7	R8
Plot 1 (H)	445.72	0	0	1.905	0	1.905	0	19
Plot 2 (H)	17.78	17.4625	16.51	15.875	15.875	17.145	17.4625	64.72
Plot 3 (C)	17.78	17.145	16.51	15.875	15.875	17.145	17.78	19
Plot 4 (L)	17.145	3.175	15.875	0	15.24	0	19	
Plot 5 (H)	17.78	15.24	0	0	0	15.875	0	19
Plot 6 (L)	17.4625	17.4625	17.145	15.875	17.145	18.415	79.96	
Plot 7 (C)	17.145	72.34	17.145	15.875	15.24	247.6	17.78	308.56
Plot 8 (L)	18.415	12.065	17.145	0	15.24	0	19	
Plot 9 (C)	16.8275	17.145	0.635	0.9525	0	15.875	0	19

## **VITA**

Angel Renea Peters Dyer was born in Lawrenceburg, Tennessee on August 28, 1978. She attended schools in the Lawrence County Public School System, where she graduated from Loretto High School in May, 1996. She entered Tennessee Technological University, Cookeville, Tennessee in August 1996 and in December, 1999, she received the Bachelor of Science in Environmental Agriscience with a minor in biology. She entered the Master's program in Biosystems Engineering Technology at The University of Tennessee, Knoxville in January, 2000, officially receiving her Master's degree in December, 2001.